Clinical and Protein Correlations of Prophylactic Treatments for Preeclampsia

Dr. Renuka Shanmugalingam

School of Medicine

Doctor of Philosophy (PhD) Submission

STATEMENT OF AUTHENTICATION

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text, I hereby declare that I have not submitted this material, either in full or part, for a degree at this or any other institute.

In accordance with the Graduate Research School of Western Sydney University's **Thesis as A Series of Papers** policy (1), this thesis consists of **a minimum of 4 original scholarly research articles** that have been accepted for publication.

Signed: 

Date: 25th of July 2020

Renuka Shanmugalingam

School of Medicine

Western Sydney University

ABSTRACT

The prophylactic benefit of aspirin in the prevention of preeclampsia is now widely recognized and increasingly utilized. However, its mechanism of action and optimal clinical utility in the prevention of preeclampsia remains a subject of ongoing research.

This thesis examined the mechanism of action of aspirin in preventing preeclampsia with specific focus on its anti-inflammatory role, through the 15-epilipoxin-A_4, aspirin triggered lipoxin (ATL), pathway. This thesis also examined for the influence of dose of aspirin in pregnancy through a pharmacokinetic assessment, and the influence of inadequate adherence to aspirin on the desired clinical outcomes in high-risk pregnancy. Additionally, this thesis also examined for factors that influenced women's adherence to aspirin in pregnancy.

A non-interventional, multi-centre, longitudinal cohort study was prospectively undertaken to achieve the aims of this thesis. In keeping with recent data, this thesis demonstrated that aspirin is an effective prophylactic intervention for preeclampsia amongst high-risk women when adequate adherence is observed. In addition to its known anti-platelet effect, the outcomes of this thesis suggest that aspirin is likely to play a role in placental development through its ATL mediated anti-inflammatory effect in high-risk pregnant women. Additionally, the studies in this thesis also suggest the potential need for aspirin dose adjustment in pregnancy given its altered pharmacokinetic in pregnancy. Importantly, this thesis also demonstrates the importance of adequate adherence with aspirin and emphasizes on the importance of assessment of adherence to aspirin in pregnancy, both in clinical and research settings.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

This thesis was made possible through the financial support from PEARLs and the Women's Health Initiative Translational Unit (WHITU) of South Western Sydney Local Health District (SWSLHD).

Firstly, I would like to express my sincere gratitude to my supervisors and role models, Professor Angela Makris and Professor Annemarie Hennessy for their tireless mentorship, guidance, and support. Their advice and wisdom have helped me grow both professionally and personally. In meeting them, I have found role models who constantly challenge me to be better and I will always cherish the relationship I share with them. I would also like to thank Dr. XiaoSuo Wang, Dr. Gerald Munch, Dr. Zelalam Mengesha, Dr. Penelope Motum, Dr. Ian Fulcher, Stephanie Notaras, Gaksoo Lee and Roshika Kumar for their much-valued collaboration and friendship through the duration of this candidature and beyond. I would also like to thank my peers in the Vascular Immunology Research group, Dr. Suzanne Pears, Dr. Katrina Chau and Dr. Shikha Aggarwal for their comradeship: May the force be with you!

The recruitment phase of this project would not have been possible without the support and enthusiasm of the midwives of SWSLHD and my colleagues in the Department of Renal Medicine, SWSLHD. I will always appreciate their contributions towards this project. I would also like to thank all the women who participated in this project. Their struggles, hope and commitment will continue to drive us to find the much-needed answers on preeclampsia.

This thesis would not have been possible without the unconditional love, encouragement, patience, and support from my husband, Rajeev, and daughter, Remina. Thank you for always being my source of strength and courage. I would also like to thank my parents, in-laws, and extended family for their unwavering belief in me over the years.

PUBLICATIONS, PRESENTATIONS AND AWARDS

PUBLICATIONS IN RELATION TO THE THESIS

1) **Aspirin in the prevention of preeclampsia: the conundrum of how, who and when.**

   Renuka Shanmugalingam, Angela Makris, Annemarie Hennessy.

   Journal of Human Hypertension, 33, 1–9 (2019).

   DOI: https://doi.org/10.1038/s41371-018-0113-7

2) **A pharmacokinetic assessment of optimal dosing, preparation and chronotherapy of aspirin in pregnancy.**

   Renuka Shanmugalingam, XiaoSuo Wang, Gerald Munch, Ian Fulcher, Gaksoo Lee, Katrina Chau, Bei Xu, Roshika Kumar, Annemarie Hennessy, Angela Makris.

   American Journal of Obstetric and Gynaecology (AJOG), 22, 255.E1-E9 (2019).

   DOI: https://doi.org/10.1016/j.ajog.2019.04.027

3) **The 15-epilipoxin-A4 pathway (ATL) with prophylactic aspirin in preventing preeclampsia: a longitudinal-cohort study.**

   Renuka Shanmugalingam, XiaoSuo Wang, Penelope Motum, Ian Fulcher, Gaksoo Lee, Roshika Kumar, Annemarie Hennessy, Angela Makris.

   Journal of Clinical Endocrinology and Metabolism (JCEM), Accepted July 2020 (IN-PRESS).

4) **The clinical influence of non-adherence with prophylactic aspirin in preventing preeclampsia in high-risk pregnancies: A multi-centre prospective, observational, cohort study**.

Renuka Shanmugalingam, XiaoSuo Wang, Penelope Motum, Ian Fulcher, Gaksoo Lee, Roshika Kumar, Annemarie Hennessy, Angela Makris.

Hypertension, 75,1125–1132 (2020).

DOI: https://doi.org/10.1161/HYPERTENSIONAHA.119.14107

5) Factors that influence adherence to aspirin therapy in the prevention of preeclampsia amongst high-risk pregnant women: A mixed method analysis.

Renuka Shanmugalingam, Zelalem Mengesha, Stephanie Notaras, Pranee Liamputtong, Ian Fulcher, Gaksoo Lee, Roshika Kumar, Annemarie Hennessy, Angela Makris.

PLoS ONE 15(2), e0229622 (2020).

DOI: https://doi.org/10.1371/journal.pone.0229622

INVITED AUTHORSHIP ARTICLES IN RELATION TO THE THESIS

1) **Tackling pre-eclampsia: getting ahead of the game.**

   Renuka Shanmugalingam, Angela Makris, Annemarie Hennessy

   How To Treat Edition of Australian Doctors, June Edition (2019).

2) **Eclampsia and hypertensive emergency in pre-eclampsia.**

   Renuka Shanmugalingam, Angela Makris, Annemarie Hennessy

   O&G Magazine: Calling A Code, 19 (2017).

PUBLICATIONS RELEVENT TO PHD (NOT DIRECTLY RELATED TO THESIS)

1) **Vertebral artery dissection in pre-eclampsia: a case series and literature review.**

   Renuka Shanmugalingam; Nina Rezapour; Siang Chye Chuah; Thi Mong Vo; Roy Beran; Annemarie Hennessy; Angela Makris

   BMC Pregnancy and Childbirth 16, 164 (2016).

   DOI: 10.1186/s12884-016-0953-5

2) **Pregnancy induced atypical haemolytic uremic syndrome: a new era with Eculizumab.**

   Renuka Shanmugalingam, Danny Hsu, Angela Makris

   Obstetric Medicine, 11, 28-13 (2018).

   DOI: https://doi.org/10.1177/1753495X17704563

3) **The effect of acetyl salicylate acid (aspirin) on trophoblast-endothelial interaction in an in-vitro preeclampsia model.**

Bei Xu, Renuka Shanmugalingam, Suzanne Pears, Annemarie Hennessy, Angela Makris.

Journal of Reproductive Immunology, 124, 54-61 (2017).

DOI: https://doi.org/10.1016/j.jri.2017.10.044

4) **RNAi modulation of placental sflt1 for the treatment of preeclampsia.**

Turanov AA, Lo A, Hassler MR, Makris A, Ashar-Patel A, Alterman JF, Coles AH, Haraszti RA, Roux L, Godinho BMDC, Echeverria D, Pears S, Iliopoulos J, Shanmugalingam R, Ogle R, Zsengeller ZK, Hennessy A, Karumanchi SA, Moore MJ, Khvorova A.

Nature Biotechnology, 36, 1164–1173 (2018).

DOI: https://doi.org/10.1038/nbt.4297

5) **Galectin-1 related modulation of trophoblast endothelial interactions by integrins α1 and β1.**

Bei Xu, Renuka Shanmugalingam, Katrina Chau, Angela Makris, Annemarie Hennessy.

Reproductive Sciences, 27, 1097–1109 (2020).

DOI: https://doi.org/10.1007/s43032-019-00046-z

CONFERENCE PRESENTATIONS

1) **A pharmacokinetic assessment of aspirin through timed analysis of plasma salicylate acid level: an analysis of difference in gender, dose and preparation of aspirin.**

   Renuka Shanmugalingam, XiaoSuo Wang, Gerald Munch, Ian Fulcher, Gaksoo Lee, Katrina Chau, Bei Xu, Roshika Kumar, Annemarie Hennessy, Angela Makris.

- National meeting oral presentation; Society of Obstetric Medicine Australia and New Zealand meeting (SOMANZ), Cairns 2018.
- International meeting poster presentation; International Society of the Study of Hypertension in Pregnancy (ISSHP) Amsterdam 2018.

2) **A cohort study utilising a biochemical assessment of aspirin compliance vs resistance in high-risk pregnant women.**

   Renuka Shanmugalingam, XiaoSuo Wang, Penelope Motum, Ian Fulcher, Gaksoo Lee, Roshika Kumar, Annemarie Hennessy, Angela Makris

- National meeting oral Presentation; Australia and New Zealand Society of Nephrology (ANZSN), Sydney 2018.
- International meeting poster presentation; International Society of the Study of Hypertension in Pregnancy (ISSHP) Amsterdam 2018.

3) **A mixed method analysis on factors that influences adherence to aspirin therapy in the prevention of pre-eclampsia amongst high-risk pregnant women.**

Renuka Shanmugalingam, Zelalem Mengesha, Stephanie Notaras, Pranee Liamputtong, Ian Fulcher, Gaksoo Lee, Roshika Kumar, Annemarie Hennessy, Angela Makris

- National meeting oral presentation; Society of Obstetric Medicine Australia and New Zealand (SOMANZ) meeting, Melbourne 2019.
- International meeting oral presentation; Asia-Pacific Congress of Hypertension (APCH) annual meeting, Brisbane 2019.

4) **Effect of aspirin on placental growth factor (PlGF) in pregnancy and the development of preeclampsia.**

Renuka Shanmugalingam, XiaoSuo Wang, Penelope Motum, Ian Fulcher, Gaksoo Lee, Roshika Kumar, Annemarie Hennessy, Angela Makris

- National meeting oral presentation; Society of Obstetric Medicine Australia and New Zealand (SOMANZ) meeting, Melbourne 2019.
- International meeting oral presentation; Asia-Pacific Congress of Hypertension (APCH) annual meeting, Brisbane 2019.

5) Assessment of the aspirin triggered lipoxin pathway (ATL) and its influence on the cytokine profile of high-risk pregnant women.

Renuka Shanmugalingam, XiaoSuo Wang, Penelope Motum, Ian Fulcher, Gaksoo Lee, Roshika Kumar, Annemarie Hennessy, Angela Makris.

- National meeting poster presentation; Society of Obstetric Medicine Australia and New Zealand (SOMANZ) meeting, Melbourne 2019.
- International meeting poster presentation; Asia-Pacific Congress of Hypertension (APCH) annual meeting, Brisbane 2019. APCH Student Poster Prize Finalist.

INVITED SPEAKER PRESENTATIONS

1) **Prophylactic use of aspirin in the prevention of preeclampsia.**

   Annual Scientific Meeting, Australian Diabetes in Pregnancy Society (ADIPS)

   Sydney 2019.

2) **Tackling preeclampsia: Getting ahead of the game.**

   South Western Sydney Local Health District General Practitioner Continuous Professional Development (CPD) Education Meeting

   Sydney 2017.

3) **Effect of aspirin on placental growth factor (PlGF) in pregnancy and the development of preeclampsia.**

   International Winner Presentation, British and Irish Hypertension Society (BIHS) Meeting

   Glasgow 2020.

   (Postponed to 2021 due to the COVID-19 pandemic)

AWARDS

- High Blood Pressure Research Council Australia (HBPRCA) Best Student Oral Presentation Award

  Asia Pacific Council of Hypertension (APCH), Brisbane 2019.

- Young Investigator Student Travel Grant

  Asia Pacific Council of Hypertension (APCH), Brisbane 2019.

- Andrew Phippard Memorial Award for Best Oral Scientific Presentation

  Society of Obstetric Medicine Australia and New Zealand (SOMANZ),

  Melbourne 2019.

- Andrew Phippard Memorial Award for Best Oral Scientific Presentation

  Society of Obstetric Medicine Australia and New Zealand (SOMANZ),

  Cairns 2018.

- Young Investigator Travel Award

  International Society of Study of Hypertension in Pregnancy (ISSHP),

  Amsterdam 2018.

- Three Minute Thesis (3MT) 2017
  - Winner, Heart Research Institute (HRI), Sydney (Heats)
  - Winner and People's Choice Award Winner, School of Medicine Heats, Western Sydney University (WSU)
  - Runner up, University Finals, Western Sydney University (WSU)

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND ACRONYMS

◦C	degrees Celsius
µL	microlitre
Φ	Phi correlation
2D	Two dimensional
3D	Three dimensional
6-keto-PGF1α	6-keto prostaglandin F 1-alpha
15*R*-HETE	15R-hydroxyeicosatetraenoic acid
AA	Acetic acid
CAN	Acetonitrile
ADP	Adenosine 5'-diphosphate
ANOVA	Analysis of variance
aPL	Antiphospholipid antibody
ATL	Aspirin triggered lipoxin/15-epilipoxin-A_4
AUC(t-24)	Area under the curve time 0-24 hours
BMI	Body mass index
CI	Confidence interval
CL	Clearance
Cmax	Point of maximum concentration
COL/ADP	Collagen/adenosine
COL/EPI	Collagen/epinephrine
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1

COX-2	Cyclooxygenase-2
CPD	Continuing professional development
CT	Closure time
CTB-ORG	Cytotrophoblast Organoid
D4-SA	Deuterated salicylic acid
DBP	Diastolic blood pressure
EC	Enteric coated
En-Lipoxin-A_4	Endogenous Lipoxin A_4
ELISA	Enzyme linked immunosorbent assay
EPI	Epi-nephrine bitartrate
ERK	Extracellular-signal-regulated kinase
EVT	Extravillous trophoblast
FA	Formic acid
FMF	Fetal Maternal Foundation
G	Gauge
GFR	Glomerular Filtration Rate
GP	General Practitioner
GP IIB/IIIA	Glycoprotein IIB/IIIA
HCP	Health care providers
HLA-C	Human leukocyte antigen-C
HELLP	Haemolysis, Elevated Liver enzymes, Low Platelet count
HPLC	High performance liquid chromatography
HR	Hazards ratio
HR-AA	Aspirin adherent high-risk pregnant women

HR-NA	Non-aspirin high-risk pregnant women
HTR8	Human trophoblast cell line
HUVEC	Human umbilical vein endothelial cells
IFN-γ	Interferon gamma
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-1β	Interleukin-1Beta
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-8	Interleukin-8
IPA	Impedance platelet aggregometry
ISSHP	International Society of the Study of Hypertension in Pregnancy
IUGR	Intrauterine growth restriction
KIR	Killer immunoglobulin receptors
LCMS	Liquid chromatography mass spectrometry
LGA	Large for gestation age
Lipoxin A_4-d5	Deuterated lipoxin A_4
LPS	Lipopolysaccharide
MAP	Mean arterial pressure
MESOR	Midline estimating statistics of rhythm
Mg	Milligram
mL	Millilitre

Mm	Millimetre
mmHg	millimetres of Mercury
MoMs	Multiple of the Median
MP A	Mobile phase A
MP B	Mobile phase B
MMP 3	Matrix Metallopeptidase 3
MRM	Multiple reaction monitoring
mRNA	messenger Ribonucleic Acid
N_2	Nitrogen
NATA	National Association of Testing Authorities
NF-Kb	Nuclear factor kappa-light-chain- enhancer of activated B cells
Ng	Nanogram
NICE	National Institute for Health and Care Excellence
NK	Natural killer
NO	Nitric oxide
Non-EC	Non enteric coated
OR	Odds ratio
PAPP-A	Pregnancy-associated plasma protein-A
PCV	Packed cell volume
PFA-100	Platelet function analyser
Pg	Picogram
PG12	Prostaglandin-2
pH	Potential Hydrogen
PI	Pulsatility index

PlGF	Placental growth factor
Plt	Platelet
PMN	Polymorphonuclear neutrophils
Rpm	Revolutions per minute
RR	Risk reduction
SA	Salicylic acid
SBP	Systolic blood pressure
sFlt-1	Soluble fms-like tyrosine kinase -1
sEng	Soluble endoglin
SGA	Small for gestation age
SMAQ	Simplified Medication Adherence Questionnaire
SOMANZ	Society of Obstetric Medicine Australia and New Zealand
SPE	Solid phase extraction
SPSS	Statistical Package for Social Sciences
SWSLHD	South Western Sydney Local Health District
t½	Elimination half life
TFA	Trifluoroacetic acid
Tmax	Time of maximum concentration
TNF-α	Tumour Necrosis Factor Alpha
TXA2	Thromboxane A-2
TXB2	Thromboxane B-2
V	Voltage
v/v	Concentration of solution
VCAM-1	Vascular cell adhesion molecule-1

Vd	Volume of distribution
VE-cadherin	Vascular endothelial cadherin
vWF	von Willebrand factor

CHAPTER 1

INTRODUCTION

Publication in relation to this chapter:

REVIEW PAPER:

ASPIRIN IN THE PREVENTION OF PREECLAMPSIA: THE CONUNDRUM OF HOW, WHO AND WHEN.

Renuka Shanmugalingam, Angela Makris, Annemarie Hennessy.

Journal of Human Hypertension 33, 1–9 (2019).

DOI: https://doi.org/10.1038/s41371-018-0113-7

1.1 Preeclampsia and its pathophysiology

Preeclampsia is a hypertensive disorder of pregnancy that affects 8 – 10% of all pregnancies globally and is the indication for 20% of labour inductions and 15% of caesarean sections annually (2). It is defined as the onset of maternal hypertension after 20 weeks of gestation with clinical and/or biochemical features of end organ impairment (Figure 1). Whilst the general incidence of preeclampsia remains static, the incidence of maternal and fetal morbidity and mortality, particularly in developing countries, is increasing and remains the second commonest cause of maternal mortality internationally (2-4).

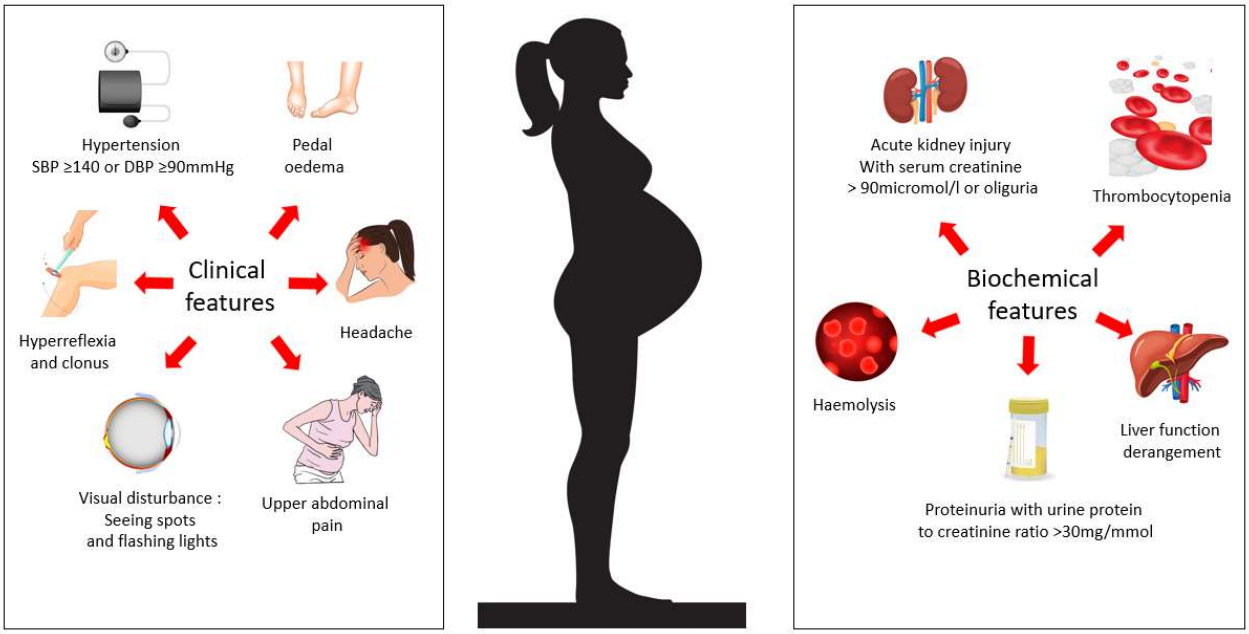


Figure 1: Clinical and biochemical features of Preeclampsia.

Adapted from: Shanmugalingam et al. Tackling Preeclampsia – A three stage management strategy. How To Treat Edition of Australian Doctors (2019).

SBP: systolic blood pressure, DBP: diastolic blood pressure

The understanding of the pathophysiology of preeclampsia continues to grow, however, at the time of this thesis, it remains a condition of heterogenous theories. The most commonly cited theory describes impaired placental development in the first trimester that later results in the clinical syndrome (5, 6)– this is best simplified into a two-stage model (Figure 2).

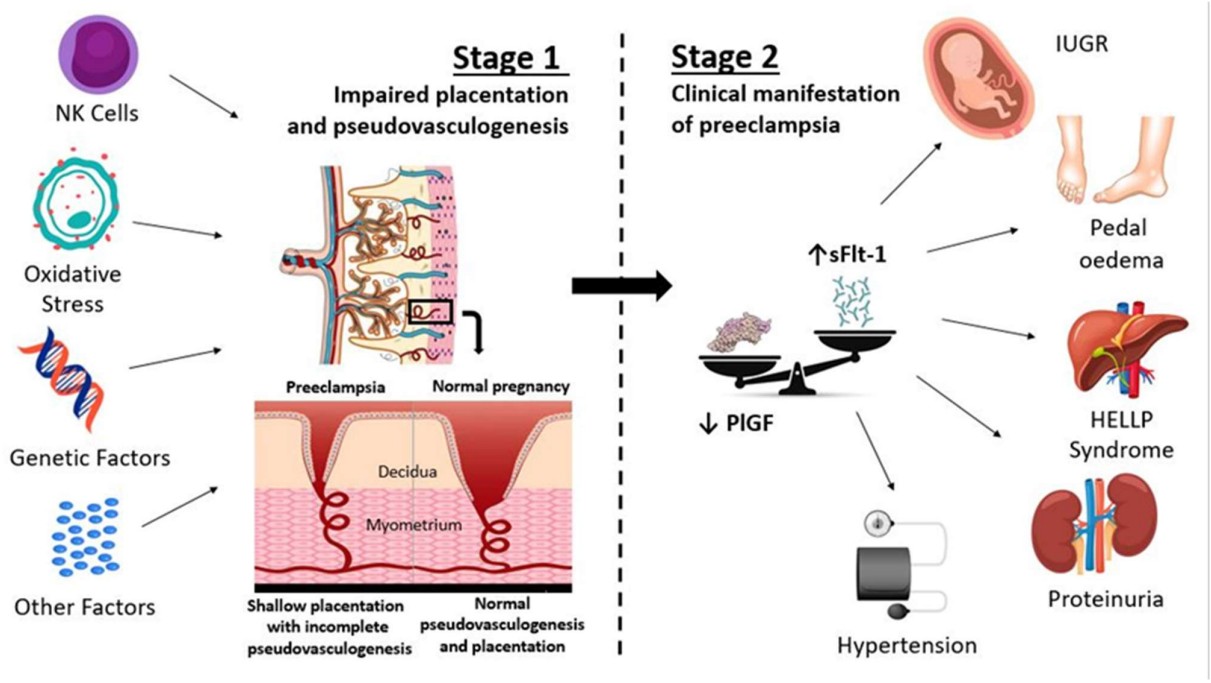


Figure 2: Two stage pathophysiology of preeclampsia.

Adapted from: Shanmugalingam R et al, Aspirin in the prevention of preeclampsia: the conundrum of how, who and when. Journal of Human Hypertension 33, 1–9 (2019).

PlGF: placental growth factor, sFlt-1: soluble fms-like tyrosine kinase -1, HELLP: Haemolysis, Elevated Liver enzymes, Low Platelet count, IUGR: intrauterine growth restriction.

1.1.1 Stage 1: Impaired placentation

Stage one of the pathophysiology describes impaired cytotrophoblast invasion and pseudovasculogensis of the maternal uterine arteries. In normal pregnancy, the maternal spiral arteries are invaded by placental cytotrophoblast cells that replaces the endothelial lining of the spiral arteries and initiates pseudovasculogenesis, resulting in remodelling of the vessel wall (7). These alterations result in vasodilatation of the spiral arteries, allowing for an increase in placental blood flow with reduced resistance (8, 9). In preeclampsia, the cytotrophoblastic invasion of maternal spiral arteries is impaired, resulting in narrow spiral arteries that perfuses the placental bed with higher resistance (5).

Cellular control of cytotrophoblast invasion depends on interactions between maternal decidua and fetal trophoblast, and is determined by local oxygen tension and immune-mediated interactions (10). *In-vitro* studies have demonstrated that increased oxygen tension inhibits cytotrophoblasts from differentiating into cells with “invasive” phenotype and reduces expression of vascular endothelial (VE)-cadherin, an endothelial junction molecule, that helps regulate cytotrophoblast invasion (11).

In addition to oxygen tension, immune mechanisms are also thought to play a role in cytotrophoblast invasion and pseudovasculogenesis. Redman *et al* described placentation as nature's transplant, drawing parallel to a semi-allogenic transplant, except, it is the placenta, and not the fetus that constitutes the 'transplant' in pregnancy (11). The placenta plays an important role in maintaining a balance; allowing for trophoblast invasion and adequate pseudovasculogenesis, whilst also preventing

uncontrolled trophoblast invasion (12). The maternal immune system maintains this balance by preventing overly robust trophoblast invasion whilst also preventing an exaggerated immune response in reaction to the trophoblastic invasion. In normal pregnancy, there is a predominance of a Th2 cytokine profile that consists of anti-inflammatory properties. The Th2-type cells secrete interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-10 (IL-10) and has been shown to contribute towards trophoblast invasion and differentiation along with placental proliferation and angiogenesis (20, 21). Additionally, IL-10 has been shown to be a strong suppressor of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interferon-gamma (IFN-ɣ) with additional anti-apoptotic features (22). In women at risk of preeclampsia, there is a predominance of Th1 pro-inflammatory cytokine profile, often from the first trimester, resulting in an abnormal Th1/Th2 balance (23-25). Cytokines interleukin-1 (1L-1), interleukin-1β (1L-1β), interleukin-2 (IL-2), interleukin-8 (IL-8), TNF-α and IFN-ɣ are characteristic of Th1-type immunity and are associated with cell-mediated cytotoxic and inflammatory response. Therefore, the abnormal Th1/Th2 maternal cytokine balance in the first trimester is thought to play a role in impaired placentation and angiogenesis (11).

Genetic studies suggest that the susceptibility to preeclampsia may be influenced by polymorphic human leukocyte antigen-C (HLA-C) ligands and killer immunoglobulin receptors (KIRs) present on natural killer (NK) cells (5, 7). These data suggest that alterations in NK cell signalling may mediate the abnormal placentation noted in preeclampsia (12). It is hypothesized that the shallow placentation and inadequate

transformation of spiral arteries that occurs in Stage 1 of the disease subsequently leads to placental ischemia and the maternal syndrome of preeclampsia (Stage two) (7, 13).

1.1.2 Stage 2: Clinical manifestation of the syndrome

Two endogenous anti-angiogenic proteins of placental origin—circulating soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) have a role in contributing towards the pathogenesis of preeclampsia in response to placental ischemia in stage two of the disease process (14, 15). sFlt-1 is a splice variant protein of vascular endothelial growth factor (VEGF) receptor Flt-1 and acts as a potent antagonist to VEGF and placental growth factor (PlGF). Plasma sFlt-1 concentrations have been found to be increased in women with preeclampsia approximately 2-5 week preceding the onset of clinical symptoms (6, 14, 16) along with a decrease in plasma PlGF concentrations (6, 16). In animal models, increasing circulating sFlt-1 resulted in a syndrome of hypertension, proteinuria, and glomerular endotheliosis, resembling preeclampsia (Figure 1) (5, 14, 15). The increased understanding of the sFlt-1/PlGF imbalance in the second stage of the disease process has led to the clinical utility of this ratio in predicting preeclampsia in women with suspicious clinical features (17-20).

Whilst the role of sFlt-1 and PlGF imbalance in the second stage of preeclampsia is well recognized, PlGF's additional role in the first stage of the disease process is continuing to strengthen. PlGF is a member of the VEGF family and is predominantly expressed in the placenta, although it is also expressed at low levels in many other tissues. Like VEGF, PlGF is pro-angiogenic as it enhances the activity of VEGF by competitively binding to VEGFR-

1 receptors. The role of PlGF in placental development is likely to be in the promotion of development and maturation of the placental vascular system through its influence on differentiation of uterine NK cells and proliferation of trophoblast cells (20, 21). In normal pregnancy, maternal plasma concentration of PlGF increases throughout gestation, peaking at approximately 26–30 weeks gestation with some variation due to smoking status, ethnicity, and pre-existing diabetes (20, 22). However, women at risk of developing preeclampsia and intrauterine growth restriction (IUGR) have low first trimester plasma PlGF concentration with this now being recognized as a biomarker of poor placentation (23-25). Consequently, PlGF is now used as a validated, commercially available, maternal biomarker as part of the first trimester screening algorithm to identify women who are at risk of developing preeclampsia (25, 26).

1.2 The use of aspirin in the prevention of preeclampsia

The understanding of the thromboxane-2 (TXA2) and prostaglandin-2 (PGI2) imbalance in preeclampsia led to the trial use of aspirin in managing preeclampsia in 1978 (21-23). Subsequently, aspirin was observed to have a prophylactic role in preventing preeclampsia in 1985, when Beaufils *et al* used a combination of aspirin (150 mg) and dipyridamole (300 mg) in first trimester and demonstrated a reduction in the rate of preeclampsia in the treatment group (0/48 vs 6/45; $p<0.05$) (24). Similarly, in 1991, the EPREDA trial proposed an added benefit of aspirin (150 mg daily) in reducing the rate of IUGR in comparison to placebo (20/156 [13%] vs 19/73 [26%]; $p<0.02$) (25). However, subsequent studies on prophylactic aspirin were contradictory, with equivocal or absence of benefit in preventing preeclampsia (26-28). These studies, however, were confounded by varying doses of aspirin, heterogenous inclusion criteria and varied

timing of aspirin initiation. In the 2000s, aspirin re-emerged as a promising prophylactic agent (29-31). Between 2007 and 2010, Bujold *et al* and Askie *et al* demonstrated a benefit in the use of aspirin (RR 0.47: 95% CI 0.34-0.65) (30, 31). In 2017, Rolnik *et at* demonstrated a significant risk reduction of up to 70% when 150mg of aspirin is commenced prior to 16 weeks of gestation through a landmark multicentre, placebo controlled, randomized control trial (29). Whilst these studies have demonstrated variable outcomes, collectively, they provide useful safety data in demonstrating a lack of aspirin related complications in both the mother and fetus (26, 27, 30, 32).

Risk stratifying women for preeclampsia is challenging. Current clinical guidelines suggest risk stratification based on clinical history (22, 23), however, a meta-analysis demonstrated that clinical history alone has a detection rate of 37% for early-onset preeclampsia and 28.9% for late-onset preeclampsia (33). In nulliparous women, clinical history alone detects 37% of preeclampsia for a false positive rate of 5% (34). Prior history of preeclampsia was the second strongest predictor factor, which is not applicable to nulliparous women (35). Mean arterial pressure (MAP), compared to systolic or diastolic reading alone has been discussed as a clinical predictor of preeclampsia. A prospective screening study utilising MAP (expressed as log multiple of the median) measured between 11^{+0} and 13^{+6} weeks gestation and maternal variables demonstrated a preeclampsia detection rate of 62.5% for a false-positive rate of 10% (36, 37). A predictive testing model incorporating maternal factors and second trimester uterine artery pulsaltility index (PI) demonstrated a detection rate for early and late preeclampsia of 100% and 56.4%, respectively for a false positive rate of 10% (36). More recently, the combination of maternal placental biomarkers; pregnancy associated

plasma protein A (PAPP-A) and PlGF along with MAP and clinical history has been shown to be sensitive, detecting 96% of cases of preeclampsia requiring delivery before 34 weeks and 54% of all cases of preeclampsia with a false-positive rate of 10% (38). Whilst screening algorithms have been shown to effectively identify women at risk, particularly nulliparous women, its use is often limited by accessibility to the required resources. Therefore, many centres rely on clinical history for risk assessment. In keeping with this, the current guidelines recommend the use of clinical history in risk stratifying women (39-41). This, however, may change based on the accessibility and cost- benefit analysis of the new screening markers.

The importance of timing of initiation of aspirin is now recognized (30, 42). Bujold *et al*'s meta-analysis demonstrated that commencing low-dose aspirin prior to 16 weeks of gestation significantly reduced the rate of preeclampsia (RR 0.47: 95% CI 0.34-0.65) and IUGR (RR 0.44: 95% CI 0.30-0.65)(30). The observed benefit was lost when aspirin was commenced after 16 weeks of gestation (preeclampsia: RR 0.81: 95% CI 0.63-1.03 and IUGR: RR 0.98: 95% CI 0.87-1.10) (30). Contrary to this, another meta-analysis suggested that the 16 weeks gestation cut-off was not significant (43). Meher *et al* conducted an individual participant dataset analysis of the PARIS (Perinatal Antiplatelet Review of International Studies) Collaboration dataset and suggested that the outcomes with low-dose aspirin were consistent when commenced before or after 16 weeks of gestation (RR 0.90: 95% CI 0.79 - 1.03) for <16 weeks and for ≥16 weeks (RR 0.90: 95% CI 0.83 - 0.98) (interaction test p = 0.98)(31, 43). The analysis of the PARIS Collaboration data, however, only demonstrated a modest overall preeclampsia reduction of ~10% with the use of prophylactic aspirin (43). The current guidelines suggest initiating aspirin before 16

weeks of gestation (39-41), however, recent studies suggest earlier initiation of aspirin (29, 44, 45). This, however, has not been assessed with a prospective randomised trial at the time of this thesis.

1.3 The gaps in the literature

1.3.1 Mechanism of action of aspirin

Aspirin is a non-selective, irreversible cyclooxygenase-1 (COX-1) inhibitor (Figure 3). It prevents the production of TXA2 in platelets by acetylating a serine residue at position 529 of the COX-1 isoform. This results in an anti-thrombotic effect with a decrease in the TXA2/PGI2 ratio (46).

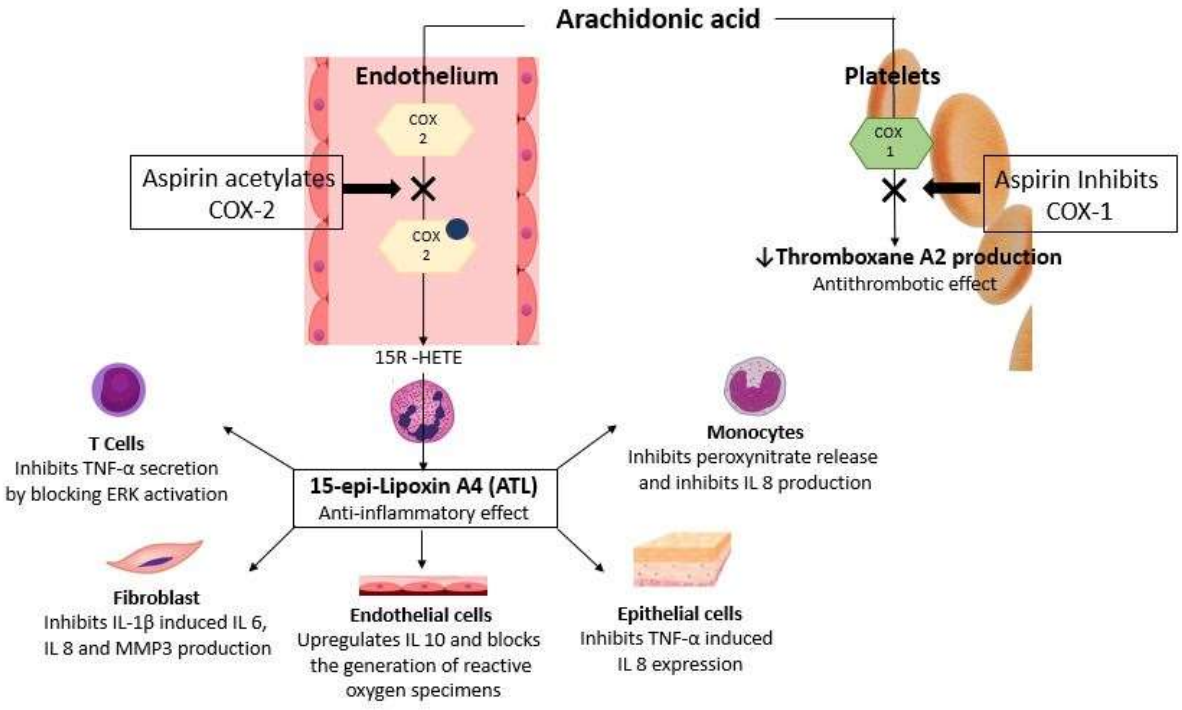


Figure 3: Mechanisms of action of aspirin in the prevention of preeclampsia.

Adapted from Shanmugalingam R et al, Aspirin in the prevention of preeclampsia: the conundrum of how, who and when. Journal of Human Hypertension 33, 1–9 (2019).

15R-HETE: 15R-hydroxyeicosatetraenoic acid, ATL: Aspirin triggered lipoxin/15-epilipoxin-A4, COX-1: Cyclooxygenase-1, COX-2: Cyclooxygenase-2, ERK: Extracellular-signal-regulated kinase, IL-1β: Interleukin 1-beta, IL 6: Interleukin 6, IL 8: Interleukin 8, IL 10: Interleukin 10, MMP3: Matrix Metallopeptidase 3, TNF-α: Tumour Necrosis Factor Alpha

Early studies on the mechanism of aspirin's action in preeclampsia demonstrated an aspirin-induced decrease of TXA2 concentration and alteration of the TXA2/PGI2 ratio (47, 48). Roberts *et al* noted a dose mediated response with TXB2 (inactive metabolite of TXA2) and prostacyclin production in both non-pregnant female and male volunteers (49). Their study demonstrated that 50 mg of aspirin daily reduced TXB2 plasma concentration in almost 95% of their volunteers without an alteration in prostacyclin concentration. The use of 100-300 mg of aspirin a day, however, completely reduced TXB2 concentration and partially reduced prostacyclin concentration by 27% (49). When examined in pregnant women, Sibai *et al* demonstrated a dose response reduction in TXA2 plasma concentration (of up to 98%) after 1 week of 80 mg aspirin therapy (50). It was then hypothesized that aspirin improves placental blood flow and minimizes risk of placental thrombosis by reducing TXA2 and PGI2 concentrations. Studies that examined the correlation between TXA2/PGI2 concentration and maternal uterine artery pulsatile indexes (PI) demonstrated that aspirin reduced platelet aggregation and inhibited vasoconstriction with enhanced uterine blood flow (51). At that point, clinically, however, aspirin, had only demonstrated a modest benefit in the prevention of preeclampsia, with a risk reduction of 10- 20% despite the postulated modulation of the TXA2/PGI2

imbalance (52, 53). One explanation for this variation is that the TXA2/PGI2 imbalance is not the only pathogenic pathway for preeclampsia. There may also be a wide inter-patient variation in the degree of aspirin effect in addition to a gestation specific effect. This is partially answered in the observations that the preventative effect of aspirin is greater with early onset preeclampsia than late onset preeclampsia (29) and that the prophylactic benefit of aspirin is most evident when commenced prior to 16 weeks of gestation; with 16 weeks corresponding with the time at which placentation is complete (42). This suggests that aspirin potentially, has greater influence on placental development and less so on maternal biochemical dysregulation (42, 44).

In 1989, Claria *et al* described the generation of endogenous 15-epilipoxin A_4, better known as aspirin-triggered lipoxins (ATL) with the use of aspirin (54). The biosynthesis of ATL occurs through the cyclooxygenase-2 (COX-2) pathway (Figure 3). Aspirin acetylates COX-2, switching its catalytic activity and enabling the conversion of endogenous arachidonic acid to 15*R*-hydroxyeicosatetraenoic acid (15*R*-HETE) (54, 55). The acetylated form of COX-2 remains active in vascular endothelial cells and mucosal epithelial cells to produce 15*R*-HETE. The 15*R*-HETE generated by endothelial and epithelial cells is then transformed by leukocyte 5-lipoxygenase into 15-epilipoxin-A_4 and 15-epilipoxin-B_4. Given that ATL is the endogenous 15*R* enantiomers of endogenous Lipoxin-A_4 (En-Lipoxin-A_4), it shares many bioactivities with En-Lipoxin-A_4, including downregulation of polymorphonuclear neutrophils (PMN) dependent acute inflammation (56, 57). ATL is 50% less effectively enzymatically converted to its biologically inactive group of 15-oxo-metabolites in comparison to En-Lipoxin-A_4.

Therefore, consequently, ATL is found to have a 2-fold greater half-life compared to En-Lipoxin-A_4 (57).

Given its anti-inflammatory properties, ATL has been shown to block the generation of reactive oxygen species in endothelial cells, inhibit nuclear factor kappa-light-chain-enhancer of activated B cells (NF-Kb) activation and tumour necrosis factor alpha (TNF-α) secretion in activated T cells (Figure 3) (55, 58-60). This is supported by studies where the use of exogenous ATL have demonstrated positive outcomes in experimental models of inflammatory conditions such as peritonitis (61), colitis (62), ischemic reperfusion injury (61) and angiogenesis (56). The endogenous generation of ATL with aspirin consumption was first confirmed by Fiorucci *et al* in 2003 (63). This was further demonstrated by Chiang *et al* who showed a rise in ATL concentration with low dose aspirin (81 mg) (0.25 ± 0.63 ng/ml, p = 0.04).

More recently, En-Lipoxin-A_4 and ATL have been shown to have a role in reproductive medicine. Xu *et al*'s described a two-fold reduction En-Lipoxin-A_4 in women with established preeclampsia (64). This was also demonstrated in a lipopolysaccharide (LPS) induced preeclampsia rat model (64, 65). Furthermore, low first trimester plasma En-Lipoxin-A_4 concentration has also been proposed as a potential screening biomarker in identifying women who are at risk of intrauterine growth dysregulation (66). Cell culture studies have demonstrated that exogenous ATL has the potential to reverse the inflammatory process observed in preeclampsia by upregulating IL-10 and nitric oxide (NO) whilst downregulating the generation of TNF -α (67, 68). Given this, the role of ATL

production with aspirin, in high-risk women with En-Lipoxin-A_4 dysregulation is of growing interest, however, remains unclear (69).

1.3.2 Clinical use of aspirin – influence of dose, adherence, and resistance

The relatively modest benefit of prophylactic aspirin in some obstetric studies have raised questions on optimal aspirin dosing, aspirin resistance and non-adherence amongst high-risk pregnant women. Initial studies that demonstrated prophylactic benefit of aspirin, prescribed a daily dose of 150-300 mg (24). Subsequent studies, however, used lower dose ranging from 75 – 150 mg daily (30, 42), whilst more recent studies suggest better clinical outcomes with the use of 150 mg daily (44, 70). Most guidelines at the time of this thesis do not specify a recommended dose of aspirin but suggest a range of 75 – 150 mg with 100 mg being the most commonly recommended (39, 71). The use of 150 mg in recent studies, however, has resulted in a shift towards the use of 150 mg in some guidelines (72). Nevertheless, there remains a significant paucity in clinical and pharmacokinetic data that directly compares the use of 100 mg to 150 mg of aspirin in pregnant women to support such a change in clinical practice. It, therefore, remains unclear if the use of 150 mg daily results in better bioavailability of aspirin and consequently, better clinical outcomes.

Aspirin non-responsiveness or resistance, refers to a lack of anti-platelet action or desired clinical effect with the use of aspirin (73). Multiple factors such as platelet turnover, dose of aspirin and patient's weight have been shown to contribute towards

aspirin non-responsiveness in the non-pregnant population (Figure 4) (73, 74). Non-obstetric cardiology studies have demonstrated a 2 to 3 fold higher incidence of morbidity and mortality with aspirin non-responsiveness, suggesting the need to further examine its actual prevalence (75, 76). Subsequent studies, however, attributed 50% of aspirin non-responsiveness to inadequate adherence, suggesting inadequate adherence as the strongest confounder in aspirin non-responsiveness (77, 78).

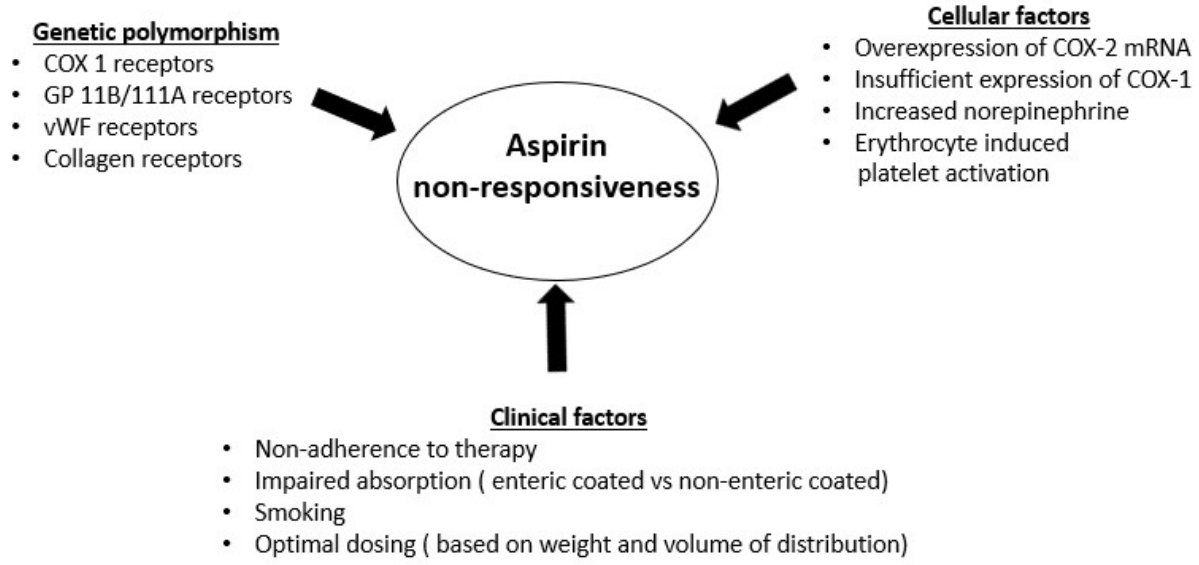


Figure 4: Factors thought to contribute toward towards aspirin non-responsiveness.

Adapted from Shanmugalingam R et al, Aspirin in the prevention of preeclampsia: the conundrum of how, who and when. Journal Human Hypertension 33, 1–9 (2019).

COX 1: Cyclooxygenase-1, COX 2: Cyclooxygenase-2, GP IIB/IIIA: Glyocprotein IIB/IIIA, mRNA: messenger ribonucleic acid, vWF: von Willebrand factor

In obstetrics, the actual prevalence of clinical aspirin resistance or non-responsiveness in the pregnant cohort is unknown. Caron *et al*'s study showed that approximately 30% of pregnant women demonstrated a lack of laboratory response to aspirin at 81 mg, based on platelet function analyzer (PFA-100) assessment (79). The lack of PFA-100 response was overcome with a higher dose of aspirin (162 mg daily) (79). It was, however, unclear if the observed lack of biochemical response with 81mg of aspirin was a result of true aspirin resistance or lack of adherence as demonstrated by non-obstetric aspirin studies. Non-adherence with prescribed medications amongst pregnant women is reported to vary between 20-80% (83-85). Yet, only 30% of all obstetric aspirin studies over the last 45 years have assessed for adherence with aspirin. Even so, adherence was inconsistently defined and assessed in these studies (80, 81). Recent data suggests that high-risk women will have to be at least 90% adherent with their prescribed aspirin to benefit from it (81). Most studies have utilised indirect, qualitative measure of adherence through self-reporting questionnaires to assess adherence (80, 81). Whilst qualitative assessment provides a convenient and cost effect method of assessment, its reliability is highly questionable, given the potential for reporting and recall bias (82). The use of quantitative assessment of adherence with aspirin, through platelet aggregation makers or metabolite markers of aspirin are more reliable methods of assessment of adherence (83, 84). However, its use is limited by the cost and need for laboratory expertise. Given the variability in the current literature, the actual prevalence of aspirin non-adherence amongst high-risk pregnant women and its influence on their desired clinical outcome remains undetermined.

Other factors that may contribute towards aspirin non-responsiveness includes difference in aspirin preparation: enteric coated (EC) vs non-enteric coated (Non-EC), effect of smoking and presence of gene polymorphism or cellular dysregulation (Figure 4) (85-87). The consensus on the recommended preparation of aspirin in pregnancy remains unclear. Enteric coated (EC) aspirin delays and reduces systemic absorption by ~ 20% in healthy non-pregnant volunteers (88-90). These factors, however, have not been examined in pregnancy and its influence on the clinical efficacy of aspirin in high-risk pregnancies remains unknown.

1.4 The rationale of this thesis: Aims and Objectives

This thesis was aimed at answering the research questions relating to an alternative mechanism by which aspirin reduces the risk of preeclampsia and to examine factors (aspirin dose, adherence, and resistance) that influences the desired obstetric outcomes.

- **Chapter 4 (Scholarly Research Paper 1): Mechanism of action of aspirin**

  This chapter examines the difference in longitudinal En-Lipoxin-A_4 concentration in normal pregnancy compared to high-risk pregnancy and the influence of aspirin on maternal plasma concentration of ATL, PlGF and selected cytokine profile of high-risk pregnancy.

- **Chapter 5 (Scholarly Research Paper 2): Pharmacokinetics of aspirin in pregnancy**

  The chapter examines the pharmacokinetics of aspirin in pregnancy.

- **Chapter 6 (Scholarly Research Paper 3): Aspirin resistance and non-adherence in pregnancy**

  This chapter examines the prevalence of aspirin resistance and non-adherence amongst high-risk pregnant women and its influence on obstetric outcomes.

- **Chapter 7 (Scholarly Research Paper 4): Factors that influence adherence to aspirin in pregnancy**

  This chapter examines patient reported factors that influences adherence with aspirin in pregnancy.

Journal of Human Hypertension
https://doi.org/10.1038/s41371-018-0113-7

REVIEW ARTICLE

Aspirin in the prevention of preeclampsia: the conundrum of how, who and when

Renuka Shanmugalingam[1,2] · Annemarie Hennessy[1,2] · Angela Makris[1,2]

Received: 26 July 2018 / Accepted: 28 August 2018

CHAPTER 2

METHODS

2.1 Longitudinal cohort study

2.1.1 Participant recruitment

This study was conducted as a non-interventional, observational, longitudinal cohort study. Pregnant women were opportunistically recruited from three outpatient high-risk pregnancy clinics across the South Western Sydney Local Health District (SWSLHD), Australia. The three hospitals were Liverpool, Bankstown, and Campbelltown Hospitals (Figure 5). Ethics approval was obtained (South Western Sydney Local Health District (SWSLDH) human ethics committee) (HE 16/184) and participants provided written informed consent to participate in this study.

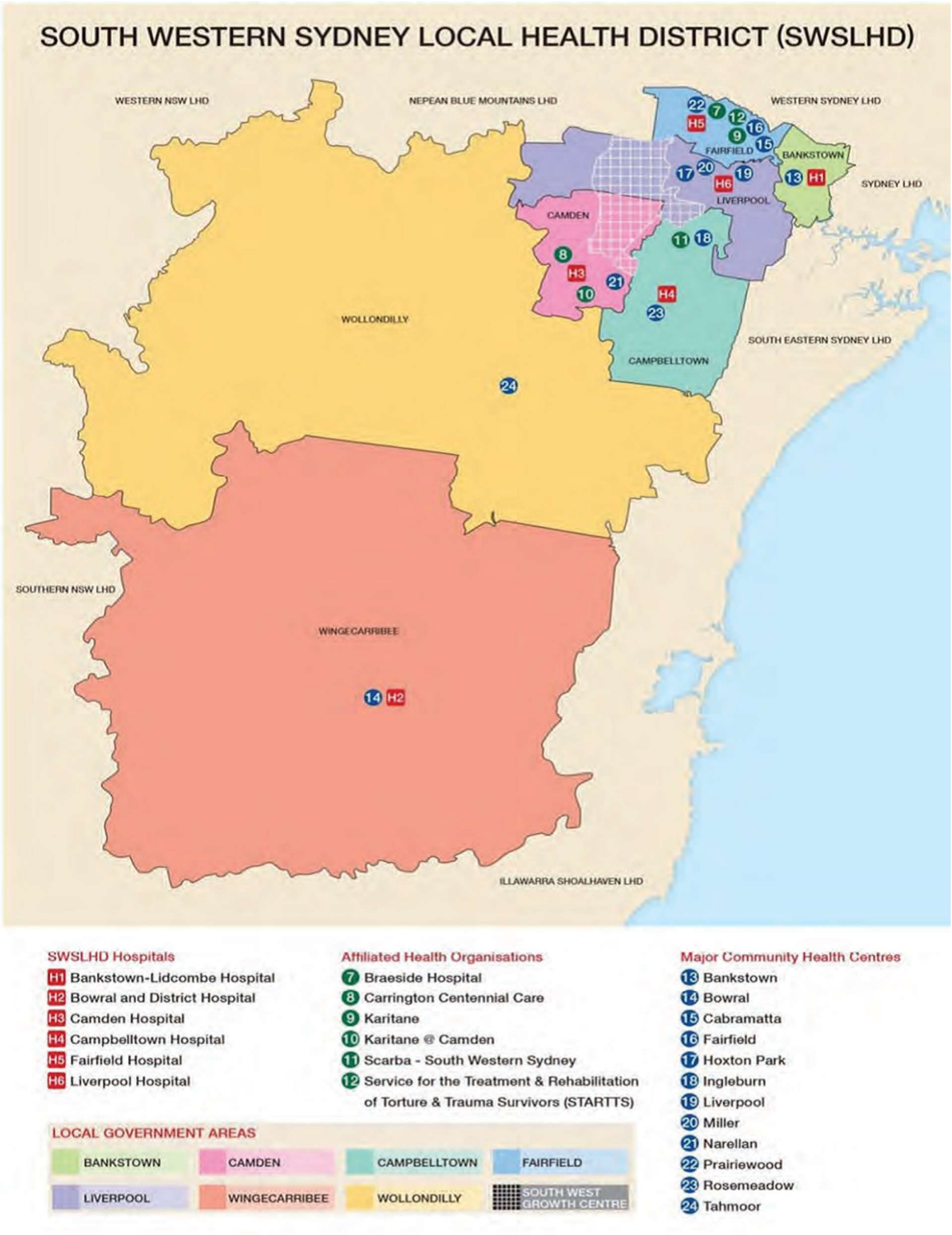


Figure 5: Area map of the South Western Sydney Local Health District

Adapted from SWSLHD planning unit

https://www.swslhd.health.nsw.gov.au/planning/swslhdcommunities.html

Liverpool Hospital is a tertiary referral unit with an annual delivery rate of 4000 women. Bankstown and Campbelltown Hospitals are both outer metropolitan centres with an annual delivery rate of 2500 and 3500 respectively (91) . All pregnant women at the high-risk clinics were risk stratified by their treating clinicians based on the National Institute for Health and Care Excellence, UK (NICE) clinical guidelines or the Fetal Medicine Foundation (FMF) first trimester screening algorithm (Table 1). Based on the NICE guidelines, the clinicians commenced aspirin in women who were found to have 1 major risk factor or at least 2 moderate risk factors (35). The FMF screening scans were performed in private medical imaging facilities external to the hospital. The treating clinicians commenced women on aspirin based on their risk as identified by the FMF algorithm. Women were advised to take either 100 mg or 150 mg of aspirin by their clinicians based on the centre specific practise. A large study favouring the use of 150 mg of aspirin (29) was published mid-way through the recruitment of this longitudinal study, however, given the lack of strong recommendation from clinical guidelines, some centres within the SWSLHD continued to prescribe 100 mg of aspirin. There were no alteration to the initial prescribed dose of aspirin (no cross over between 100 mg and 150 mg of aspirin). All women who participated in this study underwent their antenatal care at a SWSLHD public hospital.

The inclusion and exclusion criteria were as below:

Inclusion criteria:

- Risk stratified as high- risk for preeclampsia by treating clinician
- ≤ 20 weeks gestation at time of recruitment (estimated gestation as verified by dating scan)

Exclusion criteria:

- ≥ 20^{+1} weeks of gestation at time of recruitment
- Non-English-speaking
- Unable to provide written informed consent
- < 16 years of age
- Commenced on aspirin prior to recruitment

NICE Guidelines (35)	FMF First trimester screening (92)
Major risk factors • Chronic hypertension • Autoimmune conditions • Chronic kidney disease • Previous preeclampsia • Type 1 or type 2 diabetes **Moderate risk factors** • First pregnancy • Age 40 years or older • Pregnancy interval of more than 10 years • BMI of ≥ 35 kg/m^2 • Family history of preeclampsia • Multi-fetal pregnancy.	FMF first trimester screening with combined MoMs of maternal MAP, uterine artery PI, PlGF and PAPP-A into an algorithm with a cut off of 1:100.

Table 1: Risk stratification criteria.

NICE: National Institute for Health and Care Excellence, FMF: Fetal-Maternal Medicine, BMI: Body mass index, MoMs: Multiple of the Median, MAP: Mean arterial pressure, PI: pulsatility index, PlGF: placental growth factor, PAPP-A: pregnancy-associated plasma protein-A

2.1.2 Sample size

Sample size was calculated based on the assumption that the untreated high-risk pregnant women have a preeclampsia-risk rate of 40% and that aspirin treated high-risk pregnant women will have a 50% reduction in preeclampsia-risk compared to the untreated group (93). Thus, approximately 60 women in each group (aspirin and non-aspirin) group would be needed to have 80% power assuming an alpha (two tailed) of 0.05. The target recruitment number was inflated to 220 women in total to allow for a predicted 20% dropout in both groups and a high prevalence (50%) of inadequate adherence.

2.1.3 Study protocol

Blood samples and clinical data were collected prospectively at baseline (prior to first dose of aspirin). Subsequently, serial blood samples, clinical data and self-reported adherence assessment was conducted at 16, 20, 24, 28, 32 and 36 weeks of gestation (Figure 6). High-risk pregnant women who were not prescribed aspirin, either due to aspirin intolerance or delayed presentation (between 16^{+1}-20 weeks of gestation) (Figure 6) followed the same protocol and served as the control high-risk pregnancy group.

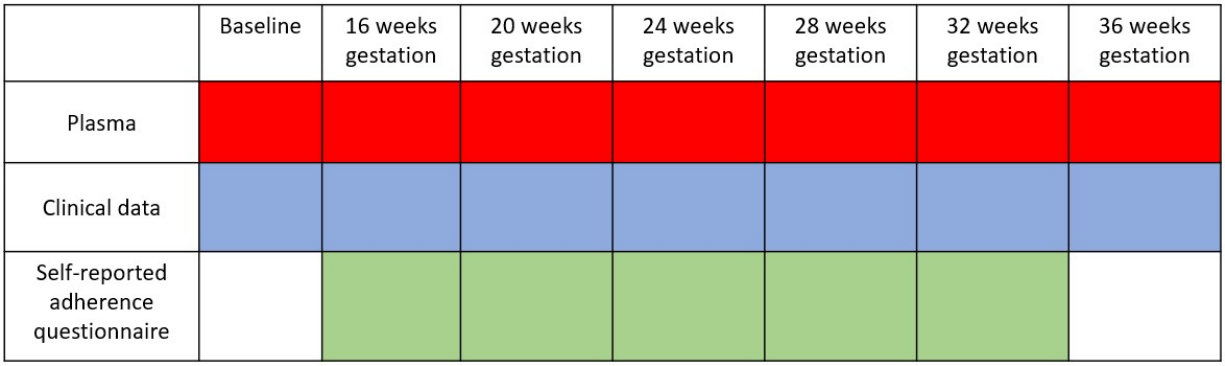


Figure 6: Illustrative representation of study protocol.

Blood samples were collected via a 21FG Vacutainer Push Button needle (Becton, Dickinson and Company USA) into 3 x 4 ml VACUETTE® K2EDTA tubes (Greiner Bio-One International) and 2 x 3.8 ml tubes containing 0.38 ml of 0.129 mol/l buffered sodium citrate (pH 5.5) for PFA-100 analysis. Samples in two of the three VACUETTE® K2EDTA tubes were centrifuged immediately at 3000 rpm for 10 minutes, plasma was aliquoted (220 µL) and stored at -80^{0}C until analysis. The third VACUETTE® K2EDTA tube and both sodium citrate tubes were sent to the South Western Sydney pathology service at Liverpool Hospital, NSW, Australia (National Association of Testing Authorities, Australia (NATA) accredited) at room temperature for a full blood count and PFA-100 assessment within 4 hours of collection. The investigators were blinded to the PFA-100 results till after study completion. Clinical data were collected in real time at each time point. Details on assessment of self-reported adherence is as elaborated under Chapter 2.4.1

2.2 Biochemical analysis

2.2.1 Magnetic Luminex® analysis

Simultaneous quantitative analysis of plasma IL-1β, TNF-α, IL-8, IL-10 and IFN-γ were performed with the use of the Magnetic Luminex® Performance multiplex human high sensitivity cytokine premixed kit A (R&D Systems, Minnesota, USA). Standards, reagents and undiluted plasma samples were prepared and assayed in duplicates based on the manufacturer's manual with the use of HydroFlex® microplate washer (Tecan Trading AG, Switzerland) (94). Briefly, cytokine-specific antibodies were pre-coated on the magnetic microplates followed by the addition of 100 μL of undiluted plasma sample into each well. Biotin-antibody cocktails, specific to the cytokines, were added to each well. Streptavidin-Phycoerythrin conjugated secondary antibody was used for detection. Prepared microplates were analysed with Luminex® 100/200® system (Luminex Corporation, Texas, USA).

Quantitative data for each cytokine was derived based on the microparticle regions specified by the manufacturer (94) and the respective standard curves by utilizing xPONENT® software (Luminex Corporation, Texas, USA) and presented in pg/mL. Samples with intra and inter-assay variation of more than 10% were repeated.

2.2.2 Platelet Function Analyzer (PFA-100) analysis

PFA-100 analysis refers to the assessment of primary haemostasis under sheer stress with a benchtop automated instrument, PFA-100 analyzer®. The PFA-100® (Dade

Behring, Düdingen, Switzerland) instrument is composed of a microprocessor-controlled device and single-use test cartridges. The test cartridges consist of a sample reservoir, a capillary and a membrane coated with 2 μg equine type I collagen and either 10 μg epinephrine bitartrate (EPI cartridge) or 50 μg adenosine 5'-diphosphate (ADP cartridge). Blood samples were analysed in duplicates within 4 hours of collection. Women's platelet count was assessed prior to PFA-100 analysis. Samples from women with platelet count of $<100 \times 10^9$/L were not assessed to minimize erroneous results.

Prior to sample analysis, test cartridges were left in room temperate for 5 minutes following which, test cartridges were placed into the cassette one at a time. Blood samples were mixed by gentle inversion (3-4 times by hand) prior to pipetting 800 μL of blood into the reservoir of the cartridge. The sample is then aspirated through a capillary with a diameter of 200 mm with constant negative pressure resulting in high shear forces (5000–6000s-1). The capillary ends in a membrane aperture with a diameter of 150 mm. Platelets adhere at the aperture where they are activated by collagen and aggregates. The two agonists, epinephrine and ADP enhances aggregation. Finally, a platelet plug occludes the aperture and blood flow stops. The time measured in seconds from the beginning of the test until formation of an occluding platelet plug is called closure time (CT). If an occluding platelet plug does not form after 300 seconds, the analysis is stopped. Samples were tested in duplicates with both reagents (EPI and ADP). Samples with an inter- and intra-assay variation of more than 10% were not reported and were repeated by the investigators. CT reference ranges of 80-170 seconds for COL/EPI and 60-120 seconds for COL/ADP were used based on the manufacture's and the SWSLHD pathology internal validation (Figure 7).

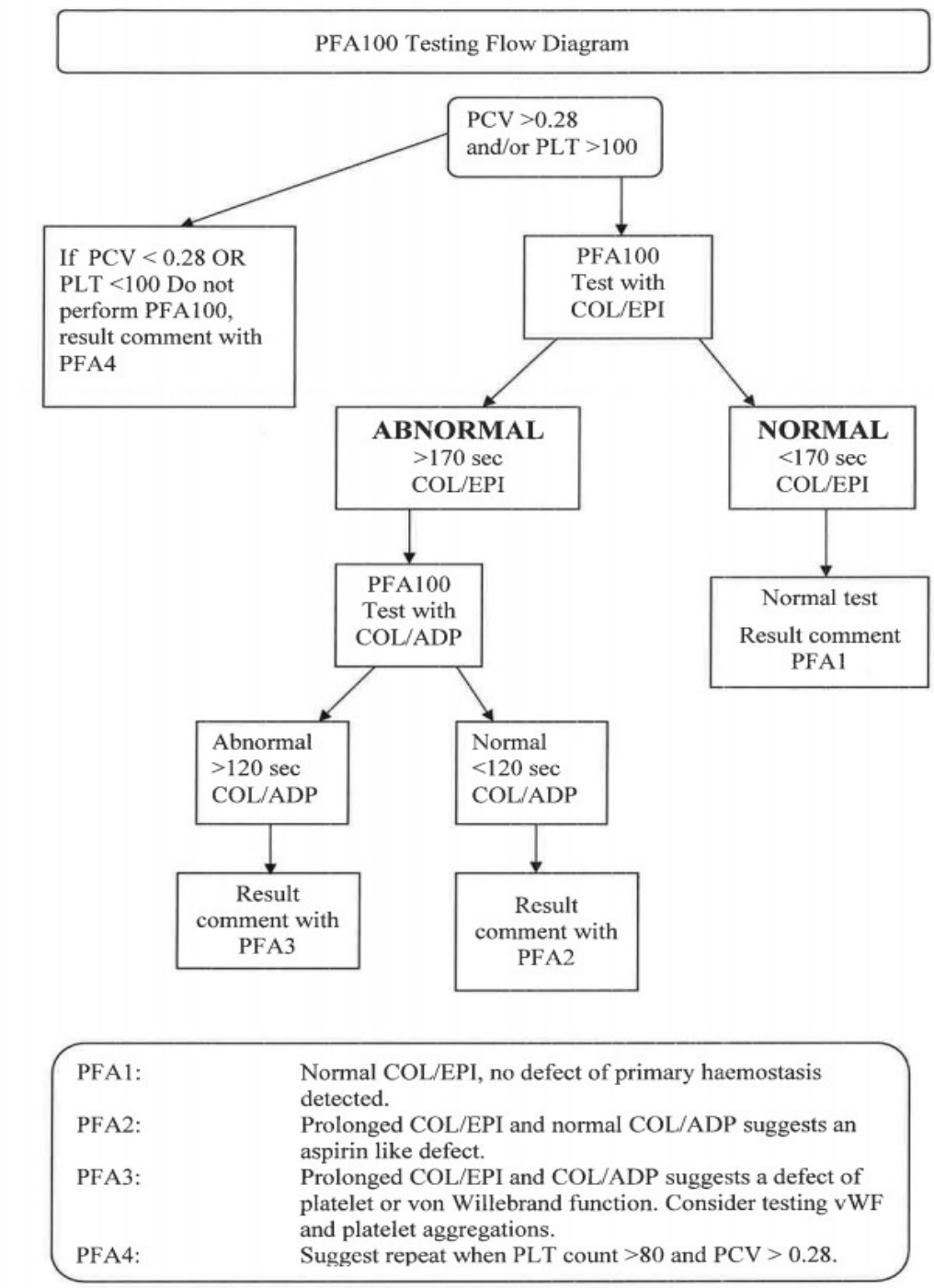


Figure 7: Flow chart of Platelet Function Analyzer (PFA-100) closure time (CT) analysis.

Adapted from SWSLHD pathology laboratory manual (95).

PFA 100: Platelet Function Analyzer 100, PCV: packed cell volume, PLT: platelet, COL/ADP: collagen/adenosine, COL/EPI: collagen/epinephrine

2.2.3 Placental growth factor (PlGF) analysis

Enzyme linked immunosorbent assay (ELISA) were performed using commercial kits for PlGF (R&D Systems, MN, USA). Lyophilised human PlGF standard provided by the manufacturer was reconstituted using proprietary calibrator diluent RD5K. This was used to produce a dilution series (in duplicates) for a standard curve ranging from 0 to 1000 pg/mL. Pre-coated 96-well plates were then filled with 100 µL of proprietary assay diluent RD1-22 followed by duplicates of 100 µL of prepared standard or plasma sample. Plate were then covered with plastic film and incubated for 2 hours at room temperature on an orbital shaker. The plates were then washed 3 times in an auto washer using 400 µL of proprietary wash buffer solution per well, following which, 200 µL of conjugate (horseradish peroxidase linked polyclonal antibody specific for human PlGF, included in the kit) was added to each well. The plates were then covered with plastic film again and incubated for 1 hour at room temperature on an orbital shaker. Following which, the plates were washed for a final time (as specified above). Subsequently, 200 µL of substrate (hydrogen peroxide and tetramethylbenzidine, included in the kit) was added to each well and incubated for 30 minutes in a darkened environment, in room temperature. As a final step, 50 µL of an acid stop solution (2N sulphuric acid, included in the kit) was added to each well. The plates were then shaken for 5 seconds within the spectrophotometer (Sunrise, Tecan, Germany) prior to being read at 450 nm with wavelength correction at 540 nm. Samples with an inter and intra-assay variation of more than 10% were repeated.

2.3 Liquid chromatography mass spectrometry (LCMS)

2.3.1 Plasma salicylic acid (SA) detection

2.3.1.1. Sample preparation for analysis

Standards were prepared in duplicates using 100 µL of blank human plasma with known concentrations of salicylic acid (SA) (Sigma Aldrich, Castle Hill, NSW, Australia). Diluted SA (in 100% methanol) was added to blank plasma in standard concentrations of 0 ng/mL, 10 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL, 200 ng/mL and 500 ng/mL. All standard preparation were spiked with a fixed concentration of 125 ng deuterated salicylic acid (D4-SA) (Santa Cruz Biotechnology, CA, USA) as internal control and vortexed for 1 minute. Samples from participants, from each time points, were prepared in duplicates of 100 µL in Eppendorf tubes and spiked with a fixed concentration of 125 ng of D4-SA as internal control.

To precipitate protein, 400 µL of 100% of acetonitrile (ACN) (Lichrosolv, Merck Milipore, Baywater VIC, Australia) was added to standards and samples and vortexed for 30 seconds. Samples and standards were then centrifuged at 3000 rpm for 10 minutes (Eppendorf Microcentrifuge Model 5415R, Hamburg Germany) following which, supernatant was transferred into glass culture tubes (12 X 75 mm disposable culture tubes, Kimble, USA) and evaporated over N_2 at 60°C (Eppendorf Concentrator plus Model 22331 Hamburg) for approximately 90 minutes. Samples and standards were then reconstituted and acidified with 1 mL of 0.1% v/v formic acid (FA) (Lichrosolv, Merck Milipore, Baywater VIC, Australia) prior to solid phase extraction (SPE) with Discovery DSC-18 1 mL cartridges (Supelco, Bellefonte, PA, USA) and a vacuum manifold. Cartridges

were preconditioned with 1 mL of 100% methanol and washed with 2 mL of 0.1% v/v trifluoroacetic acid (TFA) (Lichrosolv, Merck Milipore, Baywater VIC, Australia). The sample and standards were applied to the column, washed with 1 mL of water, and eluted with 1 mL of 100% methanol. Following which, Methanol was evaporated over N_2 at 60^{o}C for 90 minutes. Samples were then reconstituted with 100 µL of 0.1% v/v FA and spun at 3000 rpm for 5 minutes prior to transfer into high performance liquid chromatography (HPLC) inserts.

2.3.1.2. Liquid chromatography mass spectrometry (LCMS) methodology

Analysis of samples were performed with Agilent 6460 LCMS (Agilent Technologies, Santa Clara, CA, USA). Standards and samples (5 µL) were injected onto a Zorbax Eclipse XDB-C18 column (4.6 X 50 mm, 1.8 µm particle size, 600 bar; Agilent Technologies, Santa Clara, CA, USA) and eluted with the gradient consisting of 0.1% v/v FA in water (mobile phase (MP A)) and 0.1% v/v FA and 90% v/v ACN in water (Mobile phase B (MP B)). The gradient was commenced at 30% of MP B, subsequently increased to 90% at 2 minutes and maintained at 90% for another 2 minutes. Gradient was then increased to 100% of MP B within 0.5 minute, maintained at 100% for another minute, decreased to 30% by 6 minutes and equilibrated for another minute. Following which, gradient was maintained at a flow rate of 0.5 ml/min. Electrospray ionization, in negative mode, was performed using the following parameters: capillary spray voltage of 3500v, drying gas flow of 10 L/min at 325°C and nebuliser pressure of 45 psi. Optimal fragmentor voltage (90v) and collision energy voltage (15v) were obtained by flow injection analysis in MS2-product ion scan mode. The following MRM ion transitions were monitored 136.9 ->93.10 (SA)

and 141.00 -> 97.00 (D4-SA). Peak areas for SA, relative to internal standard D4-SA, was used to interpolate a standard curve and calculate SA concentration in standards and samples. Duplicates with a variation of more than 10% were repeated.

2.3.2 Plasma Lipoxin-A_4 and 15-epilipoxin-A_4 detection

2.3.2.1. Sample preparation

Standards were prepared in duplicates using 100 µg/mL of 15-epilipoxin-A_4 (Sapphire Bioscience Pty Ltd, Australia) and 100 µg/mL of Lipoxin-A_4 (Sapphire Bioscience Pty Ltd, Australia). Briefly, 2 µL of each standards were added to 196 µL of 100% methanol to constitute a stock solution containing 1 µg/mL of 15-epilipoxin-A_4 and Lipoxin-A_4 respectively. Stock solution was then diluted with varying volumes of 50% methanol to obtain series of standard concentrations ranging from 0 ng/mL to 200 ng/mL. Deuterated (D5) Lipoxin-A_4 (Lipoxin A_4-d5) (Sapphire Bioscience Pty Ltd, Australia) was utilized as internal standard, in a fixed concentration of 20 ng/mL, across all standard concentrations.

Plasma samples, collected at all time points, were prepared, and analysed in duplicates. Plasma sample preparation involved protein precipitation of 50 µL of plasma with 200 µL of 100% of ACN (1:4, v/v) containing 2 ng Lipoxin A_4-d5 in Eppendorf tubes. The tubes were vortexed for 30 seconds and subsequently submerged in ice for 15 minutes prior to being centrifuged at 3000 rpm for 10 minutes. Supernatant were then transferred into glass culture tubes (12 x 75 mm disposable culture tubes, Kimble, USA) and evaporated at 45°C (Eppendorf Concentrator Model 22331 Hamburg) for approximately 90 minutes.

Standards were then reconstituted and acidified with 1 mL of 0.1% FA prior to SPE using Discovery DSC-18 1 mL cartridges (Supelco, Bellefonte, PA, USA) on a vacuum manifold. SPE cartridges were preconditioned with 1 mL of 100% Methanol and washed with 2 mL of 0.1% FA. Following which, samples were applied to the column and washed with 1 mL of 0.1% FA prior to elution with 600 µL of 100% Methanol twice. Methanol eluate were then evaporated at 45°C for 90 minutes. Samples were then reconstituted with 100 µL of 20% Methanol containing 0.05% Acetic Acid (AA) followed by a final spin at 1400 rpm for 10 minutes prior to transfer into HPLC vial inserts.

2.3.2.2. Liquid chromatography mass spectrometry (LCMS) methodology

LCMS analysis were performed with Shimadzu triple quadrupole mass spectrometer LCMS8050 coupled with UHPLC Nexera X2 series (Shimadzu Corp. Kyoto, Kyoto Prefecture, Japan). Separation of En-Lipoxin-A_4 and ATL in plasma were achieved with a Phenomenax Kinetex® C18 (150 x 2.1 mm, 1.7 µm) (Phenomenax Pty Ltd, NSW, Australia) column fitted with a SecurityGuard™ ULTRA Cartridges UHPLC C18 (2.1 mm) (Phenomenax Pty Ltd, NSW, Australia), with MP A containing 0.05% AA in water and MP B consisting of 100% Methanol. Injection volume of 5 µL was used with a total run of 24 minutes at a flow rate of 0.5 mL/min. The gradient was started at 59% of MP B and maintained isocratic for 20 minutes. Gradient was subsequently increased to 95% within 0.5 minutes and maintained for 2 minutes before returning to 59% in the next half minute. Tandem MS was performed using electrospray ionization, in negative mode, with the following parameters: Nebulizing gas flow of 3 L/min and heating gas flow of 10 L/min at 300°C. Drying gas flow was set at 10 L/min with heat block temperature of

400°C. The optimal fragmentor voltage and respective collision energy voltage for each molecule were obtained by flow injection analysis using Optimizer. The following MRM ion transitions were monitored m/z 351->217 and 351->115 for En-Lipoxin-A_4 and ATL with additional channels of m/z 315->135 for En-Lipoxin-A_4 and m/z 315->59 for ATL, respectively. Lipoxin-A_{4-} d5 peak was used as an internal standard to identify the corresponding peak of En-Lipoxin-A_4 and quantify both molecules by fitting their peak area ratios to the standard curve. Linear regression curves were constructed using the ratios of peak areas for En-Lipoxin-A_4 and ATL relative to the internal standard and presented in pg/mL. Data acquisition was performed using LabSolutions software (version 5.91) and qualitative and quantitative analysis of data was conducted using accompanied browser software. Duplicates with a variation of more than 10% were repeated.

2.4 Assessment of adherence

2.4.1 Self-reported adherence with aspirin

Qualitative assessment of adherence was conducted through a questionnaire generated based on the Simplified Medication Adherence Questionnaire (SMAQ) (Table 2) (96-98). Women undertook this assessment at each time point for the duration of aspirin therapy (Figure 6). The investigators (research midwives) verbally questioned women at each time point. Adherence through qualitative assessment was defined as a negative response to questions 2 and 3 in the qualitative questionnaire in ≥90% of the time points.

Questions	Response
1) Are you on aspirin?	Yes/No
2) Have you missed any of your regular aspirin?	Yes/No
3) Have you missed your regular aspirin in the last 7 days	Yes/No
4) In the last 7 days, how many doses of aspirin have you missed?	Number:
5) When was your last dose of aspirin	Date…… Time……

Table 2: Self-reported adherence questionnaire used at every time point of follow-up

2.4.2 Biochemical assessment of adherence with aspirin

Biochemical evidence of adequate adherence with aspirin was defined as an appropriate PFA-100 CT (CT reference ranges of 80-170 seconds for COL/EPI and 60-120 seconds for COL/ADP))(95) with detectable plasma SA in ≥90% of the time points. The cut-off of ≥90% was based on published literature (81). Biochemical evidence of intermittent adherence was defined as appropriate PFA-100 response and detectable plasma SA in <90% of the time points. Women were defined as aspirin resistant if there was a lack of appropriate PFA-100 response despite detectable plasma SA (Figure 8).

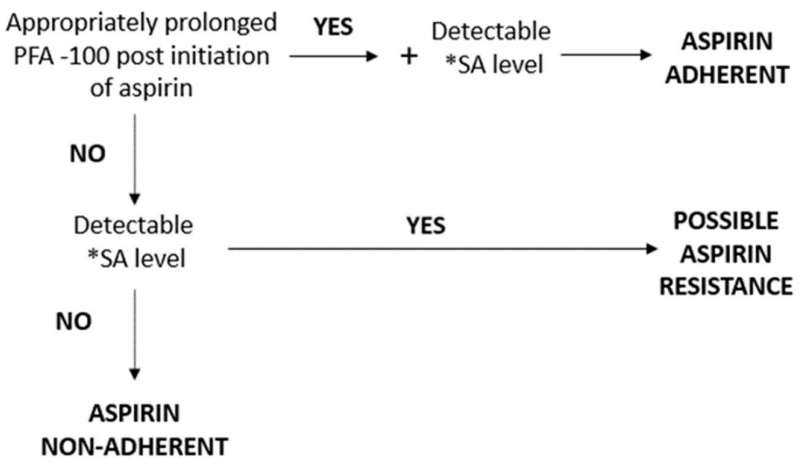


Figure 8: Illustrative representation of assessment of biochemical adherence.

Adapted from Shanmugalingam et al. The clinical influence of non-adherence with prophylactic aspirin in preventing preeclampsia in high-risk pregnancies. Hypertension, 75,1125–1132 (2020).

*PFA-100 = Platelet function analyzer 100, *SA: Salicyclic acid*

2.5 Mixed methods analysis: Quantitative and qualitative study

High-risk pregnant women from the longitudinal study who were prescribed aspirin (n=154) were invited to participate in a sequential-exploratory designed mixed methods study. Women in the non-aspirin group were excluded. Electronic invitations, via email, were sent out to women from October 2018 – June 2019, to participate in this anonymous, non-compulsory survey that consisted mainly of multiple-choice responses with free text boxes for additional responses.

Anonymous response was utilized to facilitate honest and non-biased responses. Questions for the quantitative study were generated based on factors that are known to influence adherence with medications in pregnancy (Table 3) (99, 100).

Question	**Question type**
How do you rate your understanding of why you were asked to take aspirin in your recent pregnancy?	Graded response (Scale of 1-5)
Who first advised you to take aspirin in your pregnancy?	5 Multi-choice, single-response option with free text for additional response
How satisfied were you with your doctor's/midwife's explanation on the need for aspirin in your pregnancy?	Graded response (Scale of 1-4)
Can you please elaborate on what could have been done better with the explanation provided to you?	Free text response
Were you worried about taking aspirin in your pregnancy?	Yes/No response
What were your concerns with taking aspirin in pregnancy	9 Multi-choice, multi-response option with free text for additional response
Did you feel comfortable discussing your concerns with your doctor/midwife?	Yes/No response
If no, why did you not feel comfortable discussing your concerns?	Free text response
How satisfied were you with the discussion with your pharmacist on the use of aspirin in pregnancy?	Graded response (Scale of 1-4)
How satisfied were you with your discussion with your obstetrician on the use of aspirin in pregnancy?	Graded response (Scale of 1-4)
Did you do your own research to understand the need for aspirin in pregnancy?	Yes/No response
What was/were your source(s) of information?	4 Multi-choice, multi-response option with

	free text for additional response
Did your research change your decision on taking aspirin in pregnancy?	Yes/No response
How often did you miss your aspirin in your pregnancy?	1 of 7 Multi-choice, single-response option
What was/were the reason(s) you missed your aspirin?	8 Multi-choice, multi-response option with additional free text response
What strategies might have helped you take your aspirin consistently without missing it?	5 Multi-choice, multi-response option with additional free text response.
Did you have to take other medications/vitamins in your recent pregnancy and if so, how many medications were you taking?	4 Multi-choice, single-response option with additional free text response
Did you have difficulty taking other medications (other than aspirin)?	Yes/No response
How often did you miss your other medications (besides aspirin) in your pregnancy?	1 of 7 Multi-choice, single-response option

Table 3: Questions used in quantitative study

At the end of the questionnaire (quantitative assessment), women were invited to participate in a one-to-one, face-to-face interview for the qualitative assessment. Women were advised to express their interest by sending an email to the primary investigator. Purposeful selection of interview participants, based on participant's obstetric history and biochemically observed adherence to aspirin (101) , was done to gain maximum and balanced variation in the sample. Open ended questions for the qualitative interview were generated based on current literature and the data obtained from the quantitative survey (Table 4)(80, 102-104).

Theme 1: Knowledge *(probing questions in italic)*

1) What is your understanding of preeclampsia

2) What is your understanding on why you were asked to take aspirin?

3) How did you feel about taking aspirin in your pregnancy?

- *Were you worried? If so, what were your concerns?*
- *Did you talk to anyone about your concern?*
- *What did your family and friends think about you being on aspirin?*
- *Did you know anyone else on aspirin? Did this reassure you?*

4) Did you do your own research about using aspirin in pregnancy?

- *What was your source?*
- *Was your source helpful? Did it scare you?*
- *What you have preferred as a source of information?*
- *What is your take on the information available on the internet?*
- *Did your research change your decision on taking aspirin? if so, how and why?*

Theme 2: Compliance

1) How many medications did you have to take during your pregnancy?

2) How did you feel about taking medications in pregnancy?

- *Did you feel safe taking medications in pregnancy?*
- *Did you feel like you knew enough about why you needed the medications?*

3) How often would you have missed medications

- *What was the reason you missed them?*
- *How often would you have missed aspirin?*
- *What was the reason for this?*
- *Did you experience any side effects?*
- *Were there any restrictions that made it difficult?*

  (Ie: taking it at a particular time/specific instructions / tablet size or configuration)

4) What would have helped you take your medications without missing them?

- *What strategies have you tried to use to minimize the times you missed your pills? Was this effective? IF no, why?*

Theme 3: Quality improvement
1) Were you happy with the explanation your doctor provided with need for aspirin in your pregnancy? - *Did you discuss the use of aspirin in pregnancy with your renal doctors? Were you satisfied with this interaction?* - *Did you discuss the use of aspirin with your obstetrician? Were you satisfied with this interaction?* - *Did you discuss the use of aspirin with your GP? Were you satisfied with this interaction?* - *Did you discuss the use of aspirin with your pharmacist? Were you satisfied with this interaction?*

Table 4: Questions used in qualitative study

Semi-structured interviews were conducted by a single experienced co-investigator who was not involved with the clinical care of the women. Interviews were audio-recorded with short key notes taken during interviews to allow for member checking at the end of the interview. Duration of the interviews ranged from 45 to 75 minutes. Participants were provided with a verbal summary of the interview and the opportunity to request amendments to the interpretations. Interviews were ceased at the point of data saturation, which was set at the point at which no new data emerged from interviews.

2.5.1 Data analysis

Audio recorded interviews were transcribed verbatim. No preconceived codes or categories were used. A six-stage sequential qualitative analysis was undertaken in analysing the transcription with the use of NVivo® (v.12 QRS International Pty Ltd) (105). The six stage analysis was based on Braun and Clarke's thematic analysis which

involved (a) familiarity with data set, (b) generating initial codes, (c) formation of themes, (d) review of themes, (e) defining themes and (f) data reporting (106).

Transcripts were re-analysed through the six-stage analysis process by a second investigator to ensure agreement of codes and themes. Results of the quantitative and qualitative studies were combined through a process of triangulation that enabled the investigators to connect and interpret both data sets simultaneously through convergence and corroboration with the use of NVivo® (107, 108). Phi correlation (φ) of data was done through SPSS (v.25 Chicago,IL, USA). Statistical significance was set at 0.05. A φ value of 0.7 – 1 was representative of a strong correlation, 0.3 – 0.69 of a moderate correlation and values of <0.3 were representative of a weak correlation.

2.6 Definitions used

Clinical definitions and outcomes assessed were based on the Society of Obstetric Medicine, Australia and New Zealand (SOMANZ) and International Society of Study of Hypertension in Pregnancy (ISSHP) clinical guidelines (39, 71, 72) and are as elaborated in Table 5. Both international (ISSHP 2018) and national (SOMANZ 2014) level guidelines were utilized to ensure standardization of definitions used.

Outcomes	**Definition**
Preeclampsia	Onset of hypertension after 20 weeks of gestation accompanied by one or more of the following features of end organ impairment: <u>Renal</u> • Significant proteinuria – a spot urine protein/creatinine ratio ≥ 30 mg/mmol • Serum or plasma creatinine > 90 μmol/L • Oliguria: urine output <80 mL/4 hr <u>Haematological</u> • Thrombocytopenia <100,000 /μL • Haemolysis: schistocytes or red cell fragments on blood film, raised bilirubin, raised lactate dehydrogenase >600 mIU/L, decreased haptoglobin • Disseminated intravascular coagulation <u>Liver</u> • Raised serum transaminases • Severe epigastric and/or right upper quadrant pain. <u>Neurological</u> • Convulsion (eclampsia) • Hypereflexia with sustained clonus • Persistent, new headache • Persistent visual disturbances (photopsia, scotomata, cortical blindness, posterior reversible encephalopathy syndrome, retinal vasospasm) • Stroke

	Pulmonary oedema Intrauterine growth restriction (IUGR)
Early onset-preeclampsia	The onset of preeclampsia (as described above) ≤ 34 weeks of gestation
Late onset-preeclampsia	The onset of preeclampsia (as described above) >34 weeks of gestation
Intrauterine growth restriction (IUGR)	Intra-uterine estimated fetal weight of <10th centile
Preterm delivery	Delivery prior to 37 weeks of gestation
Increase in antenatal antihypertensives	A cumulative increase of ≥50% (through the duration of study) in the dose of antenatal antihypertensive or the addition of additional antihypertensive agents to maintain antenatal BP target of ≤140/90mmHg
Decrease in antenatal antihypertensives	A cumulative reduction of ≥50% (through the duration of study) in the dose of antenatal antihypertensives or the cessation of one or more antihypertensive agents.
Average SBP	Mean of all systolic blood pressure readings recorded through the duration of study
Average DBP	Mean of all diastolic blood pressure readings recorded through the duration of study
Primigravida	Woman who conceived for the first time

Table 5: Definition of clinical outcomes used

2.7 Statistical analysis

Data were analysed using statistical tests through Statistical Package for Social Sciences (SPSS®) v25 (IBM, Armonk, New York, United States) and GraphPad® Prism v8.4.1 (GraphPad Software, La Jolla, California, United States).

For the longitudinal biochemical data (Chapter 4), repeated measures analysis of variance (ANOVA) was used to analyse difference in longitudinal En-Lipoxin-A4, ATL, PlGF and cytokine concentrations. For the pharmacokinetic data (Chapter 5), one-way ANOVA (*post hoc* testing with Tukey's test), four-way ANOVA *(post hoc* testing with Tukey's test) and T-tests were utilised for analysis of mean values. For clinical data (Chapter 6), unadjusted Chi-square analysis was used to examine for difference between characteristics and obstetric outcomes. An adjusted analysis using a binary logistic-regression analysis (stepwise backward) was undertaken to assess group differences (expressed as the odds ratio (OR) and 95% confidence interval). Repeated measure ANOVA was used to examine differences between average systolic blood pressure (SBP) and diastolic blood pressure (DBP) over time and between groups. Comparison of outcomes in women with varying degree of intermittent adherence with non-aspirin control group was done through a Pearson χ^2 analysis. A Kaplan-Meier analysis was undertaken to assess pregnancy continuation (survival) between ≥90% adherent group and <90% adherent group. Agreement between self-reported and biochemical adherence was assessed with Cohen's kappa coefficient. For mixed methods data (Chapter 7), Phi correlation(φ) was utilized to correlate the combined quantitative and qualitative data to women's adherence with aspirin. Statistical significance was set at $p < 0.05$.

CHAPTER 3

PARTICIPANT CHARACTERISTICS

Pregnant women who were risk stratified as high-risk for preeclampsia were recruited from 3 high-risk pregnancy clinics across SWSLHD from December 2016 – January 2019. A total of 248 women were approached for enrolment into this study, of whom, 220 women consented to participate (Figure 9). Of the 220 women, 119 (54%) were from Liverpool Hospital, 65 (30%) from Campbelltown Hospital and 36 (16%) from Bankstown Hospital.

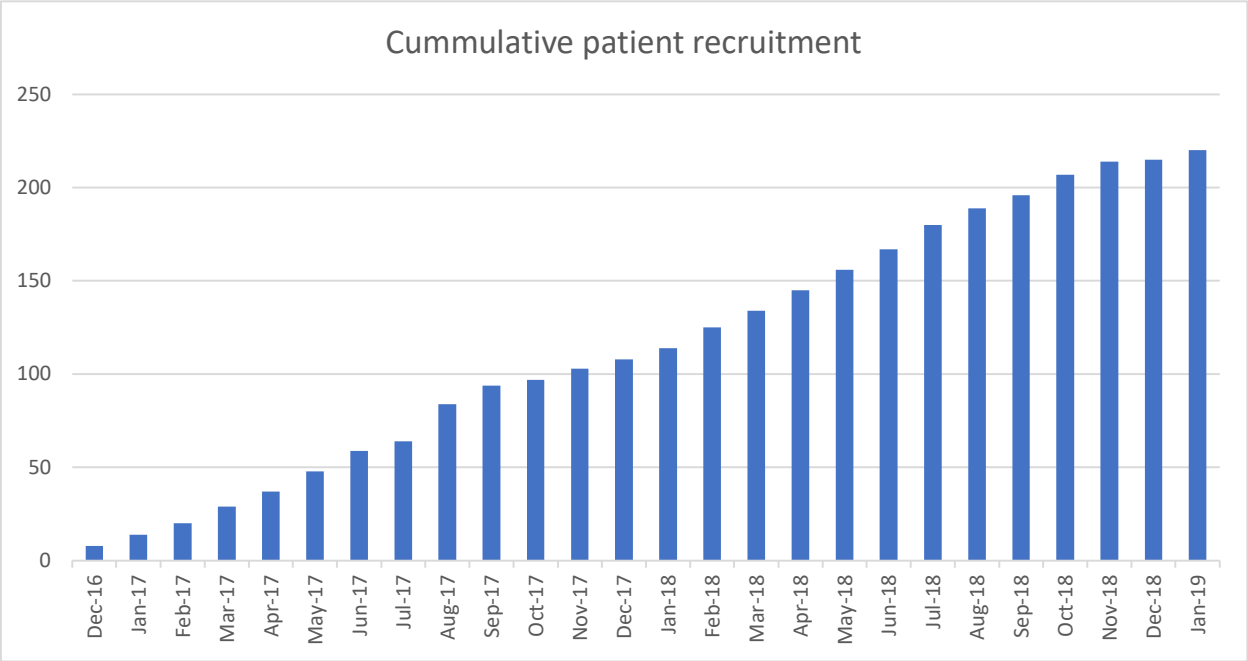


Figure 9: Illustration of cumulative recruitment of high-risk pregnant women

Of the 220 high-risk pregnant women recruited, data from 196 high-risk women were available for analysis (Figure 10). Of the 196 high-risk women, 154 high-risk women were prescribed aspirin and 42 high-risk women were not prescribed aspirin by their treating clinician. High-risk women who were not prescribed aspirin were used as the control group and are referred to, hereafter, as the 'non-aspirin group'.

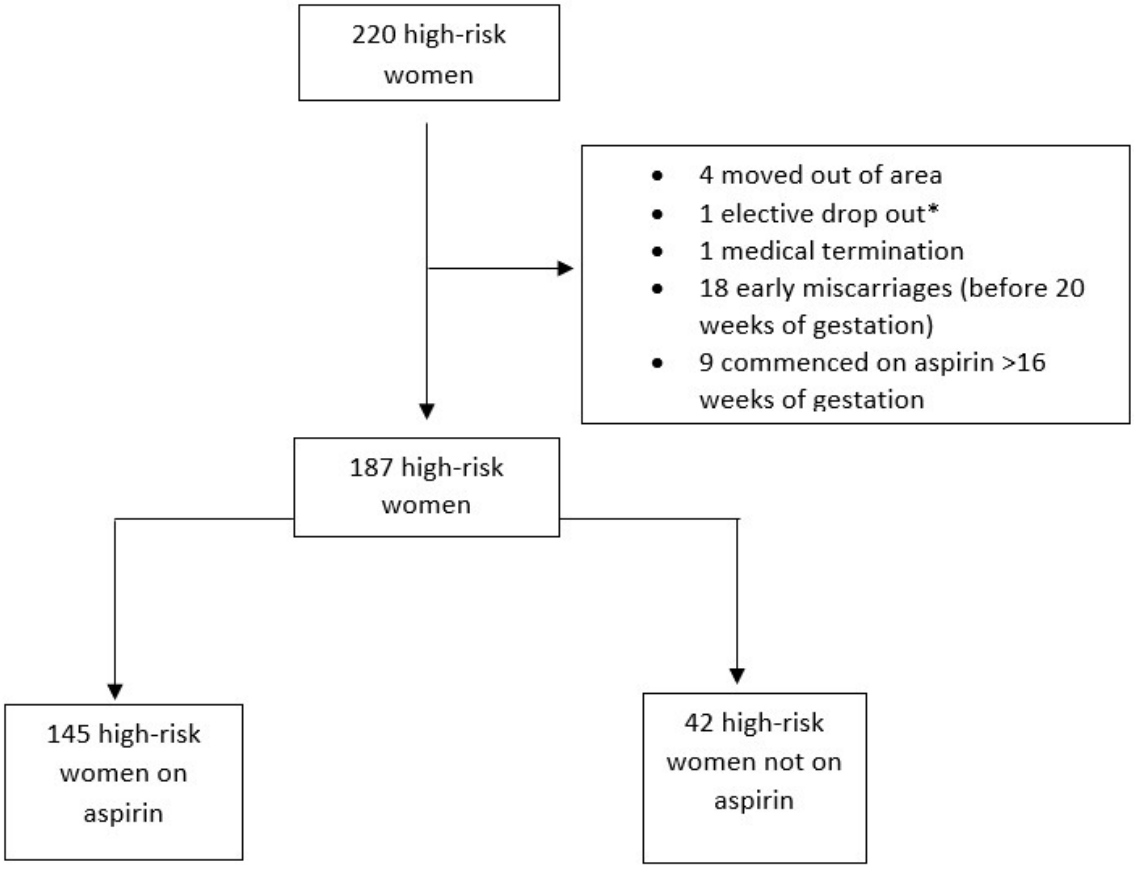


Figure 10: Outcome of recruitment of high-risk pregnant women in the longitudinal cohort study

** Patient reported difficulty to attend follow up reviews.*

Figure 11 describes the reasons women in the non-aspirin group were not prescribed aspirin by their treating clinician. The average gestation at which women were commenced on aspirin was 12 weeks (± 2 weeks). Aspirin was ceased at 34-36weeks of gestation based on centre specific practise.

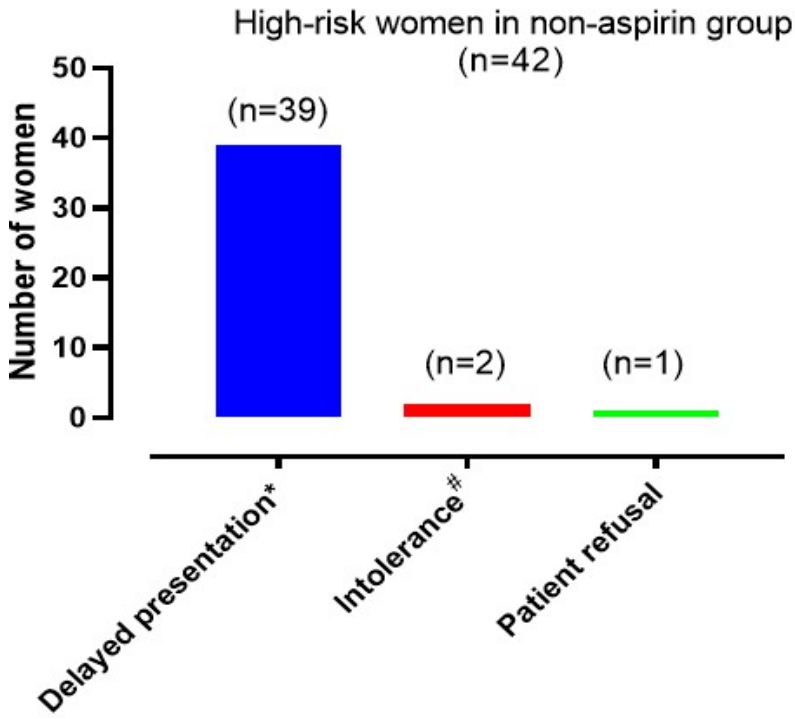


Figure 11: Reasons for non-aspirin prescription in the non-aspirin group

**Presentation to hospital between 16^{+1} and 20 weeks of gestation. # Known pre-existing intolerance of aspirin.*

Comparison of clinical characteristics of the participants are described in Table 6. The only statistically significant difference between the two groups was the proportion of women with previous preeclampsia (higher in the aspirin group (40%) in comparison to the non-aspirin group (26%)).

Characteristics	Aspirin group (n=154)	Non-aspirin group (n=42)	p-value
Age (years) *	32 (± 6.5)	31 (± 5.2)	0.4
Primigravity	18(12%)	8(19%)	0.07
Pre-pregnancy Body Mass Index (BMI) of ≥ 30	64(42%)	24(57%)	0.4
Multi-fetal pregnancy	7(4%)	1(2%)	0.7
Smoking in pregnancy	9(9%)	3(7%)	0.6
Conception through artificial reproductive therapy	14(9%)	4(10%)	0.9
§Booking systolic BP *	118 (±3)	120 (±4.1)	0.3
§Booking diastolic BP*	77 (±2.4)	74 (±3.1)	0.2
Use antihypertensives at booking§	84(55%)	24(57%)	0.4
Ethnicity			
Caucasian	81(53%)	20(48%)	
Middle Eastern	25(16%)	6(14%)	
South Asian	23(15%)	5(12%)	0.6
South East Asian	14(9%)	4(10%)	
African	6(4%)	3(6%)	
Polynesians	3(2%)	4(10%)	
Australian Aboriginals†	2(1%)	0(0%)	
**Indication for aspirin in pregnancy ** **			
Chronic hypertension	87(56%)	28(67%)	0.09
Previous preeclampsia	62(40%)	11(26%)	**0.04**
Renal disease	35(23%)	7(17%)	0.3

Type 1 or Type 2 Diabetes	12(8%)	3(7%)	0.8
Based on first trimester screening only †	9(6%)	0	0.08
Systemic lupus erythematosus	6(4%)	1(2%)	0.9

Table 6: Characteristics of participating high-risk pregnant women

**mean with standard deviation (±SD). † Analysis based on Fisher's exact test. ** Added percentages exceed 100% as some women had more than 1 medical condition. § Booking refers to the first antenatal review. BP: Blood Pressure.*

Given the growing evidence on the optimal timing of initiation of aspirin in effective prevention of preeclampsia (109), data from women who were commenced on aspirin after 16 weeks of gestation (n=9) were excluded from the final analysis.

CHAPTER 4

SCHOLARLY RESEARCH PAPER 1:

MECHANISM OF ACTION OF ASPIRIN IN THE PREVENTION OF PREECLAMPSIA

Publications in relation to this chapter:

SCHOLARLY RESEARCH PAPER 1:

THE 15-EPILIPOXIN-A4 PATHWAY (ATL) WITH PROPHYLACTIC ASPIRIN IN PREVENTING PREECLAMPSIA: A LONGITUDINAL-COHORT STUDY.

Renuka Shanmugalingam, XiaoSuo Wang, Penelope Motum, Ian Fulcher, Gaksoo Lee, Roshika Kumar, Annemarie Hennessy, Angela Makris.

Journal of Clinical Endocrinology and Metabolism (JCEM), Accepted July 2020 (IN-PRESS)

Additional publication in relation to this chapter (Manuscript in preparation):

EFFECT OF ASPIRIN ON PLACENTAL GROWTH FACTOR (PLGF) IN PREGNANCY AND THE DEVELOPMENT OF PREECLAMPSIA.

Renuka Shanmugalingam, Katrina Chau, XiaoSuo Wang, Penelope Motum, Ian Fulcher, Gaksoo Lee, Roshika Kumar, Annemarie Hennessy, Angela Makris.

4.1 Study overview

The prophylactic benefit of aspirin in the prevention of preeclampsia is increasingly recognised, however, its mechanism of action remains unclear. Given the variable clinical outcomes observed, an understanding of the mechanism of action of aspirin in preventing preeclampsia may play a role in understanding its optimal clinical utility.

Recent non-obstetric aspirin mechanistic studies have demonstrated an anti-inflammatory role of aspirin through the 15-epilipoxin-A_4 (ATL) pathway (57, 110, 111). ATL is an epimer of endogenous Lipoxin-A4 (En-Lipoxin-A4), a potent endogenous anti-inflammatory lipoxin, and has been shown to downregulate pro-inflammatory Th1 cytokines and upregulate anti-inflammatory Th2 cytokines in cell-culture and animal models (112-114). Recent obstetric studies identified a possible association between low first trimester maternal En-Lipoxin-A4 and subsequent features of placental dysfunction (65, 66) and given that ATL is an epimer of En-Lipoxin-A4, cell studies have examined the role ATL on placental development through its proposed anti-inflammatory properties (67, 68). Gil-Villa *et al* demonstrated that when exogenous ATL treated plasma from women with preeclampsia was added to human umbilical vein endothelial cells (HUVEC), adhesion of polymorphonuclear neutrophils (PMN) to HUVEC was reduced compared to the non-ATL treated plasma (68). Similarly, Alvarez *et al* demonstrated that ATL reversed antiphospholipid antibody (aPL)-induced decrease in trophoblast migration in aPL treated human trophoblast cell line (HTR8) cells. This effect appeared to be regulated through restoration of IL-6 production. However, clinical data on this remains sparse.

This chapter of the thesis aimed to examine for; (1) differences in maternal En-Lipoxin-A4 concentration in low-risk (LR) and high-risk (HR) pregnancy; (2) effect of aspirin on plasma ATL concentration in high-risk (HR) women; (3) effect of aspirin on selected circulating cytokine (specific to the pathophysiology of preeclampsia) concentration in HR women; (4) difference in ATL and cytokines with differing doses of aspirin (100 mg vs 150 mg) and (5) the observed relationship in longitudinal maternal ATL and cytokines with obstetric outcomes. Given the pro-angiogenic effect of PlGF and recent data on raised endogenous maternal PlGF concentration in relation to aspirin ingestion (115), this chapter also examined the difference in maternal PlGF concentration in relation to aspirin use and the observed effect on clinical outcomes.

Plasma samples collected from HR women recruited in the longitudinal cohort study (Chapter 2.1) were analysed for En-Lipoxin-A_4, ATL, IL-8, IL-10, TNF-α, IFN-γ, IL-1β and PlGF (Chapters 2.2 and 2.3). Samples were assessed for adequate adherence with aspirin with a two-point biochemical assessment (Chapter 2.4.2) prior to analysis. Only data from HR women with ≥90% adherence and the non-aspirin group were analysed, therefore, data presented consist of two groups: aspirin-adherent high-risk women (HR-AA) and non-aspirin control high-risk women (HR-NA). Data from women with inadequate adherence were excluded from the analysis in this chapter.

Plasma samples of LR women with normal pregnancy outcomes (n=20) were obtained from frozen plasma collection of a historic longitudinal cohort where samples were collected at 12, 20, 28 and 36 weeks of gestation between September 2003 – March 2005. LR women from the historic cohort had no clinical risk factors for preeclampsia. Given

the prolonged storage duration of the frozen plasma sample from the historic cohort, an internal control sub-analysis of En-Lipoxin-A4 plasma concentration from 5 HR women from the historic cohort (September 2003- March 2005) (total of 20 samples) were analysed against the En-Lipoxin-A4 plasma concentration of HR women from the longitudinal cohort (December 2016-January 2019). There were no statistically significant differences in all samples analysed, therefore, minimising any potential storage time related difference in plasma En-Lipoxin-A4 concentration from the historic cohort.

4.2 Summary of key findings

HR women were observed to have up to 70% lower plasma concentration of En-Lipoxin-A_4 ($p<0.001$) compared to LR women (116). HR-AA (n=82) had significantly higher concentration of plasma ATL ($p<0.001$) with lower plasma concentration of IL-8 from 12-36 weeks of gestation ($p <0.001$), TNF-α from 24-36 weeks of gestation ($P=0.02$) and an increase in plasma concentration of IL-10 from 16 – 28 weeks of gestation ($p=0.03$) compared to the HR-NA group (116). Additionally, a statistically significant rise in maternal PlGF plasma concentration by 61% at 12 weeks, 108% at 16 weeks, 116% at 20 weeks, 121% at 24 weeks, 141% at 28 weeks, 143% at 32 weeks and 160% at 36 weeks of gestation was observed in the HR-AA group compared to the HR-NA group ($p<0.001$)(Figure 12)(Table 7).

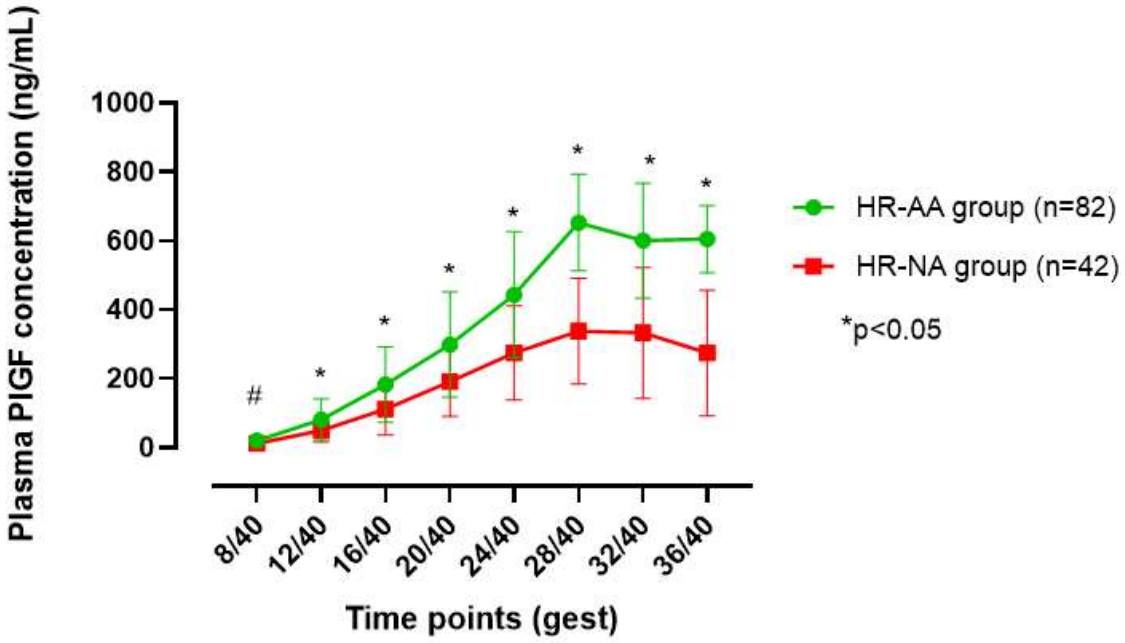


Figure 12: Plasma concentration of PlGF in aspirin adherent (HR-AA) and non aspirin high-risk (HR-NA) groups.

HR-AA: Aspirin adherent high-risk pregnant women group, HR-NA: Non-aspirin high-risk pregnant women group. PlGF: placental growth factor, # Baseline prior to initiation of aspirin.

Gestation	Plasma PlGF HR-AA (n=82) (ng/mL)	Plasma PlGF HR-NA (n=42) (ng/mL)	Difference between HR-AA and HR-NA groups (%)	p-value
8 weeks	13.5(±6)	11.4(±4)	18	0.4
12 weeks	80.8(±32)	49.8(±23)	61	**<0.001**
16 weeks	232.7(±52)	111.6(±63)	108	**<0.001**
20 weeks	348.7(±122)	161.1(±79)	116	**<0.001**
24 weeks	473.2(±124)	214.6(±113)	121	**<0.001**
28 weeks	742.7(±140)	307.9(±133)	141	**<0.001**
32 weeks	640.2(±167)	262.7(±134)	143	**<0.001**
36 weeks	634.7(±97)	244.1(±82)	160	**<0.001**

Table 7: Comparison of plasma PlGF between the aspirin adherent high-risk pregnant women group (HR-AA) and non-aspirin high-risk pregnant women group (HR-NA).

HR-AA: Aspirin adherent high-risk pregnant women group, HR-NA: Non-aspirin high-risk pregnant women group. PlGF: placental growth factor

HR-AA women who were on 150 mg of aspirin were observed to have 35% higher plasma concentration of ATL (p=0.02) and IL-10 (by 18% at 16 and 20 weeks of gestation (p=0.01)) compared to HR-AA women who were on 100 mg of aspirin. There were no statistically significant differences in plasma PlGF, IL-8, TNF-α, IL-1β and IFN-γ concentrations between 100 mg and 150 mg of aspirin (116). However, this study was underpowered for a statistically significant comparison between 100 mg and 150 mg of aspirin.

HR women who developed preeclampsia were observed to have lower En-Lipoxin-A4, ATL, and IL-10 plasma concentration with higher IL-8 plasma concentration compared to HR women who did not develop preeclampsia (116). There were no statistically significant differences in IL-1β and IFN-ϒ plasma concentration (116). HR women who developed preeclampsia were also observed to have lower plasma PlGF concentration compared to HR women who did not develop preeclampsia (Figure 13).

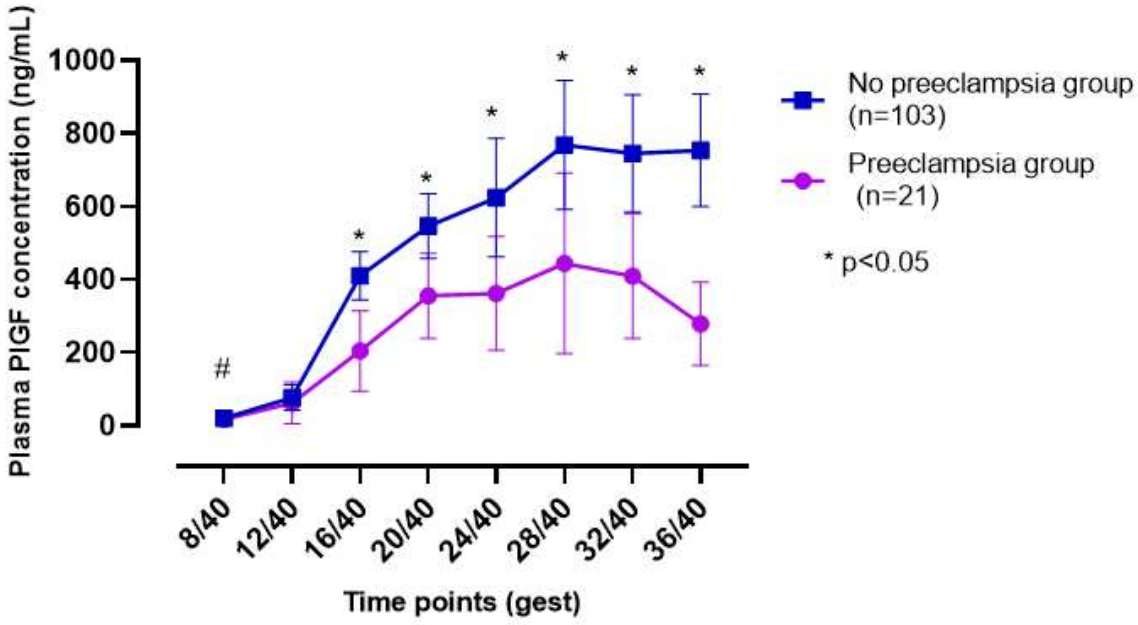


Figure 13: Comparison of plasma PlGF concentration of high-risk women (both HR-AA and HR-NA) based on clinical outcome (preeclampsia and no preeclampsia).

HR-AA: Aspirin adherent high-risk pregnant women group, HR-NA: Non-aspirin high-risk pregnant women group. PlGF: placental growth factor, # Baseline prior to initiation of aspirin.

4.3 Strengths and limitations of this study

The study is novel as the variation in plasma En-Lipoxin-A_4, detection of plasma ATL in relation to aspirin ingestion, and its corresponding alterations in selected pro-inflammatory and anti-inflammatory cytokines have not been previously examined in HR pregnant women. Additionally, stringent measures were undertaken to ensure that the observed changes were in relation to aspirin ingestion (Chapter 2.4.2). The LCMS methodology used to detect plasma En-Lipoxin-A_4 and ATL were optimized and validated internally to increase the sensitivity of detection. The comprehensive description of the methodology is detailed in Chapter 2.3.2.

The study's main limitation is that it is observational. It is hypothesized that the observed changes in the maternal plasma concentration of IL-10 and IL-8 are as a result of endogenous ATL production with adequate aspirin ingestion, however, the study does not demonstrate a direct relationship, only an association. Given the limited sample size (limited by number women who did not need adherence criteria), comparison analysis between the effects of 100 mg and 150 mg aspirin on endogenous cytokine and PlGF concentration were underpowered.

4.4 Conclusions

Plasma En-Lipoxin-A4 concentration was observed to be lower in HR women compared to LR controls. Adequate ingestion of aspirin resulted in significant elevation of endogenous ATL concentration and an observed reduction of pro-inflammatory cytokine IL-8, with an increase in anti-inflammatory cytokine IL-10. A rise in maternal plasma PlGF

concentration was also observed in relation to adequate aspirin adherence. The ongoing raise in maternal plasma PlGF concentration even after aspirin cessation suggest that the observed elevation in PlGF concentration is potentially a surrogate marker of better placentation with early initiation of aspirin.

The findings of this study suggest that aspirin potentially improves placentation, as demonstrated through the observed rise in PlGF, through its anti-inflammatory properties via the ATL pathway. The direct effect of aspirin on placentation, however, further exploration.

The 15-epilipoxin-A4 pathway (ATL) with prophylactic aspirin in preventing preeclampsia: a longitudinal-cohort study

Renuka Shanmugalingam[1,2,3,4], XiaoSuo Wang[5], Penelope Motum[3,6], Ian Fulcher[7] Gaksoo Lee[3,7], Roshika Kumar[7], Annemarie Hennessy[1,2,3,4], Angela Makris[1,2,3,4,8]

1 Department of Renal Medicine, South Western Sydney Local Health District, NSW AUSTRALIA

2 School of Medicine, Western Sydney University, NSW AUSTRALIA

3 Women's Health Initiative Translational Unit (WHITU), Ingham Institute For Applied Medical Research and South Western Sydney Local Health District, NSW AUSTRALIA

4 Vascular Immunology Research Group, Heart Research Institute (HRI), Sydney, NSW, AUSTRALIA

5 Bosch Mass Spectrometry Faculty, University of Sydney, NSW AUSTRALIA

6 Department of Haematology, South Western Sydney Local Health District, NSW AUSTRALIA

7 Department of Obstetrics and Gynaecology, South Western Sydney Local Health District, NSW AUSTRALIA

8 South Western Sydney Clinical School, University of New South Wales (UNSW), NSW AUSTRALIA

Short title

ATL pathway with prophylactic aspirin in preeclampsia

Key words

Aspirin, ATL, preeclampsia, Lipoxin-A4, cytokines

Corresponding author and person to whom reprint request should be addressed

Dr. Renuka Shanmugalingam
Department of Renal Medicine
Liverpool Hospital, Elizabeth St
NSW 2170

Source of funding

Partial financial assistance was received from the PEARLS Foundation, Australia and the SWSLHD funded Women's Health Initiate Translational Unit (WHITU), Australia

Disclosures summary

Nil

Abstract

Introduction: The benefit of aspirin in preventing preeclampsia is increasingly recognised, however, its mechanism of action remains unclear. Non-obstetric studies have described an anti-inflammatory effect of aspirin through 15-epilipoxin-A4(ATL). However, the anti-inflammatory mechanism of aspirin in the prevention of preeclampsia remains unknown.

Objective/Hypothesis: To examine the (1) difference in longitudinal endogenous lipoxin-A4(En-Lipoxin-A4) concentration in low-risk(LR) and high-risk(HR) pregnancies, (2)effect of aspirin on endogenous ATL concentration and the associated effect on cytokine profile of HR women.

Methods: Plasma from 220 HR women were collected at 12,16,20,24,28,32 and 36 weeks of gestation. Adherence to aspirin was biochemically verified. Plasma En-Lipoxin-A4 and ATL concentrations were analysed through liquid-chromatography mass-spectrometry(LC-MS/MS) and cytokines;IL-10, TNF-α, IFN-ɣ, IL-8 and IL-1β with high-sensitivity multi-bead Luminex® assay.

Results: HR women have up to 70% lower plasma concentration of En-Lipoxin-A4($p<0.001$) compared to LR women. HR women with adequate aspirin adherence(HR-AA)(n=82) had higher plasma concentration of ATL($p<0.001$), lower concentration of IL-8 from 16-36 weeks of gestation($p<0.001$)and increased IL-10 concentration from 16 – 28 weeks of gestation ($p=0.03$) in comparison to high-risk women who were not on aspirin(HR-NA). HR-AA who did not develop preeclampsia had higher plasma En-lipoxin-A4($p<0.001$), ATL($p=0.02$) and IL-10 concentrations($p<0.001$) with lower IL-8 concentration($p=0.004$) compared to HR who developed preeclampsia.

Discussion: Plasma concentration of En-Lipoxin-A4 is lower in HR women compared to LR controls. Adequate adherence with aspirin results in an increase in ATL and IL-10 with reduced IL-8 plasma concentration. This study suggests a potential anti-inflammatory role of aspirin, through the ATL pathway with prophylactic aspirin in HR pregnant women.

Introduction

The prophylactic benefit of aspirin in preventing preeclampsia amongst high-risk pregnant(HR) women is increasingly recognized with a risk reduction of 50-68% when commenced prior to 16 weeks of gestation and with optimal adherence[1-5]. Recent meta-analysis and pharmacokinetic studies suggest potential higher risk reduction with the use of 150mgs of aspirin given its altered pharmacokinetics in pregnancy, however, the clinical data for this remains lacking[6, 7]. Whilst there is growing evidence to support its clinical utility, the mechanism by which aspirin acts as a prophylactic agent in reducing the risk of preeclampsia remains unclear and is of ongoing interest.

Early studies investigating the mechanism of aspirin in the prevention of preeclampsia demonstrated an aspirin-induced decrease in maternal thromboxane-A2(TXA2) and mediation of the unbalanced thromboxane/prostacyclin(TXA2/PGI2) ratio, in favour of prostacyclin[8-11]. It was, therefore, hypothesized that aspirin improved placental blood flow and minimized risk of placental thrombosis by mediating TXA2 and PGI2. However, current data demonstrates a gestation specific effect of aspirin with minimal prophylactic benefit when commenced after 16 weeks of gestation; a timing that correlates with completion of placental development[6]. This, therefore, suggests the possibility of additional mechanisms by which aspirin influences placentation in reducing the risk of preeclampsia.

Recent non-obstetric aspirin mechanistic studies have demonstrated an anti-inflammatory role of aspirin through the 15-epilipoxin-A4 pathway, also known as the aspirin-triggered-lipoxin(ATL) pathway[12-14]. ATL is an epimer of endogenous lipoxin-A4(En-Lipoxin-A4), a potent endogenous anti-inflammatory lipoxin, and has been shown to regulate Th1/Th2 cytokine imbalance in cell-culture and animal models[15-17]. Lipoxin-A4 has been shown to mediate inhibition of neutrophil and eosinophil chemotaxis and decreases inflammatory infiltrates *in-vivo* with downregulation of interleukin 6 (IL-6), tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ), whilst upregulating interleukin 10 (IL-10) expression[16, 18]. Given that a pro-inflammatory maternal state is known to impair cytotrophoblast implantation and placentation [19-23], an increase in maternal En-lipoxin-A4 could potentially play a role in improving the placental environment. Recent obstetric studies identified a possible association between low first trimester maternal En-Lipoxin-A4 and subsequent features of placental dysfunction[24, 25] and given that ATL is an epimer of En-Lipoxin-A4, studies have focused on examining the role ATL on placental development[26, 27]. However, clinical data on this remains sparse.

Given the paucity in the current literature, this study aimed to examine the; (1) differences in maternal En-Lipoxin-A4 concentration in low-risk(LR) and high-risk(HR) pregnancy; (2) effect of aspirin on plasma ATL concentration in HR women; (3) effect of aspirin on selected circulating cytokine concentration in HR women; (4) difference in ATL and cytokines with differing doses of aspirin (100mgs vs 150mgs) and (5) the observed relationship in longitudinal maternal ATL and cytokines with obstetric outcome.

Materials and Methods

Participant recruitment and sample collection

High-risk pregnant (HR) women were recruited over 25 months (December 2016-January 2019) from clinics across three hospitals within the South Western Sydney Local Health District (SWSLHD) as part of a longitudinal observational cohort study. Ethics approval was obtained (SWSLDH Ethics Committee HE 16/184) and women provided written informed consent. Women were assessed as high-risk for preeclampsia by their treating clinicians using the NICE clinical guidelines or the Fetal Medicine Foundation (FMF) high-risk screening program (Table 1). Inclusion criteria included (1) risk-stratified high-risk for preeclampsia by the treating clinician (2) < 20^{+1} weeks of gestation at time of enrolment. Exclusion criteria included (1) commencement of aspirin > 16^{+0} weeks of gestation (2) on aspirin at

time of recruitment (3) women of non-English speaking background (4) unable to provide written informed consent (5) > 20^{+0} weeks gestation at time of enrolment. Women were recruited at time of commencement of aspirin and were followed through to the end of their pregnancy. Blood samples and clinical data were collected at baseline (prior to first dose of aspirin) and at 16,20,24,28,32 and 36 weeks of gestation. Aspirin was ceased between 34^{+1}-35^{+6} weeks of gestation in accordance with local practice. HR women who were not prescribed aspirin either due to aspirin intolerance or delayed presentation (between 16^{+1}-20^{+0} weeks of gestation) were followed identically to the aspirin prescribed group and served as the control high-risk pregnancy (High risk- no aspirin: HR-NA) group. Frozen plasma samples of low-risk women (LR) with normal pregnancy outcomes (n=20) were obtained from a historic longitudinal cohort where samples were collected at 12, 20, 28 and 36 weeks of gestation between September 2003 – March 2005. Women from the historic LR cohort had no clinical risk factors for preeclampsia.

Blood samples were collected via a 21G BD Vacutainer Push Button needle(Becton, Dickinson and Company USA) into 3 x 4mls VACUETTE® K2EDTA tubes(Greiner Bio-One International) and 2 x 3.8ml tubes containing 0.38ml of 0.129mol/l buffered sodium citrate (pH 5.5) for platelet function analyser(PFA-100) analysis. Samples in two of the three VACUETTE® K2EDTA tubes were centrifuged immediately at 3000rpm for 10mins following which, plasma was aliquoted in 220µL volumes and stored at -80^{o}C until analysis. The third VACUETTE® K2EDTA tube and both sodium citrate tubes were sent to the SWSLHD pathology service at Liverpool Hospital (NATA accredited) at room temperature for full blood count and platelet function analyzer (PFA-100) assessment respectively within 4 hours of collection.

Assessment of adherence with aspirin

Biochemical evidence of adherence with aspirin was verified through a two-point biochemical assessment with the use of PFA-100 and plasma salicylate acid (SA) detection[5, 28]at all time points of the study. Adherence was defined as simultaneously prolonged PFA-100 with detectable plasma SA, at ≥90% of timepoints[5]. A value of ≥90% adherence was based on published data[1, 29].Only data from HR women with ≥90% adherence and the control group were analysed, therefore, final data presented consist of two groups: aspirin-adherent high risk women(HR-AA) and non-aspirin control high-risk women(HR-NA).

LC-MS/MS analysis of En-Lipoxin-A4 and ATL

Standards were prepared in duplicates in Eppendorf tubes using 100µg/mL of 15-epilipoxin-A4 (Sapphire Bioscience Pty.Ltd, Australia) and 100µg/mL of Lipoxin-A4 (Sapphire Bioscience Pty.Ltd, Australia). Briefly, 2µL of each standard were added into 196µL of 100% Methanol (Lichrosolv, Merck Milipore, Baywater VIC, Australia) to constitute a stock solution of 1µg/mL of 15-epilipoxin-A4 and Lipoxin-A4. Stock solution was then diluted with varying volumes of 50% methanol to obtain series of standard concentrations ranging from 0ng/mL to 200ng/mL. Deuterated lipoxin-A4(Lipoxin-A4-D5) (Sapphire Bioscience Pty.Ltd, Australia), as an internal standard, was spiked at a fixed concentration of 20ng/mL across all standards and vortexed for 30secs prior to transfer into HPLC vial inserts.

Plasma preparation, in duplicates, involved protein precipitation of 50µL of plasma with 200µL of 100% of acetonitrile (ACN)(1:4, vol:vol)(Lichrosolv, Merck Milipore, Baywater VIC, Australia) containing 2ng Lipoxin-A4-D5 in Eppendorf tubes. The tubes were vortexed for 30secs and submerged in ice for 15mins prior to being centrifuged at 16,168g for 10mins (Eppendorf Microcentrifuge Model 5415R, Hamburg Germany). The supernatant were then transferred into glass culture tubes (12 x 75mm disposable culture tubes, Kimble, USA) and evaporated at 45°C using an Eppendorf Concentrator (Model 22331 Hamburg) for 90mins. Standards were then reconstituted and acidified

with 1mL of 0.1% formic acid(FA)(Lichrosolv, Merck Milipore, Baywater VIC, Australia) prior to solid phase extraction using Discovery DSC-18 1mL (SPE) cartridges (Supelco, Bellefonte, PA, USA) on a vacuum manifold. SPE cartridges were preconditioned with 1mL of 100% methanol, then washed with 2mL of 0.1% FA. Following which, samples were applied to the cartridge and washed with 1mL of 0.1% FA prior to elution with 600µL of 100% methanol twice. The methanol eluate were then evaporated at 45°C for 90mins. Dried samples were then reconstituted with 100µL of 20% Methanol containing 0.05% Acetic Acid (AA) followed with a final spin at 1.4 x104rpm for 10mins prior to transfer into HPLC vial inserts.

LC-MS/MS analysis were performed on Shimadzu triple quadrupole mass spectrometer LCMS8050 coupled with UHPLC Nexera X2 series (Shimadzu Corp. Kyoto, Kyoto Prefecture, Japan). Separation of En-lipoxin-A4 and ATL in plasma was achieved with a Phenomenax Kinetex® C18 (150 x 2.1mm, 1.7µm)(Phenomenax Pty Ltd, NSW, Australia) column fitted with a SecurityGuard™ ULTRA Cartridges UHPLC C18 (2.1mm)(Phenomenax Pty Ltd, NSW, Australia), with mobile phase A containing 0.05% AA in water and mobile phase B consisting of 100% Methanol. Injection volume of 5µL was used with a total run of 24mins at a flow rate of 0.5mL/min. The gradient was commenced at 59% of mobile phase B and maintained isocratic for 20min. Following which the gradient was increased to 95% within 0.5mins and maintained for 2 mins before returning to 59% in the next half minute. Tandem MS was performed using the following parameters; negative electrospray ionization, nebulizing gas flow of 3L/min with heating gas flow of 10L/min and interface temperature of 300°CThe optimal fragmentor voltage and respective collision energy voltage for each molecule were obtained by flow injection analysis using Optimizer. The following MRM ion transitions were monitored; m/z 351->217 for En-Lipoxin-A4 and 351->115 for ATL with additional channels of m/z 315->135 for En-Lipoxin-A4 and m/z 315->59 for ATL. Epimers of ATL and En-Lipoxin-A4 were separated on LC at different retention times, though they share the same MRM ion transitions. The Lipoxin-A4-D5 peak was used as internal standard to identify the corresponding peak of En-Lipoxin-A4 and quantify levels of both molecules by fitting their peak area ratios to the standard curve. Linear regression curve was constructed using the ratios of peak areas for En-Lipoxin-A4 and ATL relative to the internal standard and presented in pg/mLs. Data acquisition was performed using LabSolutions software (version 5.91) and qualitative and quantitative analysis of data was conducted using accompanied browser software. Duplicates with a variation of more than 10% were repeated.

Cytokine analysis with Magnetic Luminex® Performance Assay multiplex kits.

Simultaneous quantitative analysis of interleukin-1β(IL-1β), TNF-α, Interleukin-8(IL-8), IL-10 and IFN-γ concentrations were measured with the use of the Magnetic Luminex® Performance multiplex human high sensitivity cytokine premixed kit A(R&D Systems, Minnesota, USA). Standards, reagents and undiluted 100µL of plasma were prepared and assayed in duplicate based on the manufacturer's manual with the use of HydroFlex® microplate washer(Tecan Trading AG, Switzerland)[30]. Prepared microplates were analysed with the Luminex® 100/200® system (Luminex Corporation, Texas, USA). Quantitative data for each cytokine were derived based on the microparticle regions specified by the manufacturer[30] and the respective standard curves with the use of the xPONENT® software(Luminex Corporation, Texas, USA) and presented in pg/ml. Samples with intra and inter-assay variation of more than 10% were repeated.

Definition of preeclampsia

Clinical diagnosis of preeclampsia was defined as the onset of hypertension (≥140/90mmHg) after 20 weeks of gestation with clinical and/or biochemical features of end organ impairment in keeping with the current guidelines[31, 32].

Statistical analysis

Sample size was calculated based on the assumption that untreated HR women have a preeclampsia-risk rate of 40% and that aspirin treated HR women will have a 50% reduction in preeclampsia-risk compared to the HR-NA group. The initial total recruitment target number of 120 was inflated to 220 women (in total) to allow for a predicted 20% dropout and a high prevalence (50%) of intermittent adherence with aspirin.

Repeated measures ANOVA was used to analyse difference in longitudinal En-Lipoxin-A4, ATL and cytokine concentrations. Chi-square analysis and t-test were used for assessment of clinical characteristics. Statistical analysis was performed with SPSS v25 with data reported as mean (±SD standard deviation) and significance set at <0.05. Results were analysed per protocol based on adherence. Aspirin adherence analysis was undertaken to ensure adequate exposure to aspirin in analysing results obtained and clarify relationships where ingestion was verified.

Results

A total of 220 HR women were recruited, with complete data from 187 women available for analysis; of which 145 HR women were prescribed aspirin and 42 were not prescribed aspirin (HR-NA) (Figure 1). Of the 145 HR women who were prescribed aspirin, data from 82 women who met adherence criteria were analysed (HR-AA) and within this group, 54(66%) were on 100mgs of aspirin and 28(34%) were on 150mgs of aspirin. Data from 63 HR women who did not meet aspirin adherence criteria were excluded.

Samples from 20 women in the historic LR group were analysed for plasma En-Lipoxin-A4. Clinical characteristics of women in the historic LR group and all prospectively examined HR women (n=187) are as describe in Table2. There was a higher proportion of primigravids (65% vs 18%, p=0.001) and women who were smoking in pregnancy (25% vs 6%, p=0.003) in the historic LR cohort compared to the HR women cohort (Table 2).

Comparison of clinical characteristics between HR-AA(n=82) and HR-NA(n=42) women demonstrated a higher percentage of women with previous preeclampsia in the HR-AA group compared to the HR-NA group (48% vs 26%, p=0.03)(Table 3). There were no other significant clinical differences between both groups (Table 3).

Plasma En-Lipoxin-A4 concentration in LR and HR groups

Adjusted longitudinal analysis of plasma En-Lipoxin-A4 concentration between both groups of women demonstrated a 64-70% lower plasma En-Lipoxin-A4 concentration in the HR group compared to the LR group at all gestation time points of analysis (p<0.001)(Figure 2a)(Supplementary Table 1)[33].

Plasma ATL concentration in HR-AA and HR-NA groups

Baseline comparison of plasma ATL concentration between HR-AA and HR-NA groups (pre-aspirin therapy plasma concentration) did not demonstrate a statistically significant difference (Figure 2b). On initiation of aspirin, HR-AA women had up to a 19-fold elevation in plasma ATL concentration for the duration of aspirin therapy in comparison to the HR-NA women (p<0.001) (Figure 2b)(Supplementary Table 2)[33]. HR-AA women on 150mgs of aspirin had a 21±4% (p<0.001) higher plasma ATL concentration compared to HR-AA women on 100mgs of aspirin through the duration of aspirin therapy (Figure 2c)(Supplementary Table 3).

Effect of aspirin on selected cytokine profile

The cytokines examined were IL-8, TNF-α, IFN-γ and IL-1β and 1L-10. HR-AA women had lower plasma concentration of IL-8 by 43% at 16 weeks (p<0.001), 51% at 20 weeks (p<0.001), 48% at 24 weeks (p<0.001), 42% at 28 weeks (p<0.001), 51% at 32 weeks(p<0.001) and 47% at 36 weeks (p<0.001) compared to HR-NA women(Figure 3a)(Supplementary Table 4)[33]. Lower plasma concentration of TNF-α; by 10% at 28 weeks(p=0.03), 11% at 32 weeks (p=0.02) and 9% at 36 weeks (p=0.04) were observed in the HR-AA group (Figure 3b) compared to the HR-NA group. There were no differences in plasma TNF-α concentration (p=0.08) between 16 – 24 weeks of gestation and in IL-1β and IFN-γ concentrations between both groups at all time points (Figure 3c). HR-AA women also demonstrated higher plasma concentration of IL-10; by 87% at 16 weeks (p<0.001), 58% at 20 weeks (p<0.001), 43% at 24 weeks (p<0.001) and 29% at 28 weeks (p<0.001)(p=0.02) (Figure 3d) compared to HR-NA group.

HR-AA women on 150mgs of aspirin demonstrated higher IL-10 plasma concentration at 16 weeks (by 15%, p=0.01) and 20 weeks of gestation (by 13%, p=0.02) compared to HR-AA women on 100mgs aspirin (Supplementary Figure 1c) (Supplementary Table 3)[33] . There was no difference in IL-10 plasma concentration between differing aspirin doses between 24-36 weeks of gestation. Furthermore, there were no aspirin dose related effect on the concentrations of IL-8 and TNF-α at all time points of assessment (Supplementary Table 5 and Supplementary Figure 1)[33].

Obstetric outcome in relation to plasma En-lipoxin-A4, ATL and cytokine levels

This sub-analysis examined for difference between the HR-AA and HR-NA groups only. 6 women in the HR-AA group and 15 women in the HR-NA women developed preeclampsia[5]. They were observed to have significantly lower En-lipoxin-A4 concentration at all time points (p<0.001) and lower ATL concentration (in HR-AA group)(p=0.02) for the duration of aspirin therapy (Figures 4a and b).

Plasma IL-8 concentration was noted to be lower in the HR-AA who did not develop preeclampsia (p= 0.004) (Figure 4c) and similarly, plasma IL-10 concentration was higher in HR-AA women who did not develop preeclampsia(p<0.001)(Figure 4d).

Discussion

Our study demonstrates that HR women have up to 70% lower plasma En-lipoxin-A4 concentration compared to LR women through the duration of their pregnancy. In response to adequate aspirin therapy, HR-AA women were observed to have a significant increase in endogenous ATL concentration for the duration of aspirin therapy. An assessment of the effect of aspirin on selected cytokines demonstrated a significant and sustained reduction in pro-inflammatory IL-8 and increased anti-inflammatory IL-10 plasma concentration in the HR-AA group compared to the HR-NA group. A comparison between 100mgs and 150mgs of aspirin demonstrated that HR women who were on 150mgs of aspirin had a 21±4% higher concentration of ATL through the duration of aspirin therapy with modestly increased plasma concentration of IL-10 (13-15%) between 16-20 weeks of gestation. The obstetric outcomes of the women in this observational cohort study have been published[5]. We observed a lower rate of preeclampsia (7% vs 36%, p<0.001), intrauterine growth retardation (5% vs 21%, p<0.001) and pre-term delivery (8% vs 26%, p<0.001) in the HR-AA group compared to the HR-NA group. Based on our clinical data[5], whilst most women who developed preeclampsia were found to have late onset preeclampsia (onset ≥ 34 weeks of gestation), 2% of women from the HR-AA group and 7% of women from the HR-NA group developed early onset preeclampsia (onset < 34 weeks of gestation). Further analysis of plasma En-lipoxin-A4, endogenous ATL and cytokine concentrations in relation to rate of preeclampsia suggests that HR-AA who did not develop preeclampsia had higher

plasma En-lipoxin-A4, ATL and IL-10 concentrations with lower plasma concentration of IL-8 in comparison to HR women who developed preeclampsia, suggesting a potential anti-inflammatory effect of prophylactic aspirin (Figure 4). The increased TNF-α concentration in third trimester (24-36 weeks of gestation) in HR-NA group is likely reflective of the higher rate of preeclampsia in this group in comparison to the HR-AA and less-likely due to an aspirin-related effect (Figure 3b)[5].

Endogenous Lipoxin-A4 (En-Lipoxin-A4) is a lipid mediator that elicits anti-inflammatory actions via its receptor, formyl peptide receptor 2 (FPR2/ALX) and is thought to be the " regulator" of inflammation[18, 34]. Human reproductive studies have shown that En-Lipoxin-A4 and its receptor FPR2/ALX regulates inflammatory events in the human endometrium and decidua of early pregnancy[34, 35]. Clinical studies, however, have demonstrated contradictory findings and this is likely attributed to the variation in the gestation at which the maternal En-Lipoxin-A4 concentration were examined and the cohort of women studied. Lipa *et al* demonstrated that low maternal first-trimester En-Lipoxin-A4 concentration was associated with intrauterine fetal growth disturbance, in keeping with our results, suggesting that low first-trimester En-Lipoxin-A4 concentration played a role in placental dysregulation[24]. In established preeclampsia (mainly third trimester), Perruci *et al* demonstrated elevated En-Lipoxin-A4 concentration suggesting a physiological anti-inflammatory response to the pro-inflammatory pathogenesis of established preeclampsia[36]. Perruci *et al*, however, excluded women with chronic conditions, a group that is known to have lower plasma En-Lipoxin-A4 concentration. In keeping with non-obstetric studies that have demonstrated lower En-Lipoxin-A4 in patients with chronic conditions[37, 38], our study found lower En-Lipoxin-A4 concentration in the HR group, in which 88% of women had pre-existing chronic conditions, through the duration of study (16-36 weeks of gestation) compared to the LR group in whom none had underlying chronic conditions. A sub-analysis of En-Lipoxin-A4 concentration in HR women who developed preeclampsia (both early and late onset preeclampsia) against HR women who didn't develop preeclampsia demonstrated lower plasma concentration in the preeclampsia group at all times points (Figure 4a). Higher BMI has been suggested to influence plasma En-Lipoxin-A4 concentration[36] however a comparison of characteristics between the low En-Lipoxin-A4 group (HR group) and high En-Lipoxin-A4 group (LR group) did not demonstrate any difference in our cohort (Table 2). Further sub-analysis of En-Lipoxin-A4 of all women (LR and HR) in relation to BMI did not demonstrate a difference in En-Lipoxin-A4 concentration between women with BMI of <30 and ≥30 (Supplementary Table 6)[33] .The LR group used in our study had a higher number of women who smoked in their pregnancy in comparison to women in the HR group. The influence of smoking on En-Lipoxin-A4 concentration remains unknown.

ATL is an endogenous 15R-enantiomer of En-Lipoxin-A4 and shares many of the bioactivities evoked by En-lipoxin-A4 with a 2-fold greater half-life. Chiang *et al* demonstrated that ingestion of 81mgs of aspirin in a male predominant cohort, resulted in plasma ATL concentration of approximately 3ng/mls (3000pg/mls)[39]. Our study observed plasma ATL concentration of up to 7000pg/mls with 100mgs of aspirin and up to 8500pg/mls with 150mgs of aspirin. The difference in plasma ATL concentration identified is likely confounded by a dose-dependent response and method of plasma ATL detection used (ELISA in Chiang *et al*'s study and high sensitivity LC/LCMS in our study). Non-obstetric studies have demonstrated upregulation of IL-10 and down-regulation of TNF-α and 1L-8 with the use of exogenous ATL[40-42]. Similarly, reproductive cell culture studies have demonstrated ATL related reversal in the inflammatory process observed in preeclampsia by upregulating IL-10 and nitric oxide (NO) whilst downregulating TNF-α[27]. However, knowledge on the influence of endogenous ATL in HR obstetric subjects remains limited and the dose of exogenous ATL used to produce this effect is not parallel with the endogenous plasma ATL concentration observed with the use of aspirin. Our study demonstrated that endogenous ATL concentration in HR women with adequate aspirin ingestion was associated with a sustained increase in plasma concentration of IL-10 and reduced concentration of IL-8 compared to HR women were not on aspirin.

Dose-dependent effects of 100mgs and 150mgs of aspirin were examined in this study. Whilst we observed higher endogenous ATL concentration with the use of 150mgs of aspirin compared to 100mgs of aspirin, we did not observe a meaningful difference in plasma IL-10 and IL-8 concentration between both doses of aspirin. The lack of difference may be attributable to the small sample size in both groups and the potential physiological longitudinal variation in IL-10[22, 23]. More recent metanalysis and pharmacokinetic studies[6, 7] have demonstrated the need for >100mgs of aspirin in the prevention of preeclampsia, however, pharmacodynamic and clinical studies with a larger sample size and adequate power, are required in examining this.

Strengths and limitations

Our study is novel in that the variation of En-lipoxin-A4 and the detection of ATL with aspirin ingestion and its corresponding alteration in selected pro-inflammatory and anti-inflammatory cytokines have not been previously examined in women at risk of developing preeclampsia. Additionally, we verified adequate aspirin adherence and included only those women found to be adherent in our analysis to ensure that the observed ATL and cytokines changes were in relation to aspirin ingestion. The LC-MS/MS methodology used to detect plasma En-lipoxin-A4 and ATL was optimized and validated by our group to increase the sensitivity of detection. Given the prolonged storage duration of the plasma sample used for the En-lipoxin-A4 level in the LR group, we conducted an internal control analysis of plasma En-lipoxin-A4 concentration in plasma samples of 5 HR women from the historic cohort (years 2003-2005) against the plasma En-lipoxin-A4 concentration of the HR women in our cohort (years 2016-2019). There was no statistically significant difference in the plasma En-Lipoxin-A4 concentration between samples analysed, therefore, minimising any potential storage time related difference in En-Lipoxin-A4 concentration from the historic cohort.

Our study has several limitations. We hypothesize that the observed changes in the maternal plasma IL-10 and IL-8 concentrations were a result of endogenous ATL production with adequate aspirin ingestion, however, our study does not demonstrate a causal relationship, only by association. The study was also not adequately powered for the *post hoc* analysis comparing differences in cytokine concentrations between different aspirin doses. Further cell culture studies (endothelial and trophoblast cells) examining the direct effect of aspirin on endogenous ATL and its effect on cytokines, endothelial nitric oxide (eNOS) and cell invasion may help better understand the relationship between aspirin, endogenous ATL and its effect on placentation in HR women.

Conclusion

Our study suggests that HR pregnant women have lower En-lipoxin-A4 concentration. Adequate adherence with prophylactic aspirin amongst these women demonstrated an increased plasma concentration of ATL and an observed increase in plasma IL-10 and reduction in IL-8 concentrations. This was clinically associated with a lower rate of preeclampsia[5]. Therefore, suggesting a possible anti-inflammatory role of prophylactic aspirin in the prevention of preeclampsia.

Acknowledgments

The authors would like to thank the participants of this study, the PEARLS Foundation, the Women's Health Initiative Translational Unit (WHITU), the Department of Renal Medicine SWSLHD and the Department of Obstetrics and Gynaecology SWSLHD, NSW Australia for their support in conducting this study.

Data availability

All data generated or analysed during this study are included in this published article or in the data repositories for supplementary data (https://doi.org/10.6084/m9.figshare.12578666.v1)

References

1. Wright D, Poon LC, Rolnik DL, Syngelaki A, Delgado JL, Vojtassakova D, et al. Aspirin for Evidence-Based Preeclampsia Prevention trial: influence of compliance on beneficial effect of aspirin in prevention of preterm preeclampsia. Am J Obstet Gynecol. 2017;217(6):685 e1- e5.
2. Wright D, Syngelaki A, Akolekar R, Poon LC, Nicolaides KH. Competing risks model in screening for preeclampsia by maternal characteristics and medical history. Am J Obstet Gynecol. 2015;213(1):62 e1-10.
3. Roberge S, Sibai B, McCaw-Binns A, Bujold E. Low-Dose Aspirin in Early Gestation for Prevention of Preeclampsia and Small-for-Gestational-Age Neonates: Meta-analysis of Large Randomized Trials. Am J Perinatol. 2016.
4. Rolnik DL, Wright D, Poon LC, O'Gorman N, Syngelaki A, de Paco Matallana C, et al. Aspirin versus Placebo in Pregnancies at High Risk for Preterm Preeclampsia. N Engl J Med. 2017;377(7):613-22.
5. Shanmugalingam R, Wang X, Motum P, Fulcher I, Lee G, Kumar R, et al. Clinical Influence of Nonadherence With Prophylactic Aspirin in Preventing Preeclampsia in High-Risk Pregnancies: A Multicenter, Prospective, Observational Cohort Study. Hypertension. 2020;75(4):1125-32.
6. Roberge S, Bujold E, Nicolaides KH. Meta-analysis on the effect of aspirin use for prevention of preeclampsia on placental abruption and antepartum hemorrhage. Am J Obstet Gynecol. 2018;218(5):483-9.
7. Shanmugalingam R, Wang X, Münch G, Fulcher I, Lee G, Chau K, et al. A pharmacokinetic assessment of optimal dosing, preparation, and chronotherapy of aspirin in pregnancy. Am J Obstet Gynecol. 2019.
8. Schiff E, Peleg E, Goldenberg M, Rosenthal T, Ruppin E, Tamarkin M, et al. The use of aspirin to prevent pregnancy-induced hypertension and lower the ratio of thromboxane A2 to prostacyclin in relatively high risk pregnancies. N Engl J Med. 1989;321(6):351-6.
9. Viinikka L, Hartikainen-Sorri AL, Lumme R, Hiilesmaa V, Ylikorkala O. Low dose aspirin in hypertensive pregnant women: effect on pregnancy outcome and prostacyclin-thromboxane balance in mother and newborn. Br J Obstet Gynaecol. 1993;100(9):809-15.
10. Roberts MS, Joyce RM, McLeod LJ, Vial JH, Seville PR. Slow-release aspirin and prostaglandin inhibition. Lancet (London, England). 1986;1(8490):1153-4.
11. Sibai BM, Mirro R, Chesney CM, Leffler C. Low-dose aspirin in pregnancy. Obstetrics and gynecology. 1989;74(4):551-7.
12. Solheim S, Arnesen H, Eikvar L, Hurlen M, Seljeflot I. Influence of aspirin on inflammatory markers in patients after acute myocardial infarction. Am J Cardiol. 2003;92(7):843-5.
13. Renda G, Zurro M, Romano M, De Caterina R. Aspirin-triggered lipoxin in patients treated with aspirin and selective vs. nonselective COX-2 inhibitors. Br J Clin Pharmacol. 2010;69(3):303-6.
14. Romano M, Cianci E, Simiele F, Recchiuti A. Lipoxins and aspirin-triggered lipoxins in resolution of inflammation. Eur J Pharmacol. 2015;760:49-63.
15. Leonard MO, Hannan K, Burne MJ, Lappin DW, Doran P, Coleman P, et al. 15-Epi-16-(para-fluorophenoxy)-lipoxin A(4)-methyl ester, a synthetic analogue of 15-epi-lipoxin A(4), is protective in experimental ischemic acute renal failure. J Am Soc Nephrol. 2002;13(6):1657-62.
16. Takano T, Fiore S, Maddox JF, Brady HR, Petasis NA, Serhan CN. Aspirin-triggered 15-epi-lipoxin A4 (LXA4) and LXA4 stable analogues are potent inhibitors of acute inflammation: evidence for anti-inflammatory receptors. J Exp Med. 1997;185(9):1693-704.

17. Medeiros R, Kitazawa M, Passos GF, Baglietto-Vargas D, Cheng D, Cribbs DH, et al. Aspirin-triggered lipoxin A4 stimulates alternative activation of microglia and reduces Alzheimer disease-like pathology in mice. Am J Pathol. 2013;182(5):1780-9.
18. Zhangye Xu MD, Feng Zhao, M.M., Feng Lin, M.M., Huiqiu Xiang, M.M., Ni Wang, M.M., Duyun Ye, M.M., Yinping Huang, M.M. Preeclampsia is associated with a deficiency of lipoxin A4, an endogenous anti-inflammatory mediator. Fertility and Sterility. 2014;102(1):282–90.e4.
19. Redman CW, Sargent IL. Immunology of pre-eclampsia. Am J Reprod Immunol. 2010;63(6):534-43.
20. Kumar A, Begum N, Prasad S, Agarwal S, Sharma S. IL-10, TNF-alpha & IFN-gamma: potential early biomarkers for preeclampsia. Cell Immunol. 2013;283(1-2):70-4.
21. Taylor BD, Ness RB, Klebanoff MA, Zoh R, Bass D, Hougaard DM, et al. First and second trimester immune biomarkers in preeclamptic and normotensive women. Pregnancy Hypertens. 2016;6(4):388-93.
22. Hennessy A, Pilmore HL, Simmons LA, Painter DM. A deficiency of placental IL-10 in preeclampsia. J Immunol. 1999;163(6):3491-5.
23. Makris A, Xu B, Yu B, Thornton C, Hennessy A. Placental deficiency of interleukin-10 (IL-10) in preeclampsia and its relationship to an IL10 promoter polymorphism. Placenta. 2006;27(4-5):445-51.
24. Lipa M, Bomba-Opon D, Lipa J, Bartnik P, Bartoszewicz Z, Wielgos M. Lipoxin A4 (LXA4) as a potential first trimester biochemical marker of intrauterine growth disorders. J Matern Fetal Neonatal Med. 2017;30(20):2495-7.
25. Xu Z, Zhao J, Zhang H, Ke T, Xu P, Cai W, et al. Spontaneous miscarriages are explained by the stress/glucocorticoid/lipoxin A4 axis. J Immunol. 2013;190(12):6051-8.
26. Alvarez AM, Mulla MJ, Chamley LW, Cadavid AP, Abrahams VM. Aspirin-triggered lipoxin prevents antiphospholipid antibody effects on human trophoblast migration and endothelial cell interactions. Arthritis Rheumatol. 2015;67(2):488-97.
27. Gil-Villa AM, Norling LV, Serhan CN, Cordero D, Rojas M, Cadavid A. Aspirin triggered-lipoxin A4 reduces the adhesion of human polymorphonuclear neutrophils to endothelial cells initiated by preeclamptic plasma. Prostaglandins Leukot Essent Fatty Acids. 2012;87(4-5):127-34.
28. New South Wales Health Pathology (NSWHP)-South-Liverpool Haematology Platelet Aggregometry & PFA manual.
29. Shanmugalingam R, Wang X, Motum P, Fulcher I, Lee G, Kumar R, et al. The clinical influence of non-adherence with prophylactic aspirin in preventing preeclampsia in high-risk pregnancies : A multi-centre prospective, observational, cohort study. Hypertension (IN PRESS)2019.
30. Systems RD. Magnetic Luminex Perfomance Assay. Human High Sensitivity Cytokine Premixed Kit A Catalog Number FCSTM09.
31. Brown MA, Magee LA, Kenny LC, Karumanchi SA, McCarthy FP, Saito S, et al. The hypertensive disorders of pregnancy: ISSHP classification, diagnosis & management recommendations for international practice. Pregnancy Hypertens. 2018;13:291-310.
32. Lowe SA, Bowyer L, Lust K, McMahon LP, Morton M, North RA, et al. SOMANZ guidelines for the management of hypertensive disorders of pregnancy 2014. Aust N Z J Obstet Gynaecol. 2015;55(5):e1-29.
33. Renuka S, XiaoSuo W, Penelope M, Ian F, Gaksoo L, Roshika K, et al. V3 Supplementary tables JCEM.docx2020.
34. Macdonald LJ, Boddy SC, Denison FC, Sales KJ, Jabbour HN. A role for lipoxin A_4 as an anti-inflammatory mediator in the human endometrium. Reproduction. 2011;142(2):345-52.
35. Maldonado-Pérez D, Golightly E, Denison FC, Jabbour HN, Norman JE. A role for lipoxin A4 as anti-inflammatory and proresolution mediator in human parturition. FASEB J. 2011;25(2):569-75.
36. Perucci LO, Santos PC, Ribeiro LS, Souza DG, Gomes KB, Dusse LM, et al. Lipoxin A4 Is Increased in the Plasma of Preeclamptic Women. Am J Hypertens. 2016;29(10):1179-85.

37. Ringholz FC, Buchanan PJ, Clarke DT, Millar RG, McDermott M, Linnane B, et al. Reduced 15-lipoxygenase 2 and lipoxin A4/leukotriene B4 ratio in children with cystic fibrosis. Eur Respir J. 2014;44(2):394-404.
38. Yu D, Xu Z, Yin X, Zheng F, Lin X, Pan Q, et al. Inverse Relationship between Serum Lipoxin A4 Level and the Risk of Metabolic Syndrome in a Middle-Aged Chinese Population. PLoS One. 2015;10(11):e0142848.
39. Chiang N, Hurwitz S, Ridker PM, Serhan CN. Aspirin has a gender-dependent impact on antiinflammatory 15-epi-lipoxin A4 formation: a randomized human trial. Arterioscler Thromb Vasc Biol. 2006;26(2):e14-7.
40. Prescott D, McKay DM. Aspirin-triggered lipoxin enhances macrophage phagocytosis of bacteria while inhibiting inflammatory cytokine production. Am J Physiol Gastrointest Liver Physiol. 2011;301(3):G487-97.
41. Pouliot M, Serhan CN. Lipoxin A4 and aspirin-triggered 15-epi-LXA4 inhibit tumor necrosis factor-alpha-initiated neutrophil responses and trafficking: novel regulators of a cytokine-chemokine axis relevant to periodontal diseases. J Periodontal Res. 1999;34(7):370-3.
42. Serhan CN, Clish CB. Aspirin triggered lipid mediators: novel inhibitors of leucocyte trafficking. Thorax. 2000;55 Suppl 2:S10-2.

Legends for Figures and Tables

Figure 1: Recruitment of high-risk pregnant women. *due to difficulty in attending research follow-up appointments. HR: High-risk pregnancy, HR-AA: High-risk pregnancy aspirin adherent, HR-NA: High-risk pregnancy not on aspirin

Figure 2: (A)Difference in endogenous plasma Lipoxin-A4 (En-Lipoxin-A4) concentration between women in the low-risk pregnancy (LR) group (historic cohort) and high-risk pregnancy (HR) group. (B) A comparison of endogenous plasma 15-epilipoxin-A4 (ATL) concentration between high-risk pregnancy women who met aspirin adherence criteria (HR-AA) and high-risk pregnant women who were not on aspirin (HR-NA). (C) A sub-comparison of plasma ATL concentration between 100mg and 150mg of aspirin in high-risk pregnant women who met aspirin adherence criteria (HR-AA). *statistically significant at <0.05. # High-risk women in the non-aspirin (HR-NA) group did not have plasma assessment at 12 weeks of gestation due to delayed presentation. ^ Pre-aspirin baseline plasma ATL concentration (HR-AA) comparison between the high- risk aspirin adherent group (at 12 weeks of gestation) and baseline level in the high-risk non-aspirin group(HR-NA)(at 16 weeks of gestation) was not statistically significant.

Figure 3: (A) Comparison of plasma IL-8 concentration between non-aspirin (HR-NA) and aspirin adherent (HR-AA) high-risk pregnancy groups. (B) Comparison of plasma TNF-α concentration between HR-NA and HR-AA groups. (C) Comparison of plasma IL-1β concentration between HR-NA and HR-AA groups. (D) Comparison between plasma IFN-γ concentration between HR-NA and HR-AA groups and (E) Comparison between plasma IL-10 concentration between HR-NA and HR-AA groups.

*statistically significant at <0.05. # High-risk women in the non-aspirin group did not have plasma assessment at 12 weeks of gestation due to delayed presentation.

Figure 4: (A) Comparison of plasma En-Lipoxin-A4 concentration between preeclampsia and non-preeclampsia groups. (B) Comparison of plasma 15-epilipoxin-A4 (ATL) concentration between preeclampsia and non-preeclampsia groups. (C) Comparison between plasma IL-8 concentration between preeclampsia and non-preeclampsia groups and (D) Comparison between plasma IL-10 levels between preeclampsia and non-preeclampsia groups.*statistically significant at <0.05. For Figures 4 (B-D), HR-AA women with no preeclampsia is represented by the green solid line with solid dot. HR-AA women with preeclampsia is represented by green dotted line with solid square. HR-NA women with no preeclampsia is represented by red solid line with solid dot. HR-NA women with preeclampsia is represented by red dotted line with solid square.

Table 1: Screening methods used to by clinicians to risk-stratify pregnant women in this study. *NICE guidelines recommend the use of aspirin in women with 1 major risk factor or ≥2 moderate risk factors. # The FMF screening algorithm recommends the use of aspirin in women with a ratio of <1:100.

Table 2: (A) Clinical characteristics comparison between women in the historic low-risk pregnancy (LR) group and prospectively examined all high-risk pregnancy (HR) group. (B) Subgroup analysis of aspirin-adherent high-risk pregnant women (HR-AA) and non-aspirin high-risk pregnant women (HR-NA).‡ All high-risk (HR) group consist of all high-risk women from the longitudinal study (HR-AA, HR-NA and HR women who were prescribed aspirin but did not meet adherence criteria). #HR group consist only of HR-AA and HR-NA with exclusion of HR women who did not meet adherence criteria. * mean with standard deviation (±SD). † Analysis based on Fisher's exact test.** added percentages exceed 100% as some women had more than 1 medical conditions. ± First trimester screening were done in private screening units based on a combined MoMs algorithm of maternal mean arterial pressure (MAP), uterine artery pulsatility index (PI) and maternal serum placental growth factor (PlGF) and pregnancy-associated plasma protein A (PAPP-A) with a cut off of 1:100. N/A (not applicable) as no women in the low-risk group had indications for aspirin.

NICE Guidelines*	Fetal-Maternal Medicine (FMF) First trimester screening#
Major risk factors • Chronic hypertension • Autoimmune conditions • Chronic kidney disease • Previous preeclampsia • Type 1 or type 2 diabetes Moderate risk factors • first pregnancy • age 40 years or older • pregnancy interval of more than 10 years • body mass index (BMI) of 35 kg/m2 or more at first visit • family history of pre-eclampsia • multi-fetal pregnancy.	FMF first trimester screening combined multiple of median (MoMs) of maternal mean arterial pressure (MAP), uterine artery pulsatility index (PI) and maternal serum placental growth factor (PlGF) and pregnancy-associated plasma protein A (PAPP-A) into an algorithm with a cut off of 1:100.

Table 1: Screening methods used to by clinicians to risk-stratify pregnant women in this study. *NICE guidelines recommend the use of aspirin in women with 1 major risk factor or ≥2 moderate risk factors. # The FMF screening algorithm recommends the use of aspirin in women with a ratio of <1:100.

	(A) Total Study Group (n=207)			(B) HR Groups (n=124)[#]		
Characteristics	**LR group (n=20)**	**All HR group (n=187)‡**	**p-value (LR vs all HR)**	**HR-AA group (n=82)**	**HR-NA group (n=42)**	**p-value (HR-AA vs HR-NA)**
Age (years) *	32 (±5.3)	33 (±5.5)	0.9	32 (± 5.5)	31 (± 5.2)	0.4
Primigravity	13(65%)	33(18%)	**0.001**	14(17%)	8(19%)	0.8
Pre-pregnancy Body Mass Index (BMI) of ≥ 30	8(40%)	86(46%)	0.5	34(41%)	24(57%)	0.06
Multifetal Pregnancy	1(5%)	8(4%)	0.8	6(7%)	1(2%)	0.7
Smoking in pregnancy	5(25%)	7(4%)	**0.003**	4(5%)	3(7%)	0.6
Conception through artificial reproductive therapy	2(10%)	16(9%)	0.08	9(11%)	4(10%)	0.9
Ethnicity			0.11			0.6
Caucasian	11(55%)	94(50%)		39(48%)	20(48%)	
Middle Eastern	3(15%)	30(16%)		15(18%)	6(14%)	
South Asian	3(15%)	27(14%)		10(12%)	5(12%)	
South East Asian	3(15%)	18(10%)		12(15%)	4(10%)	
African†	0	9(5%)		4(5%)	3(6%)	
Polynesians†	0	7(4%)		2(2%)	4(10%)	
Australian Aboriginals†	0	2(1%)		0(0%)	0(0%)	
Indication for aspirin in pregnancy **						
Chronic hypertension	N/A	110(59%)		44(54%)	28(67%)	0.09
Previous preeclampsia		80(43%)		39(48%)	11(26%)	**0.03**
Renal disease		41(22%)		21(26%)	7(17%)	0.3
Type 1 or Type 2 Diabetes		15(8%)		7(9%)	3(7%)	0.8
Based on first trimester screening only± †		8(4%)		6(7%)	0(0%)	0.08
Systemic lupus erythematosus		5(3%)		2(2%)	1(2%)	0.9

Table 2: (A) Clinical characteristics comparison between women in the historic low-risk pregnancy (LR) group and prospectively examined all high-risk pregnancy (HR) group. (B) Subgroup analysis of aspirin-adherent high-risk pregnant women (HR-AA) and non-aspirin high-risk pregnant women (HR-NA).

‡ All high-risk (HR) group consist of all high-risk women from the longitudinal study (HR-AA, HR-NA and HR women who were prescribed aspirin but did not meet adherence criteria). #HR group consist only of HR-AA and HR-NA with exclusion of HR women who did not meet adherence criteria. * mean with standard deviation (±SD). † Analysis based on Fisher's exact test.** added percentages exceed 100% as some women had more than 1 medical conditions. ± First trimester screening were done in private screening units based on a combined MoMs algorithm of maternal mean arterial pressure (MAP), uterine artery pulsatility index (PI) and maternal serum placental growth factor (PlGF) and pregnancy-associated plasma protein A (PAPP-A) with a cut off of 1:100. N/A (not applicable) as no women in the low-risk group had indications for aspirin.

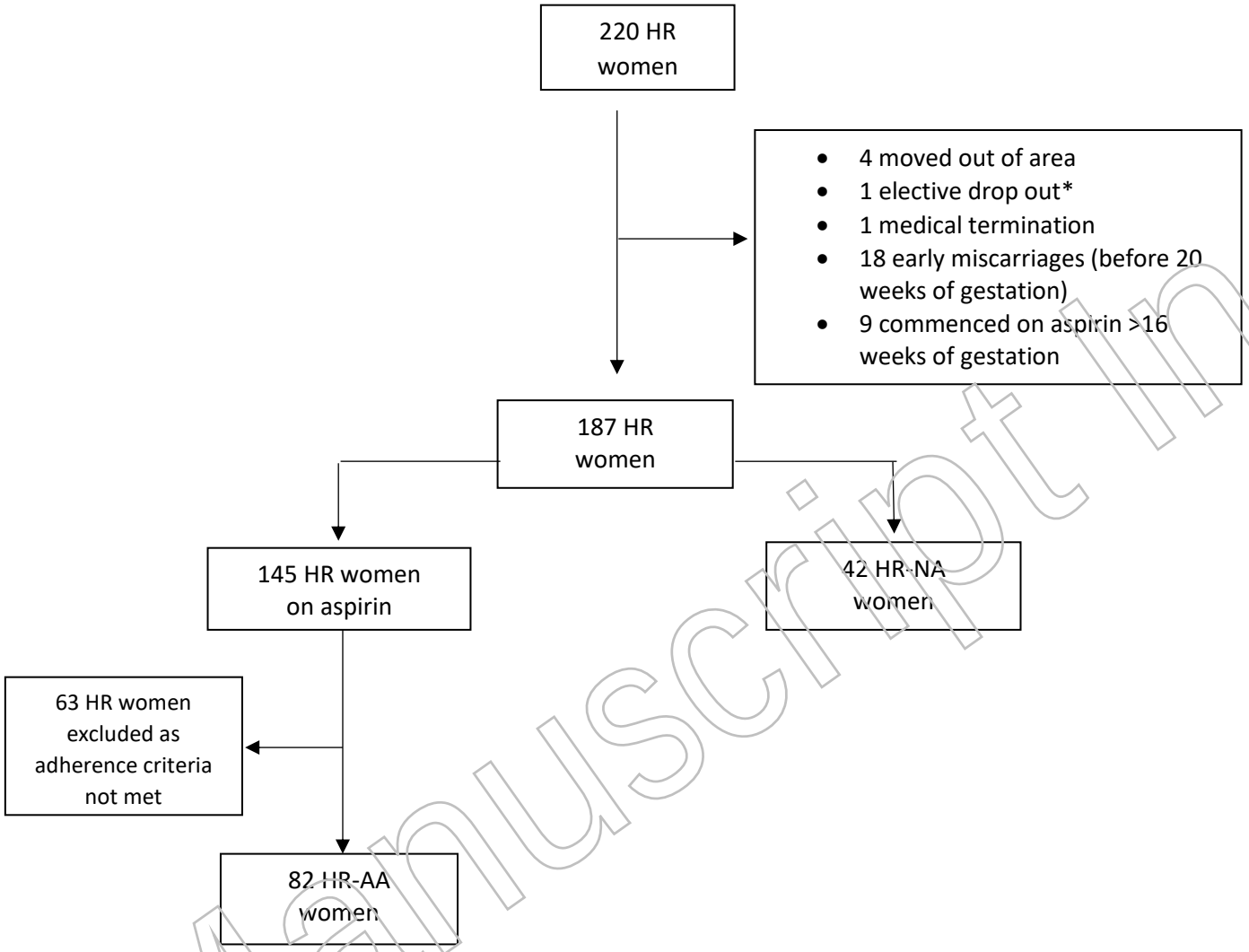


Figure 1: Recruitment of high-risk pregnant women . *due to difficulty in attending research follow-up appointments.

HR: High-risk pregnancy, HR-AA: High-risk pregnancy aspirin adherent, HR-NA: High-risk pregnancy not on aspirin

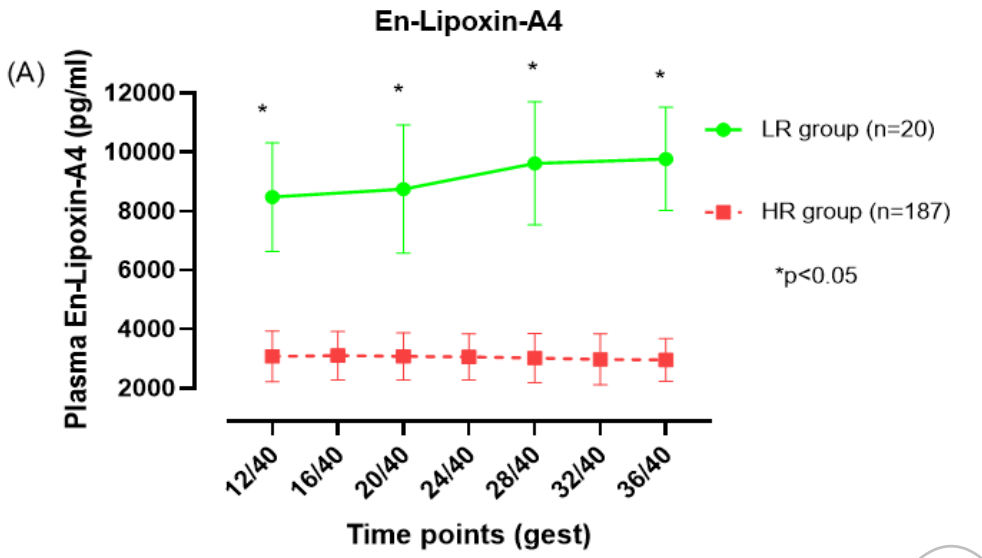
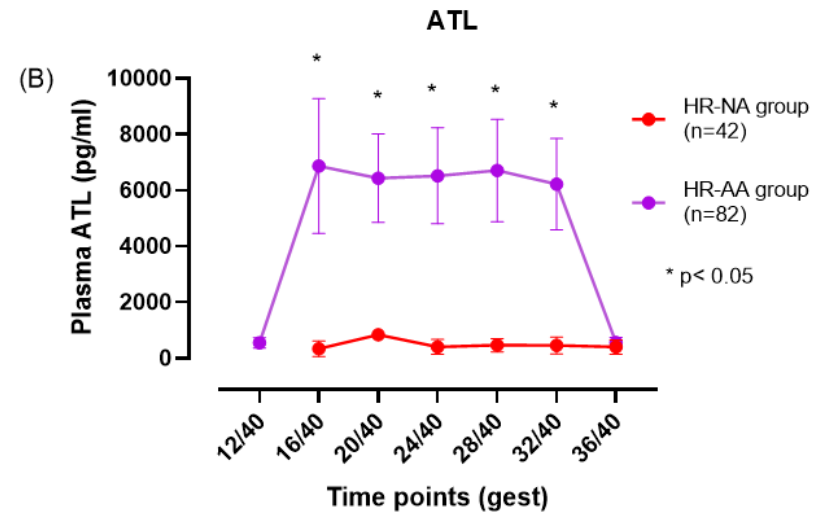
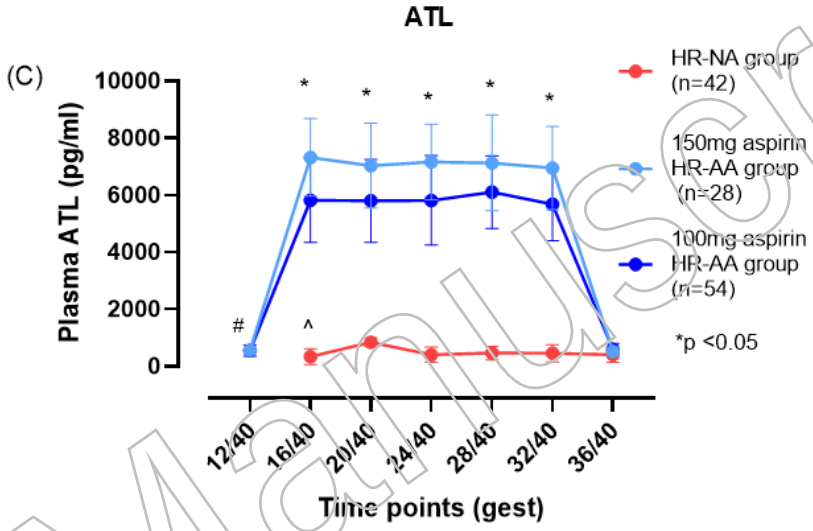


Figure 2: (A) Difference in endogenous plasma Lipoxin-A4 (En-Lipoxin-A4) concentration between women in the low-risk pregnancy (LR) group (historic cohort) and high-risk pregnancy (HR) group. (B) A comparison of endogenous plasma 15-epilipoxin-A4 (ATL) concentration between high-risk pregnancy women who met aspirin adherence criteria (HR-AA) and high-risk pregnant women who were not on aspirin (HR-NA). (C) A sub-comparison of plasma ATL concentration between 100mg and 150mg of aspirin in high-risk pregnant women who met aspirin adherence criteria (HR-AA).

*statistically significant at <0.05. # High-risk women in the non-aspirin (HR-NA) group did not have plasma assessment at 12 weeks of gestation due to delayed presentation. ^ Pre-aspirin baseline plasma ATL concentration (HR-AA) comparison between the high- risk aspirin adherent group (at 12 weeks of gestation) and baseline level in the high-risk non-aspirin group(HR-NA)(at 16 weeks of gestation) was not statistically significant.





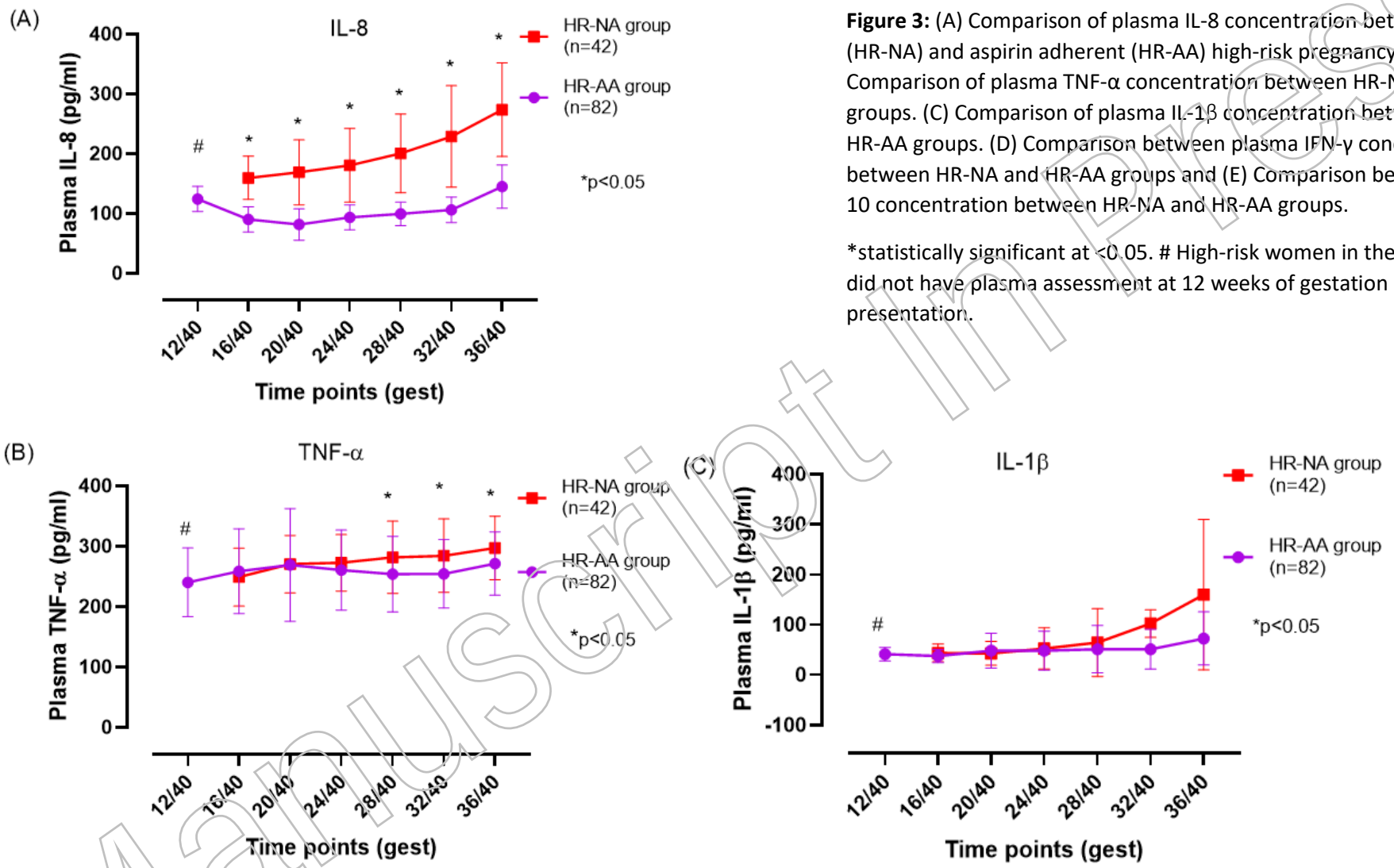
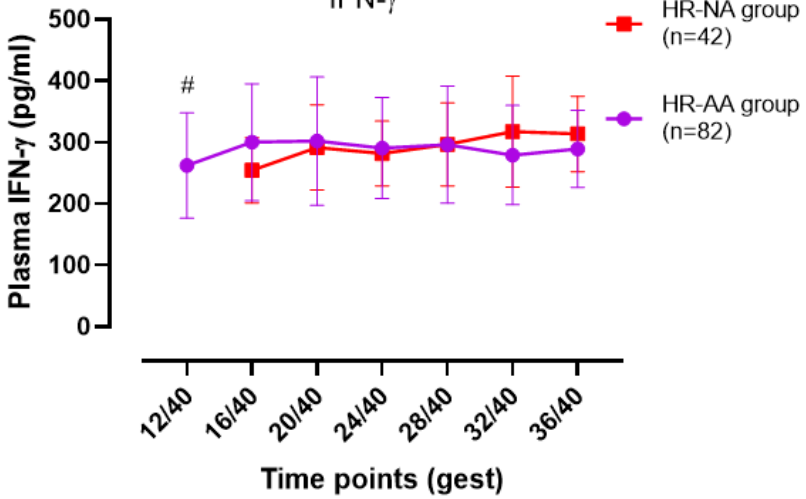


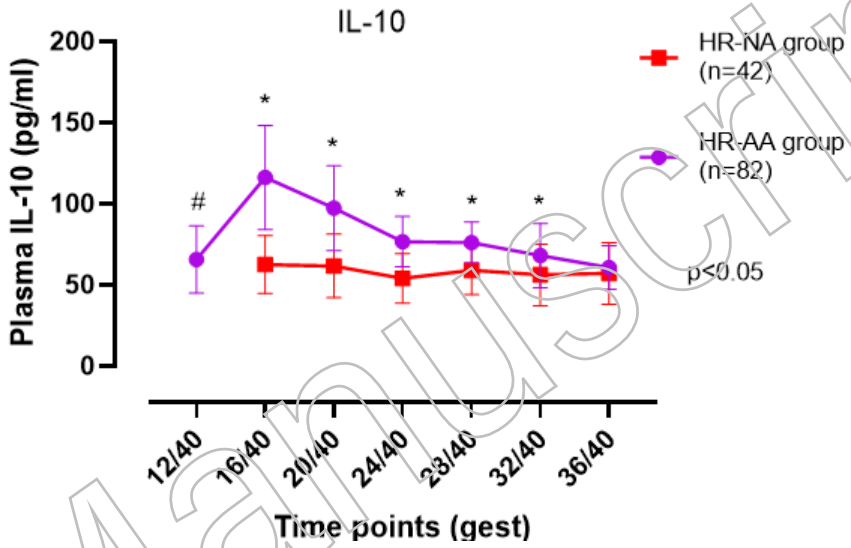
Figure 3: (A) Comparison of plasma IL-8 concentration between non-aspirin (HR-NA) and aspirin adherent (HR-AA) high-risk pregnancy groups. (B) Comparison of plasma TNF-α concentration between HR-NA and HR-AA groups. (C) Comparison of plasma IL-1β concentration between HR-NA and HR-AA groups. (D) Comparison between plasma IFN-γ concentration between HR-NA and HR-AA groups and (E) Comparison between plasma IL-10 concentration between HR-NA and HR-AA groups.

*statistically significant at <0.05. # High-risk women in the non-aspirin group did not have plasma assessment at 12 weeks of gestation due to delayed presentation.

(D)



(E)



(A)

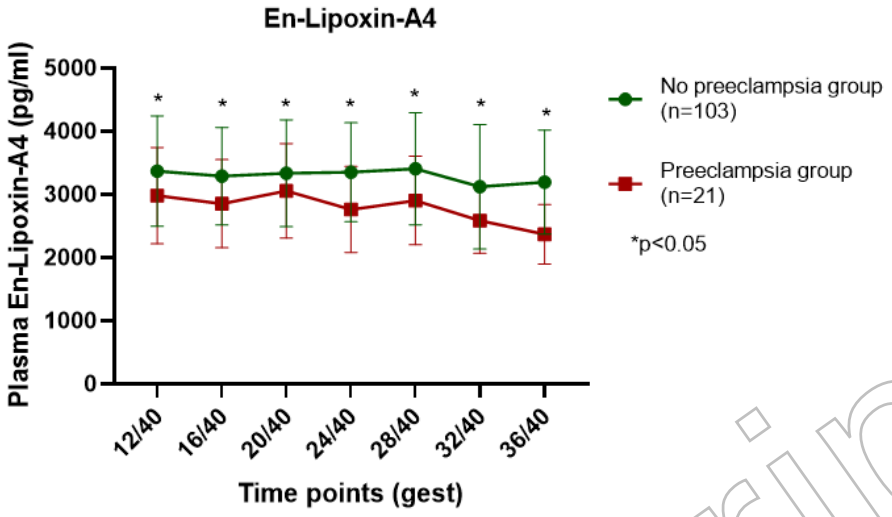
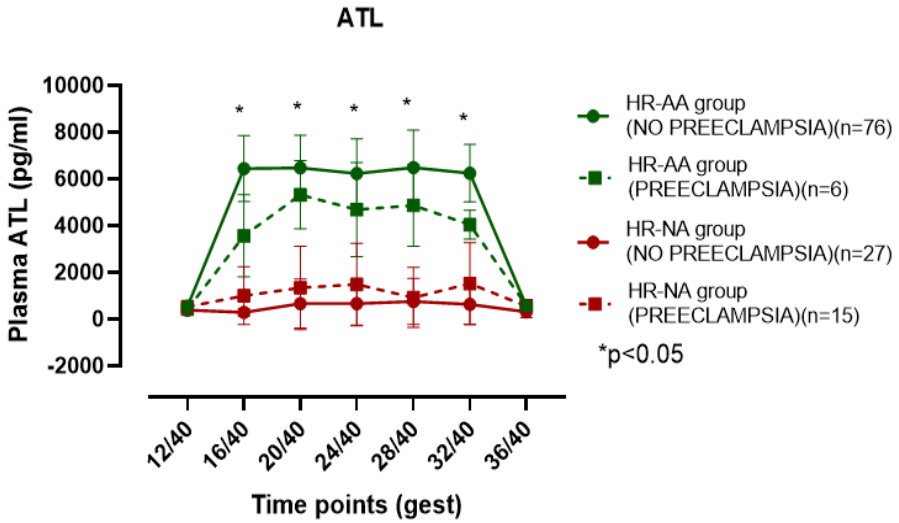


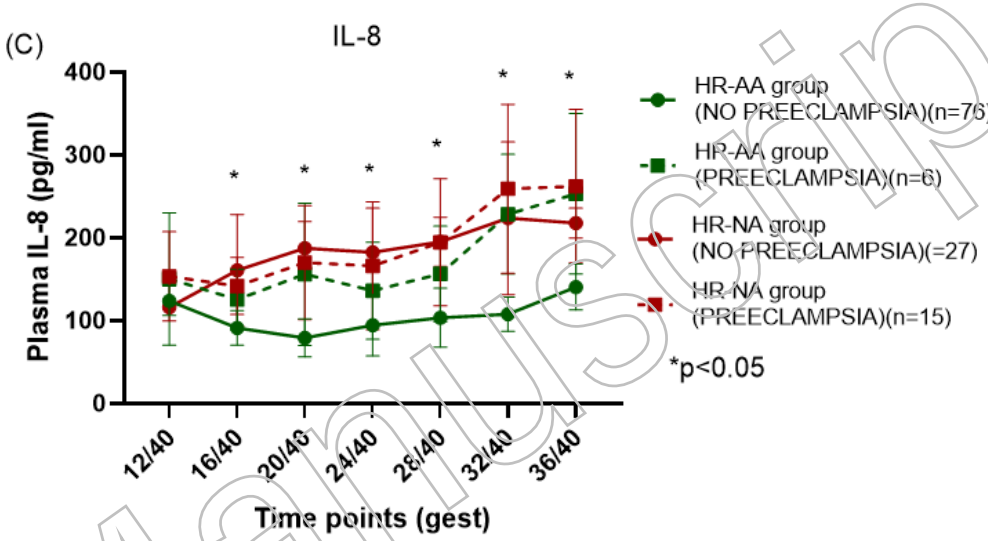
Figure 4: (A) Comparison of plasma En-Lipoxin-A4 concentration between preeclampsia and non-preeclampsia groups. (B) Comparison of plasma 15-epilipoxin-A4 (ATL) concentration between preeclampsia and non-preeclampsia groups. (C) Comparison between plasma IL-8 concentration between preeclampsia and non-preeclampsia groups and (D) Comparison between plasma IL-10 levels between preeclampsia and non-preeclampsia groups.

*statistically significant at <0.05. For Figures 4 (B-D), HR-AA women with no preeclampsia is represented by the green solid line with solid dot. HR-AA women with preeclampsia is represented by green dotted line with solid square. HR-NA women with no preeclampsia is represented by red solid line with solid dot. HR-NA women with preeclampsia is represented by red dotted line with solid square.

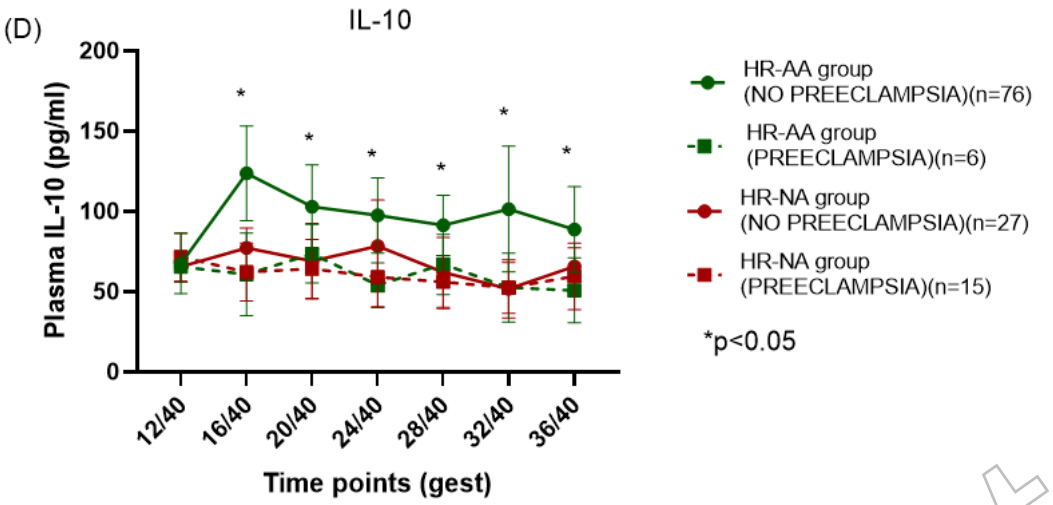
(B)



(C)



(D)



CHAPTER 5

SCHOLARLY RESEARCH PAPER 2:

PHARMACOKINETICS OF ASPIRIN

IN PREGNANCY

Publication in relation to this chapter:

SCHOLARLY RESEARCH PAPER 2*:*

A PHARMACOKINETIC ASSESSMENT OF OPTIMAL DOSING, PREPARATION AND CHRONOTHERAPY OF ASPIRIN IN PREGNANCY.

Renuka Shanmugalingam, XiaoSuo Wang, Gerald Munch, Ian Fulcher, Gaksoo Lee, Katrina Chau, Bei Xu, Roshika Kumar, Annemarie Hennessy, Angela Makris.

American Journal of Obstetrics and Gynaecology (AJOG), 22, 255.E1-E9 (2019).

DOI: https://doi.org/10.1016/j.ajog.2019.04.027

5.1 Study overview

The pharmacokinetics of medications in pregnancy is often altered and influenced by the physiological changes that occur throughout pregnancy. The altered liver metabolism and renal excretion leads to varied absorption, distribution and elimination of commonly used medications in pregnancy (117, 118). However, most pharmacokinetic data, including the pharmacokinetics of aspirin, have been extrapolated from healthy males (119, 120) and the data on its altered pharmacokinetics in pregnancy remains lacking.

At the time of this study, the optimal dose of aspirin in pregnancy remains unclear, with some data suggesting the need for dose adjustment (>100 mg), to obtain better risk reduction of preeclampsia (29, 42, 121). Current guidelines do not specify a recommended dose of aspirin but suggest a range of 75 mg – 150 mg with 100 mg being the most commonly suggested (39, 71). However, the increasing use of 150 mg since recent studies (29) is likely to impact clinical guidelines in the future (72). Nonetheless, there remains a significant paucity in clinical and pharmacokinetic data that directly compares the use of 100 mg to 150 mg of aspirin in pregnant women to support a definite change in clinical practice.

Additionally, pharmacology studies comparing varying preparations of aspirin in healthy male and female volunteers have demonstrated better platelet inhibition activity with non-EC aspirin compared to EC aspirin, which is often used for gastrointestinal protection (88). Once again, the pharmacokinetics of the various preparations of aspirin in pregnant women have not been examined and the influence on obstetric clinical outcomes remains

unknown. Another area of growing interest is the chronotherapy of daily aspirin. Hermida *et al* have demonstrated that ingestion of aspirin at bedtime results in better ambulatory blood pressure control and reduced incidence of hypertensive disorders of pregnancy amongst high-risk women (122, 123). However, the mechanism of this effect and the current definitive recommendation on this remains unclear.

This chapter of the thesis aimed to examine for; (1) the difference in pharmacokinetics of aspirin, through its major active metabolite, plasma salicylic acid (SA), in pregnant women compared to non-pregnant women, (2) the effect of dose (100 mg vs 150 mg), (3) preparation (EC vs non-EC) and, (4) chronotherapy of aspirin (morning vs night) between both groups. Plasma SA was intentionally used in place of plasma aspirin concentration given the short half-life of plasma aspirin (15 minutes) (119, 120).

Twelve HR pregnant women (from the longitudinal cohort study) and 3 non-pregnant women were enrolled into this study. Pregnant women were in one of four groups (100 mg EC, 100 mg non-EC, 150 mg non-EC morning dosing and 150 mg non-EC night dosing). Non-pregnant women undertook each of the four dosing schedules consecutively with a minimum 30-day washout period between each preparation. Blood samples were collected at baseline (pre-ingestion), 1, 2, 4, 6, 12 and 24-hours post-ingestion of aspirin (Figures 13 and 14).

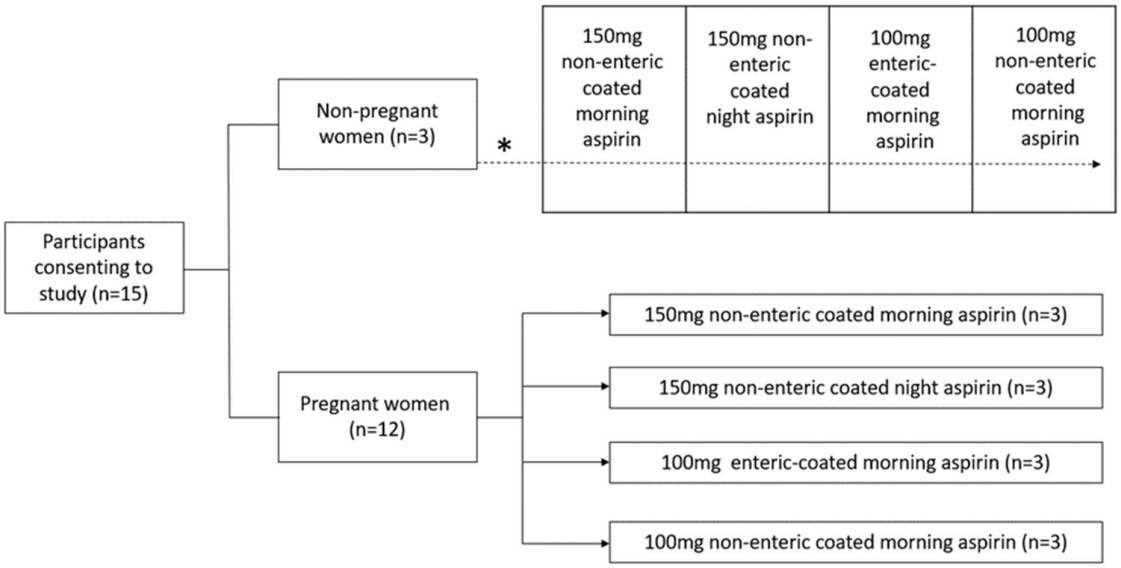


Figure 14: Illustrative representation of allocation of participants in the pharmacokinetics study.

Adapted from Shanmugalingam R. et al, A pharmacokinetic assessment of optimal dosing, preparation and chronotherapy of aspirin in pregnancy. American Journal of Obstetrics and Gynaecology (AJOG), 22, 255.E1-E9 (2019).

**Three non-pregnant females were used for all 4 subgroups in a cross-over pattern with a washout period of at least 30 days between differing aspirin preparation.*

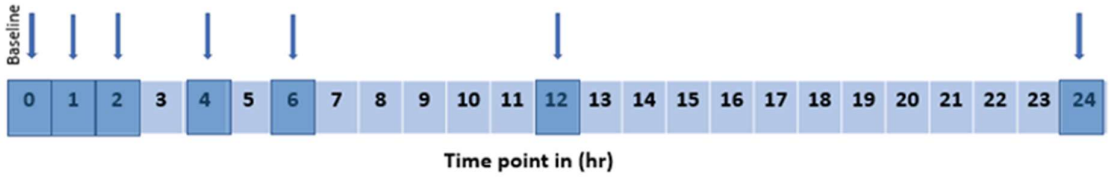


Figure 15: Time points for plasma sample collection

Time points at which plasma samples were collected are representation by the blue arrows. Baseline sample was collected prior to ingestion of daily dose of aspirin.

Plasma samples were analysed for SA through LCMS (Chapter 2.3.1). Pharmacokinetic values of area under the curve (AUC(t-24)), point of maximum concentration (Cmax), time of maximum concentration (Tmax), volume of distribution (Vd), clearance (CL) and elimination half-life (t½) (Figure 16) were obtained through a validated PKSolver® software (124) and analysed for statistical significance with SPSSv25.

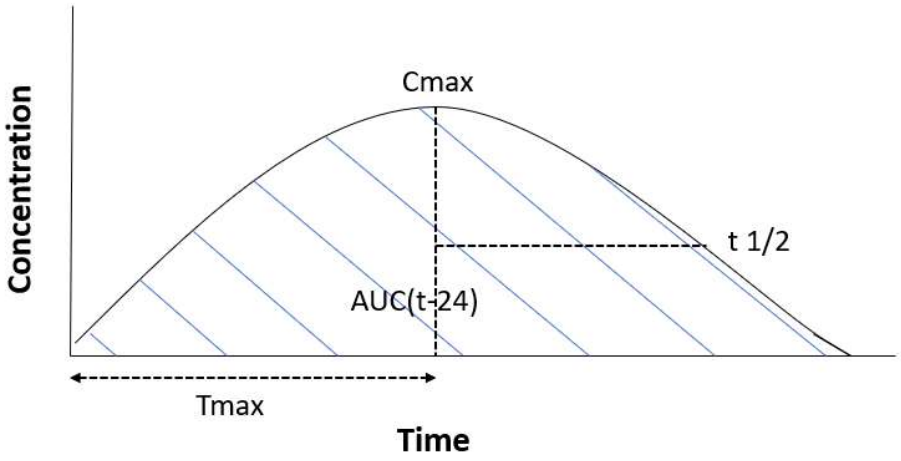


Figure 16: Illustrative representation of pharmacokinetic values assessed

AUC(t-24): area under the curve from 0-24 hours, Cmax: point of maximum concentration, Tmax: time point of maximum concentration, t ½: half life

5.2 Summary of key findings

Pregnant women were observed to have 40% ± 4% lower AUC(t-24) ($p < 0.01$), 29% ± 3% lower Cmax ($p<0.01$) and 44% ± 8% higher CL ($p<0.01$) in comparison to non-pregnant women with both 100 mg and 150 mg of aspirin. Therefore, suggesting, altered aspirin pharmacokinetics in pregnancy. The reduced AUC(t-24), with 100 mg of aspirin, however, was minimized with the use of 150 mg of aspirin in pregnant women. The AUC(t-24) achieved with 150 mg of aspirin was similar to that achieved with the use of

100 mg aspirin in non-pregnant women (Figure 17), therefore, suggesting the need for dose adjustment of aspirin in pregnancy.

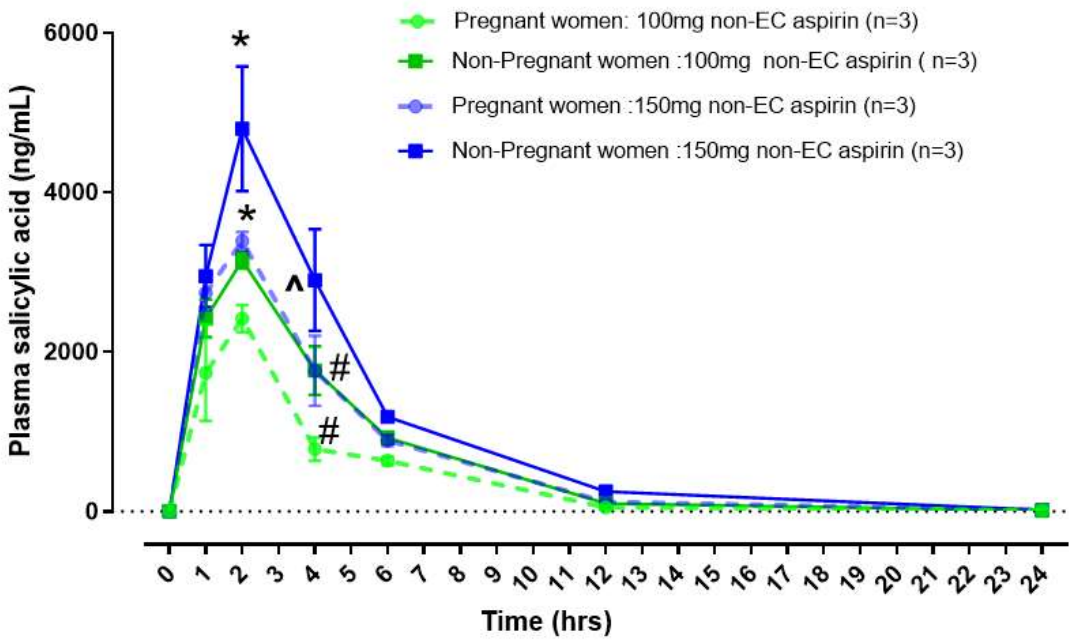


Figure 17: Illustrative representation of pharmacokinetics of aspirin in pregnant and non-pregnant women.

Adapted from Shanmugalingam R. et al, A pharmacokinetic assessment of optimal dosing, preparation and chronotherapy of aspirin in pregnancy. American Journal of Obstetrics and Gynaecology (AJOG), 22, 255.E1-E9 (2019). Pharmacokinetics of 100 mg of non-EC aspirin (green) compared to 150 mg of non-EC aspirin (blue) in pregnant (dotted lines) and non-pregnant women (solid lines). AUC(t-24) of 150 mg non-EC aspirin is 42% lower in pregnant women compared to non-pregnant women (p<0.01). # AUC of 100 mg non-EC aspirin is 38.6% lower in pregnant women compared to non-pregnant women (p<0.01). ^ The use of 150 mg of aspirin in pregnancy minimizes the pregnancy related reduction in AUC(t-24) and Cmax with the use of 100 mg of aspirin in pregnancy (125).*

There was a 4-hour delay ($p<0.01$) in Tmax, 47% ± 3% reduction in Cmax ($p<0.01$) and a 48% ± 1% increase in Vd ($p<0.01$) with the use of 100 mg EC aspirin compared to 100 mg non-EC aspirin with no difference in the overall AUC. There were no differences in pharmacokinetic values of aspirin with the comparison of morning and night dosing.

5.3 Strengths and limitations of this study

This is the first pharmacokinetic study to examine the effect of varying dose of aspirin in pregnancy in comparison to non-pregnant control women, making this a novel study at the time of this thesis. Additionally, the LCMS methodology used to detect, and measure plasma SA is unique in that it was optimized and validated for use in obstetric studies (126). The pharmacokinetic findings of this study strengthens the current data on the potential need for aspirin dose adjustment in pregnancy (121). However, further correlation with pharmacodynamic and clinical studies comparing 100 mg to 150 mg of aspirin in pregnancy will be required to better understand the optimal dose of aspirin in pregnancy.

This study has several limitations. Pregnant women with renal disease were excluded from this pharmacokinetic study. Given that SA is renally excreted, women with renal disease may have higher drug exposure. Whilst this is out of the scope of this study, the pharmacokinetics of aspirin in women with renal disease and the optimal dose in these women will require further exploration. Another limitation in this study is the small number of participants which limits the ability to examine for further confounders such as inter-gestational variation of pharmacokinetics and ingestion of aspirin in relation to

intake of food. Whilst Rymark *et al* did not demonstrate an inter-gestation variation in the pharmacokinetics of aspirin in the second and third trimester (117), the potential difference in the first trimester remains unknown and will be worth exploring in future pharmacokinetic studies. Similarly, the optimal timing of ingestion of aspirin in relation to food intake remains unclear with a recent systematic review demonstrating a lack of difference in overall bioavailability (127).

5.4 Conclusions

There is lower total drug metabolite concentration of aspirin in pregnancy compared to the non-pregnant state. This suggest a potential need for aspirin dose adjustment in pregnant women. This is likely due to the altered pharmacokinetics of aspirin in pregnancy with an increase in clearance. There was no difference in total drug metabolite concentration of aspirin between EC and non-EC aspirin nor between morning and night dosing of aspirin. However, further pharmacodynamic and randomized clinical studies are required to examine the clinical relevance of these pharmacokinetic findings.

OBSTETRICS

A pharmacokinetic assessment of optimal dosing, preparation, and chronotherapy of aspirin in pregnancy

Renuka Shanmugalingam, FRACP; XiaoSuo Wang, PhD; Gerald Münch, PhD; Ian Fulcher, RANZCOG; Gaksoo Lee, RN; Katrina Chau, PhD; Bei Xu, MBBS; Roshika Kumar, RN; Annemarie Hennessy, PhD; Angela Makris, PhD

BACKGROUND: The benefit of aspirin in preventing preeclampsia is well established; however, studies over the years have demonstrated variability in outcomes with its use. Potential contributing factors to this variation in efficacy include dosing, time of dosing, and preparation of aspirin.

OBJECTIVE: We aimed to compare the difference in pharmacokinetics of aspirin, through its major active metabolite, salicylic acid, in pregnant women and nonpregnant women, and to examine the effect of dose (100 mg vs 150 mg), preparation (enteric coated vs non−enteric-coated), and chronotherapy of aspirin (morning vs evening) between the 2 groups.

MATERIALS AND METHODS: Twelve high-risk pregnant women and 3 nonpregnant women were enrolled in this study. Pregnant women were in 1 of 4 groups (100 mg enteric coated, 100 mg non−enteric-coated, 150 mg non−enteric-coated morning dosing, and 150 mg non−enteric-coated evening dosing), whereas nonpregnant women undertook each of the 4 dosing schedules with at least a 30-day washout period. Blood samples were collected at baseline (before ingestion) and at 1, 2, 4, 6, 12, and 24 hours after ingestion of aspirin. Plasma obtained was analyzed for salicylic acid levels by means of liquid chromatography−mass spectrometry. Pharmacokinetic values of area under the curve from time point 0 to 24 hours point of maximum concentration, time of maximum concentration, volume of distribution, clearance, and elimination half-life were analyzed for statistical significance with SPSS v25 software.

RESULTS: Pregnant women had a 40% ± 4% reduction in area under the curve from time point 0 to 24 hours ($P < .01$) and 29% ± 3% reduction in point of maximum concentration ($P < .01$) with a 44% ± 8% increase in clearance ($P < .01$) in comparison to that in nonpregnant women when 100 mg aspirin was administered. The reduction in the area under the curve from time point 0 to 24 hours, however, was minimized with the use of 150 mg aspirin in pregnant women, with which the area under the curve from time point 0 to 24 hours was closer to that achieved with the use of 100 mg aspirin in nonpregnant women. There was a 4-hour delay ($P < .01$) in the time of maximum concentration, a 47% ± 3% reduction in point of maximum concentration ($P < .01$) and a 48% ± 1% increase in volume of distribution ($P < .01$) with the use of 100 mg enteric-coated aspirin compared to non−enteric-coated aspirin, with no difference in the overall area under the curve. There was no difference in the pharmacokinetics of aspirin between morning and evening dosing.

CONCLUSION: There is a reduction in the total drug metabolite concentration of aspirin in pregnancy, and therefore a dose adjustment is potentially required in pregnant women. This is likely due to the altered pharmacokinetics of aspirin in pregnancy, with an increase in clearance. There was no difference in the total drug metabolite concentration of aspirin between enteric-coated and non−enteric-coated aspirin and between morning and evening dosing of aspirin. Further pharmacodynamic and clinical studies are required to examine the clinical relevance of these pharmacokinetic findings.

Key words: aspirin, dose, pharmacokinetics, preeclampsia, pregnancy

The pharmacokinetics of medications in pregnancy is influenced by the maternal physiological changes that occurs through all three trimesters of pregnancy. These changes lead to an alteration in the absorption, distribution, and elimination of commonly used medications in pregnancy.[1,2] However, most pharmacokinetic studies of medications commonly used in pregnancy, such as aspirin, have been conducted in healthy males.[3,4]

The prophylactic use of aspirin to prevent preeclampsia has been studied over the last 40 years, but results are contradictory because of unanswered questions relating to its optimal application. The varying risk reduction of between 10% and 60% observed in previous studies has been largely attributed to the heterogeneity of studies for dosing, timing of ingestion, gestation at initiation of therapy, and type of aspirin preparation.[5−7]

Initial studies that demonstrated a prophylactic benefit of aspirin prescribed a daily dose of 150−300 mg.[8] Subsequent studies, however, argued for the use of low-dose therapy ranging from 75 to 150 mg daily,[5,9] whereas more recent studies suggest better clinical outcomes with the use of a 150-mg dose.[10,11] Current guidelines do not specify a recommended dose of aspirin but suggest a range of 75−150 mg, with 100 mg being the most commonly suggested dose.[12,13] The use of 150 mg in recent studies is likely to have an impact on clinical guidelines in the future[14]; however, there remains a significant paucity in clinical and pharmacokinetic data that directly compares the use of 100 mg to 150 mg aspirin in pregnant women to support such a change in clinical practice. It therefore remains unclear whether the use of 150 mg daily results in better bioavailability of aspirin and consequently better clinical outcomes.

Pharmacology studies comparing varying preparations of aspirin in

Cite this article as: Shanmugalingam R, Wang XS, Münch G, et al. A pharmacokinetic assessment of optimal dosing, preparation, and chronotherapy of aspirin in pregnancy. Am J Obstet Gynecol 2019;221:255.e1-9.

0002-9378/$36.00

https://doi.org/10.1016/j.ajog.2019.04.027

Click Supplemental Materials under article title in Contents at ajog.org

AJOG at a Glance

Why was this study conducted?

To address the lack of data and understanding on the pharmacokinetics of aspirin in pregnancy and its impact on the variable outcomes observed with its prophylactic role in preventing preeclampsia.

Key findings

The total drug exposure of aspirin is reduced in pregnancy compared to that in nonpregnant women and suggests the need for dose adjustment in pregnancy.

What does this add to what is known?

The physiological changes in pregnancy alters the pharmacokinetic of medication in pregnancy. Aspirin in now commonly used in high-risk pregnant women, and this finding will provide more insight into the potential need for dose adjustment in pregnancy.

healthy male and female volunteers have demonstrated better platelet inhibition activity with non−enteric-coated aspirin (non-EC) compared to enteric-coated aspirin (EC), which is often used for gastrointestinal protection.[15] Bhatt et al demonstrated a lack of platelet inhibition activity with EC aspirin in patients with diabetes, whereas others have demonstrated a lack of difference in platelet inhibition activity between the 2 preparations in healthy volunteers.[16,17] Once again, the pharmacokinetics of the various preparations of aspirin in pregnant women have not been examined, and the influence on obstetric clinical outcomes remains unknown.

Another area of growing interest is the chronotherapy of daily aspirin. Recent studies demonstrated that ingestion of aspirin at bedtime results in better ambulatory blood pressure control and reduced incidence of hypertensive disorders of pregnancy among high-risk women.[18] However, the mechanism of this effect is not understood, and the current recommendation on this remains unclear.

Based on the current gaps in the literature and clinical practice, we aimed to compare the pharmacokinetics of aspirin in pregnant vs nonpregnant women, and to examine the effect of dose (100 mg vs 150 mg), preparation (EC vs non-EC), and chronotherapy of aspirin (morning vs evening dosing) between the 2 groups.

Materials and Methods

Sample collection

Twelve pregnant women from high-risk pregnancy clinics within the South Western Sydney Local Health District (SWSLHD), NSW, Australia, gave written informed consent to participate in this study. Women were in 1 of 4 groups (100 mg EC, 100 mg non-EC, 150 mg non-EC morning dosing, and 150 mg non-EC evening dosing). Three nonpregnant women undertook each of the 4 dosing schedules with at least a 30-day washout period (Figure 1). Baseline clinical characteristics of the participants included age, ethnicity, body mass index (BMI), weight, gestation at time of study, and smoking status (Table 1). The type of aspirin consumed by healthy nonpregnant women was standardized. The aspirin consumed by pregnant participants was unaltered from their prescribed aspirin. At the time of publication, 150 mg EC aspirin was not commercially available for clinical use in Australia. Patients who were prescribed 150 mg aspirin by their clinicians were therefore advised to use half a tablet of 300 mg non-EC aspirin or one-and-a-half tablets of 100 mg non-EC aspirin. The pregnant and nonpregnant women in our study used half a tablet of 300 mg non-EC aspirin. Morning dosing of aspirin was set at 8 am ± 0.5 hour, and evening dosing was set at 2000 ± 1 hour. Ingestion and time of ingestion of aspirin were witnessed and verified by the attending investigator. Participants in this study took their aspirin right after consuming food. Women with underlying renal and liver dysfunction were excluded from this study.

Blood samples (4 mL) were collected via a 23G BD Vacutainer Push Button needle (Becton Dickinson, Franklin Lakes, NJ) at baseline (before ingestion) and 1, 2, 4, 6, 12, and 24 hours after ingestion of aspirin. Blood samples were collected into VACUETTE® K2EDTA tubes (Greiner Bio-One International) and were centrifuged immediately at 3000 rpm for 10 minutes. Plasma was then aliquoted and stored at −80°C until analysis.

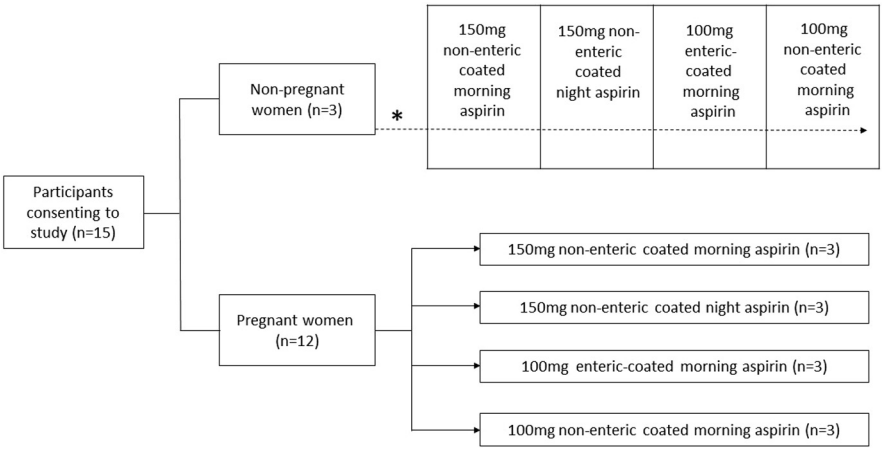
Ethics approval for this study was obtained from the SWSLDH ethics committee (HE 16/184).

Sample preparation for liquid chromatography−mass spectrometry analysis

Standards were prepared using 100 μL blank human plasma with known concentrations of salicylic acid (SA) (Sigma Aldrich, Castle Hill, NSW, Australia). Salicylic acid was dissolved and diluted with 100% methanol before spiking blank plasma with known concentrations of 0 ng/mL, 10 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL, 200 ng/mL, and 500 ng/mL. The standards were then spiked with a fixed concentration of 125 ng deuterated salicylic acid (D4-SA) (Santa Cruz Biotechnology, Santa Cruz, CA) as an internal control and vortexed for 1 minute. Similarly, 100 μL of plasma at each time point was transferred into Eppendorf tubes and spiked with a fixed concentration of 125 ng D4-SA as internal control.

To precipitate protein, 400 μL of 100% of acetonitrile (ACN) (Lichrosolv; Merck Milipore, Baywater, VIC, Australia) was added to standards and samples and vortexed for 30 seconds. Samples and standards were then centrifuged at 16,168 *g* for 10 minutes (Eppendorf Microcentrifuge Model 5415R, Hamburg Germany), after which the supernatant was transferred into glass culture tubes (12 × 75-mm disposable culture tubes) and evaporated at 45°C using an Eppendorf Concentrator (Model 22331) for

FIGURE 1
Patient distribution. A total of 12 pregnant volunteers were subdivided into 4 groups as above



*Three nonpregnant women were used for all 4 subgroups in a crossover pattern with a washout period of at least 30 days between varying aspirin exposures.

Shanmugalingam et al. Altered pharmacokinetics of aspirin in pregnancy. Am J Obstet Gynecol 2019.

approximately 90 minutes. Samples and standards were then reconstituted and acidified with 1 mL of 0.1% formic acid (FA) (Lichrosolv; Merck Milipore, Baywater, VIC, Australia).

Samples and standards underwent solid phase extraction using Discovery DSC-18 1 mL solid phase extraction cartridges (Supelco, Bellefonte, PA) with a vacuum manifold. Cartridges were preconditioned with 1 mL of 100% methanol, then washed with 2 mL of 0.1% trifluoroacetic acid (TFA) (Lichrosolv; Merck Milipore, Baywater VIC, Australia). The sample and standards were applied to the column and then washed with 1 mL of water, after which they were eluted with 1 mL of 100% methanol. The methanol eluate was then evaporated at 45°C for 90 minutes. Samples were then reconstituted with 100μL of 0.1% FA and spun at 1.4×10^4 RPM for 5 minutes before transfer into high-performance liquid chromatography (HPLC) vial inserts.

Liquid chromatography—mass spectrometry methodology

Analysis was performed on an Agilent 1290 series UHPLC system coupled with 6460A triple quadrupole mass spectrometers (Agilent Technologies, Santa Clara, CA). The separation of SA in plasma was achieved by using an Agilent Zorbax Eclipse XDB-C18 (4.6 × 50 mm, 1.8 μm) column fitted with a UHPLC Zorbax Eclipse XDB-C18 (4.6 × 5 mm, 1.8 μm) guard column, with mobile phase A containing 0.1% formic acid in water and mobile phase B consisting of 0.1% formic acid in 90% acetonitrile in water. The injection volume was 5 μL with a total run of 7 minutes at flow rate of 0.5 mL/min. The gradient was started at 30% of B and increased to 90% at 2 minutes and was then maintained at 90% for another 2 minutes. It then increased to 100% in the next 0.5 minute, maintained at 100% for another minute, decreased to 30% by 6 minutes, and re-equilibrated for another minute at 30% before the next injection. Tandem mass spectrometry was performed using electrospray ionization equipped with jet stream technology in the negative mode using the following parameters: capillary spray voltage was held at 3500 V, drying gas flow of 10 L/min with temperature set at 325°C and nebulizer pressure at 45 psi. The optimal fragmentor voltage (90 V) and collision energy voltage (15 V) was obtained by flow injection analysis in MS2-product ion scan mode. The following MRM ion transitions were monitored: 136.9 ->93.10 (SA) and 141.00 -> 97.00 (D4-SA). Peak areas for SA relative to the internal standard D4-SA was used to interpolate a standard curve and then to calculate the SA present in standard and sample.

Data analysis

Data acquisition was performed using MassHunter B.07.01, and data analysis was conducted using MassHunter qualitative and quantitative software (version B.07.00; Agilent Technologies). The pharmacokinetic parameters of maximum concentration (C_{max}), time point of maximum concentration (T_{max}), area under the curve from time point 0 to 24 hours [$AUC_{(t\text{-}24)}$], volume of distribution (Vd), clearance (CL), and elimination half-life ($t_{½}$) were determined through a 2-compartmental analysis using PKSolver.[19] One-way analysis of variance (post hoc testing with Tukey test), 4-way analysis of variance (post hoc testing with Tukey test), and *t* tests were used for analysis of mean values with SPSS v25 software.

Results

The characteristics of the participants are described in Table 1. There was no statistically significant difference in the clinically relevant characteristic between the participants. This included age, weight, BMI, and gestation in pregnant women. None of the participants were smokers, and none were on a proton pump inhibitor or histamine H2 receptor antagonist.

Effect of varying dose of aspirin

The 150-mg non-EC group had a 40% ± 6% higher $AUC_{(t\text{-}24)}$ ($P = .01$) and 31% ± 2% higher C_{max} ($P = .02$) compared to 100-mg non-EC group for both pregnant and nonpregnant women (Figure 2 and Table 2). There was no difference in the $t_{½}$ and T_{max} in pregnant and nonpregnant women regardless of dosage. However, the mean $AUC_{(t\text{-}24)}$ was 41% ± 2% and 34% ± 4% lower in pregnant women in both the 100-mg non-EC and 150-mg non-EC aspirin groups, respectively ($P < .01$), with a 43% ± 8% increase in CL, in comparison to that in nonpregnant women. Similarly, the C_{max} was 25% ± 2% and

TABLE 1
Participant characteristics

	100 mg enteric-coated aspirin		100 mg non–enteric-coated aspirin		150 mg non–enteric-coated aspirin, morning dosing		150 mg non–enteric-coated aspirin, evening dosing	
	Pregnant women (n = 3)	Nonpregnant women (n = 3)[a]	Pregnant women (n = 3)	Nonpregnant women (n = 3)[a]	Pregnant women (n = 3)	Nonpregnant women (n = 3)[a]	Pregnant women (n = 3)	Nonpregnant women (n = 3)[a]
Age (mean)	32.2 ± 2	37.4 ± 5	31.4 ± 1	37.4 ± 5	31.8 ± 3	37.4 ± 5	33.2 ± 2	37.4 ± 5
Weight (kg) (mean)	71.8 ± 5	75.6 ± 7	72.9 ± 4	75.6 ± 7	70.8 ± 9	75.6 ± 7	73.9 ± 5	75.6 ± 7
BMI (mean)	25.7 ± 2	26.9 ± 2	26.4 ± 1	26.9 ± 2	26.1 ± 2	26.9 ± 2	26.8 ± 2	26.9 ± 2
Gestation (wk) (Mean)	24.6 ± 1	N/A	26.1 ± 1	N/A	25.2 ± 1	N/A	26.3 ± 1	N/A
Pre-existing comorbidities	Hypertension (2) Prior preeclampsia (2) SLE (1)	N/A	Hypertension (3) Type 2 DM (1) Prior preeclampsia (2)	N/A	Hypertension (2) SLE (1) Prior preeclampsia (3)	N/A	Hypertension (2) Prior preeclampsia (2) Type1 DM (1)	N/A
Ethnicity	White (2) South East Asian (1)	Mediterranean (2) South Asian (1)	White (1) South Asian (1) Middle Eastern (1)	Mediterranean (2) South Asian (1)	White (2) South Asian (1)	Mediterranean (2) South Asian (1)	White (1) South East Asian (1) African (1)	Mediterranean (2) South Asian (1)
Smoking status	Nonsmoker	Nonsmoker	Nonsmoker	Nonsmoker	Nonsmoker	Nonsmoker	Nonsmoker	Nonsmoker

Data are mean ± standard deviation. Numbers in parentheses are numbers of women.
BMI, body mass index; *DM*, diabetes mellitus; *N/A*, not applicable; *SLE*, systemic lupus erythematosus.
[a] Nonpregnant female participants were examined in a crossover pattern with a washout period of at least 30 days between aspirin groups.
Shanmugalingam et al. Altered pharmacokinetics of aspirin in pregnancy. Am J Obstet Gynecol 2019.

31% ± 3% lower in pregnant women in both the 100-mg non-EC and 150-mg non-EC aspirin groups, respectively ($P < .01$).

When comparing 150 mg in pregnancy against a regular dose of aspirin in nonpregnant women (100 mg), the difference in the pharmacokinetics between groups was minimal, with a difference of 5% in the $AUC_{(t\text{-}24)}$ and 2.3% in the C_{max}. This suggests that a higher dose of aspirin (150 mg) minimizes the pregnancy-related reduction in total drug exposure of 100 mg aspirin (Figure 2 and Table 2).

Effect of varying preparation of aspirin

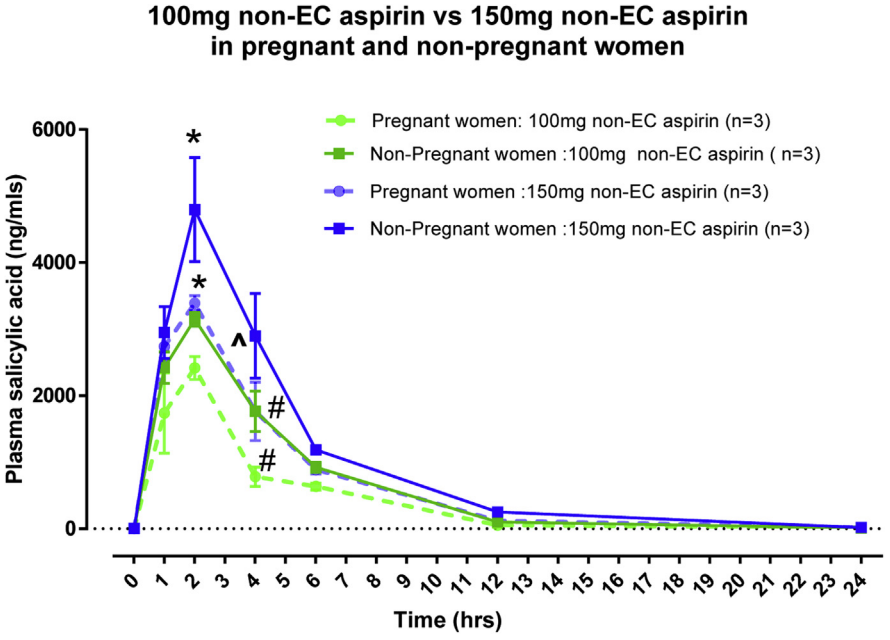
There was no difference in the $AUC_{t\text{-}24}$ and $t_{½}$ between the 100-mg EC and non-EC dosing. There was, however, a 4-hour delay ($P < .01$) in the T_{max} and a 47% ± 2% reduction in C_{max} ($P < .01$) as well as a 48% ± 1% increase in Vd ($P < .01$) with the use of 100 mg EC aspirin compared to non-EC aspirin (Figure 3 and Table 3).

Once again, pregnant women had a 43% ± 2% lower $AUC_{t\text{-}24}$ ($P = .03$) and a 27% ± 1% lower C_{max} ($P = .01$) respectively, with a 44% ± 1% increase in CL ($P < .01$) in both the EC and non-EC preparations of 100 mg aspirin compared to that in nonpregnant women (Figure 3 and Table 3).

Effect of chronotherapy of aspirin

In examining the influence of chronotherapy on the pharmacokinetics of aspirin, we compared morning (8 am ± 0.5 hour) and evening (8 pm ± 1 hour) dosing of 150 mg aspirin in pregnant and nonpregnant women. There was no difference in the $AUC_{(t\text{-}24)}$, C_{max}, T_{max}, and $t_{½}$ half between the 2 dosing regimens, indicating a lack of influence of chronotherapy on the pharmacokinetics of aspirin (Figure 4 and Table 4). We once again found that pregnant women had a 35% ± 4% lower $AUC_{(t\text{-}24)}$ ($P = .01$) and 24% ±1% lower C_{max} ($P = .02$) with a 46% ± 4% increase in CL compared to that in nonpregnant women in both morning and evening dosing of 150 mg non-EC aspirin groups (Figure 4 and Table 4).

FIGURE 2
Pharmacokinetics of 100 mg non−enteric-coated (non-EC) aspirin (green) compared to 150 mg non-EC aspirin (blue) in pregnant (dotted lines) and nonpregnant women (solid lines)



*Area under the curve from time point 0 to 24 hours [$AUC_{(t-24)}$] of 150 mg non-EC aspirin is 42% lower in pregnant women compared to nonpregnant women ($P < .01$). #Area under the curve of 100 mg non-EC aspirin is 38.6% lower in pregnant women compared to nonpregnant women ($P < .01$). ^Use of 150 mg aspirin in pregnancy minimizes the pregnancy-related reduction in the area under the curve (AUC) and the point of maximum concentration (C_{max}) with the use of 100 mg aspirin in pregnancy.

Shanmugalingam et al. Altered pharmacokinetics of aspirin in pregnancy. Am J Obstet Gynecol 2019.

A 4-way analysis of variance examining for multifactor relationship did not demonstrate a statistically significant 4-way interaction among dose, chronotherapy, pregnancy, and preparation ($F_{3,16} = 0.773$, $P = .5$).

Comment

Our study demonstrated a consistent reduction in total drug metabolite concentration of aspirin (measured through SA) in pregnancy, with an average 40% ± 4% reduction in $AUC_{(t-24)}$ and 29% ± 3% reduction in C_{max} with a 44% ± 8% increase in CL in comparison to that in nonpregnant women when 100 mg aspirin was administered. The reduced AUC with the use of 100 mg, however, was minimized with the use of 150 mg aspirin in pregnant women. Use of the 150-mg dose in pregnant women achieved total drug metabolite concentration similar to that from 100 mg aspirin in nonpregnant women. This therefore suggests that the physiological changes, such as increase in clearance, that occur in pregnancy highlight the potential need for aspirin dose adjustment in pregnancy. The only pharmacokinetic study of aspirin in pregnancy to date was conducted in 1994 by Rymark et al, who demonstrated that 75 mg aspirin in pregnant women in the second and third trimesters had a lower SA plasma peak

TABLE 2
Comparison of mean pharmacokinetic values between pregnant and nonpregnant women with both doses of non−enteric-coated (non-EC) aspirin

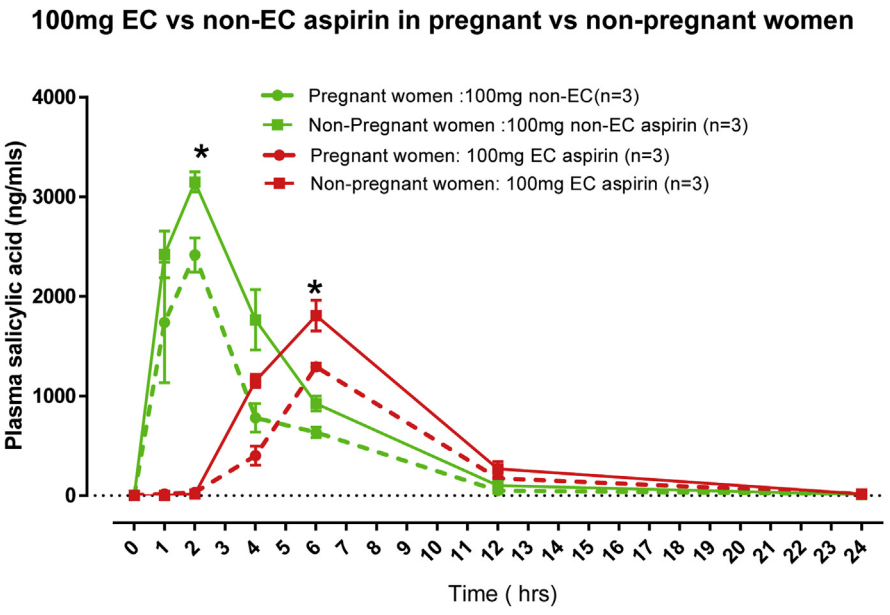
	100 mg Non-EC aspirin			150 mg Non-EC aspirin			
	Pregnant women (n=3)	Nonpregnant women (n=3)	*P* value[a]	Pregnant women (n=3)	Nonpregnant women (n=3)	*P* value[a]	*P* value[b]
$AUC_{(t-24)}$ (ng/mL*h)	12187.3 ± 100	21096.6 ± 532	<.01	19993.3 ± 240	29288.3 ± 430	<.01	.01
Tmax (h)	2	2	NS	2	2	NS	NS
C_{max} (ng/mL)	2417.2 ± 71	3214.7 ± 98	<.01	3289.3 ± 117	4797.5 ± 182	<.01	.02
t½ (h)	2.5 ± 0.3	2.7 ± 0.2	NS	2.6 ± 0.2	2.6 ± 0.4	NS	NS
Vd mg/(ng/mL)	0.08 ± 0.005	0.06 ± 0.005	NS	0.09 ± 0.002	0.07 ± 0.003	NS	NS
CL mg/(ng/mL)/h	0.006 ± 0.001	0.003 ± 0.001	<.01	0.009 ± 0.001	0.005 ± 0.001	<.01	NS

Data are mean ± standard deviation.
$AUC_{(t-24)}$, area under the curve from time point 0 to 24 hours; *CL*, clearance; *C_{max}*, point of maximum concentration; *NS*, not significant; *T_{max}*, time of maximum concentration; *t½*, elimination half-life; *Vd*, volume of distribution.

[a] *P* value represents the comparison between pregnant and nonpregnant women in the 100 mg non-EC aspirin group and the 150 mg non-EC aspirin groups, respectively; [b] *P* value represents the comparison between 100 mg non-EC aspirin and 150 mg non-EC aspirin.

Shanmugalingam et al. Altered pharmacokinetics of aspirin in pregnancy. Am J Obstet Gynecol 2019.

FIGURE 3
Pharmacokinetics of 100 mg enteric-coated (EC) aspirin (red) and 100 mg non—enteric-coated (non-EC) aspirin (green) in both pregnant (dotted lines) and nonpregnant (solid lines) women



*EC aspirin demonstrated a 4-hour delay in time of maximum concentration (T_{max}) ($P < .01$) and a 47% reduction in point of maximum concentration (C_{max}) ($P < .01$) compared to non-EC aspirin, with no difference in area under the curve from time point 0 to 24 hours [$AUC_{(t\text{-}24)}$] ($P =$ not significant).

Shanmugalingam et al. Altered pharmacokinetics of aspirin in pregnancy. Am J Obstet Gynecol 2019.

compared to that in nonpregnant women, with no intertrimester variation.[1] Despite the widespread use of aspirin in pregnancy, its altered pharmacokinetics in pregnancy and the impact of this on optimal dosing and clinical outcomes have not been adequately examined and remain unclear. This evidence suggests that we should stop extrapolating pharmacokinetic data from nonpregnant women and should undertake the relevant studies in pregnant women.

Aspirin is absorbed rapidly from the stomach and intestine by passive diffusion and is rapidly hydrolyzed, with a short half-life of 15 minutes, into salicylic acid.[3,4] Salicylic acid is responsible for aspirin's anti-inflammatory effects.[20] With repeated dosing, salicylic acid has been found to be the principal substance in plasma, with a half-life of 2 hours.[21] For these reasons, we chose to examine the pharmacokinetics of aspirin through detection of its hydrolyzed product, salicylic acid, as did Rymark et al. About 50—70% of salicylic acid in the blood is bound to albumin, whereas the rest remains in the active, ionized state.[3,20] The protein binding of salicylic acid is concentration dependent, and therefore saturation of binding sites leads to more free salicylic acid and eventually to toxicity.[20] The volume of distribution (Vd) of salicylic acid is low, at 0.1—0.2 L/kg, indicating that the majority of salicylic acid is confined to the plasma.[4] This is consistent with what we demonstrate in this study. As for its metabolism, 80% of salicylic acid is metabolized in the liver through conjugation with glycine to form salicyluric acid and with glucuronic acid to form 2 different glucuronide esters.[20]

TABLE 3
Comparison of mean pharmacokinetic values between pregnant and nonpregnant women in enteric-coated (EC) and non—enteric-coated (non-EC) 100-mg aspirin

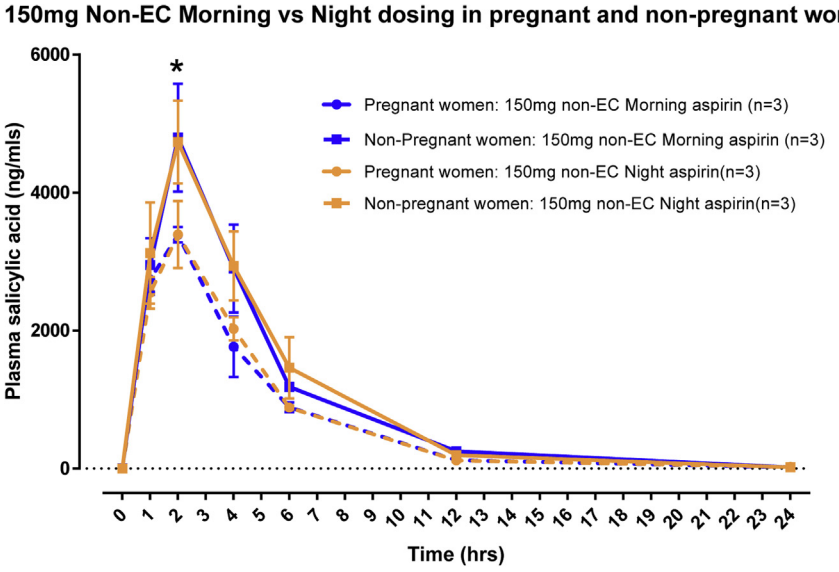
	100 mg Non-EC aspirin			100 mg EC aspirin			
	Pregnant women (n = 3)	Nonpregnant women (n = 3)	*P* value[a]	Pregnant women (n = 3)	Nonpregnant women (n = 3)	*P* value[a]	*P* value[b]
$AUC_{t\text{-}24}$ (ng/mL*h)	12187.3 ± 100	21096.6 ± 532	.03	11927.3 ± 104	22007.3 ± 118	.03	NS
Tmax (h)	2	2	NS	6	6	NS	<.01
Cmax (ng/mL)	2417.2 ± 71	3214.7 ± 98	.01	1275 ± 97	1808.3 ± 154	.01	<.01
t½ (h)	2.5 ± 0.3	2.7 ± 0.2	NS	2.6 ± 0.1	2.5 ± 0.3	NS	NS
Vd mg/(ng/mL)	0.08 ± 0.005	0.06 ± 0.005	NS	0.16 ± 0.01	0.13 ± 0.01	NS	<.01
CL mg/(ng/mL)/h	0.006 ± 0.001	0.003 ± 0.001	<.01	0.007 ± 0.001	0.004 ± 0.001	<.01	NS

Data are mean ± standard deviation.
$AUC_{(t\text{-}24)}$, area under the curve from time point 0 to 24 hours; *CL*, clearance; C_{max}, point of maximum concentration; *NS*, not significant; T_{max}, time of maximum concentration; t½, elimination half-life; *Vd*, volume of distribution.

[a] *P* value represents the comparison between pregnant and nonpregnant women in the 100-mg enteric-coated (EC) aspirin group and the non—enteric-coated (non-EC) group, respectively; [b] *P* value represents the comparison between 100 mg EC and non-EC aspirin.

Shanmugalingam et al. Altered pharmacokinetics of aspirin in pregnancy. Am J Obstet Gynecol 2019.

FIGURE 4
Pharmacokinetics of morning (blue) and evening (purple) dosing of 150 mg non—enteric coated (non-EC) aspirin in pregnant (dotted line) and nonpregnant (solid line) women



*There was no difference in the pharmacokinetics of morning and evening dosing of 150 mg aspirin.

Shanmugalingam et al. Altered pharmacokinetics of aspirin in pregnancy. Am J Obstet Gynecol 2019.

Salicylic acid is excreted mainly by the kidneys as salicyluric acid (75%), free salicylic acid (10%), salicylic phenol (10%), and acyl glucuronides (5%).[3,4]

In pregnancy, there is a progesterone-driven delay in gastrointestinal motility which results in a 30% increase in gastric emptying time and an approximately 40% reduction in gastric acidity.[22,23] These changes can result in ionization of weak acids such as aspirin and could potentially affect its absorption. Similarly, there have been arguments that the use of enteric-coated aspirin can also reduce its absorption.[15] Evidence to validate this assumption, however, has been contradictory. Our assessment on the influence of enteric coating demonstrated an expected delay in T_{max} with reduced C_{max} and an increase in Vd with no overall difference in $AUC_{(t\text{-}24)}$ between EC and non-EC aspirin. However, the influence of EC aspirin on the pharmacodynamic of aspirin in pregnancy remains unknown and is subject to further research.

A stronger influence on the alteration of pharmacokinetics in pregnancy is the variation in drug distribution and elimination. The physiological plasma expansion of 50% in pregnancy often results in altered drug volume of distribution (Vd) and is most prominent toward the end of the first trimester.[24,25] The total mean increase in the maternal body volume is approximately 8 L, and the resulting volume expansion results in a decrease in peak serum concentration (C_{max}), particularly of hydrophilic agents such as salicylate acid. The increased circulating estrogen and progesterone in pregnancy can either increase or decrease hepatic metabolism of drugs based on its stimulatory or inhibitory influence on the cytochrome P450 isoenzymes and uridine 5'-diphosphoglucuronosyltransferase isoenzyme activity.[24,26,27] Glucuronidation activity may be a critical determinant of aspirin efficacy; however, the enzymes responsible for this conjugation have yet to be

TABLE 4
Comparison of mean pharmacokinetic values between pregnant and nonpregnant women in both morning and evening dosing of 150 mg non—enteric-coated (non-EC) aspirin

	150 mg Non-EC aspirin, morning dosing			150 mg Non-EC aspirin, evening dosing			
	Pregnant women (n = 3)	Nonpregnant women (n = 3)	*P* value[a]	Pregnant women (n = 3)	Nonpregnant women (n = 3)	*P* value[a]	*P* value[b]
AUC $_{t\text{-}24}$ (ng/mL*h)	19993.3 ± 240	29288.3 ± 430	.01	21417.7 ± 428	30048.2 ± 296	.01	NS
Tmax (h)	2	2	NS	2	2	NS	NS
Cmax (ng/mL)	3289.3 ± 117	4797.5 ± 182	.02	3439.6 ± 256	4276.5 ± 238	.02	NS
t½ (h)	2.6 ± 0.2	2.6 ± 0.4	NS	2.8 ± 0.5	2.5 ± 0.5	NS	NS
Vd mg/(ng/mL)	0.09 ± 0.002	0.07 ± 0.003	NS	0.08 ± 0.01	0.07 ± 0.01	NS	NS
CL mg/(ng/mL)/h	0.009 ± 0.001	0.005 ± 0.001	<.01	0.008 ± 0.001	0.003 ± 0.001	<.01	NS

Data are mean ± standard deviation. *$AUC_{(t\text{-}24)}$*, area under the curve from time point 0 to 24 hours; *CL*, clearance; *C_{max}*, point of maximum concentration; *NS*, not significant; *T_{max}*, time of maximum concentration; *t½*, elimination half-life; *Vd*, volume of distribution.

[a] *P* value represents the comparison between pregnant and nonpregnant women in the morning and evening dosing groups, respectively; [b] *P* value represents the comparison between morning and evening dosing of aspirin.

Shanmugalingam et al. Altered pharmacokinetics of aspirin in pregnancy. Am J Obstet Gynecol 2019.

identified and characterized.[28] Therefore, the influence of pregnancy on the hepatic metabolism of salicylic acid remains subject to further research.

The relationship between increased creatinine clearance and drug excretion in pregnancy is well recognized and has been demonstrated through various studies, including ours, which demonstrated a 45% increase in clearance of SA.[24,29,30] Renal drug excretion is dependent on the glomerular filtration rate (GFR), tubular secretion, and resorption. In pregnancy, GFR is increased by 50% from the first trimester and continues to increase until the last week of pregnancy.[24,31] Therefore, the clearance of drugs and metabolites that are excreted renally, such as salicyluric acid and salicylic acid, is thought to parallel the change in GFR in pregnancy, leading to lower C_{max} and $AUC_{(t\text{-}24)}$ as demonstrated in our study.[32] Plasma protein binding of drugs is known to reduce in pregnancy because of reduced albumin concentration and estrogen-induced reduced protein-binding of drugs.[29,33] Decreased protein binding increases the concentration of free drug and consequently increases the clearance of drugs and metabolites such as salicylates via the increased renal clearance in pregnancy. The net effect of reduced protein binding, however, is counterbalanced by the increased renal clearance of drugs and metabolites such as salicylates.

Although our study did demonstrate a reduced total drug metabolite concentration of aspirin in pregnancy with a potential need for dose adjustment in pregnancy, the clinical relevance of this will need to be examined through a randomized clinical study in pregnancy that directly compares the clinical outcomes with the use of 100 mg and 150 mg aspirin. In addition, more data on the safety of 150 mg aspirin in pregnancy is required. Our study did not demonstrate a difference in the pharmacokinetics between EC and non-EC aspirin in the pregnant and nonpregnant states. Similarly, we did not demonstrate a difference in the pharmacokinetics of aspirin in relation to chronotherapy. The current thought on the mechanism of aspirin in the prevention of preeclampsia focuses on 2 pathways that potentially influence placentation. This involves aspirin's influence on platelet aggregation (through the cyclooxygenase [COX]−1 pathway) and its immunomodulatory effect through the COX-2 pathway.[34,35] Given this, in translating our pharmacokinetic findings to clinical studies, there is a need to further examine a dose-dependent response to platelet aggregation and the immune pathway of preeclampsia. In addition to this, there is a need to examine its overall effect on the pertinent end-point—namely, the clinical outcomes of these women and their infants.

Our study has a few limitations. The study excluded pregnant women with renal disease. Given that salicylate acid is renally excreted, women with renal disease can potentially have higher drug exposure. Although this is beyond the scope of our study, the pharmacokinetics of aspirin in women with renal disease and the optimal dose in these women will require further exploration. Another limitation to our study is the small number of participants. Although our post hoc power analysis was close to 100% to address the main aim of this study, the sample size limited our ability to examine for further confounders such as intergestational variation of pharmacokinetics and ingestion of aspirin in relation to intake of food. Although Rymark et al did not demonstrate an intergestational variation in the pharmacokinetics of aspirin in the second and third trimesters,[1] the potential difference in the first trimester remains unknown and will be worth exploring in future pharmacokinetic studies. Similarly, the optimal timing of aspirin ingestion in relation to food intake remains unclear, with a recent systematic review demonstrating a lack of difference in overall bioavailability.[36] This, again, is worth exploring in future pharmacokinetic studies.

In conclusion, the physiological changes that occur in pregnancy alter the pharmacokinetics of aspirin in pregnancy, suggesting the need for aspirin dose adjustment in pregnancy. ■

Acknowledgments

We would like to thank the volunteers for participating in our study. We would also like the thank the Department of Renal Medicine of SWSLHD, Department of Obstetrics and Gynaecology of SWSLHD, Women's Health Initiative Translational Unit, SWSLHD (WHITU), and the PEARLS foundation for their support in conducting this study.

References

1. Rymark P, Berntorp E, Nordsjo P, Liedholm H, Melander A, Gennser G. Low-dose aspirin to pregnant women: single dose pharmacokinetics and influence of short term treatment on bleeding time. J Perinat Med 1994;22:205–11.
2. Alsmadi MM, Idkaidek N. Optimization of drugs pharmacotherapy during pregnancy using physiologically based pharmacokinetic models—an update. Curr Drug Metab 2018;19:972–8.
3. Levy G. Comparative pharmacokinetics of aspirin and acetaminophen. Arch Intern Med 1981;141:279–81.
4. Nagelschmitz J, Blunck M, Kraetzschmar J, Ludwig M, Wensing G, Hohlfeld T. Pharmacokinetics and pharmacodynamics of acetylsalicylic acid after intravenous and oral administration to healthy volunteers. Clin Pharmacol 2014;6:51–9.
5. Bujold E, Roberge S, Lacasse Y, et al. Prevention of preeclampsia and intrauterine growth restriction with aspirin started in early pregnancy: a meta-analysis. Obstet Gynecol 2010;116:402–14.
6. Askie LM, Duley L, Henderson-Smart DJ, Stewart LA, Group PC. Antiplatelet agents for prevention of pre-eclampsia: a meta-analysis of individual patient data. Lancet 2007;369:1791–8.
7. Rolnik DL, Wright D, Poon LC, et al. Aspirin versus placebo in pregnancies at high risk for preterm preeclampsia. N Engl J Med 2017;377:613–22.
8. Beaufils M, Uzan S, Donsimoni R, Colau JC. Prevention of pre-eclampsia by early antiplatelet therapy. Lancet 1985;1:840–2.
9. Roberge S, Nicolaides K, Demers S, Hyett J, Chaillet N, Bujold E. The role of aspirin dose on the prevention of preeclampsia and fetal growth restriction: systematic review and meta-analysis. Am J Obstet Gynecol 2017;216:110–20.
10. Rolnik DL, Wright D, Poon LCY, et al. ASPRE trial: performance of screening for preterm preeclampsia. Ultrasound Obstet Gynecol 2017;50:492–5.
11. Park F, Russo K, Williams P, Pelosi M, Puddephatt R, Walter M, et al. Prediction and prevention of early-onset pre-eclampsia: impact of aspirin after first-trimester screening. Ultrasound Obstet Gynecol 2015;46:419–23.
12. Tranquilli AL, Dekker G, Magee L, et al. The classification, diagnosis and management of the hypertensive disorders of pregnancy: a revised statement from the ISSHP. Pregnancy Hypertens 2014;4:97–104.
13. Lowe SA, Bowyer L, Lust K, McMahon LP, Morton M, North RA, et al. SOMANZ guidelines for the management of hypertensive disorders of

pregnancy 2014. Aust N Z J Obstet Gynaecol 2015;55:e1–29.
14. Brown MA, Magee LA, Kenny LC, et al. The hypertensive disorders of pregnancy: ISSHP classification, diagnosis & management recommendations for international practice. Pregnancy Hypertens 2018;13:291–310.
15. Cox D, Maree AO, Dooley M, Conroy R, Byrne MF, Fitzgerald DJ. Effect of enteric coating on antiplatelet activity of low-dose aspirin in healthy volunteers. Stroke 2006;37:2153–8.
16. Karha J, Rajagopal V, Kottke-Marchant K, Bhatt DL. Lack of effect of enteric coating on aspirin-induced inhibition of platelet aggregation in healthy volunteers. Am Heart J 2006;151:976.
17. Bhatt DL, Grosser T, Dong JF, et al. Enteric coating and aspirin nonresponsiveness in patients with type 2 diabetes mellitus. J Am Coll Cardiol 2017;69:603–12.
18. Ayala DE, Ucieda R, Hermida RC. Chronotherapy with low-dose aspirin for prevention of complications in pregnancy. Chronobiol Int 2013;30:260–79.
19. Zhang Y, Huo M, Zhou J, Xie S. PKSolver: an add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel. Comput Methods Programs Biomed 2010;99:306–14.
20. Levy G. Clinical pharmacokinetics of aspirin. Pediatrics 1978;62:867–72.
21. Netter P, Faure G, Regent MC, Procknal JA, Levy G. Salicylate kinetics in old age. Clin Pharmacol Ther 1985;38:6–11.
22. Macfie AG, Magides AD, Richmond MN, Reilly CS. Gastric emptying in pregnancy. Br J Anaesth 1991;67:54–7.
23. Chiloiro M, Darconza G, Piccioli E, De Carne M, Clemente C, Riezzo G. Gastric emptying and orocecal transit time in pregnancy. J Gastroenterol 2001;36:538–43.
24. Anderson GD. Pregnancy-induced changes in pharmacokinetics: a mechanistic-based approach. Clin Pharmacokinet 2005;44:989–1008.
25. Costantine MM. Physiologic and pharmacokinetic changes in pregnancy. Front Pharmacol 2014;5:65.
26. Koren G, Pariente G. Pregnancy-associated changes in pharmacokinetics and their clinical implications. Pharm Res 2018;35:61.
27. Tsutsumi K, Kotegawa T, Matsuki S, et al. The effect of pregnancy on cytochrome P4501A2, xanthine oxidase, and N-acetyltransferase activities in humans. Clin Pharmacol Ther 2001;70:121–5.
28. Kuehl GE, Bigler J, Potter JD, Lampe JW. Glucuronidation of the aspirin metabolite salicylic acid by expressed UDP-glucuronosyltransferases and human liver microsomes. Drug Metab Dispos 2006;34:199–202.
29. Pariente G, Leibson T, Carls A, Adams-Webber T, Ito S, Koren G. Pregnancy-associated changes in pharmacokinetics: a systematic review. PLoS Med 2016;13:e1002160.
30. Chamberlain A, White S, Bawdon R, Thomas S, Larsen B. Pharmacokinetics of ampicillin and sulbactam in pregnancy. Am J Obstet Gynecol 1993;168:667–73.
31. Davison JM, Dunlop W. Renal hemodynamics and tubular function normal human pregnancy. Kidney Int 1980;18:152–61.
32. Feghali M, Venkataramanan R, Caritis S. Pharmacokinetics of drugs in pregnancy. Semin Perinatol 2015;39:512–9.
33. Perucca E, Crema A. Plasma protein binding of drugs in pregnancy. Clin Pharmacokinet 1982;7:336–52.
34. Gil-Villa AM, Norling LV, Serhan CN, Cordero D, Rojas M, Cadavid A. Aspirin triggered-lipoxin A4 reduces the adhesion of human polymorphonuclear neutrophils to endothelial cells initiated by preeclamptic plasma. Prostaglandins Leukot Essent Fatty Acids 2012;87:127–34.
35. Cadavid AP. Aspirin: the mechanism of action revisited in the context of pregnancy complications. Front Immunol 2017;8:261.
36. Moore RA, Derry S, Wiffen PJ, Straube S. Effects of food on pharmacokinetics of immediate release oral formulations of aspirin, dipyrone, paracetamol and NSAIDs—a systematic review. Br J Clin Pharmacol 2015;80:381–8.

Author and article information

From the School of Medicine (Drs Shanmugalingam, Hennessy, and Makris), Western Sydney University, NSW, Australia; Department of Renal Medicine (Drs Shanmugalingam, Hennessy, and Makris, and Ms Lee), South Western Sydney Local Health District, NSW, Australia; Heart Research Institute (Drs Shanmugalingam, Chau, Xu, Hennessy, and Makris), University of Sydney, NSW, Australia; Women's Health Initiative Translational Unit (WHITU) (Drs Shanmugalingam, Hennessy, and Makris, and Ms Lee), South Western Sydney Local Health District, NSW, Australia; Bosch Mass Spectrometry Facility (Dr Wang), Bosch Institute, Faculty of Medicine and Health, University of Sydney, NSW, Australia; Pharmacology Unit, School of Medicine (Dr Münch), Western Sydney University, NSW, Australia; Department of Obstetrics and Gynaecology (Dr Fulcher and Ms Kumar), Liverpool Hospital, NSW, Australia.

Received Jan. 8, 2019; revised April 16, 2019; accepted April 24, 2019.

The authors report no conflicts of interest.

Presented in part as an oral presentation at the 2018 SOMANZ (Society of Obstetric Medicine Australia and New Zealand) annual scientific meeting, July 13—15, 2018, Cairns, Brisbane, Australia. Dr Shanmugalingam was awarded the SOMANZ Andrew Phippard Memorial Award for the best oral scientific presentation for this paper. Presented as a poster presentation at the 2018 Biannual congress of the International Society of Hypertension In Pregnancy (ISSHP), Oct. 6—9, 2018, Amsterdam, Netherlands.

Corresponding author: Renuka Shanmugalingam, FRACP. Renuka.shanmugalingam@health.nsw.gov.au

CHAPTER 6

SCHOLARLY RESEARCH PAPER 3:

CLINICAL INFLUENCE OF NON-ADHERENCE TO ASPIRIN IN HIGH-RISK PREGNANCY

Publication in relation to this chapter:

SCHOLARLY RESEARCH PAPER 3:

CLINICAL INFLUENCE OF NONADHERENCE WITH PROPHYLACTIC ASPIRIN IN PREVENTING PREECLAMPSIA IN HIGH-RISK PREGNANCIES: A MULTICENTER, PROSPECTIVE, OBSERVATIONAL COHORT STUDY.

Renuka Shanmugalingam, XiaoSuo Wang, Penelope Motum, Ian Fulcher, Gaksoo Lee, Roshika Kumar, Annemarie Hennessy, Angela Makris.

Hypertension, 75,1125–1132 (2020).

DOI: https://doi.org/10.1161/HYPERTENSIONAHA.119.14107

6.1 Study overview

Whilst the understanding on the prophylactic use of aspirin in preeclampsia prevention continues to improve, there remains the need to optimize the prescription and uptake of aspirin to maximize the observed risk reduction. The variable risk reduction with obstetric aspirin studies historically have been attributed to study heterogeneity with inconsistent dose, gestation of initiation of aspirin and risk stratification. Recent studies have better addressed these factors (38, 42, 70, 121, 125), however, the clinical burden of non-adherence with aspirin in pregnancy and its influence on the expected preeclampsia risk-reduction remains unclear. This is particularly important given that non-adherence to medications in pregnancy is reported to occur in 60-70% of women and is associated with poorer maternal and fetal outcomes (128, 129).

This chapter of the thesis aimed to examine for; (1) the prevalence of aspirin non-adherence amongst high-risk pregnant women and its influence on obstetric outcomes and (2) the correlation between quantitative and qualitative assessment of adherence with aspirin. HR women who were recruited into the longitudinal observational cohort study (Chapter 2.1) underwent both quantitative and qualitative assessment of adherence, in the form of PFA-100 and plasma SA assessment (Chapter 2.4.2) and self-reported assessment (Chapter 2.4.1) respectively.

6.2 Summary of key findings

Of the 220 high-risk women recruited, no women were aspirin resistant and 63 (44%) women demonstrated inadequate adherence of <90% (101). Adjusted logistic regression

analysis demonstrated that women with inadequate adherence (<90% adherence) had higher incidence of early-onset preeclampsia (17% vs 2%) (OR=1.9; 95% CI 1.1-8.7;p=0.04), late-onset preeclampsia (41% vs 5%)(OR=4.2; 95% CI 1.4-19.8;p=0.04), IUGR (29% vs 5%)(OR=5.8; 95% CI 1.2-8.3;p=0.001), preterm delivery (27% vs 10%)(OR=5.2; 95% CI 1.5-8.7;p=0.008) and higher likelihood of increase in antihypertensives antenatally (60% vs 10%)(OR=4.6; 95% CI 1.2-10.5;p=0.003)(101). Kaplan-Meier analysis demonstrated a lower incidence of premature delivery with a longer duration pregnancy (HR=0.3; 95% CI 0.2-0.5;p<0.001)(Figure 18) in the adherent group (101).

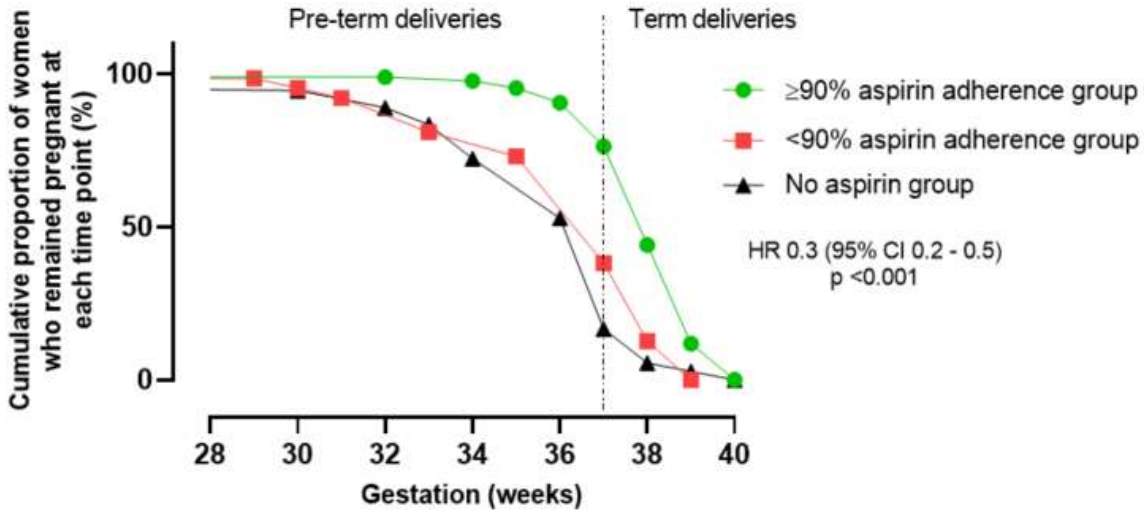


Figure 18: Kaplan-Meier analysis of preterm delivery between all three groups of analysis.

Adapted from Shanmugalingam et al. Clinical influence of nonadherence with prophylactic aspirin in preventing preeclampsia in high-risk pregnancies: a multicenter, prospective, observational cohort study. Hypertension, 75, 1125–1132 (2020).

Obstetric outcomes were also compared between the non-aspirin control group (non-aspirin group) and the group with inadequate adherence, where adherence was treated as an ordinal (<30% vs 31-60% vs 61-89% adherence) rather than dichotomous variable. Analysis comparing the four groups did not demonstrate any difference in the examined obstetric outcomes (101). An analysis between both methods of adherence assessment (quantitative; biochemical assessment and qualitative; self-reported adherence) demonstrated only a moderate agreement with ƙ=0.48, SE=0.029, p<0.0001(101).

Nine women who were commenced on aspirin after 16 weeks of gestation (between 16^{+1} and 20^{+0} weeks of gestation) were excluded for the purpose of the main analysis. However, a descriptive sub-analysis of these women demonstrated that 6 women (67%) met adherence criteria and of these 6 women, 2 developed late onset preeclampsia and 3 developed early onset preeclampsia despite being adherent, indicating the importance of timing of initiating aspirin (Table 8).

	Aspirin commenced after 16 weeks gestation & adherence criteria met (n=6)	**Aspirin commenced before 16 weeks of gestation & adherence criteria met (n=82)**
Early onset preeclampsia	3 (50%)	2(2%)
Late onset preeclampsia	2(30%)	4(5%)
Intra-uterine growth restriction	3(50%)	4(5%)
Preterm delivery	3(50%)	8(10%)

Table 8: Clinical outcomes in high-risk women who were commenced on aspirin after 16 weeks of gestation (between 16+1 and 20+0 weeks of gestation)

6.3 Strengths and limitations of this study

This study is novel in assessing for obstetric outcomes in a multi-centre, demographically diverse, cohort of high-risk pregnant women based on a reliable quantitative assessment of adherence with aspirin. Additionally, this study also assessed the clinical impact of intermittent adherence on obstetric outcome and agreement between quantitative and self-reported adherence with aspirin.

Limitations of this study include the variation in aspirin doses. In the aspirin adherent group, 72% of women were on 100 mg and 28% were on 150 mg of aspirin. This variation is due to the change in evidence during the period of this study (29). There was no cross over in dose taken by any woman. Given the small numbers of women in each subgroup, an analysis to compare obstetric outcomes between the two doses was underpowered and could not be performed. There was, however, no observed difference in maternal or fetal complications with both doses of aspirin (101) . This study did not assess for reliability of semi-qualitative method of adherence which is occasionally used in obstetric and non-obstetric studies. Another limitation is the exclusion of women of non-English speaking background. Language barrier has been shown to influence adherence with medications in non-obstetric studies (130) and, therefore, further studies examining the relationship between language and adherence as assessed by semi-qualitative and quantitative methods in obstetric studies would be beneficial.

6.4 Conclusions

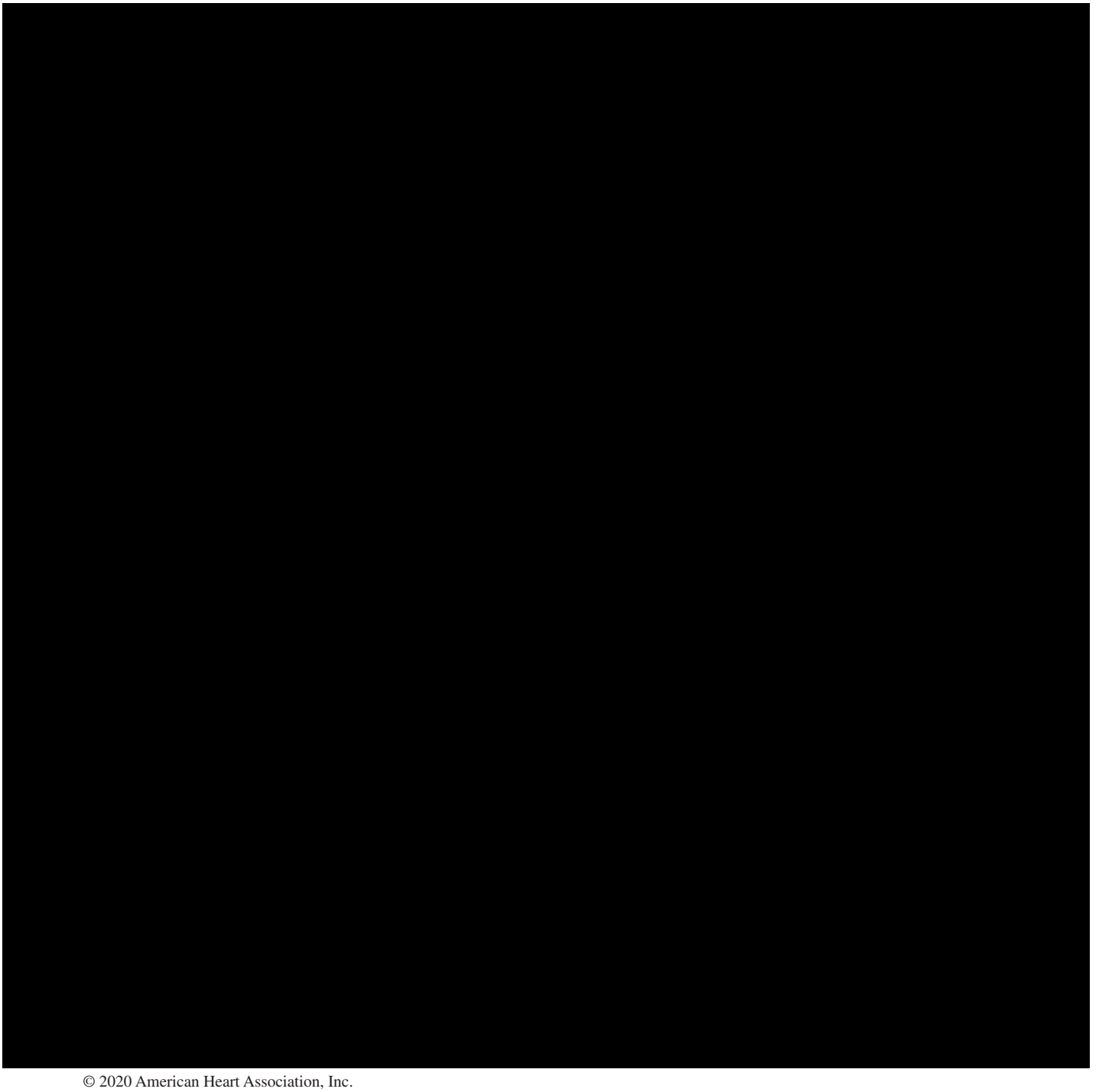
The data observed is in keeping with the current literature in supporting the prophylactic effect of aspirin with an absolute risk reduction of preeclampsia of 51%(NNT=2) in a high-risk cohort, but only where adherence was ≥90%. Women who were prescribed but <90% aspirin adherent, had a higher rate of preeclampsia, IUGR, delivery before 37 weeks of gestation and increase in antihypertensive requirements in pregnancy. This study also demonstrates that self-reported adherence does not accurately reflect actual adherence with only moderate agreement between both methods of assessment. Recent obstetric aspirin meta-analyses have shown the importance of initiating aspirin before 16 weeks of gestation. The sub-analysis described in section 6.2 demonstrated similar findings despite adequate adherence with aspirin (Table 8).

This study demonstrated the high, and often underrecognized prevalence of aspirin non-adherence amongst high-risk women and the significance of inadequate adherence on the desired preeclampsia risk reduction. This, therefore, highlights the importance of assessing for adherence to aspirin through a validated and reliable method in both clinical practice and research.

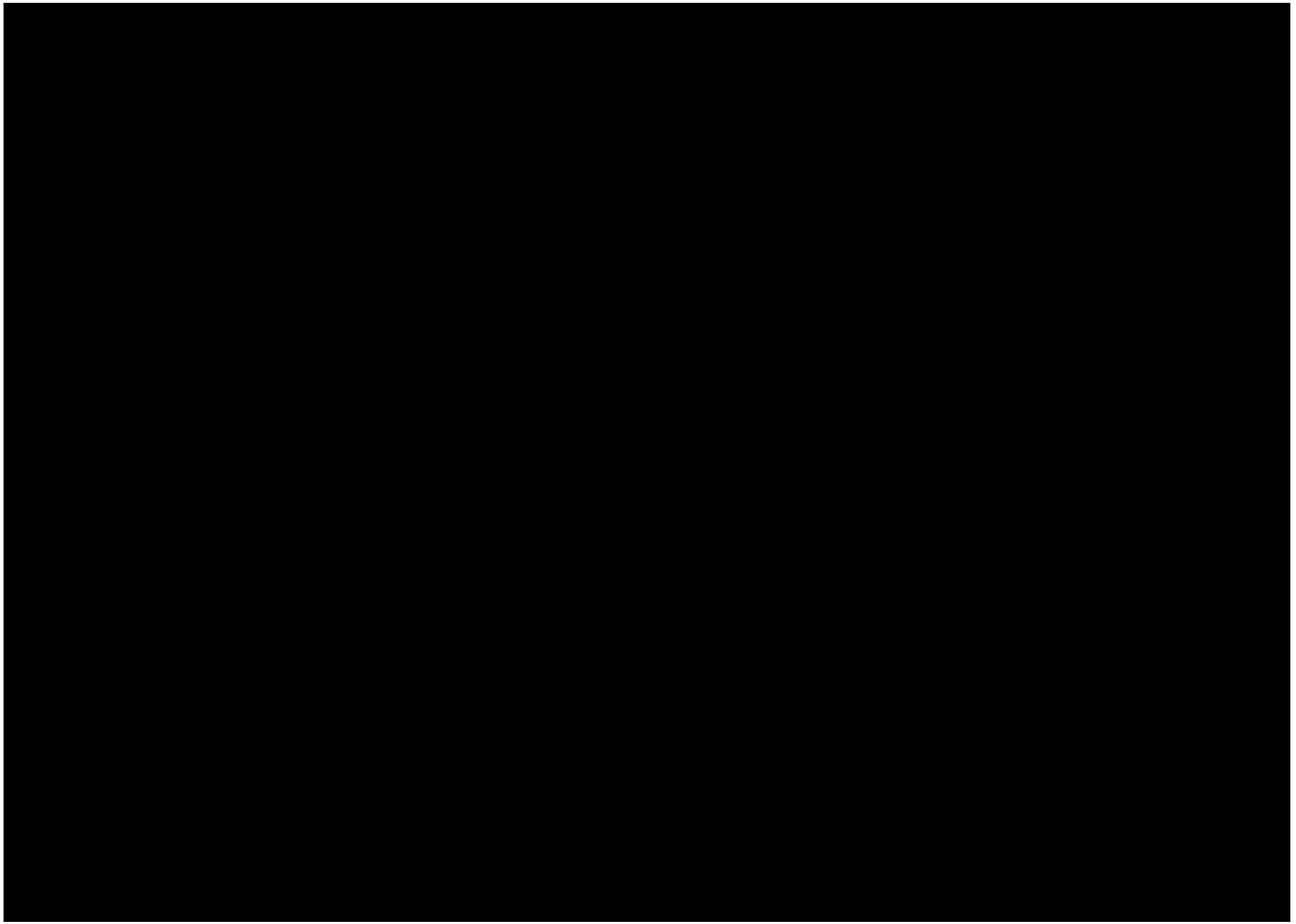
Clinical Influence of Nonadherence With Prophylactic Aspirin in Preventing Preeclampsia in High-Risk Pregnancies

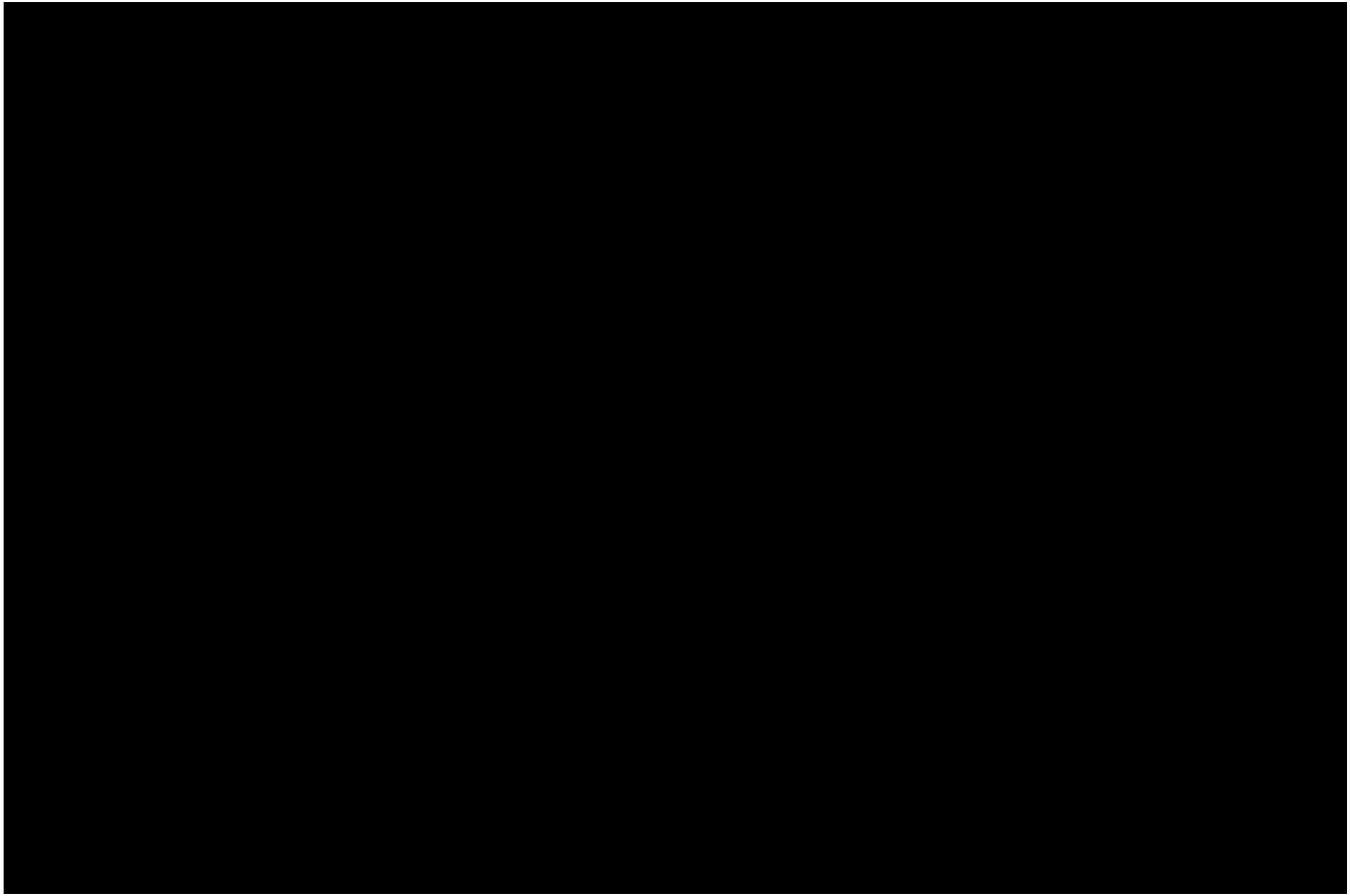
A Multicenter, Prospective, Observational Cohort Study

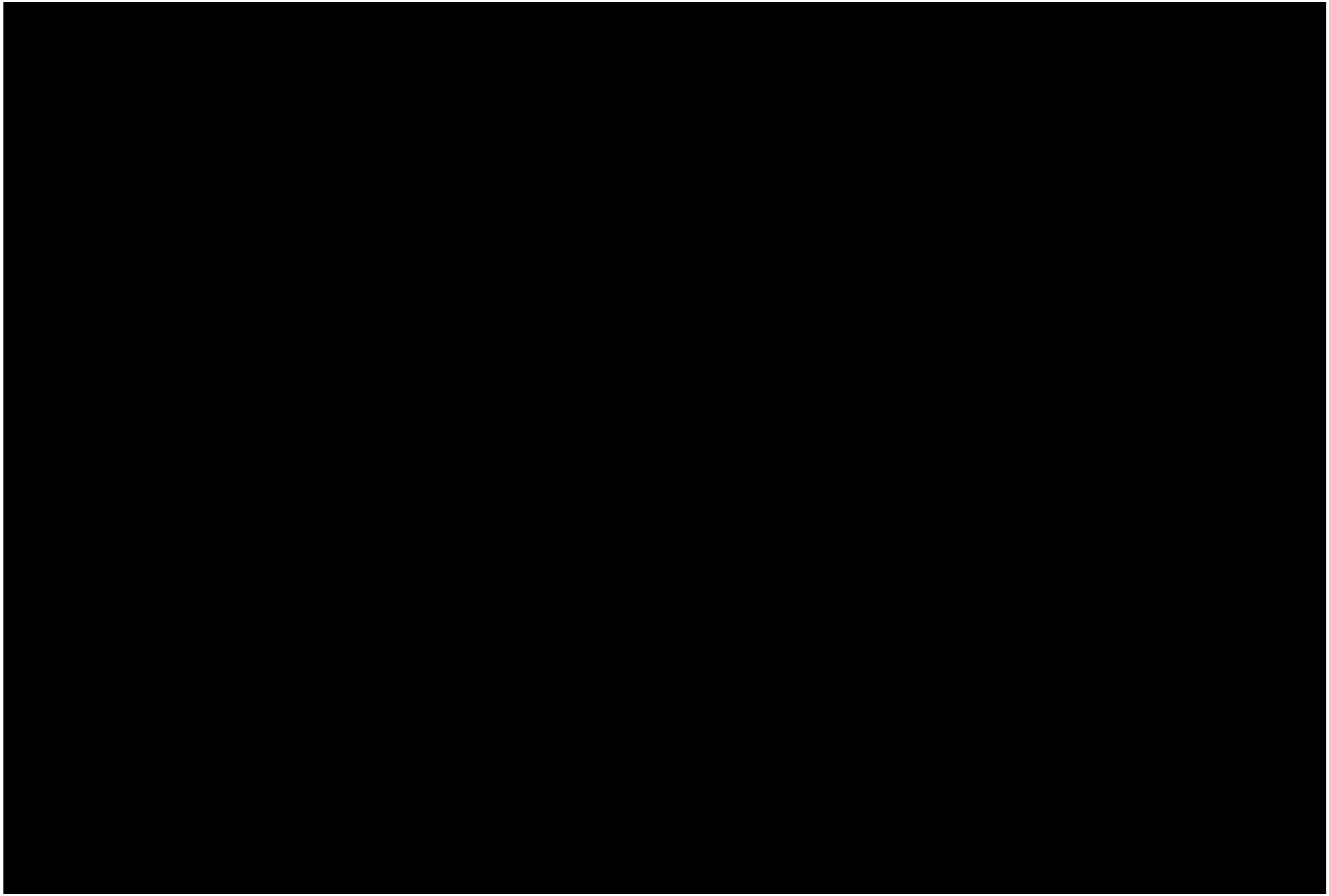
Renuka Shanmugalingam, XiaoSuo Wang, Penelope Motum, Ian Fulcher, Gaksoo Lee, Roshika Kumar, Annemarie Hennessy, Angela Makris

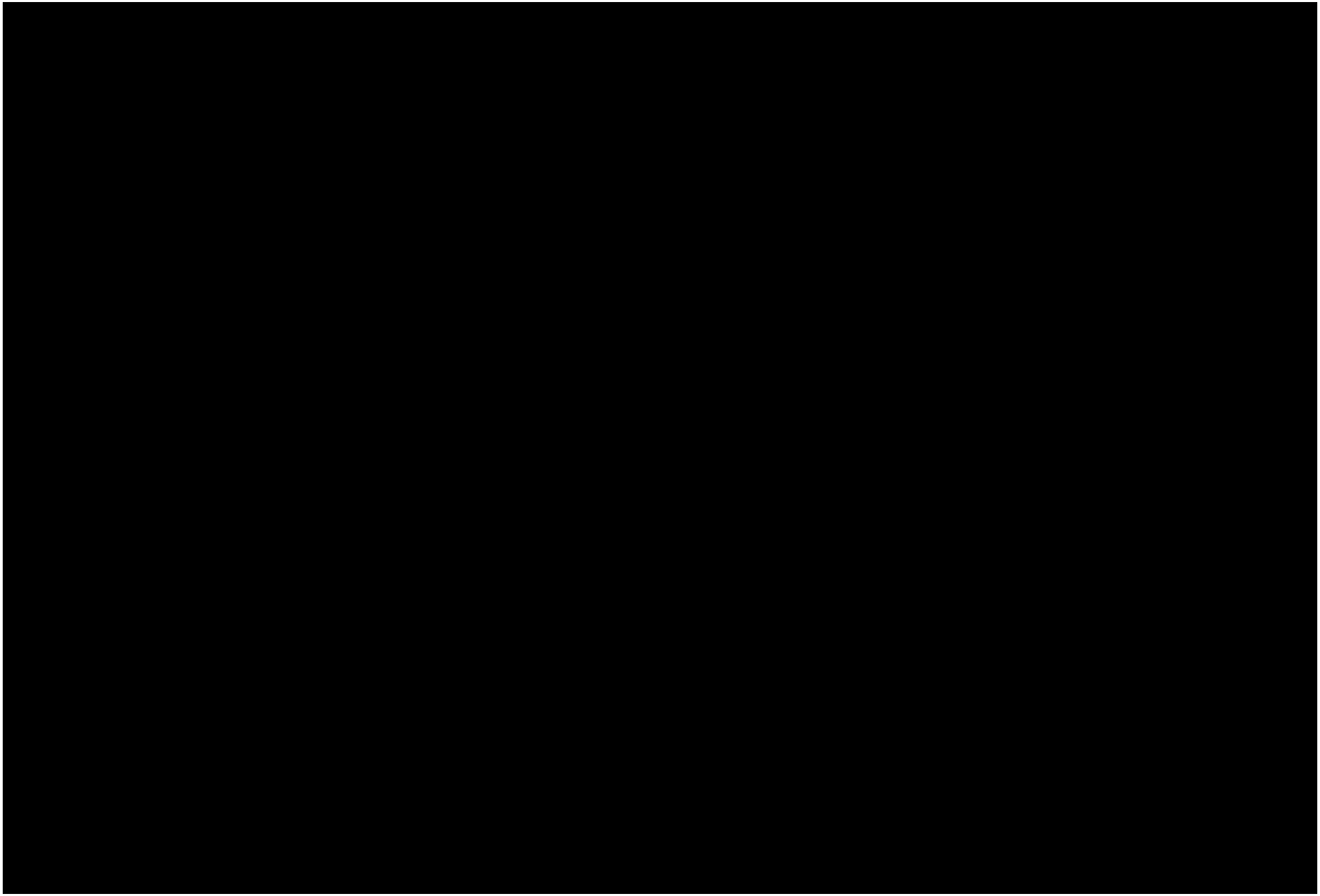


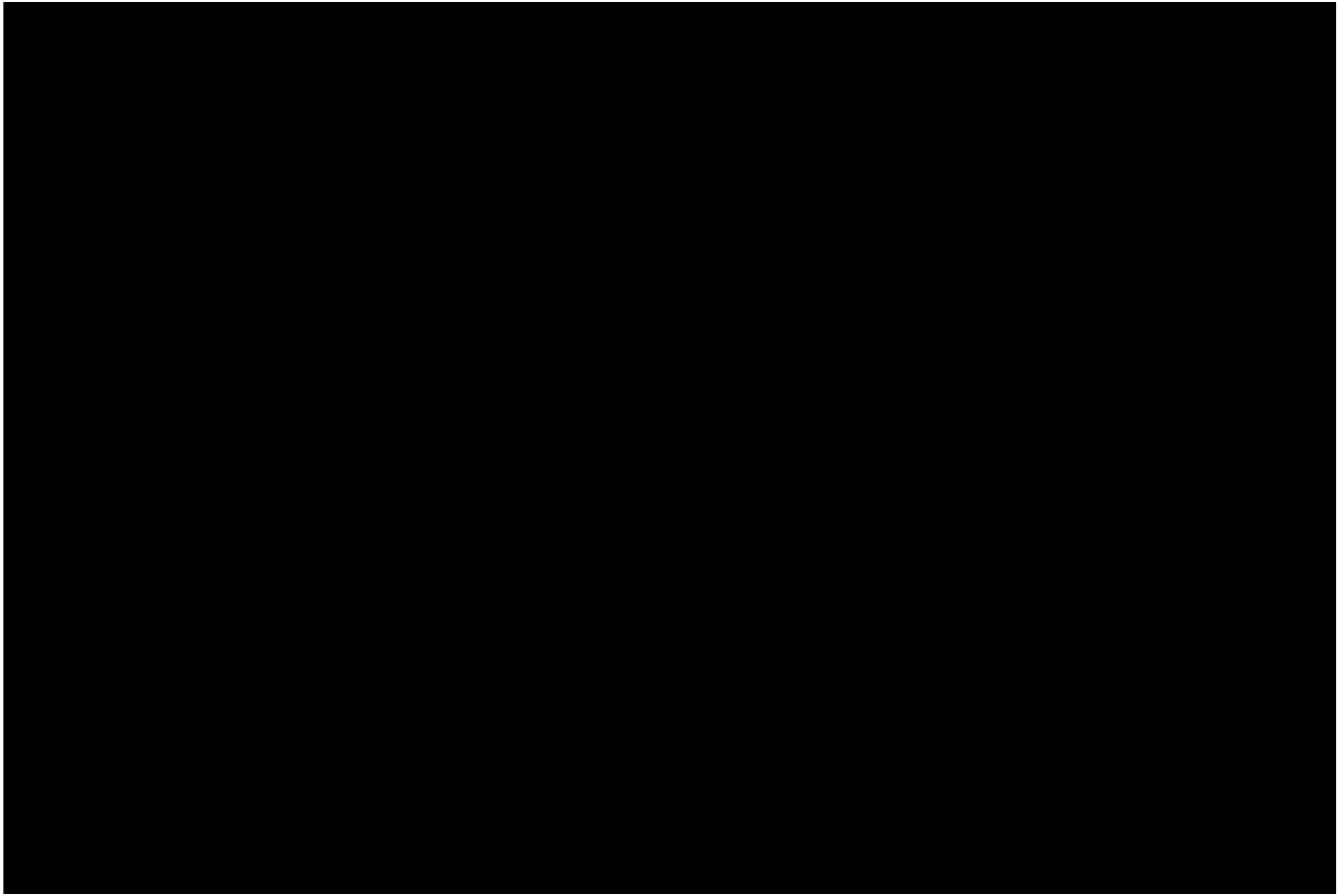
Hypertension **is available at https://www.ahajournals.org/journal/hyp** **DOI: 10.1161/HYPERTENSIONAHA.119.14107**

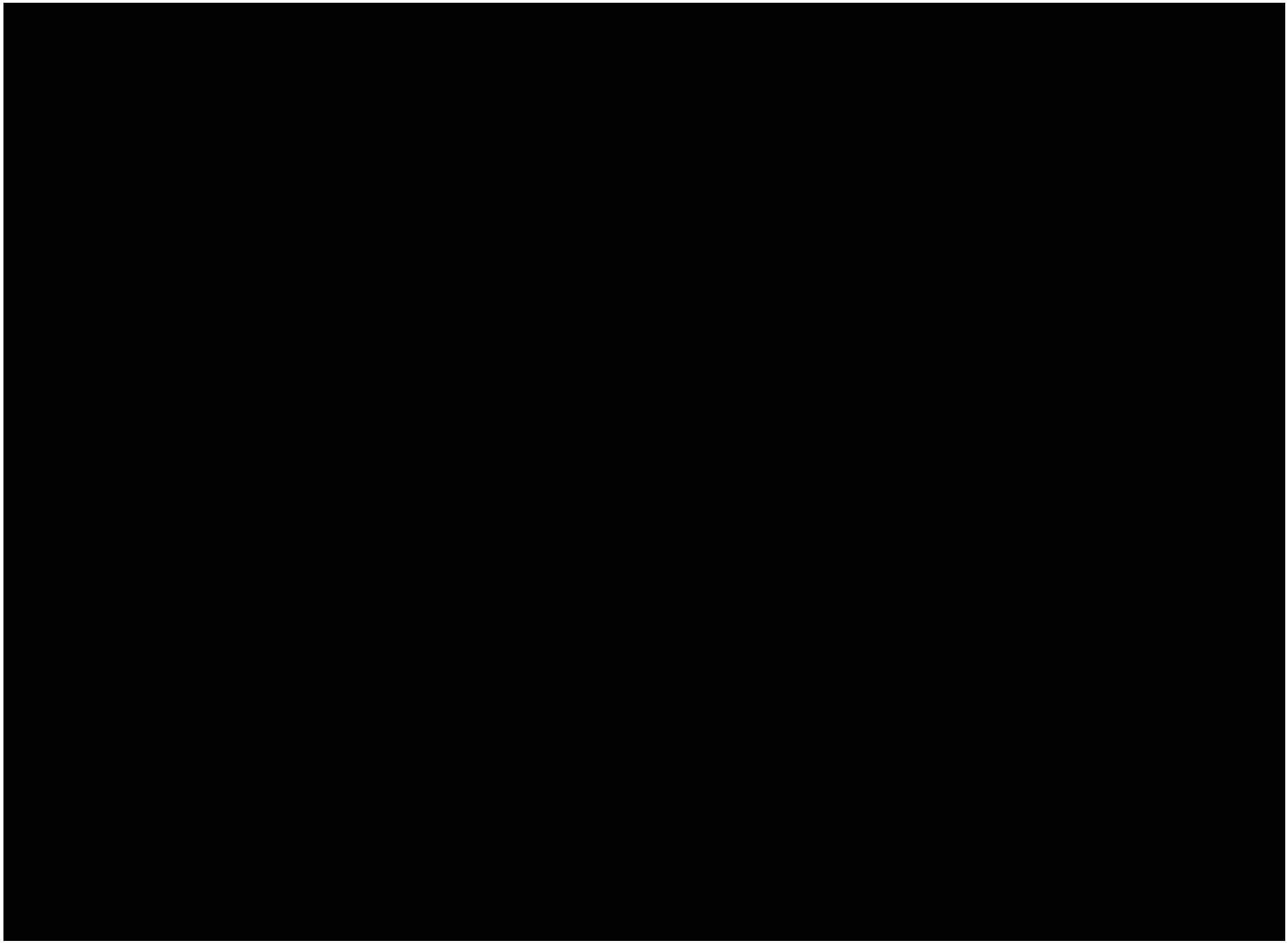


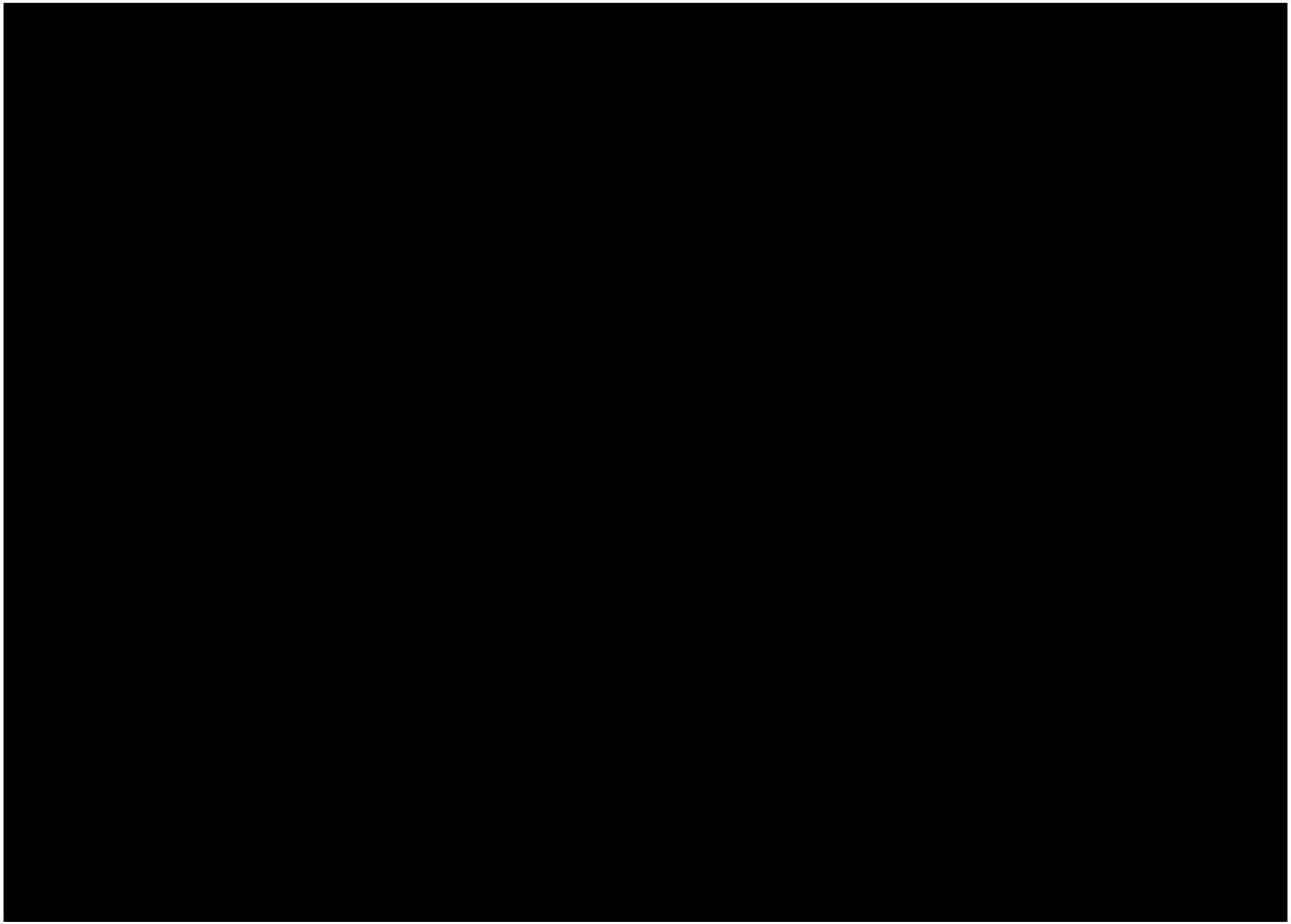


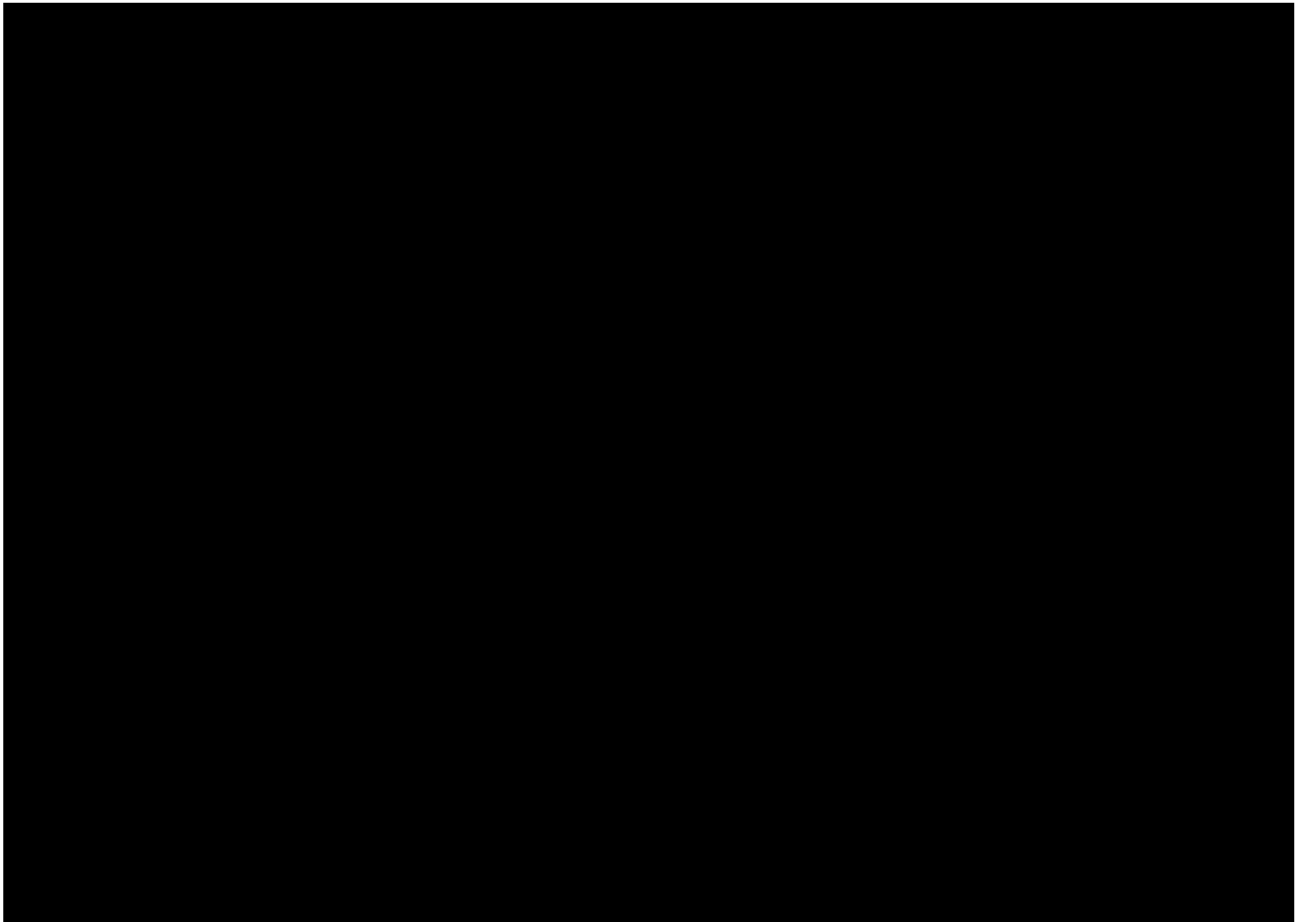












CHAPTER 7

SCHOLARLY RESEARCH PAPER 4:

FACTORS THAT INFLUENCE ADHERENCE TO ASPIRIN AMONG HIGH-RISK PREGNANT WOMEN

Publication from this study:

SCHOLARLY RESEARCH PAPER 4*:*

FACTORS THAT INFLUENCE ADHERENCE TO ASPIRIN THERAPY IN THE PREVENTION OF PREECLAMPSIA AMONGST HIGH-RISK PREGNANT WOMEN: A MIXED METHOD ANALYSIS.

Renuka Shanmugalingam, Zelalem Mengesha, Stephanie Notaras, Pranee Liamputtong, Ian Fulcher, Gaksoo Lee, Roshika Kumar, Annemarie Hennessy, Angela Makris.

PLoS ONE 15(2), e0229622 (2020)

DOI: https://doi.org/10.1371/journal.pone.0229622

7.1 Study overview

Non-adherence to medications in pregnancy is increasingly recognized and results in higher rates of potentially preventable maternal and fetal morbidity and mortality (129). The study described in Chapter 6 demonstrated that 44% of the high-risk pregnant women in the longitudinal cohort study were not adequately adherent with prophylactic aspirin. Consequently, this was associated with a higher incidence of preeclampsia, preterm delivery and intrauterine growth restriction (101). The factors that influences adherence to aspirin in pregnancy, from the women's perspective, remain poorly studied and understood.

This chapter of the thesis aimed to examine for the factors, from the women's perspective, that influenced adherence with prophylactic aspirin in their pregnancy. A sequential-exploratory designed mixed methods study involving a quantitative (n=122) and qualitative (n=6) survey was conducted. Women recruited in the longitudinal cohort study (Chapter 3 and Chapter 6) (101) were invited to participate in an electronic anonymous quantitative study and face-to-face qualitative interview as described in Chapter 2.5.

7.2 Summary of key findings

The study identified factors that both positively and negatively influenced adherence to aspirin amongst high-risk pregnant women. Two key themes, from the women's perspective, that influenced adherence to aspirin in pregnancy were; (1) pill burden and

non-intention omission (2) communication and relationship with their health care provider (HCP) (Figure 17)(131).

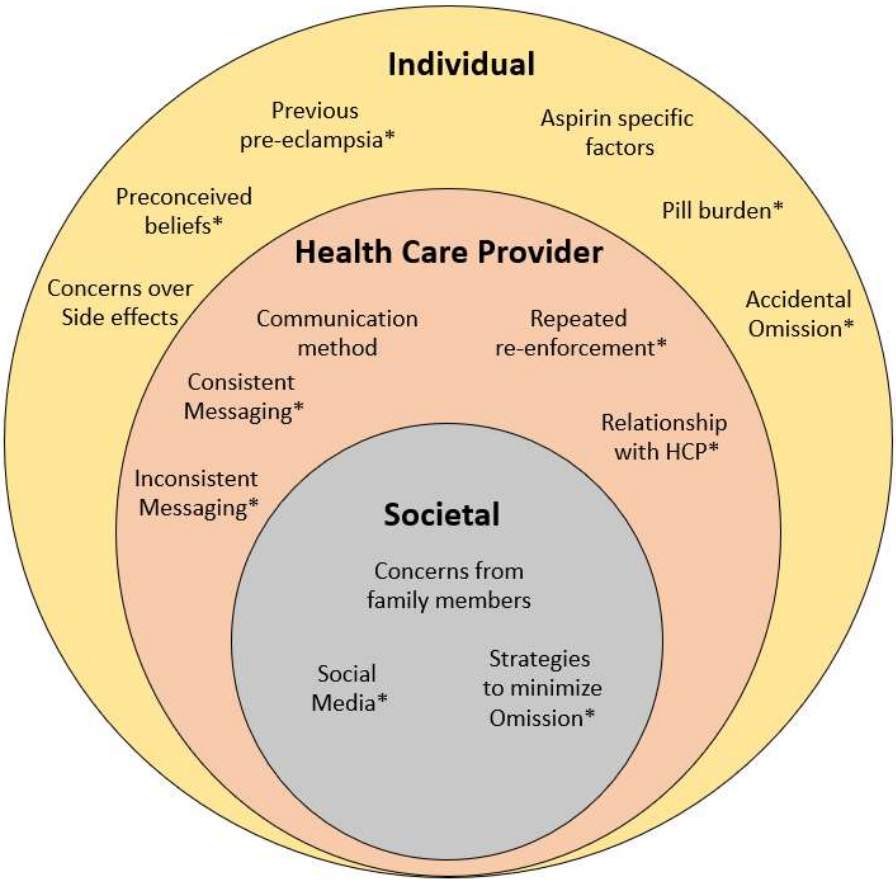


Figure 19: Patient reported factors that influenced adherence with aspirin in pregnancy.

** indicates factors that statistically correlated with adherence*

Pill burden and its associated non-intentional omission both strongly correlated with reduced adherence (Φ=0.8, p=0.02,Φ=0.8, p<0.01) whilst the use of reminder strategies were found to strongly minimize accidental omission and improve adherence (Φ=0.9, p<0.01)(131). Consistent communication amongst HCPs and good patient-HCP

relationship were found to correlate with improved adherence (Φ=0.7, p=0.04, Φ=0.9, p=<0.01) and were found to play an important role in alleviating factors that had potentials to negatively influence adherence to aspirin in pregnancy (131). The findings of this study highlights the importance in recognizing the impact of pill burden in pregnancy and the need to counsel women on the utility of reminder strategies to minimize non-intentional omission. This study also emphasizes the importance a positive patient-HCP relationship through effective and consistent communication to achieve better maternal and fetal outcomes in high-risk pregnancies.

7.3 Strengths and limitations of this study

At the time of this thesis, a mixed methods study in analysing factors that influences adherence to aspirin in pregnancy, from a patient's perspective had never been undertaken. Additionally, the patient population in this study consist of women from a multicultural background with variation in education and socio-economic background.

This study, however, has several limitations. Given the nature of both the quantitative and qualitative study, non-English speaking women were excluded given the inability to access multilingual translational service for research. However, language barrier has been significantly demonstrated as a factor that influences adherence mainly due breakdown in communication (130). This is often overcome with the use of a medical interpreter, however, the use of an interpreter may hamper forming a therapeutic relationship with HCPs and reduce the potential for patients to discuss their concerns (132). Additionally, studies using electronic questionnaires carry a risk of selection bias

towards the more literate population. Further mixed methodology analysis on the influence of medical literacy and language barrier, particularly in the migrant population will be instrumental. This study may also be confounded by recall bias given that women participated, on an average of 12.4 (± 7.4) months post-partum in the qualitative study, and 14.8 (± 2.8 months) post-partum in the quantitative study. Whilst it would have been ideal to unblind the participant's adherence in the quantitative study, women were allowed to participate in the quantitative study anonymously to allow and encourage them to provide honest and unbiased response to better understand their experience on the use of aspirin in pregnancy.

7.4 Conclusion

This study demonstrates factors within two themes that influences the use of aspirin amongst high-risk pregnant women; (1) pill burden and accidental omission (2) communication and relationship with HCPs. It highlights the need for clinicians to be aware of the consequences of pill burden in pregnancy and the importance of counselling patients on the utility of reminder strategies whilst applying repeated re-enforcement to minimize non-intentional omission of essential medications. This study also demonstrates the need for HCPs to recognize the importance of building a positive relationship with their patients to achieve the desired maternal and fetal outcomes.

PLOS ONE

RESEARCH ARTICLE

Factors that influence adherence to aspirin therapy in the prevention of preeclampsia amongst high-risk pregnant women: A mixed method analysis

Renuka Shanmugalingam[1,2,3,4]*, **Zelalem Mengesha**[5], **Stephanie Notaras**[2], **Pranee Liamputtong**[6], **Ian Fulcher**[7], **Gaksoo Lee**[4,7], **Roshika Kumar**[7], **Annemarie Hennessy**[1,2,3,4], **Angela Makris**[1,2,3,4,8]

1 Department of Renal Medicine, South Western Sydney Local Health District, Liverpool, NSW, Australia, **2** School of Medicine, Western Sydney University, Penrith, NSW, Australia, **3** Vascular Immunology Group, Heart Research Institute, University of Sydney, Newtown, NSW, Australia, **4** Women's Health Initiative Translational Unit (WHITU), Ingham Institute For Applied Medical Research and South Western Sydney Local Health District, Liverpool, NSW, Australia, **5** Research and Social Policy Team, Uniting Australia, Sydney, NSW, Australia, **6** School of Health Sciences and Translational Health Research Institute, Western Sydney University, Penrith, NSW, Australia, **7** Department of Obstetrics and Gynaecology, Liverpool Hospital, Liverpool, NSW, Australia, **8** School of Medicine, University of New South Wales, Sydney, NSW, Australia

* Renuka.shanmugalingam@health.nsw.gov.au

OPEN ACCESS

Citation: Shanmugalingam R, Mengesha Z, Notaras S, Liamputtong P, Fulcher I, Lee G, et al. (2020) Factors that influence adherence to aspirin therapy in the prevention of preeclampsia amongst high-risk pregnant women: A mixed method analysis. PLoS ONE 15(2): e0229622. https://doi.org/10.1371/journal.pone.0229622

Editor: Vijayaprasad Gopichandran, ESIC Medical College & PGIMSR, INDIA

Received: November 13, 2019

Accepted: February 10, 2020

Published: February 27, 2020

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: Supportive funds were received from the PEARLS Foundation and WHITU. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript

Competing interests: The authors have declared that no competing interests exist.

Abstract

Background

Non-adherence with medications in pregnancy is increasingly recognized and often results in a higher rate of preventable maternal and fetal morbidity and mortality. Non-adherence with prophylactic aspirin amongst high-risk pregnant women is associated with higher incidence of preeclampsia, preterm delivery and intrauterine growth restriction. Yet, the factors that influences adherence with aspirin in pregnancy, from the women's perspective, remains poorly understood.

Objective

The study is aimed at understanding the factors, from the women's perspective, that influenced adherence with prophylactic aspirin in their pregnancy.

Study design

A sequential-exploratory designed mixed methods quantitative (n = 122) and qualitative (n = 6) survey of women with recent high-risk pregnancy necessitating antenatal prophylactic aspirin was utilized. Women recruited underwent their antenatal care in one of three high-risk pregnancy clinics within the South Western Sydney Local Health District, Australia. The quantitative study was done through an electronic anonymous survey and the qualitative study was conducted through a face-to-face interview. Data obtained was analysed against women's adherence with aspirin utilizing phi correlation (φ) with significance set at <0.05.

Results

Two key themes, from the women's perspective, that influenced their adherence with aspirin in pregnancy were identified; (1) pill burden and non-intention omission (2) communication and relationship with health care provider (HCP). Pill burden and its associated non-intentional omission, both strongly corelated with reduced adherence ($\Phi = 0.8$, $p = 0.02$, $\Phi = 0.8$, $p<0.01$) whilst the use of reminder strategies minimized accidental omission and improved adherence ($\Phi = 0.9$, $p<0.01$). Consistent communication between HCPs and a good patient-HCP relationship was strongly associated with improved adherence ($\Phi = 0.7$, $p = 0.04$, $\Phi = 0.9$, $p = <0.01$) and more importantly was found to play an important role in alleviating factors that had potentials to negatively influence adherence with aspirin in pregnancy.

Conclusion

This study identified factors that both positively and negatively influenced adherence with aspirin amongst high-risk pregnant women. Is highlights the importance in recognizing the impact of pill burden in pregnancy and the need to counsel women on the utility of reminder strategies to minimize non-intentional omission. Importantly, it emphasizes on the importance of a positive patient-HCP relationship through effective and consistent communication to achieve the desired maternal and fetal outcomes.

Introduction

Medication adherence in a health care setting is defined as the extent to which an individual takes medications and executes lifestyle changes in accordance to the recommendations from a health care provider [1, 2]. Non-adherence with medications, either by delayed or omitted doses, is reported in up to 50% of non-pregnant patients with chronic illness [3, 4] and is associated with higher morbidity and mortality [2, 4]. Non-adherence to medications during pregnancy is reported to occur in 40–60% patients and is associated with both significant maternal and fetal co-morbidities [5–7]. Pregnant women view prescribed medications differently and adherence with medications has been found to vary with the state of disease in pregnancy, uncertainty of the benefit of the medication, expected side effects and fear of teratogenic effects of medications [8–10]. This issue is particularly prominent with the use of prophylactic and maintenance therapy in managing maternal comorbidities, especially when the acuity of the maternal medical issue is not present [5, 11, 12].

Prophylactic use of aspirin is recommended in women who are at risk of developing pre-eclampsia and has been shown to have a risk reduction of 60–80% [13–15]. However, recent data suggest that the prophylactic benefit is best observed with $\geq$90% adherence with aspirin [16]. The problem, however, lies in adherence with aspirin therapy in pregnancy [17–19]. Our observational cohort study, which assessed for adherence quantitatively through plasma salicyclic acid detection and platelet function analyser-100 (PFA-100) assessment, demonstrated that 44% of high-risk women were <90% adherent with aspirin therapy [20]. Women with inadequate adherence demonstrated aa higher rate of both early and late onset preeclampsia with a higher rate of intrauterine fetal growth restriction (IUGR) and preterm delivery in comparison to women who were $\geq$90% adherent with aspirin therapy [20, 21]. This demonstrated that non-adherence to prophylactic aspirin increases the risk of potentially avoidable maternal and fetal complications in pregnancy.

Multiple factors are known to contribute towards both intentional and non-intentional non-adherence with medications in pregnancy [9, 10, 22]. However, an assessment on influencers of non-adherence, particularly with the use of prophylactic aspirin in pregnancy, has not been undertaken. Our study was aimed at examining the factors, from a patient's perspective, that influences adherence with prophylactic aspirin in pregnancy and identifying factors that may enhance adherence and thus improve pregnancy outcomes.

Method

We utilized an sequential-exploratory designed mixed methods approach, combining both quantitative and qualitative analysis [23]. Ethics approval for this study was obtained from the Ethics and Research Committee of South Western Sydney Local Health District NSW, Australia (HE 16/184). Women provided written informed consent to participate.

High-risk pregnant women who were on aspirin as part of a multi-centre longitudinal cohort study [20] within the South Western Sydney Local Health District (SWSLHD), NSW, Australia, were invited to participate in an online survey (SurveyMonkey®). Women included in this study were risk stratified as high-risk for preeclampsia based on the current guidelines [24, 25] and were prescribed aspirin (100mgs or 150mgs) by their treating clinicians. Women who were of non-English speaking background and who were unable to provide written informed consent were excluded.

Electronic invitations, via email, were sent out to 154 women, from October 2018 –June 2019, to participate in this anonymous, non-compulsory survey that consisted mainly of multi-choice responses with additional free text boxes for additional responses. As part of the quantitative questionnaire, women were required to specify the extend of adherence (self-reported) with prescribed aspirin during their recent pregnancy. Anonymous response was utilized to facilitate honest and non-biased responses. Questions for the quantitative study were generated based on the validated adherence barrier questionnaire (ABQ) [26] and factors that are known to influence adherence with medications in pregnancy (S1 Table) [27, 28].

Women were also invited to express their interest in participating in a one-to-one, face-to-face interview for the qualitative assessment. Purposeful selection of the interview participants was done based on the participant's obstetric history and biochemically observed adherence to aspirin [20, 21] to gain maximum variation in the sample. The qualitative study was designed based on the quantitative data with the aim of further exploring the data obtain. Open ended questions for the qualitative interview were generated based on the current literature on medication adherence in pregnancy in addition to the data obtained from the quantitative survey (S2 Table). The semi-structured interviews were conducted by a single experienced co-investigator who was not involved with the clinical care of these women during their pregnancy. The interviews were conducted on an average of 12 months post-partum and were audio-recorded with short key notes taken during interviews to allow for member checking at the end of the interview. Participants were provided with a verbal summary of the interview and the opportunity to request amendments to the interpretations. The duration of the interviews ranged from 45 to 75 minutes. Interviews were ceased at the point of data saturation, which was set at the point at which no new data emerged from interviews.

Adherence for the purposes of the later thematic analysis was defined based on graded self-reported adherence with aspirin of $\geq$90% for the duration of the pregnancy as specified in the quantitative survey. A value of $\geq$90% was based on current data [16]. Audio recorded interviews were transcribed verbatim. No preconceived codes or categories were used. A six-stage sequential qualitative analysis was undertaken in analysing the transcription with the use of NVivo® (v.12 QRS International Pty Ltd) [29]. Transcripts were re-coded by a second

investigator to ensure agreement. Results of the quantitative and qualitative studies were combined through a process of triangulation that enabled the investigators to connect and interpret both data sets simultaneously through convergence and corroboration with the use of NVivo® [30, 31]. Phi correlation(φ) of data was done through SPSS (v.25 Chicago, IL, USA). Data was analysed using the appropriate test based on the data distribution and statistical significance was set at 0.05. A φ value of 0.7–1 is representative of a strong correlation, 0.3–0.69 of a moderate correlation and values of <0.3 are representative of a weak correlation.

Results

Of the 154 invitations sent out, 122 women (79%) completed the survey. Thirty-six women expressed interest in participating in the qualitative study, of whom, 18 were identified through purposeful selection to attend the interviews that were conducted at Liverpool Hospital, NSW, Australia. Interviews were ceased at point of qualitative data saturation which was achieved after the sixth interview. Three of these 6 women in the qualitative study were noted to have >90% adherence in the longitudinal study [20] with <90% adherence in the remaining three women. Characteristics of the women invited to participate in the quantitative study and those in the qualitative study are described in Tables 1 and 2 respectively. The quantitative survey demonstrated that 65 (53%) of the women reported ≥90% adherence with aspirin in their pregnancy.

Table 1. Characteristics of participants invited to participate in quantitative study (*).

Characteristics	Quantitative study invitations (n = 154)
Age (years) **	33 (± 5.6)
Duration in months since delivery (months)**	12.4 (± 7.4)
Primigravity	28 (17%)
Secondary education level	19 (12%)
Tertiary education level (Higher and Vocational)	135 (88%)
Smoking in pregnancy	8 (5%)
Ethnicity	
Caucasian	78 (51%)
Middle Eastern	24 (16%)
South Asian	22 (14%)
South East Asian	19 (12%)
African	6 (4%)
Polynesian	3 (2%)
Australian Aboriginal	2 (1%)
Indication for aspirin in most recent pregnancy§	
Chronic hypertension	90 (53%)
Previous pre-eclampsia	74 (44%)
Renal disease	35 (21%)
Type 1 or Type 2 Diabetes	13 (8%)
Based on first trimester screening only	8 (5%)
Systemic lupus erythematoses	5 (3%)
Anonymously self-reported adherence of ≥90% with aspirin	65 (53%)#

*Given the anonymous nature of this survey the characteristics of the women who participated (79% of total women invited) could not be isolated. Presented here are the characteristics of all women invited to participate except for #, which is based on the response from women who participated in the survey.

**Mean with standard deviation (± SD). §Some women had more than one medical condition.

https://doi.org/10.1371/journal.pone.0229622.t001

Table 2. Characteristics of participants in qualitative study.

Characteristics	Qualitative study participants (n = 6)
Age (years)*	30.3 (±3.7)
Duration since delivery (months)*	14.8 (±2.5)
Primigravity	1 (17%)
Secondary education level	2 (33%)
Tertiary education level (Higher and Vocational)	4 (67%)
Smoking in pregnancy	0 (0%)
Ethnicity	
Caucasian	4 (67%)
South Asian	1 (17%)
Middle Eastern	1 (17%)
Indication for aspirin in pregnancy**	
Chronic hypertension	2 (33%)
Type 1 or Type 2 Diabetes	1 (17%)
Renal disease	1 (17%)
Previous pre-eclampsia	4 (67%)

*Mean with standard deviation (±SD).

** Some women had more than one medical condition.

https://doi.org/10.1371/journal.pone.0229622.t002

Based on the combined analysis, we identified factors that influenced women's adherence with aspirin therapy in pregnancy. Results are present thematically: (1) Pill burden and non-intentional omission (2) Communication and relationship with health care providers (HCPs) (Table 3)

Pill burden and non-intentional omission: *"I had so many pills and aspirin was another pill..it was a struggle"*

On average, women in our study were on 3.5 (range 2–6) medications at any one time in their pregnancy with repeated dosing over 24 hours (average: 12 hourly). Of the women who missed

Table 3. Summary of factors that influenced adherence with prophylactic aspirin.

Themes	Factors	Effect on adherence with aspirin	P value*
Pill burden and non-intentional omission	Pill burden	Reduced	Φ = 0.8, p = 0.02
	Non-intentional omission	Reduced	Φ = 0.8, p<0.01
	Strategizes to minimize non-intentional omission	Improved	Φ = 0.9, p<0.01
Communication and relationship with Health Care Providers (HCPs)	Consistent messaging between HCPs	Improved	Φ = 0.7, p = 0.04
	Repeated re-enforcement by HCPs	Improved	Φ = 0.8, p = 0.02
	Influence of social media and women support group	Improved	Φ = 0.7, p = 0.03
	Positive relationship with HCPs	Improved	Φ = 0.9, p<0.01

*Based on phi coefficient relationship (φ)

https://doi.org/10.1371/journal.pone.0229622.t003

more than 1 dose of aspirin (<90% adherent) in pregnancy, 42 (74%) reported pill burden as an issue (φ = 0.8, p = 0.02) and this was associated with a higher rate of patient reported non-intentional omission of aspirin (φ = 0.8, p<0.01). This was further evident in the qualitative data where women who reported non-intentional omission were on an average of 6 pills a day in comparison to women with good adherence with aspirin (average of 2 pills a day):

"*I was taking lots of other vitamins and things, so yeah, I don't think I fully grasped how important it was to take aspirin every day. It was definitely not something I prioritized. I didn't like taking medications in general. I feel like there was a lot towards the end. I got a little bit hysterical." (Interview participant 1, aspirin non-adherent group, 29 yo)*

"*To be honest with you, I was taking a lot. There was a time where I was taking eight per day; that included things like Elevit, Vitamin D, my blood pressure medication and aspirin, yeah, so a lot. I had to take my diabetic medication, Aspirin, Macrolide, Folate and Vitamin D and Calcium so yeah it was hard to keep track of all of it." (Interview participant 4, aspirin non-adherent group, 32 yo)*

Non-intentional omission was also strongly related to difficulty with taking aspirin at night as instructed by their doctor in 20 (35%) of women (φ = 0.8, p<0.01). The qualitative study further indicated that the need to adhere to specified timing of aspirin ingestion (bedtime dosing), along with pill burden was associated with increased non-intentional omission of aspirin:

"*I had all my medications besides my diabetes medication and aspirin cause I had to take that at specific times especially at night. When I got into bed and forgot to take all my medications, I went 'I'm not getting back out of bed. I'm exhausted. Yeah I'm in bed for the night now. I know later on I'm going to get out of bed a million times. No I'm not getting out just to take medication'." (Interview participant 4, aspirin non-adherent group, 32 yo)*

A total of 65 (53%) women reported good adherence with aspirin either with no omission 45(37%) or only 1 omitted dose 20(16%) through the duration of their pregnancy. The strategies utilized to minimize accidental omission included the use of a mobile phone reminder application in 42(65%), pill box in 19(29%) and a medication routine 9(14%). The use of these strategies strongly correlated with aspirin adherence (φ = 0.9, p<0.01) and was supported by the qualitative responses in which women elaborated on the reminder strategies they utilized to overcome issues in relation to bedtime dosing of aspirin:

"*I had a reminder strategy. So usually I would take it when I brush my teeth, as a reminder. So I just made sure that they were there and when I went to brush my teeth, I would see it and take it." (Interview participant 3, aspirin adherent group, 34 yo)*

"*I put all my medications in my room in my drawer so I knew when I went to bed I pulled out the drawer and get all my medications out ready and yeah go to bed. I knew where all my medication was, and I had to take it." (Interview participant 6, aspirin adherent group, 30 yo)*

Therefore, whilst pill burden and non-intentional omission of medications were key factors that negatively influenced adherence, the use of reminder strategies was an effective influencer in overcoming this.

Communication and relationship with Health Care Providers (HCPs): *"I had a good rapport with my doctor early on which helped me trust the healthcare system"*

Consistent communication between the women's HCP positively impacted their uptake of aspirin. Most women, 101(83%), were first advised to take aspirin by their renal/obstetric physician, 33 (27%) by an obstetrician or midwives and 20 (16%) by general practitioners or based on previous recommendation. Of these women, 59 (48%) discussed the use of aspirin with a second HCP. Consistent message from HCPs on the indication of aspirin in pregnancy strongly correlated to women's uptake and adherence with aspirin (φ = 0.7, p = 0.04). The

importance of consistent messaging and the negative impact of inconsistent messaging between HCPs was evident through the qualitative data in which women elaborated on how to information they obtained from multiple HCPs influenced their adherence with aspirin (both positively and negatively):

"*When I was told by the first doctor, I was still a bit sceptical and it's only when I saw the second and third doctor, it sunk in and I thought, it must be important as they are all saying the same thing. It then made sense. It works well when doctors communicate the same thing, it gives us confidence." (Interview participant 2, aspirin adherent group, 34 yo)*

"*The chemist told me that I should not take aspirin while I was pregnant despite my doctor's advice. This made my husband very concerned and discouraged me from taking the aspirin (Interview participant 1, aspirin non-adherent group, 29 yo)*

Additionally, 52(43%) of women reported that repeated re-enforcement on adherence with aspirin by HCPs emphasized on its importance. This strongly correlated with adherence (φ = 0.8, p = 0.02) and was supported by the qualitative data:

"*I remember my doctor saying not to forget to take my medications, especially the aspirin, so actually I do recall her saying that to me and made it think it must be important for her to say that." (Interview participant 6, aspirin adherent group, 30 yo)*

Communication on the use of aspirin in pregnancy with other high-risk women via social media and forums was found to strongly correlate with uptake and adherence with aspirin in 33(27%) of women (φ = 0.7, p = 0.03). This was also evident in the qualitative data in which these women elaborated on the positive impact of social media in reassuring them on the use of aspirin in pregnancy:

"*Speaking to other women that have been through it (preeclampsia) and that are going through it–you know finding friends who are on or who have taken aspirin in pregnancy, who are going through similar things gave me comfort in taking it." (Interview participant 6, aspirin adherent group, 30 yo)*

Women's good relationship with their HCPs played an important role in alleviating factors that had potentials to negatively influence adherence with aspirin in pregnancy. These factors included inconsistent messaging among HCP, especially from pharmacist which was reported in 36 (30%) of women, concerns regarding maternal and fetal side effects of aspirin 39 (32%) and discouragement from family and friends 18 (15%). However, none of these factors significantly correlated with adherence (φ = 0.1–0.4) as 111 (91%) of women reported having a good relationship with their primary HCPs and were happy to discuss their concerns on the use of aspirin in pregnancy (φ = 0.9, $p<0.01$). This was further corroborated by women who participated in the interviews:

"*The chemist kept telling me that I should not take aspirin while I was pregnant despite my doctor's advice. This made my husband and mother very concerned and they discouraged me from taking the aspirin. My husband was unhappy and came with me to my appointment to talk about this with my doctor. My doctor spent a lot of time to talk to us about it and put our mind at ease. She also called the chemist after we left." (Interview participant 6, aspirin adherent group,30 yo)*

"*My previous pregnancy was horrible because I never had that connection with my doctor. So you know, there was a difference this time, the relationship that I had with my doctor made a huge difference. They had time to listen and answer my question. I knew I was under better care. I had trust in them." (Interview participant 3, aspirin adherent group, 34)*

Discussion

Our study identified two key themes, from a patient's perspective, that influenced adherence with prophylactic aspirin in pregnancy: 1) Pill burden and non-intentional omission and 2)

Communication and positive relationship with HCPs (Table 3). We previously demonstrated that, from the women's demographic characteristic perspective, women who had previous pre-eclampsia and tertiary level education had a higher rate of adherence with aspirin [20, 21].

Pill burden and non-intentional omission of medications are commonly associated with non-adherence in both pregnant and non-pregnant patients [2, 5, 25]. Our study demonstrated that these two factors were strongly correlated with suboptimal aspirin adherence, particularly when women were put on a bedtime regimen. In keeping with this, other studies have described restrictions around dosing requirements such as 'take with food', 'take with an empty stomach', 'take away from other medications' and 'take at night' as common contributing factors towards non-intentional medication omission [32, 33]. However, the use of electronic reminders and pill boxes as reminders has been shown to minimize accidental medication omission [34] and improve adherence [22]. Our study, similarly, demonstrated a strong positive correlation between the use of reminder strategies and adherence with aspirin therapy that can be used to overcome non-intentional omission with bedtime aspirin, particular given the growing recommendation for bedtime aspirin [13, 32].

Importantly, our study reflects the importance of communication and a positive relationship between the woman and her HCPs in achieving the desired level of aspirin adherence and clinical outcomes. Pregnant women view the use of medications in pregnancy differently compared to when they are not pregnant. They often question the need for medication in pregnancy, mainly due to concerns over potential teratogenic effects [8, 9]. Therefore, unsurprisingly, a woman's understanding of the need for her medications influences her attitude towards the adherence in pregnancy and this is directly influenced by the way the need for the medication was communicated by her HCP [8]. Specific to this study, the importance of effective communication between HCPs and patients in discussing the need for aspirin and to discuss concerns regarding its use, was evident. The current literature supports the importance of a strong therapeutic relationship and trust in HCPs and patient's behaviour towards therapy [33, 34]. This relationship is often found to be closely related to the clinician's interpersonal skill, ability to provide clear explanation of correlation between therapy and disease, ability to inspire patient's trust and adequately address patient's concerns [35–38]. In our study, the relationship and interaction between the women and their HCP not only helped them understand the need for aspirin but also allowed for discussions on their concerns with regards to the use of aspirin and provide them with assurance, allowing for an improvement with adherence with prophylactic aspirin.

Similarly, re-enforcing the importance of medications and checking for adherence with therapy have been also shown to improve patient's adherence [37] and in keeping with the literature, women in our study resonated this association. Additionally, the use of social media as a domain of support network with other women has been shown to provide reassurance and help improve women's understanding on the need for therapy in pregnancy [39, 40]. This has been shown to improve adherence with the recommended therapy in pregnancy [39]. In keeping with this, our study demonstrated a positive influence of social media and support group in facilitating adherence with aspirin in pregnancy.

To our knowledge, a mixed method study in analysing factors that influences adherence with aspirin in pregnancy, from a patient's perspective has never been undertaken, hence, making this a novel study. Additionally, our patient population is vastly multicultural (Tables 1 and 2) with variation in education and socio-economic background. Our study, however, consist of a few limitations. Given the nature of both the quantitative and qualitative study, we excluded non-English speaking women given the inability to access a multilingual translational service for research. However, language barrier has been significantly demonstrated as a factor that influences adherence mainly due breakdown in communication [41]. This is often

overcome with the use of a medical interpreter, however, the use of an interpreter may hamper forming a therapeutic relationship with HCPs and reduce the potential for patients to discuss their concerns [42]. Additionally, studies using electronic questionnaires carries a risk of selection bias towards the more literate population. Further mixed methodology analysis on the influence of medical literacy and language barrier, particularly in the migrant population will be instrumental. Our data may also be confounded by re-call bias given that the women participated with an average of 12.4 (± 7.4) months post-partum in the qualitative study and 14.8 (± 2.8 months) post-partum in the quantitative study. Additionally, whist it would have been ideal to unblind the participants in the quantitative study to use their data against their established biochemical evidence of adherence with aspirin from our longitudinal cohort study [20], we chose to allow for women to participate in the quantitative study anonymously to allow and encourage them to provide honest and unbiased response to better understand their experience on the use of aspirin in pregnancy. In doing so, we found that women's self-reported adherence in this study matched what we observed biochemically in the longitudinal study. A non-anonymous patient-reported adherence assessment which we conducted as part of the longitudinal study however demonstrated that patient-reported adherence (in a non-anonymous form of qualitative assessment) only moderately correlated with their actual biochemical adherence, therefore indicating that patient-reported adherence is likely more accurate in an anonymous setting.

Conclusion

Multiple clinical, psycho-social and health care related factors are known to influence adherence in pregnancy. Our study demonstrates factors within two key themes that influences the use of aspirin in the prevention of preeclampsia amongst high-risk pregnant women; (1) pill burden and accidental omission (2) communication and relationship with HCPs. It highlights the need for clinicians to be aware of the consequence of pill burden in pregnancy and importance of counselling patients on the utility of reminder strategies whilst applying repeated re-enforcement to minimize non-intentional omission of essential medications. Our study also demonstrates the crucial need for HCPs to recognize the importance of building a positive relationship with their patients through effective and consistent communication to achieve the desired maternal and fetal outcomes in pregnancy.

Supporting information

S1 Table. Questions used in quantitative study.
(DOCX)

S2 Table. Questions used in qualitative study.
(DOCX)

Acknowledgments

The authors would like to thank the participants of this study, the PEARLS Foundation, the Women's Health Initiative Translational Unit (WHITU), the Department of Renal Medicine SWSLHD and the Department of Obstetrics and Gynaecology SWSLHD, NSW Australia for their support in conducting this study.

Author Contributions

Conceptualization: Renuka Shanmugalingam, Annemarie Hennessy, Angela Makris.

Data curation: Renuka Shanmugalingam, Zelalem Mengesha, Gaksoo Lee, Roshika Kumar.

Formal analysis: Renuka Shanmugalingam, Zelalem Mengesha.

Funding acquisition: Renuka Shanmugalingam.

Investigation: Renuka Shanmugalingam.

Methodology: Renuka Shanmugalingam, Zelalem Mengesha, Stephanie Notaras.

Supervision: Annemarie Hennessy, Angela Makris.

Writing – original draft: Renuka Shanmugalingam.

Writing – review & editing: Zelalem Mengesha, Stephanie Notaras, Pranee Liamputtong, Ian Fulcher, Annemarie Hennessy, Angela Makris.

References

1. Burkhart PV, Sabate E. Adherence to long-term therapies: evidence for action. J Nurs Scholarsh. 2003; 35(3):207. PMID: 14562485
2. World Health Organization. Adherence to long-term therapies—evidence for action. 2009.
3. Osterberg L, Blaschke T. Adherence to medication. N Engl J Med. 2005; 353(5):487–97. https://doi.org/10.1056/NEJMra050100 PMID: 16079372
4. Akincigil A, Bowblis JR, Levin C, Jan S, Patel M, Crystal S. Long-term adherence to evidence based secondary prevention therapies after acute myocardial infarction. J Gen Intern Med. 2008; 23(2):115–21. https://doi.org/10.1007/s11606-007-0351-9 PMID: 17922172
5. Sawicki E, Stewart K, Wong S, Leung L, Paul E, George J. Medication use for chronic health conditions by pregnant women attending an Australian maternity hospital. Aust N Z J Obstet Gynaecol. 2011; 51 (4):333–8. https://doi.org/10.1111/j.1479-828X.2011.01312.x PMID: 21806573
6. Nachega JB, Marconi VC, van Zyl GU, Gardner EM, Preiser W, Hong SY, et al. HIV treatment adherence, drug resistance, virologic failure: evolving concepts. Infect Disord Drug Targets. 2011; 11(2):167–74. https://doi.org/10.2174/187152611795589663 PMID: 21406048
7. Fairgrieve SD, Jackson M, Jonas P, Walshaw D, White K, Montgomery TL, et al. Population based, prospective study of the care of women with epilepsy in pregnancy. BMJ. 2000; 321(7262):674–5. https://doi.org/10.1136/bmj.321.7262.674 PMID: 10987772
8. Lupattelli A, Spigset O, Nordeng H. Adherence to medication for chronic disorders during pregnancy: results from a multinational study. Int J Clin Pharm. 2014; 36(1):145–53. https://doi.org/10.1007/s11096-013-9864-y PMID: 24162929
9. Gallinger ZR, Rumman A, Nguyen GC. Perceptions and Attitudes Towards Medication Adherence during Pregnancy in Inflammatory Bowel Disease. J Crohns Colitis. 2016; 10(8):892–7. https://doi.org/10.1093/ecco-jcc/jjw052 PMID: 26896087
10. Adhikari K, Patten SB, Lee S, Metcalfe A. Adherence to and Persistence with Antidepressant Medication during Pregnancy: Does It Differ by the Class of Antidepressant Medication Prescribed? Can J Psychiatry. 2018:706743718802809.
11. Belanger K, Hellenbrand ME, Holford TR, Bracken M. Effect of pregnancy on maternal asthma symptoms and medication use. Obstetrics and gynecology. 2010; 115(3):559–67. https://doi.org/10.1097/AOG.0b013e3181d06945 PMID: 20177287
12. Einarson A, Selby P, Koren G. Abrupt discontinuation of psychotropic drugs during pregnancy: fear of teratogenic risk and impact of counselling. J Psychiatry Neurosci. 2001; 26(1):44–8. PMID: 11212593
13. Rolnik DL, Wright D, Poon LC, O'Gorman N, Syngelaki A, de Paco Matallana C, et al. Aspirin versus Placebo in Pregnancies at High Risk for Preterm Preeclampsia. N Engl J Med. 2017; 377(7):613–22. https://doi.org/10.1056/NEJMoa1704559 PMID: 28657417
14. Roberge S, Nicolaides K, Demers S, Hyett J, Chaillet N, Bujold E. The role of aspirin dose on the prevention of preeclampsia and fetal growth restriction: systematic review and meta-analysis. Am J Obstet Gynecol. 2017; 216(2):110–20 e6. https://doi.org/10.1016/j.ajog.2016.09.076 PMID: 27640943
15. Park F, Russo K, Williams P, Pelosi M, Puddephatt R, Walter M, et al. Prediction and prevention of early-onset pre-eclampsia: impact of aspirin after first-trimester screening. Ultrasound Obstet Gynecol. 2015; 46(4):419–23. https://doi.org/10.1002/uog.14819 PMID: 25678383

16. Wright D, Poon LC, Rolnik DL, Syngelaki A, Delgado JL, Vojtassakova D, et al. Aspirin for Evidence-Based Preeclampsia Prevention trial: influence of compliance on beneficial effect of aspirin in prevention of preterm preeclampsia. Am J Obstet Gynecol. 2017; 217(6):685 e1–e5.
17. Wright D, Syngelaki A, Akolekar R, Poon LC, Nicolaides KH. Competing risks model in screening for preeclampsia by maternal characteristics and medical history. Am J Obstet Gynecol. 2015; 213(1):62 e1–10.
18. Navaratnam K, Alfirevic A, Jorgensen A, Alfirevic Z. Aspirin non-responsiveness in pregnant women at high-risk of pre-eclampsia. European journal of obstetrics, gynecology, and reproductive biology. 2018; 221:144–50. https://doi.org/10.1016/j.ejogrb.2017.12.052 PMID: 29304392
19. Abheiden CN, van Reuler AV, Fuijkschot WW, de Vries JI, Thijs A, de Boer MA. Aspirin adherence during high-risk pregnancies, a questionnaire study. Pregnancy Hypertens. 2016; 6(4):350–5. https://doi.org/10.1016/j.preghy.2016.08.232 PMID: 27939481
20. Shanmugalingam R, Wang X, Motum P, Fulcher I, Lee G, Kumar R, et al. The clinical influence of non-adherence with prophylactic aspirin in preventing preeclampsia in high-risk pregnancies: A multi-centre prospective, observational, cohort study. Hypertension (IN PRESS)2019.
21. Shanmugalingam R WX, Chau K, Xu B, Lee G, Kumar R, et al. A cohort study utilising a biochemical assessment of aspirin compliance vs resistance in high-risk pregnant women [ABSTRACT]. Pregnancy hypertension. 2018; 13:S82–S83.
22. Omonaiye O, Nicholson P, Kusljic S, Manias E. A meta-analysis of effectiveness of interventions to improve adherence in pregnant women receiving antiretroviral therapy in sub-Saharan Africa. Int J Infect Dis. 2018; 74:71–82. https://doi.org/10.1016/j.ijid.2018.07.004 PMID: 30003952
23. Creswell JW KA, Plano Clark VL, Smith KC for the Office of Behavioral and Social Sciences Research. Best practices for mixed methods research in the health sciences. National Institutes of Health. August 2011.
24. Redman CW. Hypertension in pregnancy: the NICE guidelines. Heart. 2011; 97(23):1967–9. https://doi.org/10.1136/heartjnl-2011-300949 PMID: 21990386
25. Lowe SA, Bowyer L, Lust K, McMahon LP, Morton M, North RA, et al. SOMANZ guidelines for the management of hypertensive disorders of pregnancy 2014. Aust N Z J Obstet Gynaecol. 2015; 55(5):e1–29. https://doi.org/10.1111/ajo.12399 PMID: 26412014
26. Müller S, Kohlmann T, Wilke T. Validation of the Adherence Barriers Questionnaire—an instrument for identifying potential risk factors associated with medication-related non-adherence. BMC Health Serv Res. 2015; 15:153. https://doi.org/10.1186/s12913-015-0809-0 PMID: 25884193
27. Hsu C, Lemon JM, Wong ES, Carson-Cheng E, Perkins M, Nordstrom MS, et al. Factors affecting medication adherence: patient perspectives from five veterans affairs facilities. BMC Health Serv Res. 2014; 14:533. https://doi.org/10.1186/s12913-014-0533-1 PMID: 25391694
28. Mathes T, Jaschinski T, Pieper D. Adherence influencing factors—a systematic review of systematic reviews. Arch Public Health. 2014; 72(1):37. https://doi.org/10.1186/2049-3258-72-37 PMID: 25671110
29. Kingdon C, Neilson J, Singleton V, Gyte G, Hart A, Gabbay M, et al. Choice and birth method: mixed-method study of caesarean delivery for maternal request. BJOG: an international journal of obstetrics and gynaecology. 2009; 116(7):886–95.
30. O'Cathain A, Murphy E, Nicholl J. Three techniques for integrating data in mixed methods studies. BMJ. 2010; 341:c4587. https://doi.org/10.1136/bmj.c4587 PMID: 20851841
31. Tariq S, Woodman J. Using mixed methods in health research. JRSM Short Rep. 2013; 4 (6):2042533313479197. https://doi.org/10.1177/2042533313479197 PMID: 23885291
32. Ayala DE, Ucieda R, Hermida RC. Chronotherapy with low-dose aspirin for prevention of complications in pregnancy. Chronobiol Int. 2013; 30(1–2):260–79. https://doi.org/10.3109/07420528.2012.717455 PMID: 23004922
33. Bertakis KD, Azari R. Patient-centered care is associated with decreased health care utilization. J Am Board Fam Med. 2011; 24(3):229–39. https://doi.org/10.3122/jabfm.2011.03.100170 PMID: 21551394
34. Castro EM, Van Regenmortel T, Vanhaecht K, Sermeus W, Van Hecke A. Patient empowerment, patient participation and patient-centeredness in hospital care: A concept analysis based on a literature review. Patient Educ Couns. 2016; 99(12):1923–39. https://doi.org/10.1016/j.pec.2016.07.026 PMID: 27450481
35. Mechanic D, Meyer S. Concepts of trust among patients with serious illness. Soc Sci Med. 2000; 51 (5):657–68. https://doi.org/10.1016/s0277-9536(00)00014-9 PMID: 10975226
36. Van Den Assem B, Dulewicz V. Patient satisfaction and GP trustworthiness, practice orientation and performance: implications for selection, training and revalidation. J Health Organ Manag. 2014; 28 (4):532–47. https://doi.org/10.1108/jhom-12-2012-0238 PMID: 25241598

37. Donovan JL. Patient decision making. The missing ingredient in compliance research. Int J Technol Assess Health Care. 1995; 11(3):443–55. https://doi.org/10.1017/s0266462300008667 PMID: 7591546

38. Anderson LA, Dedrick RF. Development of the Trust in Physician scale: a measure to assess interpersonal trust in patient-physician relationships. Psychol Rep. 1990; 67(3 Pt 2):1091–100.

39. Martin SL, Omotayo MO, Pelto GH, Chapleau GM, Stoltzfus RJ, Dickin KL. Adherence-Specific Social Support Enhances Adherence to Calcium Supplementation Regimens among Pregnant Women. J Nutr. 2017; 147(4):688–96. https://doi.org/10.3945/jn.116.242503 PMID: 28250195

40. Chan KL, Chen M. Effects of Social Media and Mobile Health Apps on Pregnancy Care: Meta-Analysis. JMIR Mhealth Uhealth. 2019; 7(1):e11836. https://doi.org/10.2196/11836 PMID: 30698533

41. Andreae MH, White RS, Chen KY, Nair S, Hall C, Shaparin N. The Effect of Initiatives to Overcome Language Barriers and Improve Attendance: A Cross-Sectional Analysis of Adherence in an Inner City Chronic Pain Clinic. Pain Med. 2017; 18(2):265–74. https://doi.org/10.1093/pm/pnw161 PMID: 28204760

42. Villalobos BT, Bridges AJ, Anastasia EA, Ojeda CA, Rodriguez JH, Gomez D. Effects of language concordance and interpreter use on therapeutic alliance in Spanish-speaking integrated behavioral health care patients. Psychol Serv. 2016; 13(1):49–59. https://doi.org/10.1037/ser0000051 PMID: 26349073

CHAPTER 8

SUMMARY AND FUTURE DIRECTIONS

This thesis aimed to examine for a potential alternative mechanism of action of prophylactic aspirin, through the ATL pathway, in the prevention of preeclampsia (Scholarly research Paper 1, Chapter 4) and factors that are likely to influence the rate of risk reduction of preeclampsia with the use of aspirin. The two main aspirin factors of interest in this thesis were (1) aspirin dose (Scholarly research paper 2, Chapter 5) and (2) adherence to aspirin (Scholarly research paper 3, Chapter 6). Given the observations made relating to adherence (101), this thesis also examined for factors that both positively and negatively influenced adherence to prophylactic aspirin in pregnancy (Scholarly research paper 4, Chapter 7). This chapter will discuss the findings from this thesis and suggest future research to build on the results presented.

The findings from this study will allow for an increase in interest on alternative mechanism by which aspirin may demonstrate its prophylactic benefit in preeclampsia and help optimize the clinical utility of aspirin in improving the risk reduction rate of preeclampsia.

8.1 Mechanism of action of aspirin in the prevention of preeclampsia – an assessment of the ATL anti-inflammatory pathway

To date, the mechanism by which aspirin prevents preeclampsia remains unclear. Regulation of the TXA/PGI2 ratio, with its resulting maternal anti-thrombotic effect has been previously proposed as a mechanism by which aspirin potentially reduces placental

dysfunction in the prevention of preeclampsia (47, 48). However, recent data on the lack of benefit of aspirin when commenced after 16 weeks of gestation (42) and greater prophylactic on early onset preeclampsia in comparison to late onset preeclampsia (29) suggest other potential mechanisms of action of aspirin that are specific to placental development.

In examining the potential anti-inflammatory effect of aspirin on placental development, this thesis examined the influence of ATL on selected maternal cytokines (IL-10, 1L-8, TNF-α, IFN-γ and IL-1β) based on previous cell studies that examined the effect of ATL on placentation (67, 68). This work was undertaken by performing a longitudinal cohort study of high-risk pregnant women (Chapter 2.1 and Chapter 3). In exploring the influence of ATL, this thesis first examined for the difference in En-Lipoxin-A4, an endogenous anti-inflammatory lipoxin that is an epimer of ATL, between high-risk and low-risk pregnancy. Whilst studies have demonstrated an association between low maternal first trimester En-Lipoxin-A4 and placental dysfunction related fetal growth impairment (66), the study in Chapter 4 (Scholarly research paper 1) is novel in demonstrating a significantly lower En-Lipoxin-A4 concentration (of up to 70%) in high-risk pregnancy compared to low-risk pregnancy (116). Further novel findings included demonstrating a significant detection of plasma ATL in the high-risk group in response to adequate adherence to aspirin (116). Additionally, a lower plasma concentration of IL-8 from 12-36 weeks of gestation ($p < 0.001$), TNF-α from 24-36 weeks of gestation ($P=0.02$) and an increase in IL-10 plasma concentration from 16 – 28 weeks of gestation ($p=0.03$) were observed in the aspirin adherent high-risk (HR-AA) group compared to the non-aspirin high-risk (HR-NA) group (116).

Chapter 4 of this thesis also described an observed sustained rise in maternal plasma PlGF concentration in the HR-AA group compared to the HR-NA group, even after aspirin was ceased between 34-36 weeks of gestation (Figure 12). Therefore, supporting the possibility that the increased PlGF plasma concentration in the HR-AA group is likely a surrogate marker of better placentation with adequate adherence to aspirin (115). This work also demonstrated that high-risk women who did not develop preeclampsia had higher plasma PlGF concentrations compared to high-risk women who developed preeclampsia (Figure 13).

Based on the observations of the studies in Chapter 4, adequate adherence with aspirin was found to demonstrate a significant elevation in plasma ATL concentration and was observed to be associated with lower plasma IL-8 and higher plasma IL-10 and PlGF concentrations. This also corresponded clinically, where high-risk women who did not develop preeclampsia were found to have higher plasma concentration of PlGF, IL-10 and ATL with lower plasma concentration of IL-8 in comparison to high-risk women who developed preeclampsia. These observations propose a potential anti-inflammatory effect of prophylactic aspirin through the ATL pathway in regulating maternal IL-8 and IL-10 plasma concentration. These effects, which were observed from when aspirin was commenced (before 16 weeks of gestation) is likely to have allowed for better placentation in these women, resulting in higher maternal plasma PlGF concentration, a surrogate marker for placentation. This study, however, proposes this mechanism though observational correlation only.

Further studies should focus on examining the hypothesis above with *in-vitro* placental models. Advancement at the time of this thesis, has seen the development of a human cytotrophoblast organoid (CTB-ORG) cultures that mimics the *in-vivo* structure of human placenta, express markers of trophoblast identity and secrete pregnancy hormones (133, 134). This three dimensional (3D) culture is developed from purified first-trimester cytotrophoblast preparations that are capable of self-renewal and expansion under defined culture conditions and allows for a 3D analysis of cell column formation, growth, and differentiation (133, 135). The utility of this model in examining the direct effect of ATL on extravillous trophoblast (EVT) invasion along with the local cellular expression of IL-8, 1L-10, PlGF and invasive markers such as VCAM-1 and VE-cadherin will help build on the understanding of the observations made in this study. At the time of this thesis, access, both logistically and financially, to the 3D CTB-ORGs model was limited. However, its use in the study of placental development is likely to grow in the future and replace the currently used two dimensional (2D) *in-vitro* placenta models.

8.2 Influence of aspirin dosing in pregnancy

Chapter 5 of this thesis described a novel pharmacokinetic assessment of 100 mg and 150 mg of aspirin in both pregnant and non-pregnant women through assessment of plasma SA (Scholarly research paper 2) (125). A difference in pharmacokinetic between EC and non-EC aspirin was also examined. The study demonstrated a reduced AUC(t-24) and Cmax along with an increase in CL in pregnant women (for both 100 and 150mg of aspirin) compared to non-pregnant women (125). This, therefore, suggest possible increased clearance of aspirin's active metabolite, SA, in pregnancy. The reduced AUC(t-24) in pregnant women compared to non-pregnant women with the commonly

prescribed 100mg of aspirin, however, was overcome when the dose of aspirin in pregnant women was adjusted to 150 mg (Figure 17). Therefore, suggesting a potential need to dose adjust aspirin in pregnancy to achieve similar AUC(t-24) with 100mg of aspirin in non-pregnant women. This is likely due to the altered pharmacokinetics of aspirin in pregnancy.

The pharmacokinetics of medications in pregnancy is influenced by the maternal physiological changes that occurs through all three trimesters of pregnancy. These changes lead to an alteration in absorption, distribution and elimination of commonly used medications in pregnancy (117, 118) (Figure 20).

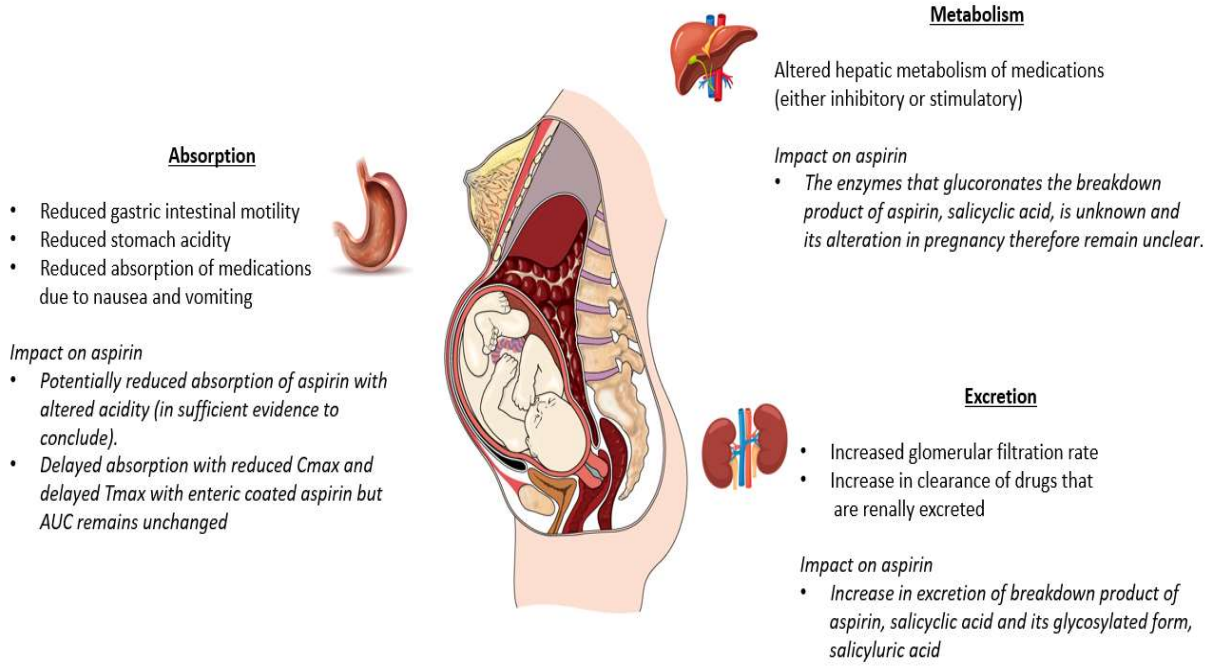


Figure 20: Illustrative representation of altered pharmacokinetics of aspirin in pregnancy

In pregnancy, there is a progesterone driven delay in gastrointestinal motility which results in a 30% increase in gastric emptying time and an approximately 40% reduction in gastric acidity (136, 137). These changes can result in ionization of weak acids such as aspirin and could potentially affect its absorption. A stronger influence on the alteration of pharmacokinetics in pregnancy is the variation in drug distribution and elimination. The physiological plasma expansion of 50% in pregnancy often results in altered drug Vd and is most prominent towards the end of the first trimester (138, 139). The total mean increase in maternal body volume is approximately 8L and the resulting volume expansion results in a decrease in Cmax particularly of hydrophilic agents, such as SA. The increased circulating estrogen and progesterone in pregnancy can either increase or decrease hepatic metabolism of drugs based on its stimulatory or inhibitory influence on cytochrome P450 isoenzymes and uridine 5'-diphospho-glucuronosyltransferase isoenzyme activity (138, 140, 141). Glucuronidation activity may be a critical determinant of aspirin efficacy, however, the enzymes responsible for this conjugation have yet to be identified and characterized (142). Therefore, the influence of pregnancy on the hepatic metabolism of salicylic acid remains subject to further research.

The relationship between increased creatinine clearance and drug excretion in pregnancy is well recognised and have been demonstrated through various studies, including this thesis which demonstrated a 45% increase in clearance of SA (138, 143, 144). Renal drug excretion is dependent on the glomerular filtration rate (GFR), tubular secretion and resorption. In pregnancy, GFR is increased by 50% from the first trimester and continues to increase until the last week of pregnancy (138, 145). Therefore, the clearance of drugs and metabolites that are renally excreted, such as salicyluric acid and

SA, are thought to parallel the change in GFR in pregnancy, hence leading to lower Cmax and AUC(t-24) as demonstrated in this thesis (146). Plasma protein binding of drugs is known to reduce in pregnancy due to reduced albumin concentration and estrogen-induced reduced protein-binding of drugs (143, 147). Decreased protein binding increases the concentration of free drug and consequently increases the clearance of drugs and metabolites such as salicylates via increased renal clearance in pregnancy. The net effect of reduced protein binding, however, is potentially counterbalanced by increased renal clearance of drugs and metabolites like SA.

When comparing the effect of preparation (EC vs non-EC aspirin) and chronotherapy, this thesis did not demonstrate a difference in overall AUC(t-24) between EC and non-EC and chronotherapy variation of aspirin (126). The study in Chapter 4 (Scholarly research article 1) demonstrated higher plasma concentration of ATL with the use of 150 mg of aspirin compared to 100 mg of aspirin (116), however this study was underpowered to make a meaningful comparison of plasma concentration of IL-8, IL-10 and PlGF between 100mg and 150mg of aspirin (116). The PFA-100 marker that was used as part of the two-point adherence assessment (Chapter 6, Scholarly research article 3), was unable to provide a quantitative assessment of the CT when the reading was >300 seconds. Given that a CT of >300 seconds was consistently observed in the group of women who were on 150 mg of aspirin, the PFA-100 value was used as a qualitative assessment of adherence along with the plasma SA detection. However, an observational difference between the average PFA-100 with 100 mg (average CT of 233 seconds) and 150 mg (>300 seconds), suggest a potential difference in the degree of platelet aggregation inhibitory effect between both doses.

Whilst the pharmacokinetic findings of this study supports the emerging recommendation of 150 mg of aspirin in the prevention of preeclampsia, future studies should focus on examining for differences in pharmacodynamic and clinical effects when comparing 100 mg and 150 mg of aspirin. Pharmacodynamic studies through assessment of the effect of aspirin on either the COX-1, COX-2 or both pathways would be useful in addressing this question. The COX-1 pathway assessment could be assessed through the gold standard quantitative marker, light transmission aggregometry (LTA), however, this method requires laboratory expertise with internal and external standardisation (148). PFA-100 and PFA-200 have been widely used in accredited clinical laboratories, however, as observed in our study, quantitative measures are limited by the inability to quantify a CT beyond 300 seconds. Non-obstetric studies have utilized plasma TXB2 and 6-keto prostaglandin F 1-alpha (6-keto-PGF1α) in examining pharmacodynamics of aspirin (120), however, the sensitivity of these markers have previously found to be variable given its short and unstable half-life . More recently, Multiplate® impedance platelet aggregometry (IPA) analyzer has been used as a rapid, comprehensive and sensitive platelet function analyzer (149), and has emerged as a potential marker in studying pharmacodynamics of anti-platelet therapy (150, 151). At the time of this thesis, access to the Multiplate® IPA was limited, however, with its growing use, its utility in obstetric aspirin pharmacodynamic studies would be of interest.

In addition to pharmacodynamic studies, future clinical studies, preferably undertaken as randomized controlled trials (RCT), directly comparing the rate of preeclampsia, IUGR and pre-term delivery between 100mg and 150mg of aspirin will be beneficial in answering the question on the optimal dose of aspirin in pregnancy. A RCT of this nature, with a large sample size, will also allow for an assessment of maternal and fetal risk of

harm with the use of 150 mg of aspirin, which has not been adequately documented in the literature at the time of this thesis

8.3 Influence of adherence to aspirin in pregnancy and factors that influences adherence to aspirin

The high prevalence of non-adherence to medications in pregnancy is well documented (128, 129), yet, the prevalence of non-adherence to prophylactic aspirin in pregnancy and its impact on obstetric outcomes remains poorly studied at the time of the thesis. The study in Chapter 6 (Scholarly research paper 3) demonstrated that 44% of the high-risk women in the longitudinal cohort study (Chapters 2.1 and 3) did not meet the two-point biochemical aspirin adherence criteria (101) . Inadequate adherence to aspirin was found to be associated with higher rates of preeclampsia, preterm delivery (Figure 18) and IUGR. This study also demonstrated that self-reported adherence, which is the commonly used qualitative assessment of adherence, only moderately correlated with biochemical verification of adherence. This study was novel in assessing for adherence with aspirin in a systematic two-point biochemical assessment and corresponding the observed adherence to clinical outcomes. This study also examined the correlation of self-reported adherence to biochemically verified adherence, providing evidence for the appropriate method of assessing adherence.

In examining for factors that influenced adherence to aspirin in pregnancy, participants from the longitudinal cohort study (Chapters 2.1 and 3) were invited to participate in a mixed methods study (Chapter 7, Scholarly research paper 4) (131). This study identified

both positive and negative factors that influenced adherence to aspirin in pregnancy, with two main themes: (1) Pill burden and accidental omission and (2) communication and relationship with HCPs. The findings of this study highlights the need for HCP to be aware of the impact of pill burden and the importance of effective and consistent communication with patient in improving adherence. Inconsistent messaging on the need and safety of aspirin in pregnancy amongst various HCP was identified as a recurring issue in this study. In identifying this, the investigators of this study (Scholarly research paper 7) along with other clinicians in SWSLHD, are currently working on initiatives to improve the uptake of aspirin in pregnancy. This includes:

1) An interventional, longitudinal cohort study on the effectiveness of aspirin and preeclampsia education and counselling

2) Education initiatives for aspirin prescribing (obstetricians, endocrinologist, nephrologist and general practitioners) and dispensing(pharmacist) HCPs to increase awareness on the use of prophylactic aspirin and emphasize on the importance of consistent messaging.

8.3.1 Effectiveness of aspirin and preeclampsia education and counselling

Based on the literate on the effectiveness of patient information sheet and other measures to improve adherence (152, 153), the team of HCPs involved in this thesis have developed an aspirin information brochure and summarized handout to improve

women's understanding on preeclampsia and the need for aspirin in pregnancy (Figures 21). The team will conduct an interventional longitudinal cohort study to assess the effectiveness of aspirin and preeclampsia education and counselling in improving the rate of adherence to aspirin in pregnancy. Enrolled high-risk women will participate in an electronic survey to establish their baseline understanding of preeclampsia and prophylactic aspirin prior to their education session with the clinician. The women will then undergo a standardized education session (based on a checklist for the prescribing clinicians) to ensure that key counselling points are discussed at the time of initiation of aspirin (Table 9), along with distribution of the aspirin information brochure (Figures 21).

Participating women will then undergo a second electronic survey 2-3 weeks following the education session to assess for effectiveness of the education session and utility of the aspirin information brochure. The second electronic survey will include an assessment of women's satisfaction with the education session and aspirin information brochure used.

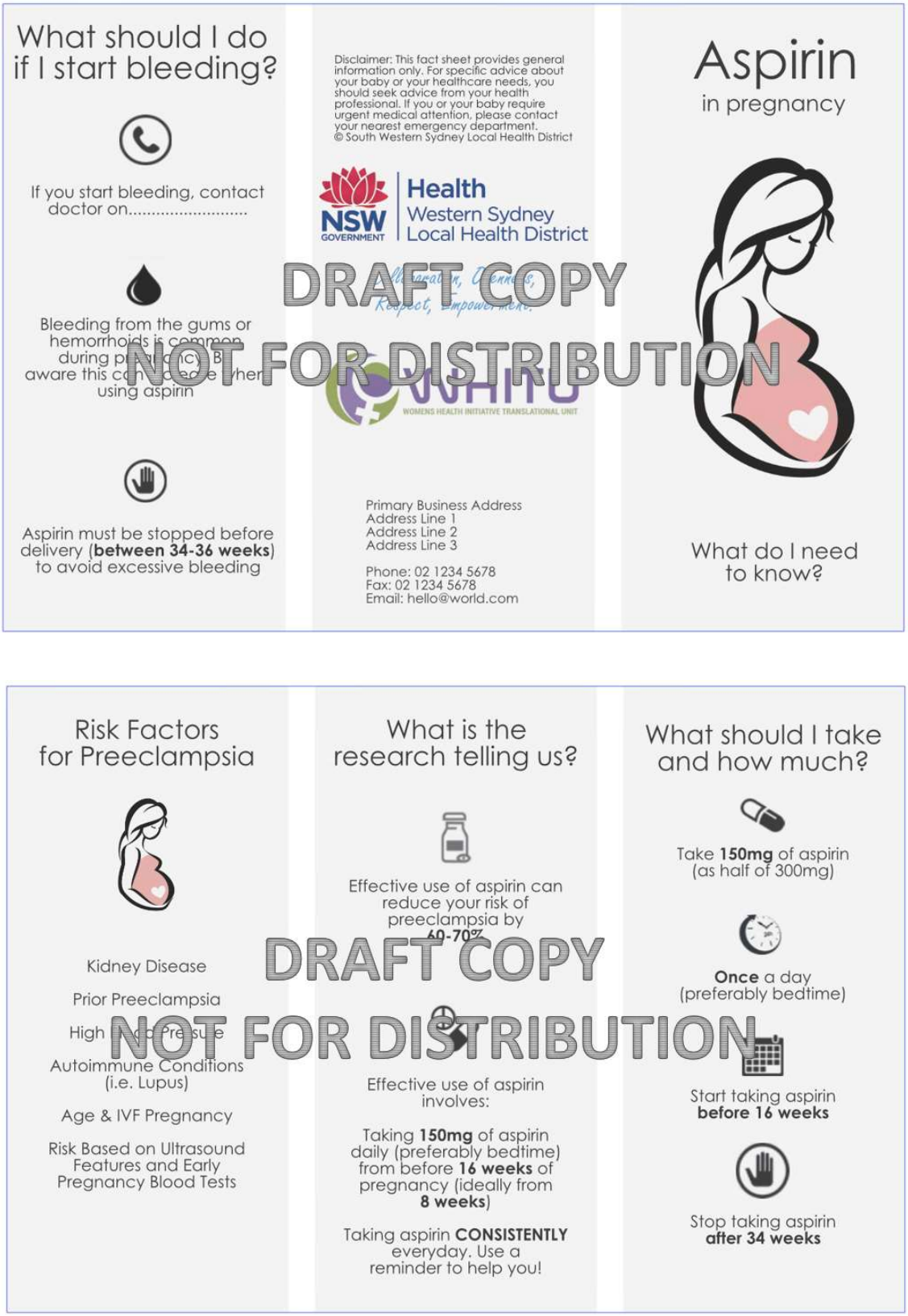


Figure 21: Aspirin information brochure

Aspirin initiation counselling checklist

- What is preeclampsia
- Why is the patient at a higher risk of preeclampsia (specific to patient)
- Evidence on the use of aspirin to reduce her risk of preeclampsia
- How to take aspirin
    - Dose
    - Timing
    - When to stop
- Side effects
- What to do if side effects occur
- Importance of adherence
- Strategies to reduce risk of accidental omission (pill box, electronic reminder)

Table 9: Aspirin counselling checklist

Concurrent biochemical adherence with aspirin, with PFA-100 and plasma SA detection as outlined in Chapter 6 will be conducted to assess for adherence to aspirin. The assessment of adherence in the SWSLHD cohort through these interventions will be compared against rate of adherence in the same cohort population without these interventions (Chapter 7). At the end of the study period, a quantitative assessment of clinician's satisfaction with the counselling intervention will be conducted.

8.3.2 Education initiatives for Health Care Providers (HCP)

The candidate of this thesis, along with the supervisors of this thesis, have conducted education sessions to HCPs at district and national level meetings and publications to increase awareness of the use of prophylactic aspirin in the prevention of preeclampsia.

This includes:

1) Prophylactic use of aspirin in the prevention of preeclampsia.
   Invited speaker at the Annual Scientific Meeting, Australian Diabetes in Pregnancy Society (ADIPS), Sydney 2019.
   R. Shanmugalingam

2) Tackling pre-eclampsia: getting ahead of the game.
   Invited authorship for How To Treat Edition of Australian Doctors
   (General Practitioner (GP) Continuing professional development (CPD) article) 2019.
   R. Shanmugalingam, A. Makris, A. Hennessy

3) The use of prophylactic aspirin in high-risk women with pre-existing Diabetes.
   Invited speaker at the SWSLHD Department of Endocrinology Meeting, Liverpool Hospital 2018.
   R. Shanmugalingam

4) Prophylactic use of aspirin in the prevention of preeclampsia.

   Invited speaker at the SWSLHD Department of Obstetrics and Gynaecology Meeting, Liverpool Hospital 2018, 2019 and 2020.

   R. Shanmugalingam and A. Makris

5) Tackling preeclampsia: Getting ahead of the game

   Invited speaker at the SWSLHD General Practitioner (GP) CPD Education Meeting, Sydney 2017.

   R. Shanmugalingam:

Future initiatives:

1) CPD article on the use of prophylactic aspirin in the prevention of preeclampsia for Australian Journal of Pharmacy, targeting pharmacist

   R.Shanmugalingam, A.Hennessy, A.Makris (manuscript in preparation)

2) Presentation at the SWSLHD Department of Renal Medicine Meeting, 2020

   R.Shanmugalingam and A.Makris

8.4 Conclusions

Aspirin is an effective prophylactic intervention for preeclampsia when adequate adherence is observed. In addition to its anti-platelet effect, aspirin is likely to play a role in placental development through its ATL mediated anti-inflammatory effect in reducing one's risk of preeclampsia. In optimizing the rate of risk reduction of preeclampsia with prophylactic aspirin, there may be a need to dose adjust aspirin in pregnancy given its altered pharmacokinetic in pregnancy. However, further pharmacodynamic and randomized controlled studies will be required to further assess this finding. Aspirin adherence should be assessed in clinical practice and future aspirin studies as adequate adherence plays an important role on the observed risk reduction of preeclampsia. The growing research interest on the use prophylactic aspirin along with the advances in *in-vitro* placental models and platelet aggregation analysis will allow for a greater understanding on ways clinicians, globally, can utilize aspirin in successfully diminishing one's risk of preeclampsia

REFERENCES

1. Thesis as a Series of Papers, Section (J), Doctorate Policy/Policies, Western Sydney University, Australia.

2. Thornton C, Dahlen H, Korda A, Hennessy A. The incidence of preeclampsia and eclampsia and associated maternal mortality in Australia from population-linked datasets: 2000-2008. Am J Obstet Gynecol. 2013;208(6):476 e1-5.

3. Jeyabalan A. Epidemiology of preeclampsia: impact of obesity. Nutr Rev. 2013;71 Suppl 1:S18-25.

4. Duley L. The global impact of pre-eclampsia and eclampsia. Semin Perinatol. 2009;33(3):130-7.

5. Maynard SE, Venkatesha S, Thadhani R, Karumanchi SA. Soluble Fms-like tyrosine kinase 1 and endothelial dysfunction in the pathogenesis of preeclampsia. Pediatr Res. 2005;57(5 Pt 2):1R-7R.

6. Levine RJ, Lam C, Qian C, Yu KF, Maynard SE, Sachs BP, et al. Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. N Engl J Med. 2006;355(10):992-1005.

7. Roberts JM, Escudero C. The placenta in preeclampsia. Pregnancy Hypertens. 2012;2(2):72-83.

8. Gerretsen G, Huisjes HJ, Hardonk MJ, Elema JD. Trophoblast alterations in the placental bed in relation to physiological changes in spiral arteries. Br J Obstet Gynaecol. 1983;90(1):34-9.

9. Robertson WB, Brosens I, Pijnenborg R, De Wolf F. The making of the placental bed. Eur J Obstet Gynecol Reprod Biol. 1984;18(5-6):255-66.

10. Hung TH, Skepper JN, Charnock-Jones DS, Burton GJ. Hypoxia-reoxygenation: a potent inducer of apoptotic changes in the human placenta and possible etiological factor in preeclampsia. Circ Res. 2002;90(12):1274-81.

11. Genbacev O, Zhou Y, Ludlow JW, Fisher SJ. Regulation of human placental development by oxygen tension. Science. 1997;277(5332):1669-72.

12. Redman CW, Sargent IL. Immunology of pre-eclampsia. Am J Reprod Immunol. 2010;63(6):534-43.

13. Zhou Y, Damsky CH, Fisher SJ. Preeclampsia is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype. One cause of defective endovascular invasion in this syndrome? J Clin Invest. 1997;99(9):2152-64.

14. Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest. 2003;111(5):649-58.

15. Makris A, Thornton C, Thompson J, Thomson S, Martin R, Ogle R, et al. Uteroplacental ischemia results in proteinuric hypertension and elevated sFLT-1. Kidney Int. 2007;71(10):977-84.

16. Zeisler H, Hund M, Verlohren S. The sFlt-1:PlGF Ratio in Women with Suspected Preeclampsia. N Engl J Med. 2016;374(18):1785-6.

17. Zeisler H, Llurba E, Chantraine FJ, Vatish M, Staff AC, Sennström M, et al. Soluble fms-like tyrosine kinase-1 to placental growth factor ratio: ruling out pre-eclampsia for up to 4 weeks and value of retesting. Ultrasound Obstet Gynecol. 2019;53(3):367-75.

18. Hodel M, Blank PR, Marty P, Lapaire O. sFlt-1/PlGF Ratio as a Predictive Marker in Women with Suspected Preeclampsia: An Economic Evaluation from a Swiss Perspective. Dis Markers. 2019;2019:4096847.

19. Bian X, Biswas A, Huang X, Lee KJ, Li TK, Masuyama H, et al. Short-Term Prediction of Adverse Outcomes Using the sFlt-1 (Soluble fms-Like Tyrosine Kinase 1)/PlGF (Placental Growth Factor) Ratio in Asian Women With Suspected Preeclampsia. Hypertension. 2019;74(1):164-72.

20. Cerdeira AS, O'Sullivan J, Ohuma EO, Harrington D, Szafranski P, Black R, et al. Randomized Interventional Study on Prediction of Preeclampsia/Eclampsia in Women With Suspected Preeclampsia: INSPIRE. Hypertension. 2019;74(4):983-90.

21. Goodlin RC, Haesslein HO, Fleming J. Aspirin for the treatment of recurrent toxaemia. Lancet (London, England). 1978;2(8079):51.

22. Walsh SW. Thromboxane production in placentas of women with preeclampsia. Am J Obstet Gynecol. 1989;160(6):1535-6.

23. Walsh SW. Physiology of low-dose aspirin therapy for the prevention of preeclampsia. Semin Perinatol. 1990;14(2):152-70.

24. Beaufils M, Uzan S, Donsimoni R, Colau JC. Prevention of pre-eclampsia by early antiplatelet therapy. Lancet (London, England). 1985;1(8433):840-2.

25. Uzan S, Beaufils M, Breart G, Bazin B, Capitant C, Paris J. Prevention of fetal growth retardation with low-dose aspirin: findings of the EPREDA trial. Lancet (London, England). 1991;337(8755):1427-31.

26. CLASP: a randomised trial of low-dose aspirin for the prevention and treatment of pre-eclampsia among 9364 pregnant women. CLASP (Collaborative Low-dose Aspirin Study in Pregnancy) Collaborative Group. Lancet (London, England). 1994;343(8898):619-29.

27. Rotchell YE, Cruickshank JK, Gay MP, Griffiths J, Stewart A, Farrell B, et al. Barbados Low Dose Aspirin Study in Pregnancy (BLASP): a randomised trial for the prevention of pre-eclampsia and its complications. Br J Obstet Gynaecol. 1998;105(3):286-92.

28. Caritis S, Sibai B, Hauth J, Lindheimer MD, Klebanoff M, Thom E, et al. Low-dose aspirin to prevent preeclampsia in women at high risk. National Institute of Child Health and Human Development Network of Maternal-Fetal Medicine Units. N Engl J Med. 1998;338(11):701-5.

29. Rolnik DL, Wright D, Poon LC, O'Gorman N, Syngelaki A, de Paco Matallana C, et al. Aspirin versus Placebo in Pregnancies at High Risk for Preterm Preeclampsia. N Engl J Med. 2017;377(7):613-22.

30. Bujold E, Roberge S, Lacasse Y, Bureau M, Audibert F, Marcoux S, et al. Prevention of preeclampsia and intrauterine growth restriction with aspirin started in early pregnancy: a meta-analysis. Obstetrics and gynecology. 2010;116(2 Pt 1):402-14.

31. Askie LM, Duley L, Henderson-Smart DJ, Stewart LA, Group PC. Antiplatelet agents for prevention of pre-eclampsia: a meta-analysis of individual patient data. Lancet (London, England). 2007;369(9575):1791-8.

32. Sibai BM, Caritis SN, Thom E, Shaw K, McNellis D. Low-dose aspirin in nulliparous women: safety of continuous epidural block and correlation between bleeding time and maternal-neonatal bleeding complications. National Institute of Child Health and Human Developmental Maternal-Fetal Medicine Network. Am J Obstet Gynecol. 1995;172(5):1553-7.

33. Poon LC, Kametas NA, Chelemen T, Leal A, Nicolaides KH. Maternal risk factors for hypertensive disorders in pregnancy: a multivariate approach. J Hum Hypertens. 2010;24(2):104-10.

34. North RA, McCowan LM, Dekker GA, Poston L, Chan EH, Stewart AW, et al. Clinical risk prediction for pre-eclampsia in nulliparous women: development of model in international prospective cohort. BMJ. 2011;342:d1875.

35. Visintin C, Mugglestone MA, Almerie MQ, Nherera LM, James D, Walkinshaw S, et al. Management of hypertensive disorders during pregnancy: summary of NICE guidance. BMJ. 2010;341:c2207.

36. Onwudiwe N, Yu CK, Poon LC, Spiliopoulos I, Nicolaides KH. Prediction of pre-eclampsia by a combination of maternal history, uterine artery Doppler and mean arterial pressure. Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology. 2008;32(7):877-83.

37. Poon LC, Kametas NA, Valencia C, Chelemen T, Nicolaides KH. Hypertensive disorders in pregnancy: screening by systolic diastolic and mean arterial pressure at 11-13 weeks. Hypertens Pregnancy. 2011;30(1):93-107.

38. Akolekar R, Syngelaki A, Poon L, Wright D, Nicolaides KH. Competing risks model in early screening for preeclampsia by biophysical and biochemical markers. Fetal Diagn Ther. 2013;33(1):8-15.

39. Lowe SA, Bowyer L, Lust K, McMahon LP, Morton M, North RA, et al. SOMANZ guidelines for the management of hypertensive disorders of pregnancy 2014. Aust N Z J Obstet Gynaecol. 2015;55(5):e1-29.

40. Redman CW. Hypertension in pregnancy: the NICE guidelines. Heart. 2011;97(23):1967-9.

41. Tranquilli AL. Introduction to ISSHP new classification of preeclampsia. Pregnancy hypertension. 2013;3(2):58-9.

42. Roberge S, Nicolaides K, Demers S, Hyett J, Chaillet N, Bujold E. The role of aspirin dose on the prevention of preeclampsia and fetal growth restriction: systematic review and meta-analysis. Am J Obstet Gynecol. 2017;216(2):110-20 e6.

43. Meher S, Duley L, Hunter K, Askie L. Antiplatelet therapy before or after 16 weeks' gestation for preventing preeclampsia: an individual participant data meta-analysis. Am J Obstet Gynecol. 2017;216(2):121-8 e2.

44. Park F, Russo K, Williams P, Pelosi M, Puddephatt R, Walter M, et al. Prediction and prevention of early-onset pre-eclampsia: impact of aspirin after first-trimester screening. Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology. 2015;46(4):419-23.

45. Zvanca ME, Bot M, Radu D, Radu N, Petca A. Impact of early supplementation with low-dose aspirin on functional first trimester parameters in low-risk pregnancies. J Matern Fetal Neonatal Med. 2017:1-6.

46. Roth GJ, Majerus PW. The mechanism of the effect of aspirin on human platelets. I. Acetylation of a particulate fraction protein. J Clin Invest. 1975;56(3):624-32.

47. Schiff E, Peleg E, Goldenberg M, Rosenthal T, Ruppin E, Tamarkin M, et al. The use of aspirin to prevent pregnancy-induced hypertension and lower the ratio of thromboxane A2 to prostacyclin in relatively high risk pregnancies. N Engl J Med. 1989;321(6):351-6.

48. Viinikka L, Hartikainen-Sorri AL, Lumme R, Hiilesmaa V, Ylikorkala O. Low dose aspirin in hypertensive pregnant women: effect on pregnancy outcome and prostacyclin-thromboxane balance in mother and newborn. Br J Obstet Gynaecol. 1993;100(9):809-15.

49. Roberts MS, Joyce RM, McLeod LJ, Vial JH, Seville PR. Slow-release aspirin and prostaglandin inhibition. Lancet (London, England). 1986;1(8490):1153-4.

50. Sibai BM, Mirro R, Chesney CM, Leffler C. Low-dose aspirin in pregnancy. Obstetrics and gynecology. 1989;74(4):551-7.

51. Haapsamo M, Martikainen H, Rasanen J. Low-dose aspirin reduces uteroplacental vascular impedance in early and mid gestation in IVF and ICSI patients: a randomized, placebo-controlled double-blind study. Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology. 2008;32(5):687-93.

52. Coomarasamy A, Papaioannou S, Gee H, Khan KS. Aspirin for the prevention of preeclampsia in women with abnormal uterine artery Doppler: a meta-analysis. Obstetrics and gynecology. 2001;98(5 Pt 1):861-6.

53. Vainio M, Riutta A, Koivisto AM, Maenpaa J. Prostacyclin, thromboxane A and the effect of low-dose ASA in pregnancies at high risk for hypertensive disorders. Acta Obstet Gynecol Scand. 2004;83(12):1119-23.

54. Claria J, Serhan CN. Aspirin triggers previously undescribed bioactive eicosanoids by human endothelial cell-leukocyte interactions. Proc Natl Acad Sci U S A. 1995;92(21):9475-9.

55. Mitchell S, Thomas G, Harvey K, Cottell D, Reville K, Berlasconi G, et al. Lipoxins, aspirin-triggered epi-lipoxins, lipoxin stable analogues, and the resolution of inflammation: stimulation of macrophage phagocytosis of apoptotic neutrophils in vivo. J Am Soc Nephrol. 2002;13(10):2497-507.

56. Fierro IM, Kutok JL, Serhan CN. Novel lipid mediator regulators of endothelial cell proliferation and migration: aspirin-triggered-15R-lipoxin A(4) and lipoxin A(4). J Pharmacol Exp Ther. 2002;300(2):385-92.

57. Romano M, Cianci E, Simiele F, Recchiuti A. Lipoxins and aspirin-triggered lipoxins in resolution of inflammation. Eur J Pharmacol. 2015;760:49-63.

58. Goh J, Baird AW, O'Keane C, Watson RW, Cottell D, Bernasconi G, et al. Lipoxin A(4) and aspirin-triggered 15-epi-lipoxin A(4) antagonize TNF-alpha-stimulated neutrophil-enterocyte interactions in vitro and attenuate TNF-alpha-induced chemokine release and colonocyte apoptosis in human intestinal mucosa ex vivo. J Immunol. 2001;167(5):2772-80.

59. Ariel A, Chiang N, Arita M, Petasis NA, Serhan CN. Aspirin-triggered lipoxin A4 and B4 analogs block extracellular signal-regulated kinase-dependent TNF-alpha secretion from human T cells. J Immunol. 2003;170(12):6266-72.

60. Nascimento-Silva V, Arruda MA, Barja-Fidalgo C, Villela CG, Fierro IM. Novel lipid mediator aspirin-triggered lipoxin A4 induces heme oxygenase-1 in endothelial cells. Am J Physiol Cell Physiol. 2005;289(3):C557-63.

61. Chiang N, Takano T, Arita M, Watanabe S, Serhan CN. A novel rat lipoxin A4 receptor that is conserved in structure and function. Br J Pharmacol. 2003;139(1):89-98.

62. Gewirtz AT, Collier-Hyams LS, Young AN, Kucharzik T, Guilford WJ, Parkinson JF, et al. Lipoxin a4 analogs attenuate induction of intestinal epithelial proinflammatory gene expression and reduce the severity of dextran sodium sulfate-induced colitis. J Immunol. 2002;168(10):5260-7.

63. Fiorucci S, Distrutti E, Mencarelli A, Morelli A, Laufor SA, Cirino G, et al. Evidence that 5-lipoxygenase and acetylated cyclooxygenase 2-derived eicosanoids regulate leukocyte-endothelial adherence in response to aspirin. Br J Pharmacol. 2003;139(7):1351-9.

64. Zhangye Xu MD, Feng Zhao, M.M., Feng Lin, M.M., Huiqiu Xiang, M.M., Ni Wang, M.M., Duyun Ye, M.M., Yinping Huang, M.M. Preeclampsia is associated with a deficiency of lipoxin A4, an endogenous anti-inflammatory mediator. Fertility and Sterility. 2014;102(1):282–90.e4.

65. Xu Z, Zhao J, Zhang H, Ke T, Xu P, Cai W, et al. Spontaneous miscarriages are explained by the stress/glucocorticoid/lipoxin A4 axis. J Immunol. 2013;190(12):6051-8.

66. Lipa M, Bomba-Opon D, Lipa J, Bartnik P, Bartoszewicz Z, Wielgos M. Lipoxin A4 (LXA4) as a potential first trimester biochemical marker of intrauterine growth disorders. J Matern Fetal Neonatal Med. 2017;30(20):2495-7.

67. Alvarez AM, Mulla MJ, Chamley LW, Cadavid AP, Abrahams VM. Aspirin-triggered lipoxin prevents antiphospholipid antibody effects on human trophoblast migration and endothelial cell interactions. Arthritis Rheumatol. 2015;67(2):488-97.

68. Gil-Villa AM, Norling LV, Serhan CN, Cordero D, Rojas M, Cadavid A. Aspirin triggered-lipoxin A4 reduces the adhesion of human polymorphonuclear neutrophils to endothelial cells initiated by preeclamptic plasma. Prostaglandins Leukot Essent Fatty Acids. 2012;87(4-5):127-34.

69. Cadavid AP. Aspirin: The Mechanism of Action Revisited in the Context of Pregnancy Complications. Front Immunol. 2017;8:261.

70. Rolnik DL, Wright D, Poon LCY, Syngelaki A, O'Gorman N, de Paco Matallana C, et al. ASPRE trial: performance of screening for preterm pre-eclampsia. Ultrasound in

obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology. 2017;50(4):492-5.

71. Tranquilli AL, Dekker G, Magee L, Roberts J, Sibai BM, Steyn W, et al. The classification, diagnosis and management of the hypertensive disorders of pregnancy: A revised statement from the ISSHP. Pregnancy Hypertens. 2014;4(2):97-104.

72. Brown MA, Magee LA, Kenny LC, Karumanchi SA, McCarthy FP, Saito S, et al. The hypertensive disorders of pregnancy: ISSHP classification, diagnosis & management recommendations for international practice. Pregnancy Hypertens. 2018;13:291-310.

73. Kuliczkowski W, Witkowski A, Polonski L, Watala C, Filipiak K, Budaj A, et al. Interindividual variability in the response to oral antiplatelet drugs: a position paper of the Working Group on antiplatelet drugs resistance appointed by the Section of Cardiovascular Interventions of the Polish Cardiac Society, endorsed by the Working Group on Thrombosis of the European Society of Cardiology. Eur Heart J. 2009;30(4):426-35.

74. Kuliczkowski W, Gasior M, Pres D, Kaczmarski J, Laszowska A, Szewczyk M, et al. Aspirin 'resistance': impact on no-reflow, platelet and inflammatory biomarkers in diabetics after ST-segment elevation myocardial infarction. Cardiology. 2015;131(1):41-50.

75. Krasopoulos G, Brister SJ, Beattie WS, Buchanan MR. Aspirin "resistance" and risk of cardiovascular morbidity: systematic review and meta-analysis. BMJ. 2008;336(7637):195-8.

76. Lee PY, Chen WH, Ng W, Cheng X, Kwok JY, Tse HF, et al. Low-dose aspirin increases aspirin resistance in patients with coronary artery disease. Am J Med. 2005;118(7):723-7.

77. Cuisset T, Frere C, Quilici J, Gaborit B, Bali L, Poyet R, et al. Aspirin noncompliance is the major cause of "aspirin resistance" in patients undergoing coronary stenting. Am Heart J. 2009;157(5):889-93.

78. Schwartz KA, Schwartz DE, Barber K, Reeves M, De Franco AC. Non-compliance is the predominant cause of aspirin resistance in chronic coronary arterial disease patients. J Transl Med. 2008;6:46.

79. Caron N, Rivard GE, Michon N, Morin F, Pilon D, Moutquin JM, et al. Low-dose ASA response using the PFA-100 in women with high-risk pregnancy. J Obstet Gynaecol Can. 2009;31(11):1022-7.

80. Abheiden CN, van Reuler AV, Fuijkschot WW, de Vries JI, Thijs A, de Boer MA. Aspirin adherence during high-risk pregnancies, a questionnaire study. Pregnancy hypertension. 2016;6(4):350-5.

81. Wright D, Poon LC, Rolnik DL, Syngelaki A, Delgado JL, Vojtassakova D, et al. Aspirin for Evidence-Based Preeclampsia Prevention trial: influence of compliance on beneficial effect of aspirin in prevention of preterm preeclampsia. Am J Obstet Gynecol. 2017;217(6):685 e1- e5.

82. OLADEJO M BS. ADHERENCE IN PREGNANCY: A SYSTEMATIC REVIEW OF THE LITERATURE. Fetal and Maternal Medicine Review2012. p. 201-29.

83. Lam WY, Fresco P. Medication Adherence Measures: An Overview. Biomed Res Int. 2015;2015:217047.

84. Navaratnam K, Alfirevic A, Jorgensen A, Alfirevic Z. Aspirin non-responsiveness in pregnant women at high-risk of pre-eclampsia. Eur J Obstet Gynecol Reprod Biol. 2018;221:144-50.

85. Abaci A, Caliskan M, Bayram F, Yilmaz Y, Cetin M, Unal A, et al. A new definition of aspirin non-responsiveness by platelet function analyzer-100 and its predictors. Platelets. 2006;17(1):7-13.

86. Bhatt DL, Grosser T, Dong JF, Logan D, Jeske W, Angiolillo DJ, et al. Enteric Coating and Aspirin Nonresponsiveness in Patients With Type 2 Diabetes Mellitus. J Am Coll Cardiol. 2017;69(6):603-12.

87. Xu ZH, Jiao JR, Yang R, Luo BY, Wang XF, Wu F. Aspirin resistance: clinical significance and genetic polymorphism. J Int Med Res. 2012;40(1):282-92.

88. Cox D, Maree AO, Dooley M, Conroy R, Byrne MF, Fitzgerald DJ. Effect of enteric coating on antiplatelet activity of low-dose aspirin in healthy volunteers. Stroke. 2006;37(8):2153-8.

89. Van Hecken A, Juliano ML, Depre M, De Lepeleire I, Arnout J, Dynder A, et al. Effects of enteric-coated, low-dose aspirin on parameters of platelet function. Aliment Pharmacol Ther. 2002;16(9):1683-8.

90. Karha J, Rajagopal V, Kottke-Marchant K, Bhatt DL. Lack of effect of enteric coating on aspirin-induced inhibition of platelet aggregation in healthy volunteers. Am Heart J. 2006;151(5):976 e7-11.

91. New South Wales (NSW) Health A. NSW Mothers and Babies Report : https://www.health.nsw.gov.au/hsnsw/Publications/mothers-and-babies-2017.pdf. 2017.

92. Poon LC, Nicolaides KH. Early prediction of preeclampsia. Obstet Gynecol Int. 2014;2014:297397.

93. Roberge S, Odibo AO, Bujold E. Aspirin for the Prevention of Preeclampsia and Intrauterine Growth Restriction. Clin Lab Med. 2016;36(2):319-29.

94. Systems RD. Magnetic Luminex Perfomance Assay. Human High Sensitivity Cytokine Premixed Kit A Catalog Number FCSTM09.

95. New South Wales Health Pathology (NSWHP)-South-Liverpool Haematology Platelet Aggregometry & PFA manual.

96. Ortega Suárez FJ, Sánchez Plumed J, Pérez Valentín MA, Pereira Palomo P, Muñoz Cepeda MA, Lorenzo Aguiar D, et al. Validation on the simplified medication adherence questionnaire (SMAQ) in renal transplant patients on tacrolimus. Nefrologia. 2011;31(6):690-6.

97. Nguyen TM, La Caze A, Cottrell N. What are validated self-report adherence scales really measuring?: a systematic review. Br J Clin Pharmacol. 2014;77(3):427-45.

98. Knobel H, Alonso J, Casado JL, Collazos J, González J, Ruiz I, et al. Validation of a simplified medication adherence questionnaire in a large cohort of HIV-infected patients: the GEEMA Study. AIDS. 2002;16(4):605-13.

99. Hsu C, Lemon JM, Wong ES, Carson-Cheng E, Perkins M, Nordstrom MS, et al. Factors affecting medication adherence: patient perspectives from five veterans affairs facilities. BMC Health Serv Res. 2014;14:533.

100. Mathes T, Jaschinski T, Pieper D. Adherence influencing factors - a systematic review of systematic reviews. Arch Public Health. 2014;72(1):37.

101. Shanmugalingam R, Wang X, Motum P, Fulcher I, Lee G, Kumar R, et al. Clinical Influence of Nonadherence With Prophylactic Aspirin in Preventing Preeclampsia in High-Risk Pregnancies: A Multicenter, Prospective, Observational Cohort Study. Hypertension. 2020;75(4):1125-32.

102. Gallinger ZR, Rumman A, Nguyen GC. Perceptions and Attitudes Towards Medication Adherence during Pregnancy in Inflammatory Bowel Disease. J Crohns Colitis. 2016;10(8):892-7.

103. Amundsen S, Øvrebø TG, Amble NMS, Poole AC, Nordeng H. Risk perception, beliefs about medicines and medical adherence among pregnant and breastfeeding women with migraine: findings from a cross-sectional study in Norway. BMJ Open. 2019;9(2):e026690.

104. Titilayo A, Palamuleni ME, Omisakin O. Sociodemographic factors influencing adherence to antenatal iron supplementation recommendations among pregnant women in Malawi: Analysis of data from the 2010 Malawi Demographic and Health Survey. Malawi Med J. 2016;28(1):1-5.

105. Kingdon C, Neilson J, Singleton V, Gyte G, Hart A, Gabbay M, et al. Choice and birth method: mixed-method study of caesarean delivery for maternal request. BJOG. 2009;116(7):886-95.

106. Braun V, Clarke V. What can "thematic analysis" offer health and wellbeing researchers? Int J Qual Stud Health Well-being. 2014;9:26152.

107. O'Cathain A, Murphy E, Nicholl J. Three techniques for integrating data in mixed methods studies. BMJ. 2010;341:c4587.

108. Tariq S, Woodman J. Using mixed methods in health research. JRSM Short Rep. 2013;4(6):2042533313479197.

109. Roberge S, Sibai B, McCaw-Binns A, Bujold E. Low-Dose Aspirin in Early Gestation for Prevention of Preeclampsia and Small-for-Gestational-Age Neonates: Meta-analysis of Large Randomized Trials. Am J Perinatol. 2016.

110. Solheim S, Arnesen H, Eikvar L, Hurlen M, Seljeflot I. Influence of aspirin on inflammatory markers in patients after acute myocardial infarction. Am J Cardiol. 2003;92(7):843-5.

111. Renda G, Zurro M, Romano M, De Caterina R. Aspirin-triggered lipoxin in patients treated with aspirin and selective vs. nonselective COX-2 inhibitors. Br J Clin Pharmacol. 2010;69(3):303-6.

112. Leonard MO, Hannan K, Burne MJ, Lappin DW, Doran P, Coleman P, et al. 15-Epi-16-(para-fluorophenoxy)-lipoxin A(4)-methyl ester, a synthetic analogue of 15-epi-lipoxin A(4), is protective in experimental ischemic acute renal failure. J Am Soc Nephrol. 2002;13(6):1657-62.

113. Takano T, Fiore S, Maddox JF, Brady HR, Petasis NA, Serhan CN. Aspirin-triggered 15-epi-lipoxin A4 (LXA4) and LXA4 stable analogues are potent inhibitors of acute inflammation: evidence for anti-inflammatory receptors. J Exp Med. 1997;185(9):1693-704.

114. Medeiros R, Kitazawa M, Passos GF, Baglietto-Vargas D, Cheng D, Cribbs DH, et al. Aspirin-triggered lipoxin A4 stimulates alternative activation of microglia and reduces Alzheimer disease-like pathology in mice. Am J Pathol. 2013;182(5):1780-9.

115. Murtoniemi K, Vahlberg T, Hämäläinen E, Kajantie E, Pesonen AK, Räikkönen K, et al. The effect of low-dose aspirin on serum placental growth factor levels in a high-risk PREDO cohort. Pregnancy Hypertens. 2018;13:51-7.

116. Shanmugalingam R WX, Motum P, Fulcher I, Lee G, Kumar Roshika, Hennessy A, Makris A. The 15-epilipoxin- A4 pathway (ATL) with prophylactic aspirin in preventing preeclampsia : a longitudinal-cohort study. Journal of Clinical Endocrinology and Metabolism (JCEM) (IN PRESS)2020.

117. Rymark P, Berntorp E, Nordsjo P, Liedholm H, Melander A, Gennser G. Low-dose aspirin to pregnant women: single dose pharmacokinetics and influence of short term treatment on bleeding time. J Perinat Med. 1994;22(3):205-11.

118. Alsmadi MM, Idkaidek N. Optimization of Drugs Pharmacotherapy During Pregnancy Using Physiologically Based Pharmacokinetic Models - An Update. Curr Drug Metab. 2018;19(12):972-8.

119. Levy G. Comparative pharmacokinetics of aspirin and acetaminophen. Arch Intern Med. 1981;141(3 Spec No):279-81.

120. Nagelschmitz J, Blunck M, Kraetzschmar J, Ludwig M, Wensing G, Hohlfeld T. Pharmacokinetics and pharmacodynamics of acetylsalicylic acid after intravenous and oral administration to healthy volunteers. Clin Pharmacol. 2014;6:51-9.

121. Roberge S, Bujold E, Nicolaides KH. Meta-analysis on the effect of aspirin use for prevention of preeclampsia on placental abruption and antepartum hemorrhage. Am J Obstet Gynecol. 2018;218(5):483-9.

122. Ayala DE, Ucieda R, Hermida RC. Chronotherapy with low-dose aspirin for prevention of complications in pregnancy. Chronobiol Int. 2013;30(1-2):260-79.

123. Hermida RC, Ayala DE, Calvo C, Lopez JE. Aspirin administered at bedtime, but not on awakening, has an effect on ambulatory blood pressure in hypertensive patients. J Am Coll Cardiol. 2005;46(6):975-83.

124. Zhang Y, Huo M, Zhou J, Xie S. PKSolver: An add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel. Comput Methods Programs Biomed. 2010;99(3):306-14.

125. Shanmugalingam R, Wang X, Munch G, Fulcher I, Lee G, Chau K, et al. A pharmacokinetic assessment of optimal dosing, preparation, and chronotherapy of aspirin in pregnancy. Am J Obstet Gynecol. 2019.

126. Shanmugalingam R, Wang X, Münch G, Fulcher I, Lee G, Chau K, et al. A pharmacokinetic assessment of optimal dosing, preparation, and chronotherapy of aspirin in pregnancy. Am J Obstet Gynecol. 2019.

127. Moore RA, Derry S, Wiffen PJ, Straube S. Effects of food on pharmacokinetics of immediate release oral formulations of aspirin, dipyrone, paracetamol and NSAIDs - a systematic review. Br J Clin Pharmacol. 2015;80(3):381-8.

128. Sawicki E, Stewart K, Wong S, Leung L, Paul E, George J. Medication use for chronic health conditions by pregnant women attending an Australian maternity hospital. Aust N Z J Obstet Gynaecol. 2011;51(4):333-8.

129. Fairgrieve SD, Jackson M, Jonas P, Walshaw D, White K, Montgomery TL, et al. Population based, prospective study of the care of women with epilepsy in pregnancy. BMJ. 2000;321(7262):674-5.

130. Andreae MH, White RS, Chen KY, Nair S, Hall C, Shaparin N. The Effect of Initiatives to Overcome Language Barriers and Improve Attendance: A Cross-Sectional Analysis of Adherence in an Inner City Chronic Pain Clinic. Pain Med. 2017;18(2):265-74.

131. Shanmugalingam R, Mengesha Z, Notaras S, Liamputtong P, Fulcher I, Lee G, et al. Factors that influence adherence to aspirin therapy in the prevention of preeclampsia amongst high-risk pregnant women: A mixed method analysis. PLoS One. 2020;15(2):e0229622.

132. Villalobos BT, Bridges AJ, Anastasia EA, Ojeda CA, Rodriguez JH, Gomez D. Effects of language concordance and interpreter use on therapeutic alliance in Spanish-speaking integrated behavioral health care patients. Psychol Serv. 2016;13(1):49-59.

133. Haider S, Meinhardt G, Saleh L, Kunihs V, Gamperl M, Kaindl U, et al. Self-Renewing Trophoblast Organoids Recapitulate the Developmental Program of the Early Human Placenta. Stem Cell Reports. 2018;11(2):537-51.

134. Turco MY, Gardner L, Kay RG, Hamilton RS, Prater M, Hollinshead MS, et al. Trophoblast organoids as a model for maternal-fetal interactions during human placentation. Nature. 2018;564(7735):263-7.

135. Knöfler M, Haider S, Saleh L, Pollheimer J, Gamage TKJB, James J. Human placenta and trophoblast development: key molecular mechanisms and model systems. Cell Mol Life Sci. 2019;76(18):3479-96.

136. Macfie AG, Magides AD, Richmond MN, Reilly CS. Gastric emptying in pregnancy. Br J Anaesth. 1991;67(1):54-7.

137. Chiloiro M, Darconza G, Piccioli E, De Carne M, Clemente C, Riezzo G. Gastric emptying and orocecal transit time in pregnancy. J Gastroenterol. 2001;36(8):538-43.

138. Anderson GD. Pregnancy-induced changes in pharmacokinetics: a mechanistic-based approach. Clin Pharmacokinet. 2005;44(10):989-1008.

139. Costantine MM. Physiologic and pharmacokinetic changes in pregnancy. Front Pharmacol. 2014;5:65.

140. Koren G, Pariente G. Pregnancy- Associated Changes in Pharmacokinetics and their Clinical Implications. Pharm Res. 2018;35(3):61.

141. Tsutsumi K, Kotegawa T, Matsuki S, Tanaka Y, Ishii Y, Kodama Y, et al. The effect of pregnancy on cytochrome P4501A2, xanthine oxidase, and N-acetyltransferase activities in humans. Clin Pharmacol Ther. 2001;70(2):121-5.

142. Kuehl GE, Bigler J, Potter JD, Lampe JW. Glucuronidation of the aspirin metabolite salicylic acid by expressed UDP-glucuronosyltransferases and human liver microsomes. Drug Metab Dispos. 2006;34(2):199-202.

143. Pariente G, Leibson T, Carls A, Adams-Webber T, Ito S, Koren G. Pregnancy-Associated Changes in Pharmacokinetics: A Systematic Review. PLoS Med. 2016;13(11):e1002160.

144. Chamberlain A, White S, Bawdon R, Thomas S, Larsen B. Pharmacokinetics of ampicillin and sulbactam in pregnancy. Am J Obstet Gynecol. 1993;168(2):667-73.

145. Davison JM, Dunlop W. Renal hemodynamics and tubular function normal human pregnancy. Kidney Int. 1980;18(2):152-61.

146. Feghali M, Venkataramanan R, Caritis S. Pharmacokinetics of drugs in pregnancy. Semin Perinatol. 2015;39(7):512-9.

147. Perucca E, Crema A. Plasma protein binding of drugs in pregnancy. Clin Pharmacokinet. 1982;7(4):336-52.

148. Paniccia R, Priora R, Liotta AA, Abbate R. Platelet function tests: a comparative review. Vasc Health Risk Manag. 2015;11:133-48.

149. Paniccia R, Antonucci E, Maggini N, Romano E, Gori AM, Marcucci R, et al. Assessment of platelet function on whole blood by multiple electrode aggregometry in high-risk patients with coronary artery disease receiving antiplatelet therapy. Am J Clin Pathol. 2009;131(6):834-42.

150. Butler K, Teng R. Pharmacokinetics, pharmacodynamics, safety and tolerability of multiple ascending doses of ticagrelor in healthy volunteers. Br J Clin Pharmacol. 2010;70(1):65-77.

151. Danese E, Fava C, Beltrame F, Tavella D, Calabria S, Benati M, et al. Relationship between pharmacokinetics and pharmacodynamics of clopidogrel in patients undergoing percutaneous coronary intervention: comparison between vasodilator-stimulated phosphoprotein phosphorylation assay and multiple electrode aggregometry. J Thromb Haemost. 2016;14(2):282-93.

152. Costa E, Giardini A, Savin M, Menditto E, Lehane E, Laosa O, et al. Interventional tools to improve medication adherence: review of literature. Patient Prefer Adherence. 2015;9:1303-14.

153. Sletvold H, Sagmo LAB, Torheim EA. Impact of pictograms on medication adherence: A systematic literature review. Patient Educ Couns. 2020;103(6):1095-103.