UNIVERSITY OF SOUTHAMPTON

FACULTY OF HEALTH SCIENCES

Changes of Blood Biomarkers Following Pre-eclampsia and Predictors of Future Cardiovascular Risk

By

Fatma.S.H. Abad

Thesis for the degree of Doctorate of Philosophy

July 2020

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF HEALTH SCIENCES

Thesis for the degree of Doctor of Philosophy

Changes of Blood Biomarkers Following Pre-eclampsia and Predictors of Future Cardiovascular Risk

By: Fatma. S.H. Abad

Background: Pre-eclampsia (P-EC) is a major cause of maternal and neonatal mortality and morbidity. Alterations in the maternal vasculature and coagulation profile may predispose women with P-EC to subsequent deleterious cardiovascular consequences. Indeed women with a history of P-EC are known to have heightened risks of future cardiovascular disease and thromboembolic complications.

Aims: To assess the relationship between circulating haemostatic factors and inflammatory cytokines in women with a previous history of P-EC.

To investigate the relationship between haemostatic, angiogenic and anti-angiogenic factors in women with a past history of P-EC.

To assess the plasma levels of Annexins A2 and A5 in pre-eclamptic women postpartum at different time intervals.

To identify clusters of differentially expressed plasma proteins in P-EC associated with the susceptibility to developing future cardiovascular diseases.

To examine miRNAs expression in P-EC post-delivery at different intervals time points.

Study-I: 26 pre-eclamptic women and 14 age-matched to healthy women. Women were included within six months to 3 years post-delivery. Plasma TF, IL-6, IL-8 and IL-10 levels increased in the P-EC group, whereas plasma TFPI and TNF-α levels were reduced. Plasma TF/TFPI ratios and IL-10 values were significantly increased in the P-EC group ($p<0.05$, $p<0.01$, respectively). There were positive and significant correlations between TFPI($r= 0.5$; $p<0.01$) and IL-10 and TF/TFPI ratio and IL-10 ($r= 0.31$; $p<0.041$).

Study-II: 21 primiparous women after a pregnancy affected by P-EC and 21 women with a previously unaffected pregnancy. Blood samples were obtained at 6-12 months postpartum.

Significant differences were not observed for VEGF, PlGF, sFlt-1, sEng, TF or TFPI between two groups.

Study-III: 66 women who had P-EC at interval years starting from 2007, and then from 2012 till 2016 and five as a control group. Findings revealed that the level of ANXA5 was reduced in P-EC cohort, and there was an increase in levels of annexin A2, particularly in late post-delivery pre-eclamptic women, although these changes were statistically insignificant. The lack of statistical significance may be due to the small number of control compare to P-EC group.

Study-IV: 5 women aged-matched with five women with normal pregnancy, results show that inflammation, immune response, blood coagulation and metabolism are dysregulated processes one-year post-delivery in women with a history of pre-eclampsia.

Study-V: Next-Generation Sequencing (NGS) technique was used during the discovery stage to identify miRNAs differentially expressed in P-EC postpartum (n=30) in comparison to five healthy control, then quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay used for confirmation and validation. 14 miRNAs that were significantly differentially expressed at a significance level of 0.05 (FDR) in the discovery experiment. The plasma miR-103a-3p were significantly differentially expressed in the validating experiment, which was downregulated in entire pre-eclamptic women with FC= -1.3; BH-adj P-value =0.033.

Conclusions: Results suggest the presence of elevated inflammatory cytokines and an imbalance of the haemostatic system in women with a past-history of P-EC, which may contribute to the known increased risk of cardiovascular disease later in life. The proteomics findings provide insight into the dysregulated cardiometabolic profile in the P-EC group. The miRNAs novel marker pave the way for the importance of miR-103a-3p in monitoring P-EC women in the future and could be a useful tool in predicting the development of cardiovascular disease in P-EC.

Table of Contents

List of Tables

List of Figures

DECLARATION OF AUTHORSHIP

I Fatma Abad declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

Changes of Blood Biomarkers Following Pre-eclampsia and Predictors of Future Cardiovascular Risk

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published in **Gynecology & Reproductive Health**
   [ISSN2639-9342] DOI: GRM-19-89
   Title: Inflammatory and Haemostatic Changes Following Pre-eclampsia: Potential Link with Development of Subsequent Cardiovascular Events?

E-Posters

59th BSH Annual Sciebtific Meeting 1-3 April 2019 [BSH-EP-070] Glasgow

Title: Plasma proteomic profiling one year postpartum of women with pre-eclampsia shows dysregulated cardiometabolic profile.

58th BSH Annual Sciebtific Meeting 16-18 April 2018 [BSH2018-I/1276] Liverpool

Title: Plasma annexins A2 and A5 in women with a past history of pre-eclampsia.

Raised plasma CD14 levels in women with a previous history of pre-eclampsia: A case-control proteomics study (Submitted to Diabetologia –Journal of the European Association for the study of Diabetes (EASD).

Participation in conferences

E-Posters

59th BSH Annual Sciebtific Meeting 1-3 April 2019 [BSH-EP-070] Glasgow

Title: Plasma proteomic profiling one year postpartum of women with pre-eclampsia shows dysregulated cardiometabolic profile.

58th BSH Annual Sciebtific Meeting 16-18 April 2018 [BSH2018-I/1276] Liverpool

Title: Plasma annexins A2 and A5 in women with a past history of pre-eclampsia.

Poster

57th Annual Scientific Meeting of the British Society for Haematology 27-29 March 2017

[BSH2017**-434] [PO-165] Brighton**

Title: Do patients with a history of pre-eclampsia have elevated levels of coagulation and angiogenic markers postpartum?

36th World Congress of the ISH hosted by the British Society for Haematology 18-21 April 2016 [Reference: ISH1619FC143] Glasgow

Title: Inflammatory and Haemostatic Changes Following Pre-eclampsia: Potential Link with Development of Subsequent Cardiovascular Events?

Signed: Fatma

Date: 01/07/2020

Acknowledgements

I would like to thank Allah (God) for all the blessings and for what I have received from him all my life. Five years have passed, and I should admit that writing an acknowledgement was harder than writing my PhD thesis in the second language. Many such emotional moments passed by since September 2014. I wish if my family were with me. However, being away from them and my home country gives me the strength to complete this study, which I am not regretting the pain of being homesick.

Firstly, I would like to express my sincere gratitude to my primary supervisor Professor Bashir Lwaleed (Professor of Experimental Pathology) you have been a tremendous mentor for me and to my secondary supervisors Dr Bas Van Rijn (University Medical Centre Utrecht, Utrecht, Netherlands) and Dr Brain Birch (Honorary Associate Professor Urology and Consultant Urologist) for their continuous support during my PhD study. I am truly blessed to have such great advisors and could not have completed this work without their support.

Besides, my advisors, I would like to thank Mrs Fiona Doyle in Level F and Shamila Tahseen at the Pathology Department Level C at Southampton General Hospital, who assisted me with blood sample collections. Without their precious support, this would not be possible to conduct the research.

I am deeply grateful for the funding that I received towards my PhD from Ministry of Higher Education and Scientific Research- Libya and special thanks for Dr Nasser Ghaith who formerly worked in the Libyan Cultural Affairs –London and Dr Ali Abdulrahim for their unconditional support and help especially during the first and final year in the PhD journey.

A big thank you for all my friends and colleagues at Tobruk Medical Centre- Libya, Dean of the University of Omar Almukhtar and all staff members at the Faculty of Medical technology Tobruk-Libya.

I would like to express my gratitude and appreciation for my best friends for life Raya, Asma, Eman, Elham, Rima and Diana for believing in me and giving the much-needed strength to push me towards my goal of completing the study.

Last but not the least, I would like to thank my family: my parents Saad and Warda, my sisters Salma, Amna, Marwa and Eman, my brothers Mohammed, Abdallah, and my beloved twins

AL -Hassan and AL- Hussain for supporting me spiritually and emotionally throughout this thesis and my life in general. It was their dream for me to get a doctorate degree.

I would like to dedicate this work in loving memory of Josephine Shaw, and to her daughter Sarah and son Robert. Josephine and her family embraced me and supported me throughout my study. I am glad to have found a new home away from home.

Definitions and Abbreviations

P-EC	**Pre-eclampsia**
CASP	**Critical Appraisal Skills Programme**
IL	**Interleukin**
TNF	**Tumour necrosis factor**
MCP-1	**Monocyte chemoattractant protein-1**
ICAM-1	**Intercellular adhesion molecule**
VCAM-1	**Vascular adhesion molecule-1**
CRP	**C-reactive protein**
FGR	**Foetal growth factor**
IUGR	**Intra uterine growth restriction**
PIGF	**Placental growth factor**
VEGF	**Vascular endothelial growth factor**
sFlt-1	**soluble fms-like tyrosine kinase 1**
sEng	**Soluble endoglin**
TF	**Tissue factor**
TFPI	**Tissue factor pathway inhibitor**
CVD	**Cardiovascular diseases**
IFNs	**Interferons**
ADP	**Adenosine diphosphate**
TXA2	**Thromboxane A2**
ISSHP	**International Society for the Study of Hypertension in Pregnancy**
EOP	**Early onset of pre-eclampsia**
LOP	**late onset of pre-eclampsia**
NO	**nitric oxide**

CO	**Carbon monoxide**
G-CSF	**Granulocyte-monocyte colony-stimulating factor**
LDL	**Cholesterol Low- density lipoprotein**
APH	**Antepartum haemorrhage**
PPH	**Postpartum haemorrhage**
PIH	**Pregnancy induced hypertension**
AFI	**Amniotic fluid index**
GDM	**Gestational Diabetes Mellitus**
ADA	**American Diabetes Association**
TVS	**Transvaginal ultrasound**
βhCG	**Beta human chorionic gonadotropin**
SBP	**Systolic blood pressure**
DBP	**Diastolic blood pressure**
Th	**T-helper cells**
Tregs	**Regulatory T cells**
VEGFR2/ VEGFR3	**Vascular endothelial growth factor receptors 2&3**
SGH	**Southampton General Hospital**
SPSS	**Statistical Package for the Social Sciences**
ELISA	**Enzyme-linked immuno-sorbent assay**
BMI	**Body mass index**
IPI	**Interpregnancy interval**
IQR	**Inter-quartile ranges**
SD	**Standard deviation**
HPLC	**High-performance liquid chromatography**
LC-MS/MS	**Liquid chromatography tandem mass spectrometry**
UFLC	**Ultra- Fast performance liquid chromatography**

UPLC	**Ultra-Performance liquid chromatography**
HP-SEC	**High Performance- Size exclusion chromatography**
MW	**Molecular weight**
GFC-SEC	**Gel filtration chromatography**
GPC-SEC	**Gel permeation chromatography**
ESI	**Electrospray ionisation**
MALDI	**Matrix-assisted laser desorption ionisation**
KDa	**Kilo Dalton**
MDa	**Millie Dalton**
SELDI	**Surface-enhanced laser desorption/ ionisation**
TOF	**Time-of-flight**
TOF-MS	**Time-of-Flight Mass Analyser**
TR	**Technical replicates**
MPa	**Mega Pascal**
TEAB	**Triethylammonium bicarbonate**
SDS	**Sodium dodecyl sulfate**
MMT	**Methyl methanethiosulfonate**
EDTA	**Ethylenediaminetetraacetic acid**
RP	**Reverse phase**
ACN	**Acetonitrile**
HCD	**Higher energy collisional dissociation**
CID	**Collision induced dissociation**
RPM	**Revolutions per minute**
FDR	**False Discovery Rate**
KEGG	**Kyoto Encyclopaedia of Genes and Genomes**
SOPIWG	**Standard Operating Procedure Integration Working Group**

iTRAQ	**Isobaric Tag for Relative and Absolute Quantitation**
IPA	**Ingenuity Pathway Analysis**
Yrs.	**Years**
DEPs	**Differentially Expressed Proteins**
EVs	**Extracellular Vesicles**
ANXA A1, A2 and A5	**Annexins A1, A2 and A5**
BSH	**British Society for Haematology**
C5	**Complement component**
PZP	**Pregnancy Zone Protein**
CD14	**Cluster of Differentiation 14**
PF4	**Platelet Factor 4**
FN1	**Fibronectin 1**
ADIPO	**Adiponectin**
LPS	**Lipopolysaccharides**
NGS	**Next Generation Sequencing**
UMIs	**Unique molecular indices**
RNA	**Ribonucleic acid**
DNA	**Deoxyribonucleic acid**
cDNA	**Complementary Deoxyribonucleic acid**
PCR	**Polymerase chain reaction**
QC	**Quality control**
CDS	**Coding DNA Sequence**
GO	**Gene Ontology (GO – Gene Ontology Consortium, 2000) is an initiative to describe genes, gene products and their attributes using vocabulary (GO terms) which is unified, hierarchical and controlled across all species. The GO terms are categorised into three GO domains: molecular function, biological process and**

	Cellular component
GO_ID	**Unique identifier for a Gene Ontology (GO) term.**
Isoforms	**Different closely related transcripts arising from the same primary transcript (and same gene or DNA sequence) by alternative splicing of exons for example. Isoforms may also be referred to as transcripts.**
LncRNA	**Long non-coding RNA**
Mappable reads	**Sequences which can be aligned to the reference genome**
mtRNA	**mitochondrial RNA**
Novel transcript	**A transcript that contains features not present in the reference annotation. A novel transcript can be both a new isoform of a known gene or a transcript without any known features. A novel transcript is most commonly a novel combination of exons or a different start site.**
Outmapped reads or high abundance reads	**For example; rRNA, mtRNA, poly-A and poly-C homopolymers**
phiX	**Libraries generated from the phiX virus used as a control in sequencing runs**
Primary transcript (pre-mRNA)	**RNA sequence transcribed from DNA. The primary transcript is then processed (e.g. by addition of 5' cap, 3'-poly-Adenylation, alternative splicing) to yield various mature RNA products such as mRNAs, ncRNAs, tRNAs, and rRNAs. Multiple primary transcripts may be transcribed from the same gene by use of different transcriptional start sites**
pri-miRNA	**Primary miRNA transcript**
BAM	**Alignment file**
BAI	**Alignment index file**
TPM	**Tags per million**
INDELS	**Insertions and deletions**
CV	**Coefficient of variation**
TMM	**Trimmed mean of M-values**
IDS	**Inhibitors of DNA binding**

RAS	**Reticular activating system**
IGFBP1	**Insulin growth factor binding protein 1**

Chapter 1 **Literature review**

1.1 Introduction

Pre-eclampsia (P-EC) is a pregnancy-specific syndrome. It is the third cause of maternal mortality and morbidity worldwide (Sibai et al. 1993; Levine et al. 1997), affecting 5–7% of all pregnancies (Walker 2000; Roberts and Cooper 2001). P-EC is believed to be of multifactorial origin. It is widely accepted that the placenta, has a major role in the development of P-EC (Petla et al. 2013). The onset, severity and progression of P-EC are affected by the maternal response to factors and proteins derived from the placenta. Pre-eclampsia is generally defined by hypertension and proteinuria after 20 weeks' gestation in a previously normotensive woman (Walker 2000; Roberts and Cooper 2001). Worldwide, this condition has a notable impact on maternal and foetal morbidity and mortality.

Research conducted into P-EC suggests that changes in the immunological system and haemostatic and endothelial status can profoundly affect maternal health, although it is not yet fully understood how these variations interlink with the aetiology and pathogenesis of P-EC. Broadening this understanding may be of value in explaining some of the pathophysiologies of the disease and monitoring its progression and consequent cardiovascular diseases later in life

During a normal pregnancy, the maternal spiral arteries are reconstructed to assist the body in coping with the increase in maternal circulation linked to placental perfusion. Upon entry of the foetal syncytial trophoblasts, the vessels dilate, enlarge and become flaccid. These changes to the blood vessels do not occur in pre-eclamptic pregnancies and, as a result, the placenta is prevented from embedding into the maternal blood vessels. This can also cause Intrauterine Growth Retardation and have a range of other effects on foetal development. The maternal immune response to fetoplacental factors is likely to be involved in orchestrating platelet activation and vascular endothelial damage characteristic of the maternal disease (Walker 2000). T-cells may also play a role in the development of P-EC induced hypertension.

1.2 Literature review

The search strategy was influenced by a need to explore the current literature about pre-eclampsia. The review methodology involved targeting national and international English-language peer-reviewed literature. A literature search was carried out using the online databases PubMed, Medline (OvidSP), Embase, and Web of Science to find studies that had investigated. Also, other search engines (i.e. Google Scholar), with additional searches conducted based on

bibliographic information from the relevant articles identified. The search included MeSH terms and synonyms for “pre-eclampsia”, “biomarkers”, “cytokines”, “inflammatory factors”, “inflammatory markers”, “cardiovascular”, “post-pregnancy”, “postnatal”, “postpartum”, “post-delivery” “inflammation in pre-eclamptic women”,”annexins”, “annexin A2”, “annexin A5” “proteomics”, “miRNAs”, “microRNA” and “cardiovascular risks in P-EC”.

1.3 Inclusion and exclusion criteria

Appendix A presents a complete list of the databases and specific search terms used; full-text articles were obtained for all abstracts that appeared eligible. The quality of the studies included was assessed using the Critical Appraisal Skills Programme (CASP) tool as a framework for evaluating studies, although this was done as formally as it would have been performed for a systematic review. The researcher reviewed the Systematic Reviews, case-control and cohort studies from significant researchers investigating the relationship between pre-eclampsia and cardiovascular disease and inflammatory biomarkers in pre-eclamptic women postnatally. The researchers then quoted relevant study results. References were managed using Endnote X8. Studies were included if the research took place between 2000 and the current year 2019. The exclusion features included material not published in English, the presence of some chronic disease, haematological disorders, hypertensive patients and those with malignant diseases.

The database was searched using the Medical Subject Headings (Mesh) and keywords (see Table 1-1)

Table 1.1 Search Strategy

Topic	Keywords	Database
Condition	"preeclampsia" OR "preeclamp*" OR "pre-eclamp*"	PubMed, Medline (OvidSP), Embase, Web of Science and Google Scholar.
Stage	"Postnatal" OR "post-delivery" OR "postpartum" OR "Post pregnancy"	
Factors	"Blood coagulation" and "inflammatory mediators" OR "cytokines" OR "Haemostatic" OR "angiogenic/anti-angiogenic" and "proteomics" or " proteomics profiles" "Annexin A2" and "Annexin A5" OR " Annexins "MicroRNA" and "miRNA"	

1.4 Search results

One of the most comprehensive studies followed a case-control design for assessing the levels of circulating cytokines, chemokines and adhesion molecules comprehensively for 60 pre-eclamptic women, 60 healthy pregnant women and 59 non-pregnant. Findings indicated that levels of interleukin (IL-1ra), tumour necrosis factor - alpha (TNFα) and monocyte chemoattractant protein (MCP-1) decreased in healthy pregnant compared to non-pregnant women reflecting anti-inflammatory changes in circulating cytokine profile. At the same time serum IL-10 was decreased and interferon gamma-induced protein (IP-10) levels elevated, in turn, possibly driving pro-inflammatory responses. In addition, to an increase in the circulating levels of the pro-inflammatory cytokines IL-6 and TNF-alpha, the chemokines IL-8, IP-10 and MCP-1, as well as the intercellular adhesion molecules (ICAM-1) and vascular cell adhesion molecule (VCAM-1), were

raised in pre-eclampsia compared with a healthy pregnancy, resulting in an overall pro-inflammatory systemic environment (Szarka et al. 2010).

There were significant correlations of increased IP-10, MCP-1, ICAM-1 and VCAM-1 concentrations of pre-eclamptic patients with blood pressure values, renal and liver function parameters, as well as with C-reactive protein (CRP), malondialdehyde, von Willebrand factor antigen and fibronectin levels. In conclusion, elevated amounts of pro-inflammatory cytokines, chemokines and adhesion molecules in the maternal circulation might play a vital role in the excessive systemic inflammatory response, as well as in the generalised endothelial dysfunction characteristics of the maternal syndrome of pre-eclampsia (Szarka et al. 2010). However, they did not specify the duration after delivery for both pre-eclamptic and healthy subjects which may effects on these markers. Therefore, the current study will run some of the pro and anti-inflammatory markers on women who have had delivered between 6 months and 3 years ago.

More recently a study was conducted by (Zhao et al. 2010) to assess the relationship between angiogenic and anti-angiogenic factors in pre-eclamptic women with/without fetal growth restriction (FGR) at three different time points. Also, they measured levels in early or late-onset pre-eclampsia and compared the results. A case-control study was designed for a total 23 pre-eclamptic women (under 32 weeks, early-onset; n= 8 and late-onset n=15 over 32 weeks of gestation) and control group (n=12). As a result, the women were divided into three groups:

1) Women with preeclampsia (early or late-onset without FGR)

2) Pre-eclamptic women with FGR

3) The control group. The blood samples were collected at three-time scales: at admission to the hospital, pre-delivery, and 48hrs post-delivery.

Findings indicated that the levels of soluble endoglin and soluble Flt-1 were increased in pre-eclamptic women compared with the control group upon admission ($p<0.001$). However, the level of placental growth factor (PIGF) or the ratio of sFlt-1/PIGF between the study and control group were insignificant.

There were no significant differences in the levels of sFlt-1 at all the time points in any of the groups. Thus, this study shows that the level of sEng, but not that of sFlt-1, was increased in pre-eclamptic pregnancies affected by FGR than those with pre-eclampsia only. Although the study was limited due to small sample size, it does suggest that the sEng level in the maternal blood was correlated better than the sFlt-1 levels in pre-eclampsia. In addition to find a significant result of

increasing the level of sEng, but not the level of sFlt-1 in early-onset pre-eclampsia compared to the late-onset group.

Two systematic reviews and meta-analysis presented the relationship between P-EC and cardiovascular diseases, both Bellamy et al. (2007) and Ahmed et al. (2014) have studied this extensively, and evidence has been provided to support this relationship. Bellamy et al. (2007) documented that previous pre-eclamptic women had twice chance of having either fatal or non-fatal ischemic heart disease. Also, the risk increased to eightfold if it is early-onset P-EC (Occurring before 34wks). Both reviews concluded that the severity of the P-EC was directly related to the risk of future heart disease. Therefore, P-EC could be considered as a prognostic risk factor for future cardiovascular disease (CVD) as both conditions share several characteristics. In this study, further proteomics profiles will carried out to study the possibility of finding single or multiple markers to predict CVD later on life for pre-eclamptic postpartum women.

Few studies have investigated the role of annexin A2 and annexin A5 in pregnant pre-eclampsia cohort. These studies have explored either one or the other. Moreover, they mostly focused on the local expression of these proteins in the placenta tissue rather than examining the whole blood levels of these proteins.

Abd El-Latif et al. (2017) conducted a case-control study to examine the levels of annexins A5 and A2 in 40 pregnant women with P-EC after 20 weeks of gestation and another 40 pregnant healthy women as a control group. They found that the level of Annexin A2 was significantly reduced in pre-eclamptic women while there were no differences in Annexin A5 between the groups. This inconsistency of the results could be attributed to the variation of the anticoagulants in the collected tubes might affect the outcomes of the chosen assays and the time and speed conditions to separate either serum or plasma as they might mask the exact levels of annexins A5 and A2.

Limited studies into pre-eclampsia have assessed levels of miRNAs expression in the plasma of pre-eclampsia post-delivery as most of the studies either have focused on late gestation or have lacked translation into relevant tissues (Akehurst et al. 2015). MicroRNAs are small noncoding RNAs that act at a posttranscriptional level to degrade target genes recognised by complementary base pairing in the 3’ untranslated region of the mRNA. miRNAs may provide a novel strategy for better understanding of consequences after experiences pre-eclampsia in women after exposure to the disease (Shukla et al. 2011). Pineles et al. (2007) was the first study to link the microRNA and pre-eclampsia, as the study aimed to investigate whether the pre-eclampsia and small gestation age are associated with changes in the placenta miRNA expression. They found the miR-182 and miR-210 were differentially expressed in the placenta of the pre-eclamptic women

compared to the control group. Our recent efforts are to study the miRNA expression in the plasma of postpartum pre-eclamptic at several time points, using the next-generation sequencing and confirmatory by using quantity polymerase chain reaction methodology.

1.5 Background

1.5.1 Haemostasis

The origin of the term “haemostasis” comes from the Greek “haem”, which means blood, and “stasis”, which is to stop or cease. Haemostasis can be defined simply as the stoppage of bleeding or haemorrhage and is a prerequisite for survival in the presence of bleeding (haemorrhage). The essential role of platelets needs to be tightly controlled to prevent excessive thrombus (clot) formation until the site of an injured blood vessel is healed, and bleeding ceases. This physiological process is very complicated but is efficient, and plays a vital role in response to vascular damage (Dusse et al. 2011). Normal haemostasis requires a balance between coagulation and fibrinolysis processes to maintain the integrity of the vasculature and physiological changes. Therefore, any defect in this complex process will enhance either haemorrhage or thrombosis.

1.5.2 Mechanism of haemostasis

Figure 1-1 shows the complex scenario of interactions that occur between blood vessels, platelets, coagulation factors, coagulation inhibitors and fibrinolysis. The four steps that take place in this process can be summarised as follows:

1. Vascular spasm or vasoconstriction: a rapid response occurs by rapid contraction of the smooth muscle inside the walls of the blood vessels to minimise the blood loss.
2. Formation of platelets to plug the site of the damaged tissue or injury. The primary haemostasis plug occurs as a consequence of the activation of the adhesion of platelets and degranulation, involving adenosine diphosphate (ADP) and thromboxane A2 (TXA2).
3. Coagulation or blood clotting: secondary haemostasis is due to the activation of a coagulation cascade of tissue factor (TF) and phospholipid via the extrinsic pathway, which makes the fibrin clot.
4. Fibrinolysis: as the name suggests, this is the removal or dissolving of blood clots by downregulation of the three serine proteases (plasmin, tissue plasminogen activator and urokinase-type plasminogen activator) (Chapin and Hajjar 2015).

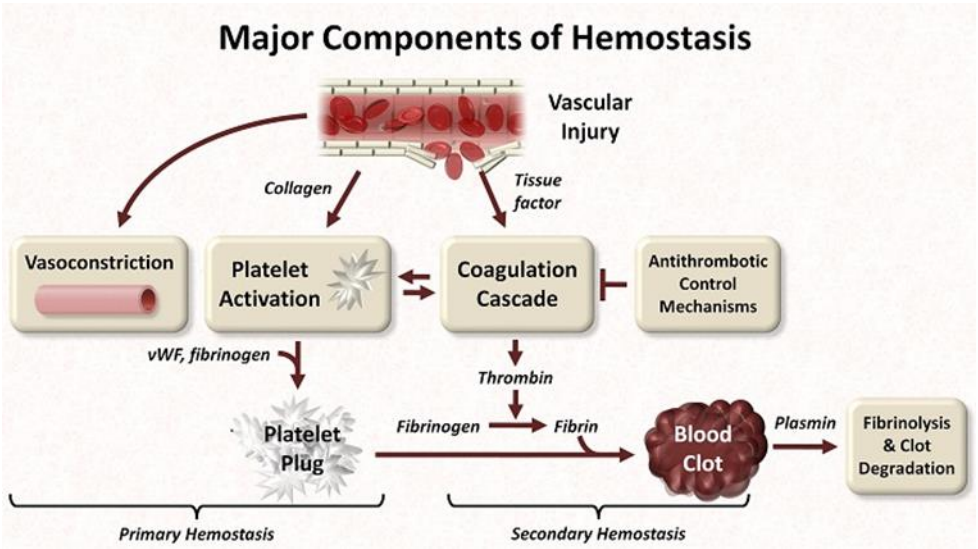


Figure 1-1 Steps involved in haemostasis. Adapted from (Heart 2017).

1.5.3 Endothelial activation

The endothelium plays a pivotal role in the regulation of vascular homeostasis as a monolayer of cells lining all blood vessels (Deanfield et al. 2007). Vascular tone and permeability, cellular adhesion, smooth muscle cell proliferation, inflammation and coagulation are some of the factors that healthy endothelial cells can regulate. In response to changes in cardiac output, endothelial function is critical for controlling blood pressure and maintaining optimal organ perfusion. For maintaining the endothelium in a quiescent state under normal physiological conditions, the role of nitric oxide (NO) is very critical. However, endothelial cells get activated due to various disturbances in NO-mediated silencing of cellular processes within the endothelium. Endothelial activation represents a switch from a quiescent state towards a phenotype that is thought to involve a host defence response (Hansson 2005; Deanfield et al. 2007). Endothelial injury is caused by prolonged endothelial activation that leads to detachment of degenerated endothelial cells and subsequent repair by endothelial progenitor cells (EPCs). Endothelial injury is characterised by circulating EPCs that are detached and was recently evaluated in pregnancy and pre-eclampsia (Gammill et al. 2007; Grundmann et al. 2008).

Elevated levels of numerous markers associated with activated endothelium (Poston 2006) ,such as von Willebrand factor (Hulstein et al. 2006), soluble adhesion molecules (Krauss et al. 1997) , end products of oxidative stress (Hubel 1999) and inflammation (Visser et al. 2007) are some of the changes characteristics associated with normal pregnancy. Pre-eclampsia in contrast to normal pregnancy, is associated with excessive endothelial activation and signs of endothelial injury, to an extent that is comparable to atherosclerosis, sepsis or end-stage renal disease

(Hansson 2005). Although some conflicting research exists over its extent and distribution, the maternal syndrome of pre-eclampsia is characterised by generalised endothelial activation that is believed to underlie most of the clinical symptoms and severe adverse complications of the disease, due to under perfusion of multiple organs including the kidney, brain, liver and the placenta (Ness and Roberts 1996; Donker et al. 2005). The cause for this state of endothelial disturbance is uncertain.

Inflammatory molecules, anti-angiogenic factors, components of the metabolic syndrome, syncytiotrophoblast microparticles and reactive oxygen species are some of the tenable biomarkers studied in the pre-ecalmpsia (Sikkema et al. 2001; Rodie et al. 2004; Levine et al. 2006; Redman and Sargent 2007). The utility of observed changes in these factors to predict the onset of clinical disease or to clearly discriminate between normal pregnancy and pre-eclampsia remains to be proven. Similar changes are consistently found in normal pregnancy, as well as in other inflammatory and vascular disorders (Hansson 2005; Redman and Sargent 2005; Deanfield et al. 2007), thus making none of these candidate markers of endothelial disturbance unique to pre-eclampsia. Rather than a specific entity driving its pathogenesis, the endothelial activation of pre-eclampsia is more likely to represent an extreme maternal host response to common pregnancy-induced inflammatory and vascular stimuli, an important aspect of its pathophysiology.

1.5.4 Coagulation Cascade

In the 1960s, Davie, Ratnoff and Macfarlane described theories outlining the concept of the “cascade” of proenzymes leading to the activation of downstream enzymes (Achneck et al. 2010).

Platelet activation and blood coagulation are complementary, equally dependent processes in haemostasis and thrombosis. Platelets interact with numerous coagulation factors, while the coagulation product thrombin is a potent platelet-activating agonist (Swieringa et al. 2018).All circulating coagulation factors exist in an inactive form, apart from small amounts of active factor VII (FVIIa). Three pathways take a role in this complex process: extrinsic, intrinsic and common. The extrinsic pathway starts when damage or injury to a blood vessel occurs. It is known that all blood vessels are coated by an intima, consisting of a monolayer of endothelial cells that rest upon a loose network of tissue called the extracellular matrix. Healthy endothelial cells participate in all aspects of vascular homeostasis and respond to a wide range of factors that regulate vascular tone and permeability, cellular adhesion, smooth muscle cell proliferation, inflammation and coagulation. Two phases begin when damage to a blood vessel occurs:

1. The initiation phase
2. The propagation phase.

The initiation phase begins when tissue factor (TF or FIII), an endothelial membrane protein interacts with FVIIa activity thereby significantly increasing its activity (Hoffman and Pawlinski 2014). The FVIIa/TF complex activates Factor IX (FIX). FIXa then activates Factor X (FX), which, together with Factor V (FV), activates a small amount of prothrombin (Factor II or FII) to thrombin (FIIa). Platelets play a vital roles in haemostasis. Platelets are generated from the nucleated precursor cells known as megakaryocytes in the bone marrow and enter the bloodstream without nuclei (Grozovsky et al. 2015). Platelet activity is mainly associated with the initiation of coagulation cascades. Platelet adhesion to the extracellular matrix is the first step in primary haemostasis. Under the conditions of high shear, von Willebrand factor (vWF) forms a bridge between exposed collagen and the platelet glycoprotein (GP) Ib-IX-V receptor complex on the platelet membrane (Jennings 2009; Clemetson 2012). Exposed collagen also binds directly to platelet GP Ia/IIa and GP VI receptors. During this process, platelets change shape and release the contents of their granules such as serotonin that is released into circulation along with other aggregating factors, consequently induce constriction of the injured blood vessels and enhance platelet aggregation to minimise the blood loss. Active GP IIb/IIIa receptor has a central role in mediating platelet aggregation. Bound fibrinogen or vWF to GP IIb/IIIa cross-links platelets and contributes to thrombus stabilisation (Berndt et al. 2014).

During the propagation phase, the small amount of thrombin activates and amplifies the coagulation process on the surface of the activated platelets in the platelet plug. Thrombin formation is accelerated by positive feedback, in which it activates FV, Factor VIII (FVIII) and Factor XI (FXI), through FIX and FX, resulting in a burst of thrombin activity (Bode 2006). Finally, thrombin activates fibrinogen, forming fibrin. However, the fibrin strands are unstable and are stabilised by FXIII, which generates covalent bonds between fibrin γ chains (Muszbek et al. 2011). The platelet plug then becomes a stable clot.

1.5.5 Normal Pregnancy

Under normal conditions, pregnancy is characterised by physiological changes that occur in different maternal systems that aim to promote the metabolic requirements of the mother along with the need of the foetus to grow (Pocock and Richards 2006). Normal gestation is associated with changes in the coagulation cascade, such as elevating the platelet turnover and accelerated platelet activation, as well as increase in clotting factors (such as FVII, V, VIII, IX, X and thrombin) (Dusse et al. 2007). Therefore, there is a tendency towards hypercoagulability during pregnancy.

This physiological compensatory process is thought to be important to decrease any bleeding issues and complications during pregnancy and at the time of delivery (Dusse et al. 2007). Nevertheless, within 4-6 weeks postnatal, this condition returns to normal levels (Hellgren 2003).

1.6 Pregnancy and adverse outcomes (pregnancy and its complications)

Certain medical conditions may complicate pregnancy, which are, in some cases, considered life-threatening if not controlled or diagnosed early either during or shortly after delivery, such as:

1. Haemorrhage

   Early pregnancy bleeding usually occurs in the first 12 weeks of pregnancy and may be caused by several factors such as; implantation bleeding, spontaneous abortion, ectopic pregnancy, trophoblastic disease and lesions of the cervix or vagina. Obstetric haemorrhage can be classified as antepartum (APH); bleeding occurring after 24 weeks gestation and before delivery, or postpartum (PPH); postpartum haemorrhage divided into a primary (within 24 hr of delivery) or secondary (between 24 hr to six weeks post-delivery) (Yang et al. 2004).

2. Premature labour and hypertensive diseases such as P-EC

   Preterm birth is frequently associated with pregnancy complications such as lower placental perfusion, increased blood pressure in the mother and preeclampsia, often resulting in intrauterine growth restriction (IUGR). Preterm labour is defined as giving birth to an alive baby before completed 37 weeks of gestation and accounts for up to 10% of deliveries worldwide (Sutherland et al. 2014). While gestational hypertensive diseases also referred as pregnancy-induced hypertension (PIH) which characterised by an elevation of the blood pressure in turns this can lead more serious condition such as pre-eclampsia (Bertagnolli et al. 2016).

3. Low amniotic fluid

   A low volume of amniotic fluid is called oligohydramnios. Obstetricians are define this as an amniotic fluid index (AFI) under five centimetre (Bachhav and Waikar 2014). The amniotic fluid that surrounds the fetus plays essential roles for its proper growth and development. It cushions the fetus from physical trauma, allows fetal lung growth, and provides a barrier against infection (Defoort 2005). Normal amniotic fluid volume varies. The average volume increases with gestational age, peaking at 800-1000 mL, which coincides with 36-37 weeks' gestation. Oligohydramnios is closely related to adverse pregnancy outcomes, such as increased fetal distress risk.

4. Gestational diabetes

Gestational Diabetes Mellitus (GDM) considers one of the most common metabolic disorders in pregnancy as the American Diabetes Association (ADA) has defined it as an impairment in the metabolism of the carbohydrates (Kintiraki and Goulis 2018).GDM occurs during the second and third trimester and is related to a high risk of both maternal and foetal complications.

5. Ectopic pregnancy

It is also known as extrauterine pregnancy which occurs when a zygote implants and grows at a site other than the uterus such as the ovary, fallopian tube, abdominal cavity or the lower part of the uterus (cervix), which connects to the vagina (Walker 2007). Therefore, early diagnosis of this condition could save the women in their first trimester as ectopic pregnancy is considered amongest the most prevalent causes of maternal mortality and morbidity. Recently, using a high-resolution technique called transvaginal ultrasound (TVS) in a combination with the assessment of serum level of beta-human chorionic gonadotropin (βhCG), has been used as an aid to the diagnosis of ectopic pregnancy (Kathpalia et al. 2018).

6. Placenta Previa

In healthy pregnancies, the placenta develops during the pregnancy to provide the oxygen, nutrition and also removing waste products of metabolism. The placenta physiologically connects to the foetus through the umbilical cord; usually, the placenta attaches to the uterus either to the top or side. However, if the placenta covers the cervix totally then it is called Placenta Previa (Karami et al. 2018). Consequently, placental previa leads to severe and possibly life-threatening condition as the vaginal haemorrhage may cause pre-term birth therefore an emergency caesarean section performed. Also, the vaginal bleeding occurs either during labour or in the first few hours post-delivery (Rowe 2014).

7. HELLP syndrome

HELLP is an abbreviation that refers to a syndrome in pregnant and postpartum women that is characterised by haemolysis, elevated liver enzymes, and low platelets (Sibai et al. 1986). The thrombocytopenia count occurring in 0.5 to 0.9% of all pregnancies and 10–20% of cases with severe pre-eclampsia (Haram et al. 2009). Therefore, all the pregnant women with upper abdomen pain irrespective of symptoms of pre-eclampsia should be considered to have HELLP

syndrome and immediate laboratory evaluation has to be done. If there is any doubt, an interdisciplinary consultation is needed.

1.7 Hypertension

Blood pressure is recorded with two numbers reading as a systolic and diastolic; the systolic reading measures the blood pressure when the heart beats and blood is pumped to the body. Whereas, the diastolic measures blood pressure when the heart is at rest between beats. It is expressed in millimetres of mercury (mmHg). Hypertension defined as an increase in the level of systolic blood pressure of ≥140mmHg and diastolic blood pressure of ≥90mmHg (Benjamin et al. 2017). It is known that in hypertension, the blood flows through the blood vessels at a higher level than normal pressure.

1.7.1 Stage of high blood pressure

Table 1.2 Stage of high blood pressure.

Stages	Systolic	Diastolic
Prehypertension	120-129 mmHg	<80 mmHg
Stage 1	130-139 mmHg	80-89 mmHg
Stage 2	140 or greater	90 mmHg

This table was adapted from (Carey et al. 2018).

1.7.2 Classification of hypertensive diseases in pregnancy

According to the revised version of the International Society for the Study of Hypertension in Pregnancy (ISSHP) classification in 2014, there are four types of the hypertensive condition in pregnancy (Tranquilli et al. 2014); 1. Chronic hypertension, 2. Gestational hypertension, 3. Pre-eclampsia or de novo, sometimes referred to as superimposed on chronic hypertension, and 4. White coat hypertension.

The following sections of this chapter study are in-depth examination of the current thinking on pre-eclampsia pathogenicity. The concept of the cardiovascular challenge of pregnancy is then discussed, together with its implications for future reproductive outcome and maternal health after delivery.

P-EC is generally defined as an inducer of hypertension and proteinuria after 20 weeks of gestation in a previously normotensive woman (Walker 2000; Roberts and Cooper 2001). In 2000, the National High Blood Pressure Education Program Working Group characterised P-EC as an increase in systolic blood pressure (SBP) ≥ 140 mmHg or diastolic blood pressure (DBP) of 90 mmHg or higher after 20 weeks of gestation on two occasions, at least 6 hours apart and proteinuria of ≥ 300 mg/dl in 24 hours of urine collection or +2 or above on the urine dipstick (Cetin et al. 2011). It should also noted that proteinuria is no longer needed to establish the diagnosis of P-EC as another maternal or uteroplacental dysfunction could coexist (Tranquilli et al. 2014). Pre-eclampsia has been categorised as mild, moderate, severe, and early and late pre-eclampsia, of which the last is a more contemporary concept(von Dadelszen et al. 2003). The early onset of pre-eclampsia (EOP) (before 34 + 0 weeks) and late-onset pre-eclampsia (LOP) (after 34 + 0 weeks) have been found to have different aetiologies and, therefore, a different clinical presentations. The key feature of P-EC is poor trophoblast invasion of maternal spiral arteries, and several experimental research studies have previously been undertaken to explain this condition.

1.8 Pathogenesis

1.8.1 Mechanism of pathogenesis

Insufficient information and lack of diagnostics criteria regarding P-EC have been the main drawbacks to making progress in the treatment and control of the disease. The definition of P-EC proposed in 2000 was designed for clinical use but maximised sensitivity to the detriment of specificity. Despite these limitations, several theoretical mechanisms have been proposed that resolve feto-placental abnormalities and clinical features of the maternal syndrome.

1.8.2 The mechanism of fetoplacental establishment

In the first stages of pregnancy, the early blastocyst (day 5) embeds itself, during the implantation process, into the inner membrane of the uterus (endometrium). This promotes bathing of the placental cotyledons in the maternal blood supply (Red-Horse et al. 2004). The extravillous cytotrophoblast cells multiply from the ends of the anchoring chorionic villi, forming a thick lining to the uterine cavity. Two weeks after blastocyst implantation, the extravillous trophoblast cells

come into contact with the decidual tissue, penetrate the decidual stroma and, upon reaching the myometrium, amalgamate into multinucleate giant cells (Lyall 2005).

The second form of invasive extravillous trophoblasts exist (interstitially), which can release vasodilators (e.g., nitric oxide [NO] and carbon monoxide [CO]), which restructure the spiral arteries, increasing the uterine vascular flow before they are eradicated by endovascular trophoblasts (Lyall 2005). Once inside the lumen of the spiral arterioles, the endovascular cytotrophoblasts invade and affix to the walls and dilate the arteries. As a consequence, the endometrial and superficial myometrial segments of the spiral arteries are lined with cytotrophoblastic cells, transforming them into soft, stretchy vessels with the ability to dilate and enlarge.

In successful pregnancies, this transformation has taken place by the second trimester. These changes to the uterine vessels are fundamental in ensuring adequate delivery of blood to the feto-placental unit. The invasion of endovascular cytotrophoblasts is restricted to the superficial portions of the spiral arterioles, which retain their endothelial lining and muscular wall, leaving them narrow-bore, high-resistance vessels ((Zhou et al. 1997). This leads to a reduction in the blood flow into the intervillous space. Pijnenborg et al. (1991) believed that this endovascular invasion denotes the beginning of pre-eclamptic problems. However, a closer examination of uterine tissues taken from caesarean patients who were sufferers of pre-eclampsia has revealed that the density of interstitial trophoblasts, together with the depth of interstitial invasion, were both greatly reduced (Kadyrov et al. 2003). It can, therefore, be suggested that a malfunction at the interstitial trophoblast invasion stage can have a detrimental effect on the body's ability to remodel the blood vessels essential for a healthy placenta. In women with pre-eclampsia, analyses of their preterm placentas have revealed vascular and occlusive lesions (Teasdale 1985; Moldenhauer et al. 2003). However, it is interesting to note that many patients with P-EC at term do not show growth abnormalities as expected, and have similar placental histomorphometry to their normotensive gestational age-matched controls.

1.8.3 Hereditary constitutional factors and pre-eclampsia

While most cases of pre-eclampsia are sporadic, it is becoming increasingly evident that genetic factors have a role in disease susceptibility (Cincotta and Brennecke 1998; Lie et al. 1998; Arngrímsson et al. 1999; Mogren et al. 1999; Moses et al. 2000; Esplin et al. 2001; Lachmeijer et al. 2001; Lachmeijer et al. 2002; Cnattingius et al. 2004; Skjærven et al. 2005). This is suggested by the following:

- A family history of pre-eclampsia in a primigravid woman is associated with a two- to fivefold higher risk of the disease (Cincotta and Brennecke 1998; Mogren et al. 1999; Carr et al. 2005; Skjærven et al. 2005). The maternal contribution to development of preeclampsia can be partially explained by imprinted genes (van Dijk et al. 2005). In a study of sisters with pre-eclampsia, it was demonstrated that the mother developed pre-eclampsia only when the foetus/placenta inherited a maternal STOX1 missense mutation on 10q22; when the foetus/placenta carried the imprinted paternal homologue, the pre-eclampsia phenotype was not expressed.
- The risk of pre-eclampsia is increased more than sevenfold in women who have had pre-eclampsia in a previous pregnancy (Duckitt and Harrington 2005).
- The spouses of men who were the product of a pregnancy complicated by pre-eclampsia are more likely to develop pre-eclampsia than spouses of men without this history (Esplin et al. 2001).
- A woman who becomes pregnant by a man whose previous partner had pre-eclampsia is at higher risk of developing the disorder than if the pregnancy with the previous partner was normotensive (Lie et al. 1998).
- The genes for sFlt-1 and Flt-1 are carried on chromosome 13. Foetuses with an extra copy of this chromosome (e.g., trisomy 13) should produce more of these gene products than their normal counterparts. In fact, the incidence of pre-eclampsia in mothers who carry foetuses with trisomy 13 is greatly increased compared with all other trisomies or with control pregnant patients (Tuohy and James 1992). In addition, the ratio of circulating sFlt-1 to PlGF is significantly increased in these women, thus accounting for their increased risk for pre-eclampsia (Bdolah et al. 2006). A large genome-wide association study (GWAS) identified a genetic risk variant with genome-wide significance, and provided convincing replication in an independent cohort (McGinnis et al. 2017). This GWAS finding provides compelling evidence that alterations in chromosome 13 near the FLT1 locus in the human foetal genome are causal in the development of pre-eclampsia. It is striking that this first well-powered unbiased GWAS focuses attention on the FLT1 genomic region, given the body of literature devoted to the role of the FLT1 pathway in pre-eclampsia pathogenesis.

1.9 Cytokines

Inflammatory processes are mediated by a group of secreted polypeptides called cytokines, with the assistance of some other soluble factors. Inflammatory cytokines can be responsible for acute inflammation or play a role in developing it. The acute phase of inflammation, which is tissues' first response to damage, can be characterised by an increase in blood flow and the permeability of vessels, together with a build-up of fluid, leucocytes, and cytokines.

By way of contrast, in the chronic inflammatory process, humoral and cellular immune responses to pathogens can be detected at the injury site. During the cellular immune responses inflammatory processes and some soluble factors influence leucocyte recruitment through increased expression of cellular adhesion molecules and chemoattraction (Akdis et al. 2011) see Figure 1-2. These soluble factors regulate the activation of the resident cells (such as fibroblasts, endothelial cells, tissue macrophages, and mast cells) and inflammatory cells, such as monocytes, lymphocytes, neutrophils, and eosinophils, creating a more systemic response to the inflammation process. The cytokines that mediate acute inflammatory reactions include tumour necrosis factor-alpha (TNF-α), interleukin 1 (IL-1), IL-6, IL-11, IL-8 and other chemokines, granulocyte-colony stimulating factor (G-CSF), and granulocyte-monocyte colony-stimulating factor (GM-CSF).

Cytokines can be divided into two groups: those known to mediate chronic inflammatory processes, such as humoral inflammation (these include: IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-13 and TGF-b); and those contributing to cellular inflammation (including: IL-1, IL-2, IL-3, IL-4, IL-7, IL-9, IL-10, IL-12, interferons [IFNs], IFN-gamma-inducing factor [IGIF], tumour growth factor-beta [TGF-β], and TNF-α and β.

1.9.1 Immunological aspects of pregnancy

The immunological theory is the most popular explanation for the pathogenesis of pre-eclampsia. During a normal pregnancy, the maternal spiral arteries are reconstructed to assist the body in coping with the increase in maternal circulation linked to placental perfusion. Upon the entry of foetal syncytial trophoblasts, the vessels dilate, enlarge and become flaccid. These changes to the blood vessels do not occur in pre-eclamptic pregnancies and, as a result, the placenta is prevented from embedding into the maternal blood vessels. This can cause intrauterine growth restrictions and have a range of other effects on foetal development. Researchers are of the view that any unrecognised foetal genes are detected by the maternal immunological system and are not tolerated. The intolerance can be between feto-placental and maternal tissue platelet activation or vascular endothelial damage or dysfunction (Walker 2011).

A growing body of research has demonstrated that increased in cytotoxic T-cell response to paternal antigens in the pre-eclamptic women compared to unaffected pregnant women (controls) (de Groot et al. 2010). A number of the hypotheses assumed that pre-eclampsia is a maternal immunologic response to a foreign foetal antigen derived from the father's sperm and that this response is decreased by prolonged exposure to paternal antigen (Tubbergen et al. 1999)

Prolonged exposure to the paternal antigen could come from a previous pregnancy or through sexual intercourse, particularly without barrier contraception (Galaviz-Hernandez et al. 2019).

Two studies of contraceptive methods and sperm exposure revealed an increased in the rate of pre-eclampsia occurrence among barrier contraceptive users (Klonoff-Cohen et al. 1989; Kenny and Kell 2018), conversely a case- control study demonstrated that using of intrauterine device is linked to small decreased risk of pre-eclampsia, particularly, if the device removed within the year prior to pregnancy (Parker et al. 2016), therefore, the role of paternal genes in the aetiology of pre-eclampsia appears to be limited.

There are many diseases and allergic reactions that manifest themselves as inflammatory abnormalities within the immune system. Trott and Harrison (2014) highlighted that T cells have a role in the development of hypertension; however, the researchers failed to provide details of the exact subsets.

CD4 T cells are classified into the following groups: T helper (Th) 1 or Th2, Th9, Th17 and Th22, depending on their activation markers and cytokine production (Figure 1-2). The Th17 cells previously mentioned produce the cytokine IL-17 and contributed to numerous autoimmune diseases, obesity, and cardiovascular disease.

The above suggests that hypertension may be mediated by multiple pro-inflammatory T cell subsets. Regulatory T (Tregs) cells, which regulate and restrain the pro-inflammatory T cells, contribute to hypertension-induced end-organ damage in mice (Trott and Harrison 2014).

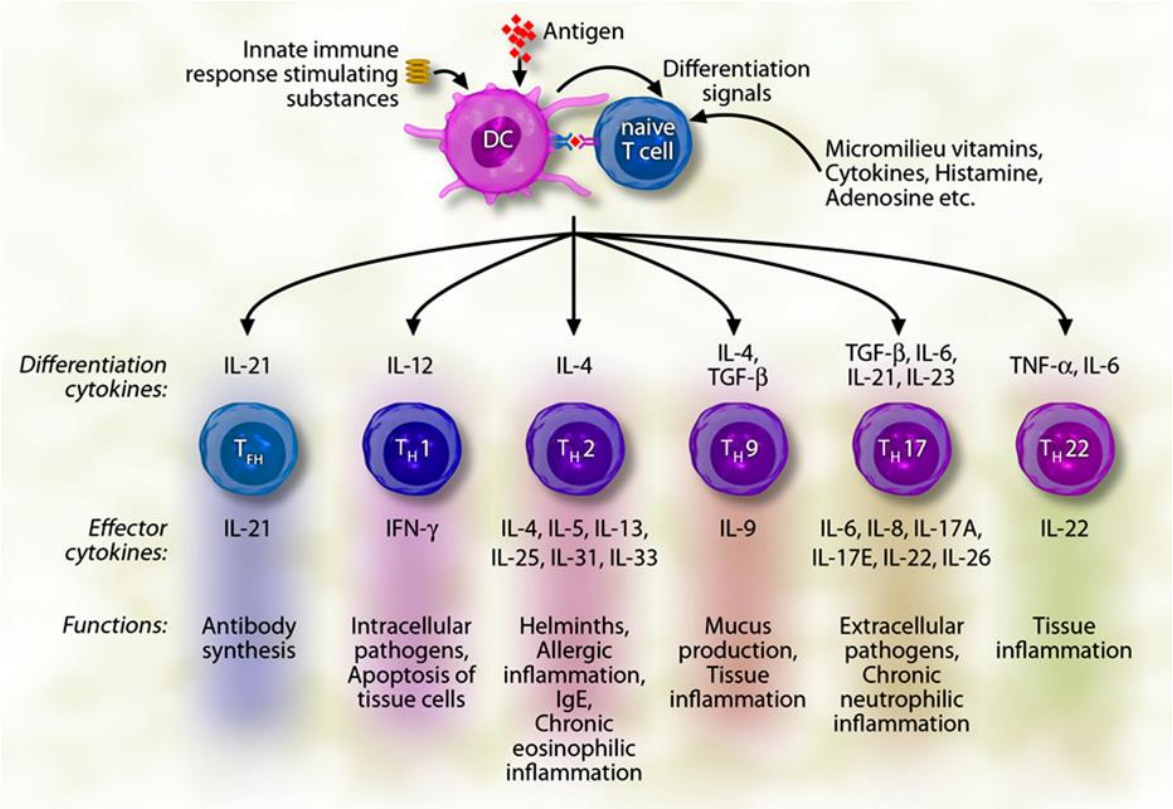


Figure 1-2 Interaction between a T cell receptor and the antigen-presenting cells (dendritic cells or accessory cells) and the stimulation of molecules on the T cell by ligands on the antigen-presenting cell, activating the T helper cells and producing various types of inflammatory responses.

The above figure was taken from (Akdis et al. 2011).

1.9.2 Long interpregnancy intervals and pre-eclampsia

Inter-pregnancy intervals (IPI) defined as the period between the delivery date of the previous live birth and the beginning of the following pregnancy (Gebremedhin et al. 2018). Several epidemiological studies have shown that both short and long IPIs are risk factors for adverse pregnancy and perinatal consequences, but the bulk of adverse complication has been associated with short intervals (Conde-Agudelo et al. 2006). According to the recommendation from the World Health Organisation, the IPI should be a minimum of 2 years (Hanley et al. 2017).

Closely-spaced pregnancies may cause depletion in some of the maternal nutrients such as folate mainly among breastfeeding mothers. Moreover, this may lead to adverse pregnancy consequences. There is a large prospective cohort study conducted in the Netherlands reported a negative relationship between short IPI and foetal growth, and that women not using folic acid supplements were at a higher risk of foetal growth restriction after a short IPI (Zhu and Le 2003; van Eijsden et al. 2008). However, a recent study demonstrated that the IPI influences the mean

arterial pressure (MAP) of the second pregnancy in women who had pre-eclampsia. Shorter IPI is linked with a more significant reduction in MAP when compared to the longer IPI. Though there was a trend toward higher pre-eclampsia recurrence with longer IPI, this tendency did not reach statistical significance (Howe et al. 2018). Moreover, IPI of ≥120 month seems to be independently correlated with a higher risk of gestational diabetes mellitus and premature membrane rupture (Lin et al. 2019).

1.10 Angiogenic factors

1.10.1 Vascular endothelial growth factor (VEGF) and placental growth factor (PlGF)

Throughout post-natal development, the vascular endothelial growth factor (VEGF) molecule plays an essential role in many biological processes and related conditions. The VEGF molecules exist and have been classified in mammals as VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). The most common of the aforementioned molecules is VEGF-A, which is interwoven with alternate isoforms possessing pro- and anti-angiogenic properties (Woolard et al. 2009). When the ligands bind, they trigger the Flt1, Flk1 (VEGFR2) and Flt4 (VEGFR3) receptors. There are also co-receptors, for example, neuropilins, that can affect the behaviour of VEGFR (Cebe Suarez et al. 2006; Favier et al. 2006; Kawamura et al. 2008; Herzog et al. 2011). These combinations of molecules and receptors play a major role in the development of blood and lymph vessels, VEGFR3 being particularly important for lymph vessels (Kukk et al. 1996). Flt1 binds VEGF-A, -B and PlGF, whereas Flk1 binds to VEGF-A and -C, and Flt4 binds VEGF-C and –D (Fischer et al. 2008).

VEGFRs are made up of three domains: extracellular, trans-membrane and the intracellular protein tyrosine kinase domain. The binding process involving VEGFRs sets in motion the tyrosine kinase activity, which opens up a range of signalling pathways between the cells with the function of regulating vascular processes. Signalling events along the Flt1 and Flk1 pathways have a regulating effect on angiogenesis, such as endothelial cell migration, proliferation, tube formation and vessel branching (Olsson et al. 2006).

The key angiogenic growth factors, VEGF and PlGF, play an important role in ensuring normal trophoblastic proliferation and implantation takes place (Ostendorf et al. 2007). Some researchers have suggested that an imbalance in these growth factors can be linked to pre-eclampsia (Thadhani et al. 2004). Impaired invasion of spiral vessels may have different aetiologies; immunological, genetic or environmental. Shallow trophoblast invasion disturbs the formation of

an environment with low resistance and the high volume of blood flow (Zhou et al. 1997). The developing placenta and, indirectly, the foetus are not offered access to appropriate gas exchange. As a result leads to ischemia in the uteroplacental compartment, and then to hypoxia (Roberts and Post 2008). Inadequate perfusion leads to ischemia-reperfusion lesions along with free radical production, placental ischemia and oxidative stress elicit inflammatory processes and cytokine release (Molvarec et al. 2011). Therefore, Maintained chronic hypoxia produces a considerable increase in inflammation. It triggers the production of angiogenic factors such as VEGF or PlGF to compensate for the shortage of blood supply by prompting angiogenesis and increasing NO production, which is anticipated in vascular endothelium stabilisation (Pratt et al. 2015). The demand that constantly grows during pregnancy makes this process inadequate results in decompensation, and disturbed balance in angiogenesis and anti-angiogenic substance production (Kwiatkowski et al. 2016).

In cancer treatments, using anti-VEGF has been linked to hypertension and proteinuria, which are often associated with the onset of pre-eclampsia, which shows these may also be relevant in understanding the condition(Ostendorf et al. 2007).

1.11 Anti-angiogenic factors

1.11.1 Soluble FMS-like tyrosine kinase

Soluble fms-like tyrosine kinase-1, also known as soluble VEGF receptor 1 or sFlt-1 is an endothelial receptor that binds to and antagonises VEGF and PlGF. By producing vasoconstriction and endothelial dysfunction (Luft 2006), sFlt-1 contributes to several features of pre-eclampsia, including hypertension and glomerular endotheliosis.

sFlt-1 is thought to be one of the key peptides involved in the development of pre-eclampsia. Maternal serum levels of sFlt-1 have been shown to be elevated in women with pre-eclampsia compared with controls, to correlate with disease severity (Chaiworapongsa et al. 2004) and to decrease markedly following delivery (Koga et al. 2003). The genetic studies have broadened and improved the understanding of several diseases pathophysiology, assisting in enhancing clinical care findings. As it is well known that sFlt-1 is located on chromosome 13 (Gray et al. 2018), there is a link between trisomy 13 and susceptibility to develop pre-eclampsia during the second and third trimester in pregnant women have foetus with trisomy 13 prone to have an abnormal placenta (Chen 2009; Silasi et al. 2011). Therefore, identification of any variant of placental sFlt-1 holds promising findings for better prediction and diagnosis of pre-eclampsia.

1.11.2 Soluble endoglin

Soluble endoglin (sEng) is a co-receptor that antagonises transforming growth factor-β1 and β3 and contributes to the anti-angiogenic environment of pre-eclampsia. sEng is highly expressed on endothelial cell membranes and syncytiotrophoblasts (Koga et al. 2003). In normal pregnancy, levels of sEng fall between the first and second trimesters, but in women who go on to develop pre-eclampsia, this reduction is blunted (Rana et al. 2007). Consistent with studies involving sFLT-1, it has also been demonstrated that levels of sEng are elevated in the sera of pregnant women with pre-eclampsia, correlate with disease severity, and fall after delivery (Venkatesha et al. 2006).

A promising discovery in terms of predicting pre-eclampsia was that levels of sEng are elevated several weeks before the development of clinical symptoms in women who developed the condition; furthermore, in patients who developed pre-term pre-eclampsia, serum sEng levels are elevated (approximately twofold) as early as gestational weeks 17-20 (Levine et al. 2006).

1.12 Metabolic changes in normal and pre-eclamptic pregnancy

Normal pregnancy is associated with considerable maternal metabolic alterations; these changes tend to be anabolic during the first two trimesters as the nutrients are stored in an early stage of pregnancy to meet the foetal and maternal requirements of late gestation and lactation. It has been recognised that the increase in insulin resistance seen in pregnancy results in increases in the maternal glucose and free fatty acid which in turn allows for greater substrate availability for foetal development (Zeng et al. 2017). In contrast, late pregnancy tends to be in a catabolic state with decreased insulin sensitivity (Martineau et al. 2015).

Metabolic syndrome defined as a cluster of common abnormalities, including hypertension, high fasting plasma glucose, dyslipidaemia, obesity and insulin resistance (Rafeeinia et al. 2014). Given the metabolic changes described in pregnancy it is not surprising that recent research has focused on the relationship between metabolic syndrome and pre-eclampsia. It is noted that there is a shift towards a pro-atherogenic state in normal pregnancy. However, this is more severe in women who develop pre-eclampsia (Martin et al. 1999; Beigh et al. 2017). The presence of metabolic syndrome can be used as a simple way to identify populations at risk of cardiovascular disease as it is a risk factor for both cardiovascular disease and diabetes. It is also referred to by the names cardiometabolic syndrome, insulin resistance syndrome, or Reavan's syndrome. The exact mechanism of metabolic syndrome is not clear yet, and it has very complex pathophysiology. Predisposing factors of metabolic syndrome encompass several factors such as

stress, obesity, sedentary lifestyle, age, history of cardiovascular disease, diabetes, schizophrenia, and other mental illnesses, rheumatic diseases, psoriasis, and psoriatic arthritis (Padmavati 2016).

1.13 Role of annexins, proteomics and miRNAs factors in pre-eclampsia

While several efforts have been made in order to understand P-EC fully, the pathogenesis of this condition remains uncertain. Thus contributes to the current lack of an effective screening method and treatment of P-EC. Therefore, it is essential to identify novel biological markers for early diagnosis and develop new strategies for treating this disorder. In this study, the researcher is aiming to explore other factors could play a role other than the inflammatory and haemostatic biomarkers, for instance, investigate the effect of annexins in women who had a history of pre-eclampsia. Annexin A5 is generally used to identify cell apoptosis as it can bind to phosphatidylserine, a marker of apoptosis on the outer leaflet of the membrane (van Engeland et al. 1998). Two annexins A2 and A5 have been selected in this project; **chapter 4** will discuss in more details the role of annexins in postpartum pre-eclamptic subjects.

For a more detailed discussion of the relationship between pre-eclampsia and metabolic syndrome see **Chapter 5**.

Other factors could be added in this project to investigate the changes in the MicroRNAs profiles in preeclampsia post-delivery at different time points since their delivery, which will be explained in-depth in **chapter 6.**

1.14 Cardiovascular Disease

Cardiovascular disease (CVD) is leading cause of mortality worldwide. It represents a group of disorders that occur as a defect in the circulatory systems, i.e. the heart and the blood vessels. (O'Donnell and Elosua 2008). The most common predisposing causes known are:

- Hypertension
- Increase in lipid profile levels especially the cholesterol Low-density lipoprotein (LDL)
- Diabetes
- Obesity
- Hereditary and another such gender, age, life/environmental style

This term cardiovascular disease refers to seven types of disorders, which include:

1. Ischemic heart disease.
2. Cerebrovascular disease.
3. Peripheral vascular disease.
4. Heart failure.
5. Rheumatic heart disease.
6. Congenital heart disease.
7. Deep vein thrombosis.

1.14.1 Pre-eclampsia and cardiovascular diseases

During pregnancy, there are some important physiological changes that occur to accommodate foetus development and which are considered necessary for a successful pregnancy, such as an increase in plasma volume and red blood cell mass, which leads to a decrease in the haemoglobin level in comparison to non-pregnant women. Also, there is an increase in the cardiac output to approximately 20% at eight weeks of gestation (Soma-Pillay et al. 2016), reaching a maximum level of 45% at 24 weeks (Sanghavi and Rutherford 2014). These changes might not be tolerated in some women and can lead to pregnancy complications. Over the past decades, it has become increasingly obvious that women with a history of pre-eclampsia are at high risk of cardiovascular disease (CVD) in the future. Pre-eclampsia continues to pose risks to the mother postpartum, with several studies reporting that women with a history of P-EC are at a greater risk of thromboembolic and cardiovascular disease several years following delivery, compared to women with previously normal pregnancies (Kestenbaum et al. 2003; Bellamy et al. 2007). Some literature has been published suggesting that the clotting and angiogenic factor changes observed during P-EC also persist postpartum. However results regarding this are inconclusive (Bremme and Blombäck 1996; He et al. 1999; Lwaleed et al. 2014). If confirmed, these haematological changes may explain the heightened risks of cardiovascular and thromboembolic disease experienced by formerly pre-eclamptic women.

American Heart Association guidelines for 2011 acknowledged that pre-eclamptic women, and those with gestational hypertension, have an independent gender-specific cardiovascular risk factor. There is much recent evidence that pre-eclampsia and cardiovascular disease share many risk factors. Bellamy et al. (2007) showed that follow-up data of up to 200.00 women with a history of pre-eclampsia show significantly increased relative risk for future chronic hypertension, ischaemic heart disease, venous thromboembolism and stroke. Overall mortality after 14.5 years

was an estimated 1.49-fold higher (95% confidence interval 1.05-2.14). Long-term maternal prognosis is especially poor for women who experienced early-onset disease, in whom long-term follow up studies revealed a 7 to 8 fold increased cardiovascular mortality, compared to a 2 fold increase in women with a history of late-onset pre-eclampsia. Moreover, recent papers on long-term cardiovascular events after pre-eclampsia demonstrated that women with previous pre-eclampsia showing increased risk of subclinical coronary artery atherosclerosis from the age of 45 years onwards (44-55yrs) (Zoet et al. 2018)

Definitive evidence on the real or main causes and consequences of pre-eclampsia remains to be resolved for the sake of mother's and children's lives in minimising the mortality and morbidity rates in developing countries.

There are several questions which have not yet been answered regarding the aetiology and the exact mechanism of this increase in cardiovascular risk. Thus, our research is focused on biomolecular markers that are thought to have a role in the postpartum course of women with pre-eclampsia. This research will help to understand the effect of P-EC on women post-delivery and susceptibility to cardiovascular diseases later in life. Next will discuss the aims of this project.

1.15 The aims of the studies

This study was conducted in postpartum pre-eclamptic women. Five hypotheses will be explored in this project:

Hypothesis I:

The objective of this study is to test the hypothesis that some inflammatory and haemostatic indices could potentially be used postpartum to predict susceptibility to future cardiovascular events in pre-eclamptic women.

Aim

To investigate the relationship between circulating levels of haemostatic factors and inflammatory cytokines in women with a history of pre-eclampsia (Paper I, Chapter II).

Hypothesis II:

To support the hypothesis that women with a medical history of pre-eclampsia have a state or tendency towards increasing levels of haemostatic and anti-angiogenic factors, including: vascular endothelial growth factor (VEGF), placental growth factor (PIGF), soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng), in combination with TF and TF pathway inhibitor (TFPI). Also, whether these factors alter postpartum in women with a history of pre-eclampsia.

Aim

To investigate the relationship between haemostatic as well as angiogenic and anti-angiogenic factors in women with a history of P-EC (Paper II, Chapter III).

Hypothesis III:

To support the hypothesis that pre-eclamptic women after one year, postpartum have alterations in their plasma proteomic profiling.

Aim

To examine the global plasma proteomic profile one year postpartum of women with pre-eclampsia (Paper III, Chapter IV).

Hypothesis IV:

To evaluate the changes in the maternal plasma levels of annexin A2 and annexin A5 in women with a history of pre-eclampsia after several years since their delivery.

Aim

To determine the plasma levels of Annexins A2 and A5 in pre-eclamptic women postpartum at different time points past since they experienced the pre-eclampsia (Chapter V).

Hypothesis V:

To support the hypothesis that there are some alterations in the genes expression occurs in women with a previous history of pre-eclampsia and the possibility of using these biomarkers as a tool to monitor the women's health.

Aim

To investigate the plasma miRNAs in postpartum pre-eclamptic women and to explore whether plasma miRNAs may serve as new biomarkers and risk factors for cardiovascular diseases development (Chapter VI).

1.16 General Experimental Design

General methods, samples size calculation and blood collections used in this work present in chapter two, however, details for specific experiments such as coagulation and angiogenic markers (Chapter 3) or plasma proteomic profiling (Chapter 4) or annexin A2 and annexin A5 and miRNA biomarkers (Chapter 5 & 6 respectively) are described in each chapter methods section. It is worth mentioning that the samples used in the first and second study were collected previously and used for all the markers measured. Then, the researcher started to recruit the participants to reach the total of pre-eclamptic blood specimens (n=66) with only five samples from the healthy non-pregnant women in the control arm.

Chapter 2

Study I

Inflammatory and Haemostatic Changes Following Pre-eclampsia: Potential Link with Development of Subsequent Cardiovascular Events?

This chapter will provide an overview about the presence of a link between women who have experienced pre-eclampsia and any imbalance of the haemostatic system and disturbance of the inflammatory pathways, which could be predictive of cardiovascular disease later in life.

Published in Gynecology & Reproductive Health (ISSN2639-9342)

http://scivisionpub.com/pdfs/inflammatory-and-haemostatic-changes-following-preeclampsia-potential-link-with-development-of-subsequent-cardiovascular-events-833.pdf

Chapter 2

2.1 Introduction

During a normal pregnancy, the maternal spiral arteries are reconstructed to assist the body in coping with the increase in maternal circulation linked to placental perfusion. Upon entry of the foetal syncytial trophoblasts, the vessels dilate, enlarge and become flaccid. These changes to the blood vessels do not occur in pre-eclamptic pregnancies and, as a result, the placenta is prevented from embedding into the maternal blood vessels. This can also cause Intrauterine Growth Retardation and have a range of other effects on foetal development. The maternal immune response to feto-placental factors is likely to be involved in orchestrating the platelet activation and vascular endothelial damage characteristic of the maternal disease (Walker 2000). T-cells may also play a role in the development of P-EC induced hypertension (Trott and Harrison 2014).

Inflammatory cytokines may play a role in the development of acute inflammation. The acute inflammatory phase, i.e. the first response to damage, is characterised by an increase in blood flow and permeability of vessels, together with a build-up of fluid, leukocytes and cytokines. In contrast, in the chronic inflammatory process, humoral and cellular immune responses to the pathogens can be detected at the injury site. Some soluble factors influence leukocyte recruitment through increased expression of cellular adhesion molecules and chemo-attractants (Cetin et al. 2011). Such soluble factors also regulate the activation of resident cells (such as fibroblasts, endothelial cells, tissue macrophages and mast cells) and together with the inflammatory cells create a systemic response. The cytokines that mediate acute inflammatory reactions include Interleukin-1 (IL-1), Interleukin-6 (IL-6), Interleukin-8 (IL-8) Interleukin-11 (IL-11), Tumour Necrosis Factor Alpha (TNF-α), and Chemokines such as Granulocyte Colony Stimulating Factor and Granulocyte Monocyte Colony Stimulating Factor.

Tissue Factor (TF) is the main cellular initiator of blood coagulation. When the endothelial lining is damaged, TF binds to FVII in the presence of calcium (TF: FVII/FVIIa) and proteolytically activates downstream coagulation factors, eventually resulting in thrombin formation and fibrin generation (Butenas et al. 2005). In vivo TF is regulated by a specific inhibitor known as Tissue Factor Pathway Inhibitor (TFPI) which, under normal conditions, is synthesised primarily by the vascular endothelium.

Previously, we studied TF and TFPI levels in women who have had P-EC compared with normal counterparts (Lwaleed et al. 2014). In the present study, we investigate the relationship between these factors and inflammatory cytokines (IL-6, IL-8, IL-10 and TNF-α) in women with a past medical history of P-EC.

2.2 Material and methods

2.2.1 Study design

This study uses a case-control design to try to identify a potential link between P-EC and the development of subsequent cardiovascular events, and whether inflammatory and haemostatic indices could be used postpartum to predict susceptibility to future cardiovascular events. The case-control design is particularly useful when there is a need to study several risk factors, and when a study must be performed relatively quickly and inexpensively. It is the most appropriate and efficient design for research into rare diseases and usually needs a smaller study population than a cohort design.

2.2.2 Ethical approval

The ethical was granted for the study by the Southampton and South West Hampshire Research Ethics Committee (REC reference number is 05/Q1702/131) (see **Appendix B**).

2.2.3 Definition of cases

In this study, the cases (study group) are women who have had P-EC previously. In the first paper, women were included within six months to 3 years post-delivery.

2.2.4 Definition of controls

Healthy women matched by age to the study group with no previous history of pre-eclampsia.

2.2.5 Recruitment strategy

The researcher reviewed the data of women who had given birth at the Princess Anne Hospital within the last years (2007, 2012-2016). The contact information was obtained from protection officers within Southampton General Hospital (SGH), including the names, addresses and contact numbers of 500 women (350 diagnosed with P-EC and 150 women in the control group). We did not have access to the patients' medical records. A study invitation letter (see **Appendix C**) was sent to 500 women with a return envelope. After receiving the reply slip. Telephone calls were made to contact women who responded to the invitation letters, and the aim and objectives of the project were explained to them. The women were invited to SGH for a blood sample to be taken. Recruitment of the controls was somewhat complicated, as the response to the initial

invitation letter was very poor. As the controls needed to match the age range of the women in the study group, they could only be invited once all the study group's samples had been completed. Given this, Sisters and female staff working at SGH were asked to participate in the study, and the response was very positive. The aim of the study was explained, and their help was sought in obtaining control samples. A number of staff and patients also offered to be involved. However, specimens were collected by previous researcher and these were used in the study I and study II.

2.2.6 Interview instrument

The researcher conducted telephone interviews with the women participating in the study. It took approximately 5-10 minutes to complete an interview. The participants were then asked to complete a general medical questionnaire (see **Appendix D**) to confirm inclusion status, then dates and times were offered to them to take a blood sample at SGH.

2.2.7 Subjects

Ethical approval was granted for the study by the Southampton and South West Hampshire Research Ethics Committee (REC reference number is 05/Q1702/131). Informed consent was obtained from all participants. The participants were asked to complete a general medical questionnaire to assess the inclusion and exclusion criteria. A case-controlled study design was used to evaluate plasma TF and TFPI levels, as well as pro-inflammatory (IL-6, IL-8 and TNF-α) and an anti-inflammatory (IL-10) cytokines in 26 women who had a history of P-EC during previous pregnancies and 14 age-matched healthy women who have never had P-EC in previous pregnancies.

2.2.8 Inclusion criteria

Inclusion criteria for the study group were that participants had experienced P-EC between January 2007 and October 2016; for the control group, participants were women within the same age range but with no history of P-EC.

2.2.9 Exclusion criteria

Exclusion criteria common for the two groups were: current pregnancy (including women who had given birth in the previous six months); chronic hypertension and obesity; the presence of cardiovascular, autoimmune and hepatic diseases; connective tissue disorders; diabetes;

coagulation disturbances; and cancer. Women on anticoagulants or corticosteroid therapy were also excluded from the study.

2.2.10 Sample size

A sample size calculation was performed, based on a 0.6 correlation coefficient between the TF, TFPI and inflammatory cytokines levels and P-EC. The p-value to assess this association was set to 5%, two-sided. The power was 0.95. Given these criteria, 30 subjects would need to be recruited in each arm, taking into account a dropout-rate of 25%.

2.2.11 Specimen collection

A 5 mL specimen of venous blood was collected using a 21-gauge needle, into vacutainer tubes containing 3.8% tri-sodium citrate. These then were centrifuged at 3000 rpm for 10 minutes at room temperature. Plasma samples then were immediately isolated and transferred into 250 µl aliquots, which then were stored at –86°C until used for batch-wise analysis. For each assay, a previously unthawed aliquot was used.

2.2.12 Assays

Commercially available enzyme-linked immuno-sorbent assay (ELISA) assays were used to measure IL-6, IL-8, IL-10 and TNF-α, according to the manufacturer's guidelines (R&D Systems, UK). The intra- and inter-assay coefficient of variations (CV) for TF and TFPI were 3.4 and 5.7%, and 3.6 and 5.9%. For cytokines these were 1.7 and 2.0%, 7.3 and 9.4%, 4.6 and 8.5%, 3.1 and 7.4%, respectively.

2.2.13 Statistical analysis

Data were analysed using the Statistical Package for the Social Sciences (SPSS), Version 23 for Windows (Statistical Analysis System, Chicago, Illinois, USA). The Shapiro-Wilk test was used to test normality. Data were not normally distributed, so results are expressed as a Box and Whisker Plot, with outliers additionally identified. Comparisons between two groups were performed by Mann-Whitney tests. P values of <0.05 were considered statistically significant. Correlations between the pro-inflammatory cytokines were assessed by Spearman correlation test. Assays results were either recorded as pg ml-1 of the original specimen for the inflammatory cytokines or ng ml-1 for TF and TFPI.

2.3 Results

2.3.1 Demographic data

The clinical and demographic characteristics of the study groups are shown in Table 2.1. No significant differences were found in the participants' age, BMI, smoking status and alcohol consumption; however, six of the participants had a family history of P-EC while eight had a previous history of hypertension. The control group also was sampled at least a year after the last delivery.

Table 2.1 Subjects' demographic and clinical data

Characteristics	Preeclampsia	Controls
Number of participants	**26**	**14**
Mean age of participants	**33.6years**	**30.5years**
Minimum to maximum age	**24-47years**	**22-43years**
Mean BMI of participants	**27.1**	**24.9**
Family history of P-EC	**6**	**None**
Family history of hypertension	**8**	**4**
Family history of type II diabetes	**4**	**7**
Family history of myocardial infarction	**5**	**5**
Family history of deep vein thrombosis	**1**	**None**
Ethnic group	**24 participants were British; 1 white European; 1 black African**	**10 participants were British;1 white European; 1 Mexican Latino; 1 black African; 1 Indian**
Current smokers	**4**	**2**
Regular exercise	**5**	**3**
Alcohol consumers	**22-average units, units consumed is 3.3 units/week**	**10 average units consumed is 3.2 units/week**
Personal history of anaemia either during, alternatively, after pregnancy	**3**	**None**
Currently on contraception	**6**	**9**

2.3.2 Inflammatory cytokines

Plasma IL-10 levels were significantly raised in women with a history of P-EC post-pregnancy compared with controls (P<0.01; Figure 2-1). Plasma IL-6 levels appeared elevated in the study group compared with controls, but this rise was not significant (Figure 2-2). On the other hand, plasma TNF-α levels tended to be reduced in the study group, compared to controls, but not reaching statistical significance (Figure 2-3). For the other inflammatory cytokines measured, we observed a trend towards raise levels in the P-EC group compared with controls. There were positive and significant correlations between the pro-inflammatory cytokines; IL-6 has strongly correlated with IL-8 and TNF-α as (r=0.32; p<0.023, r =0.4; p<0.012, respectively). No statistically significant correlations were observed between IL-8 and IL-10 (r=0.103; P<0.27), nor between TNF- and IL-10 (r=0.1; p<0.32).

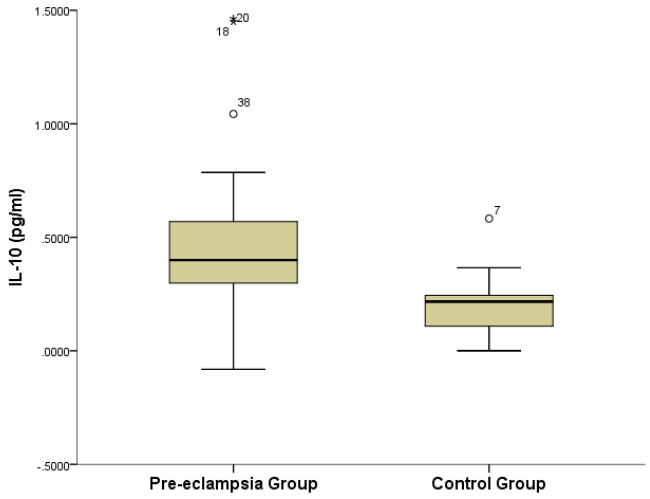


Figure 2-1 : Plasma IL-10 in women with P-EC and controls.

Results are shown in the Box and Whisker plot. The bottom and top of the 'box' represent the 25th and 75th centile, respectively, while the line within the box represents the median value. The 'whiskers' represent the range.

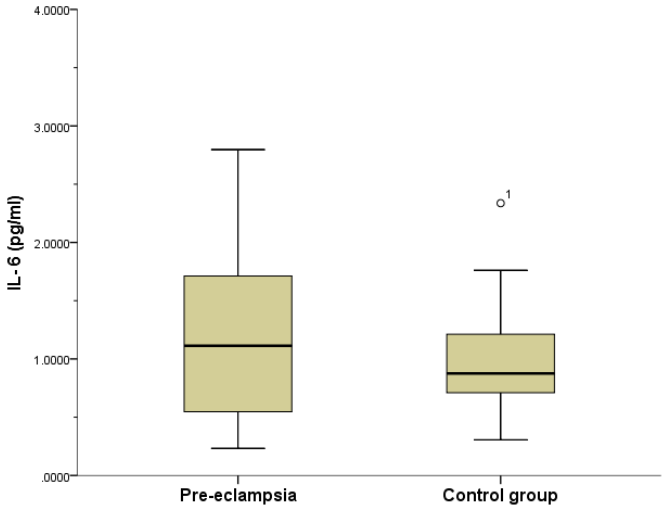


Figure 2-2 Plasma IL-6 in women with P-EC and controls

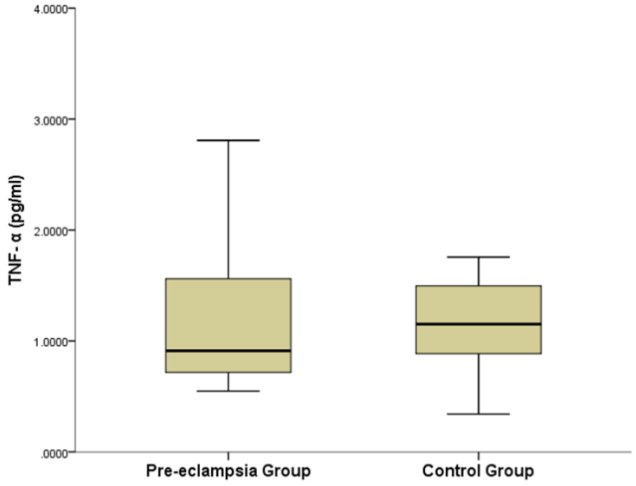


Figure 2-3 Plasma TNF-α in women with P-EC and controls

2.3.3 Tissue factor and tissue factor pathway inhibitor

There was a slight increase, albeit statistically insignificant, in plasma TF levels when P-EC group was compared the control group. On the other hand, plasma TFPI levels were slightly reduced in the P-EC group compared with controls; however, this also was not significant. Surprisingly, the TF/TFPI ratio was significantly raised when women with P-EC were compared with controls ($p<0.05$; Figure 2-4).

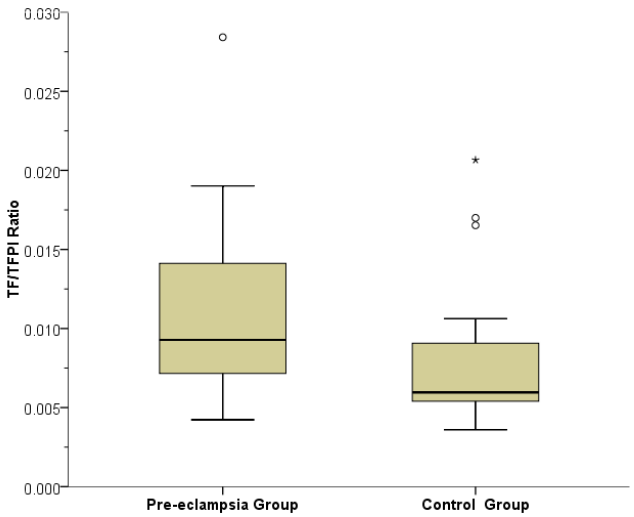


Figure 2-4 : Plasma TF/TFPI ratio in women with P-EC and controls

2.3.4 The relationship between inflammatory cytokines and tissue factor, tissue factor pathway inhibitor

There were positive and significant correlations between IL-10 and TFPI ($r= 0.5$; $p<0.01$), as well as IL-10 and TF/TFPI ($r= 0.31$; $p<0.041$).

2.4 Discussion

Changes to the immune system and haemostatic and endothelial status may have a profound effect on maternal health. However, it is not yet fully understood how such changes relate to the aetiology and pathogenesis of P-EC (ØIan et al. 1985). In this study, we show that mothers who experienced P-EC show altered levels of both inflammatory and coagulation markers postpartum. Our results have several implications for clinical practice. First, several studies have suggested that a history of P-EC is associated with an increased risk of cardiovascular disease later in life (Smith et al. 2001; Bellamy et al. 2007). Knowledge of the factors underlying this association is lacking. The findings of our study may, therefore, be relevant in broadening this understanding and may contribute to the monitoring of subsequent risk progression. Second, alterations in immune and haemostatic factors, in women with previous P-EC, during a non-pregnant state, maybe of aetiological significance in explaining maternal disease susceptibility.

The immune system pertaining to pregnant women is key in maintaining a normal healthy pregnancy. The pro-inflammatory Th1 cells which produce interleukin IL-2, interferon γ and TNF-α

are involved in cell-mediated responses and delayed-type hypersensitivity reactions, whereas the anti-inflammatory Th2 cells producing IL-4, IL-5, IL-10 and IL- 13 are involved in evoking humoral immunity. For a pregnancy to remain successful, the maternal immune response must shift from the Th1 to theTh2 phenotypes (Kumar et al. 2013). Pregnant women with P-EC have been shown to have increased concentrations of serum IL-6, IL-8 and soluble IL-4 receptors (Jonsson et al. 2006). Th1 and Th2 cytokines reciprocally regulate one another's functions (Liberman et al. 2003; Matsuzaki et al. 2005). In P-EC, the pro-inflammatory cytokines are predominantly sourced in the maternal circulation; however, both monocytes and macrophages are also known to express certain cytokines when activated by non-specific immune reactions (Rahardjo et al. 2014). Considering the immunological impact on P-EC progression, we investigated the relationship between plasma cytokines in women with a past history of P-EC and age-matched healthy women with no previous history of P-EC. All plasma cytokines measurements were made at least six months postpartum (Table 1).

IL-6 plays a vital role in regulating the body's immune response and has a significant effect on B lymphocyte differentiation and the production of acute-phase proteins, such as CRP. In P-EC, a significant increase in plasma IL-6 levels was observed between the first- and third-trimester; the same was not found in the control group (Freeman et al. 2004). Recently we demonstrated that women with a history of previous P-EC show altered levels of circulating inflammatory markers and an increased acute-phase response to influenza vaccination postpartum (van Rijn et al. 2016). In the present study, we observed raised levels of IL-6 in postpartum P-EC women compared with the control group, although this was not statistically significant.

There are many potential sources of IL-8 spanning several cell types (all nucleated cells). The main sources of IL-8 are monocytes and macrophages, as the role of IL-8 is to recruit monocytes and neutrophils, the main cells at work during an acute inflammatory response. We found a slight increase in the plasma IL-8 level of post-pregnancy pre-eclamptic women compared to their normal counterparts; this in agreement with other reports, where IL-8 has been shown to increase in pre-eclamptic subjects (Sharma et al. 2007). It is noted that TNF-α promotes apoptosis and further encourages leakage in endothelial vessels, resulting in a systemic endothelial activation response and some of the symptoms of P-EC. The low TNF-α level in postpartum pre-eclamptic women reported in our studies is in accordance with results from another study (Cackovic et al. 2008), which reported a reduced TNF-α in its patient group over controls. The reduction in TNF-α levels seen in the two studies could be attributed to its short half-life.

IL-10 acts as an immuno-regulatory cytokine balancing any increases of pro-inflammatory cytokines through its anti-inflammatory action. Bakheit et al. (2009) reported that IL-10 plays a

key role in regulating inflammatory responses in the placenta and is thought to be essential for a healthy pregnancy and that women with P-EC had raised levels of IL-10 in comparison to normotensive women. Similarly, Benian et al. (2002) found increased plasma levels of IL-10 in pre-eclamptic subjects and attributed this to the pathophysiological processes occurring in P-EC. In our study, plasma IL-10 was significantly increased in P-EC compared to controls.

Inflammatory cytokines activate coagulation through TF and protein C expression, and inhibition of fibrinolysis. Inflammation is modulated by the components of thrombin/fibrin pathway (Petäjä 2011); endothelium and monocytes/macrophages become activated and IL-1 and IL-8 secretion increases (Sower et al. 1995; van der Poll et al. 2001). Similarly, immunoglobulins could potentially exert many prothrombotic and antifibrinolytic activities, especially through interaction with mast cells (Lippi and Favaloro 2016).

Intravascular coagulation activation may play a part in the pathogenesis of P-EC (Sandset et al. 1989). It has been suggested that coagulation abnormalities may be more relevant to the fatal outcome than blood pressure (Redman 2014) and coagulation indices may be of value in monitoring P-EC progress (Howie et al. 1976). Indices of a prothrombotic state correspond with those of inflammation and may be related to the underlying vascular disease and co-morbidities (Conway et al. 2004). Vascular changes are prominent features of P-EC (Sandset et al. 1989) and both cardiovascular disease and P-EC share many risk factors (Lwaleed et al. 2014).

Previously we investigated the relationship between TF and TFPI in women who had P-EC compared to their normal counterpart and reported a significant increase in TF/TFPI ratio in women with P-EC (Lwaleed et al. 2014). Our result suggested an imbalance between TF/TFPI levels in women with the past history of P-EC post-pregnancy, and we proposed that such imbalance may contribute to the development of maternal hypercoagulable states and may predispose women with a history of P-EC to cardiovascular risks later in life (Lwaleed et al. 2014).

In the present work, we examined the relationship between TF, TFPI and TF/TFPI ratio, and pro- and anti-inflammatory cytokines in the same cohort of subjects. We observed a positive and significant correlations between IL-10 and TFPI ($r= 0.5$; $p<0.01$), as well as IL-10 and TF/TFPI ratio ($r= 0.31$; $p<0.041$). This supports the well-established phenomenon, i.e. that there is cross-talk between inflammation and haemostasis in several pathophysiological conditions (Juhan-Vague et al. 1996; O'Brien 2012). It also suggests that both anti-inflammatory cytokines and anti-TF-dependent coagulation pathway are activated in the P-EC group, arguably due to endothelial dysfunction and the development of vascular damage, both of which are known to be associated with P-EC. Indeed vascular endothelial changes are recognised as being a central process in pregnancy-induced hypertension (Granger et al. 2001).

We acknowledge that this work should be seen as a pilot study which might add beneficial information to more focused studies in the future. Despite having performed a sample size calculation, the study may have been under-powered as there are some possible associations that might have achieved statistical significance if numbers of cases had been higher.

Chapter 3

Study II

Do patients with a history of pre-eclampsia have elevated levels of coagulation and angiogenic markers postpartum?

This chapter examines the changes in the levels of angiogenic, anti-angiogenic and haemostatic markers of pre-eclamptic women after delivery. We investigate the relationship between levels of several angiogenic and anti-angiogenic factors, including vascular endothelial growth factor (VEGF), placental growth factor (PIGF), soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng), in combination with a history of severe, early onset P-EC, compared to matched controls with previously normal pregnancy, then once more again between the pre-eclampatic women and IUGR.

3.1 Abstract

Background: Pre-eclampsia (P-EC) is a pregnancy-specific disorder, characterised by placental insufficiency and endothelial dysfunction. It is a significant cause of maternal and fetal morbidity. Women with a history of P-EC have heightened risks of future cardiovascular and thromboembolic disease. In addition, pre-eclamptic patients have elevated levels of clotting and angiogenic factors; however it is unclear whether these changes persist postpartum.

Aims: The aim of this study was to investigate the relationship between haemostatic as well as angiogenic and anti-angiogenic factors in women with a past-history of P-EC, including vascular endothelial growth factor (VEGF), placental growth factor (PlGF), soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng), in combination with tissue factor (TF) and TF pathway inhibitor (TFPI), also whether these factors were altered postpartum in women with a history of P-EC.

Methods: The study followed a case-control design. Blood samples were obtained at 6-12 months postpartum from 21 primiparous women after a pregnancy affected by P-EC, and 21 women with a previously unaffected pregnancy. Plasma concentrations of each of the factors were determined using enzyme-linked immunosorbent assay.

Results: Significant differences were not observed in levels of VEGF (p=0.068), PlGF (p=0.333), sFlt-1 (p=0.910), sEng (p=0.612), TF (p=0.260) or TFPI (p=0.786) between women with and without a history of pre-eclampsia. Additionally, no significant difference was found in the TF: TFPI ratio between case and control groups (p=0.734).

Conclusion: This study does not support the hypothesis that levels of VEGF, PlGF, sFlt-1, sEng, TF or TFPI are altered in women with a history of P-EC compared to controls. However, we observed a weak positive association between all parameters measured. While we acknowledge that this is a pilot study and that the sample sizes is relatively small, our results suggest that circulating haemostatic, angiogenic and anti-angiogenic factors are not significantly altered in women with a past-history of P-EC.

Keywords: Pre-eclampsia, Angiogenic Factors, Postpartum, Intrauterine Growth Restriction, Cardiovascular Disease.

3.2 MATERIALS AND METHODS

3.2.1 Subjects

The Ethics Committee of the Tertiary Referral Centre (UMC Utrecht, the Netherlands) approved the study; Informed consent was obtained from all participants. The research protocol did not interfere with any medical recommendations or prescription.

3.2.2 Study design

The study takes a case-control design which includes a total of 42 women at 6-12 months postpartum; 21 primiparous women following a pregnancy affected by early-onset pre-eclampsia, and 21 non-pregnant women who have not had PE (control group).

3.2.3 Specimen collection

From each of the participants, a 5ml sample of venous blood was obtained using a 21-gauge needle. These were collected into vacutainer tubes containing 3.8% trisodium citrate. The blood samples were then placed in a centrifuge at a rate of 3000 rpm for 10 minutes, at room temperature. After being immediately isolated and transferred into 250µl aliquots, the plasma samples were subsequently stored at -86°C until being extracted for batch-wise analysis of the relevant haemostatic, angiogenic or anti-angiogenic factors. For each assay, a previously unthawed aliquot was used.

3.2.4 Assays

Commercially available Enzyme-Linked Immunosorbent assays were used to measure levels of VEGF, PlGF, sFlt-1, sEng, TF and TFPI in each of the samples. The intra-assay coefficient of variations for the measured markers as following; 6.7%, 7.0%, 2.6%, 2.8%, 2.3% and 4.5% respectively. Whereas the inter-assay are; 8.8%, 11.8%, 9.8%, 6.7%, 6.3% and 6.1% respectively. All assays were performed according to the manufacturers' instructions (Quantikine Human ELISA Kits; R&D Systems, UK).

3.2.5 Statistical analysis

Data were included in a database and analysed by SPSS software (version 24.0). Data normality was tested by the Shapiro-Wilk method. Results which were not normally distributed and are

expressed as medians and inter-quartile ranges (IQR). Differences between two or more groups were assessed by Mann-Whitney U test and Kruskal-Wallis test. Category variables were compared using the chi-square test. Data for TFPI were normally distributed, therefore an independent samples T-test was performed, with means and standard deviations reported. A p-value of <0.05 was considered to be statistically significant.

3.3 RESULTS

3.3.1 Demographic and clinical data

Characteristics of the study groups are summarised in Table 3.1. No significant differences were observed between the groups regarding participants' age, height, weight, BMI, systolic blood pressure, diastolic blood pressure, infant gender and whether or not they had a history of hypertension. As expected, women who had suffered from P-EC during their pregnancies had a significantly lower mean gestational age (211.10±20.40 days versus 281.86±9.45 days for controls, $p<0.01$). Consequently, formerly pre-eclamptic women had children with significantly lower mean birth weights compared to those with unaffected pregnancies (1168.57±503.93 grams versus 3574±462.12 for controls, $p<0.01$; Figure 3-1 and Figure 3-2). This is consistent with our expectations, given the effects on the offspring of mothers affected by P-EC (Szymonowicz and Yu 1987; Noris et al. 2005).

Although not statistically significant, there was a trend towards increased baseline mean diastolic blood pressure amongst the pre-eclamptic cohort (115.00 ±11.4 versus 108.75 ±8.82 for controls, $p=0.051$). However, these measurements were still within the healthy ranges for both cases and controls.

Table 3.1 Subjects' demographic and clinical data

Variable	Pre-eclampsia Group (n=21)	Control Group (n=21)	p Value
Mean Age (years)	34.84 (±4.72)	34.77(±4.78)	0.968
Mean Height (cm)	$168.58(\pm 5.85)$	171.57(±8.01)	0.223
Median Weight (kg)	$64.00(58-107)$	64(57-93)	0.587
Median BMI (kg/m²)	23.85(19.16-34.15)	$21.18(19.72-31.07)$	0.251
Mean Systolic BP (mmHg)	76.25(±6.71)	72.08(±3.97)	0.127
Mean Diastolic BP (mmHg)	115(±11.40)	108.75(±8.82)	0.051
History of previous Hypertension Yes (%) No (%)	 4(21.05%) 15(78.95%)	 1(7.14%) 13(92.86%)	 0.366
Mean Gestational age@ Delivery (days)	211.1(±20.4)	281.86(±9.45)	0.001
Mean Birth Weight (g)	1168.57(±503.93)	3574.10(±462.12)	0.001
Infant Gender Male (%) Female (%)	 14(66.66%) 7(33.33%)	 6(42.86%) 8(57.14%)	 0.169

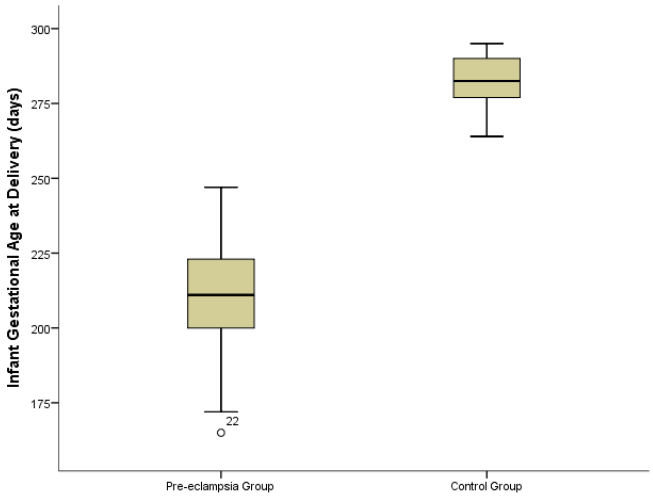


Figure 3-1 Comparison of infant gestational ages at delivery in P-EC and Control groups.

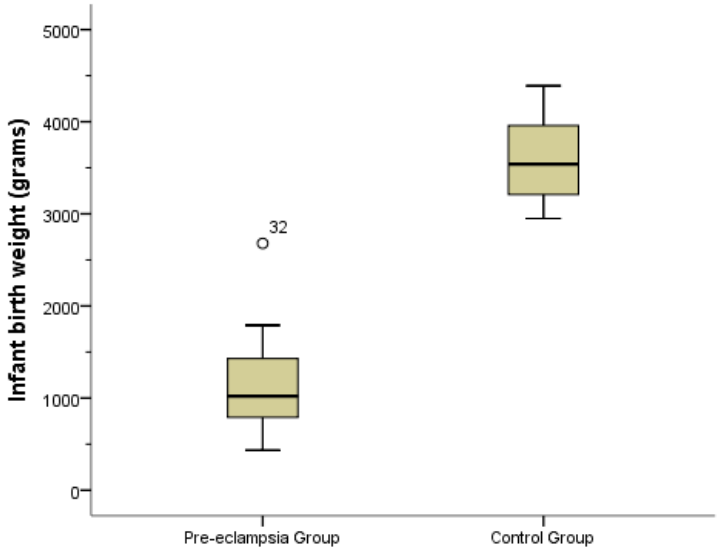


Figure 3-2 Comparing infant birth weight in study and control groups.

3.3.2 Angiogenic factor levels in women with a history of pre-eclampsia

The results indicate no change in VEGF or PlGF concentrations between case and control groups, and slight decreases between levels of sFlt-1, sEng, TF and TFPI in the formerly pre-eclamptic women compared with their healthy counterparts. Figure 3-3 illustrates the differences in the TF concentrations between the groups. None of these findings was found to be statistically significant. sFlt-1, sEng, TF and TFPI concentrations tend to reduce in concentrations amongst the pre-eclampsia group compared to controls. Such trends, however, were not statistically significant. Also, the ratio of sFlt-1 and PlGF was insignificant. As nine women out of twenty-one have had P-EC along with IUGR therefore we compared between the cohorts to see whether a history of IUGR had a significant affect on angiogenic factors postpartum (Table 3.2). There were increased levels of PlGF, sFlt-1 and TF in the group affected by IUGR compared to pre-eclamptic women (P=0.086, P=0.314 and P=0.431, respectively) whereas levels of sEng and TFPI appear to be decreased in the two groups. VEGF remained unchanged between the cohorts. These tendencies are not statistically significant

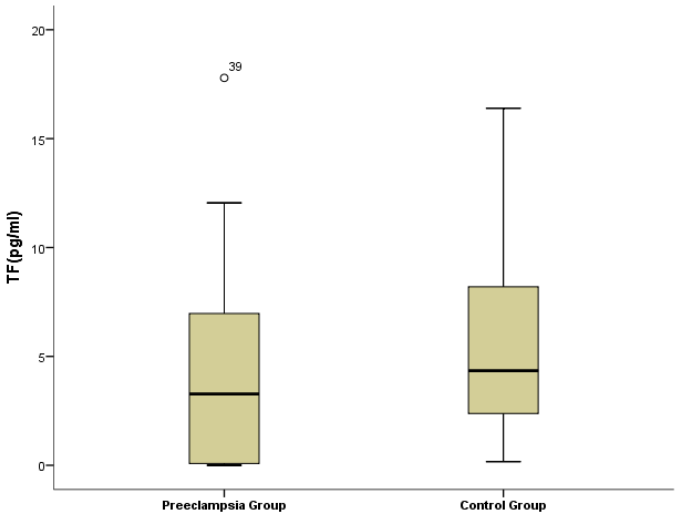


Figure 3-3 TF levels in women with P-EC and conrol groups.

Table 3.2 Comparison of all factors between women with and without a history of P-EC.

Data are reported as medians (lower and upper quartiles), except TFPI which is reported as Standard deviation (±SD)

Factors	Control Group (n=21)	P-EC Group (n=21)	p Value	Control Group (n=21)	P-EC Group (n=12)	p Value	P-EC Group (n=12)	IUGR (n=9)	p Value
VEGF	0.25 (0.25-0.25)	0.25(0.25-0.25)	0.068	0.41(0.25-0.25)	0.25(0.25-0.25)	0.148	0.25 (0.25-0.25)	0.25 (0.25-0.25)	1.000
PIGF	0.35(0.35-0.67)	0.35(0.35-0.78)	0.333	0.5(0.35-0.67)	0.5(0.35-0.78)	0.904	0.35(0.35-0.78)	0.78(0.35-0.78)	0.086
sFlt-1	8.13(0.47-19.6)	6.47(0.80-18.80)	0.910	12.6(0.47-19.6)	7.9(0.8-14.1)	0.782	1.47 (0.80-14.13)	12.3(2.22-30.0)	0.314
sEng	4.37(3.62-4.96)	4.11(3.7-4.7)	0.612	4.7(3.6-4.96)	4.2(3.7-4.69)	0.672	4.3(3.7-4.69)	4.04(3.44-4.75)	0.887
TF	4.47(2.03-9.92)	3.69(0.08-7.5)	0.260	5.8(2.0-9.9)	3.6(0.08-6.96)	0.137	3.7(0.8-6.97)	5(0.80-11.97)	0.431
TFPI	157.25 (±59.68)	152.44(±50.18)	0.786	152.38(±57.4)	153.22(±60.86)	0.970	156.8±62.5	151.26±31.79	0.934

TF/TFPI ratios in women with a history of pre-eclampsia with or IUGR compared to their counterparts

Patients with a history of P-EC showed a decreased TF/TFPI ratio, but this was not found to be statistically significant. On the other hand, there was a trend towards elevated TF/TFPI ratios amongst the groups affected by IUGR compared to pre-eclamptic women. However, this was not statistically significant see (**Table 3.3**).

Table 3.3 Comparison of TF: TFPI ratio between women with and without a history of P-EC and IUGR

Haematological Factor	Control Group (n=21)	P-EC Group (n=21)	Net Change	p-Value
TF:TFPI Ratio	0.03 (0.01-0.05)	0.02 (0.0-0.05)	Decrease	0.734
Haematological Factor	**P-EC Group (n=12)**	**IUGR Group (n=9)**	**Net Change**	**p Value**
TF:TFPI Ratio	0.02 (0.0-0.05)	0.4(0.001-0.083)	Increase	0.217

3.4 DISCUSSION

Pre-eclampsia was defined as new-onset development of hypertension (with diastolic blood pressure > 90mmHg and systolic blood pressure > 140mmHg), alongside new-onset proteinuria, dipstick 2+ or more than 300mg/24hours (Redman and Jefferies 1988). VEGF is an umbrella term for various pro-angiogenic growth factors, including PlGF. They are vital for maintaining the fenestrated endothelial in renal, liver and brain tissue, all of which can be affected in P-EC (Tjoa et al. 2010). Placental Growth Factor is expressed in the endothelial cells of the umbilical vein and trophoblast and is necessary for the angiogenesis involved in healthy placental development (Noori et al. 2010; Tjoa et al. 2010). The endothelial dysfunction underlying P-EC is thought to represent an anti-angiogenic state produced by low concentrations of VEGF and PlGF (Noori et al. 2010), which precede and coincide with disease development (Levine and Karumanchi 2005; Romero et al. 2008; Tjoa et al. 2010; Maynard and Karumanchi 2011).

This study did not find a statistically significant difference in levels of VEGF (p=0.068) or PlGF (p=0.333) between women with a history of P-EC and those with previously normal pregnancies. These findings corroborated with those of Lyall et al. (1997) who found no difference in VEGF levels at 6-12 months postpartum between formerly pre-eclamptic women and controls. Comparable results have also been reported by WikstrÖM et al. (2008) Wolf et al. (2004) and Kvehaugen et al. (2011) who measured VEGF and PlGF levels at seven days, 18 months and 5-8

years postpartum, respectively. Both Lyall et al. (1997) and WikstrÖM et al. (2008) also found that postpartum VEGF levels were significantly raised compared to pre-delivery measurements, further supporting our findings, that the decreases of VEGF during pre-eclampsia do not persist postpartum.

The only study that contradicts our findings was that of Noori and colleagues, who reported significantly elevated VEGF concentrations at 12 weeks postpartum in women who had suffered a hypertensive versus a normotensive pregnancy (Noori et al. 2010). It should be noted that this study did not differentiate between gestational hypertension and P-EC, although they have distinct pathologies for which angiogenic profiles may differ. This may account for the difference between the two studies. Also, their study did not adjust for the stage of the menstrual cycle that participants were at. It has been suggested that VEGF levels may rise with angiogenesis occurring in the ovary during follicular development, producing falsely elevated results (Noori et al. 2010).

Soluble fms-like tyrosine kinase-1 is an endothelial receptor which antagonises VEGF and PlGF. By producing vasoconstriction and endothelial dysfunction, sFlt-1 contributes to several features of P-EC, including hypertension and glomerular endotheliosis (Maynard et al. 2003; Tjoa et al. 2010). Studies have shown that placental sFlt-1 expression is elevated weeks before and during P-EC. Therefore the anti-angiogenic state increased (Maynard et al. 2003; Levine et al. 2004; Romero et al. 2008; Tjoa et al. 2010).

The present work found insignificant decrease in sFlt-1 levels amongst formerly pre-eclamptic women (median 6.47 (0.80-18.80)) versus controls (median 8.13 (0.47-19.6); p=0.910). Our results are in agreement with Noori and colleagues study who found no significant difference at 12 weeks postpartum between case and control groups, but as mentioned previously, the cohort also included women with gestational hypertension (Noori et al. 2010). sFlt-1 has a molecular weight of 110 kDa that considers too large to be filtered by the glomerulus; both PLGF and VEGF (30 and 45 kDa respectively) are easily filtered by the kidneys and excreted in the urine. Therefore, most of the studies prefer to use urinary levels of PLGF as a predictive marker for pre-eclampsia along with the serum ratio of sFlt-1/PLGF. Also, during our study, we tend to use the blood as biomarkers rather than using other types of specimens such as urine (Eddy et al. 2018).

Our findings oppose those of Powers et al. (2005) and WikstrÖM et al. (2008) who found significantly elevated sFlt-1 levels at 48 hours and seven days postpartum, respectively. The reason for these results could be the length of time after delivery at which measurements were taken. Both these studies measured sFlt-1 within days of delivery, compared to 6-12 months postpartum in our study. It could be hypothesised that a history of P-EC disrupts and slows (but

does not completely prevent) the body's mechanism of clearing sFlt-1 so that it may appear elevated in the early postpartum period. It may be the case that these patients have normalised sFlt-1 levels when followed up after a longer postpartum period. However, Wolf et al. (2004) found increased sFlt-1 concentrations in previously pre-eclamptic women at 18 months postpartum.

A reason for this could be the participant demographics; the pre-eclampsia group displayed significantly higher baseline mean blood pressures and BMIs compared to controls. And were also more likely to have a family history of Cardiovascular disease, all of which are independent risk factors for future Cardiovascular disease (Jousilahti et al. 1999), so it is not unreasonable to suggest that they also affected angiogenic marker levels in these patients.

Additionally, Kvehaugen et al. (2011) found elevated sFlt-1 levels after 5-8 years in formerly P-EC women. This could be due to the mean age of pre-eclamptic women recruited in this study (37.2±4.4years), which is slightly older than in our study (34.84±4.72years).

Soluble endoglin is a cell surface receptor which antagonises transforming growth factor-β and contributes to the anti-angiogenic environment of P-EC. It has been found to disrupt endothelial tube production, and induce vascular permeability and hypertension (Tjoa et al. 2010; Maynard and Karumanchi 2011). Studies have shown that sEng is upregulated in the circulation and placentas of pre-eclamptic women before and during its occurrence (Venkatesha et al. 2006; Jeyabalan et al. 2008; Tjoa et al. 2010; Maynard and Karumanchi 2011).

This study found a small, statistically insignificant decrease in postpartum sEng amongst the control group (median 4.37 (3.62-4.96) versus median 4.11 (3.7-4.7) for formerly pre-eclamptic women, p=0.612). Two studies were found investigating sEng levels following P-EC, both of which are supported by our findings. (Noori et al.) Also, (Kvehaugen et al.) found no significant differences in sEng levels between women with a history of P-EC and controls, when measured at 12 weeks and 5-8 years postpartum, respectively (Noori et al. 2010; Kvehaugen et al. 2011). Again, Noori and colleagues did not differentiate between gestational hypertension and P-EC, so these results should be interpreted with caution (Noori et al. 2010).

Overall, our study, in combination with the current literature does not suggest elevated levels of sEng following a pre-eclamptic pregnancy. However, our study and Noori et al. and Kvehaugen et al. studies are limited by size. Hence more extensive investigations are indicated to confirm these findings.

Tissue factor or factor III is a primary activator of the extrinsic pathway of coagulation. Tissue factor pathway inhibitor (TFPI) is its main inhibitor. TF is abundant in the placenta and decidua

and is involved in many of the mechanisms underlying P-EC such as systemic inflammation and defective placental implantation (Erez et al. 2008). Maternal plasma concentrations of TF and TFPI have been found to be elevated in women during P-EC, and are likely to contribute to the disease's hypercoagulable state (Erez et al. 2008; Rousseau et al. 2009).

We found no significant difference in TF (p=0.260), TFPI (0.786) or the TF: TFPI ratio (p=0.734) between the pre-eclamptic and control groups. There has been little literature investigating TF after pre-eclampsia. Our results partly corroborated Lwaleed and colleagues' study, which found no difference in TF and TFPI between formerly pre-eclamptic women and controls when measured within six months to three years postpartum. However, this study found an insignificant increase in TF: TFPI ratio in the IUGR group. Additionally, there was a trend towards increased TF levels amongst IUGR group, which may have reached statistical significance in a larger cohort, recent reports also suggest that a history of IUGR may increase women's risks of ischaemic heart disease, independent of pre-eclampsia (Ness and Sibai 2006).

To conclude, our study does not support the hypothesis that levels of VEGF, PlGF, sFlt-1, sEng, TF or TFPI are significantly altered 6-12 months postpartum in women with a history of severe, early-onset P-EC compared to women with previously unaffected pregnancies.

Current literature both opposes and corroborates with these results, and most studies have limitations. Further investigations into clotting and angiogenic markers following P-EC are indicated, with larger numbers of participants and longer postpartum follow-up periods. This may provide a definitive answer as to whether or not such factors play a role in the etiopathogenesis of P-EC and may explain and predict adverse cardiovascular risks in previously pre-eclamptic women.

Chapter 4

Study IV

Plasma annexins A2 and A5 in women with a past history of pre-eclampsia

Abstract

Background: Generally, pregnancy is associated with normal physiological changes in the haemostatic processes. However, it is well established that a group of pre-eclamptic women are predisposed to cardiovascular diseases later in life. The decreased anticoagulant activity of annexin A5 and the deficient profibrinolytic activity of annexin A2 have been linked to an increased risk of thrombotic events.

Methods: In the present retrospective case-control study, we investigated plasma annexins A2 and A5 levels in 5 healthy volunteers and 66 women who had pre-eclampsia (P-EC) at interval years including 2007, 2012 till 2016. Blood samples were collected in Ethylenediaminetetraacetic acid vacutainer tubes, processed and assessed for plasma annexins A2 and A5, using commercially available Enzyme-Linked Immunosorbent Assays.

Results: Plasma annexin A2 was higher in the study group compared with the controls, conversely to the plasma annexin A5, where a higher level was observed in the control group. There was no statistically significant difference in the annexins levels between the control and pre-eclamptic women group ($p = 0.12 > 0.05$ for A2, and $p = 0.086$ for A5). Annexin A2 levels showed a significant positive association with the number of years post-delivery ($r = 0.254$, $p = 0.039$). However, annexin A5 levels were negatively associated with the number of years postpartum ($r = - 0.3$, $p = 0.015$).

Conclusion: There is no significant difference between plasma annexin A2 levels and annexin A5 in the studied group. Annexin A2 showed a positive, while annexin A5 showed a negative association with the number of years postpartum. Our findings suggest that there is a slight imbalance between annexins A2 and A5 in pre-eclamptic women postpartum, this may lead to the development of cardiovascular diseases later in life. This reinforces our earlier work in relation to the raised tissue factor/ tissue factor pathway inhibitor ratio in women with P-EC.

Keywords: Annexin A2; Annexin A5; Haemostasis; Pregnancy; Pre-eclampsia; Postpartum; Cardiovascular disease

4.1 Introduction

Pre-eclampsia (P-EC) is a condition peculiar to pregnancy and commonly characterised by hypertension and proteinuria after 20 weeks of pregnancy (Roberts and Cooper 2001). Worldwide, this condition has a notable impact on maternal and foetal morbidity and mortality. Despite intensive research the aetiology of P-EC remains poorly understood. However, it has been suggested that P-EC could result from either a failure of trophoblast remodelling of uterine spiral arterioles that may lead to placental ischaemia, or due to immunological and haematological factors secreted into the circulation of the mother's circulation (Hladunewich et al. 2007). To date, the majority of studies have focused on the assessment of the coagulation and inflammatory activities. While little-published data is known about an alteration in the fibrinolytic system in P-EC women. It is well established that normal pregnancy is associated with normal physiological changes in the coagulation activities towards hypercoagulability status and the formation of placental fibrin deposits, to prevent haemorrhaging during labour. Such changes returns to normal within 4-6 weeks postpartum (Deitcher and Gardner 1999). Nevertheless, the hypercoagulability may contribute with predisposing factors, to the development of thrombotic complications associated with P-EC (Hellgren 2003). Therefore, a functional fibrinolytic system is essential to maintain the balance between the normal haemostasis and hypercoagulability status (Gohil et al. 2009). It has been demonstrated that a deficiency in the fibrinolytic activity is considered as a risk factor for increased thromboembolism in women complicated by P-EC (Sucak et al. 2006). A balance between the coagulation and the fibrinolytic systems is essential to maintain the integrity and patency of the vascular components.

Fibrinolysis is a process that removes the physiological deposit of fibrin from accumulating in the vascular system, thus preventing haemostatic clots from happening, known as clot lysis. It has been noticed that there several histological dysfunctions occurred in the placenta of many pre-eclamptic women by the presence of excessive fibrin deposition and micro-thrombi that may contribute to the disease development. Four decades ago, Synexin was the first annexin discovered and recently known as annexin A7 (Creutz et al. 1978). Annexins are a type of proteins that share structural and functional aspects (Geisow et al. 1987). To date, this family encompasses thirteen members whose primary structure has been resolved. More than 100 annexins are known to occur in many different species. Twelve proteins have been recognised in humans; these are conventionally referred to as annexin A1-13. However, the ANXA12 gene is unassigned. There are some abbreviations which denote their presence in different species for instance; the descriptor 'A' denotes their presence in vertebrates; 'B' denotes their presence in invertebrates;

'C' denotes their presence in fungi and some groups of unicellular eukaryotes; 'D' denotes their presence in plants, and 'E' their presence in protists (Gerke and Moss 2002).

Annexin A2 has been described as a cell surface co-receptor for plasminogen and its activator tissue plasminogen activator. It is binding significantly enhances cell surface plasmin generation (Hayashi et al. 2009). Additionally, annexin A2 belongs to a calcium-regulated phospholipid-binding protein family that is expressed in several cell types including vascular endothelial cells, macrophages, a wide range of tumour cells such as promyelocytic leukaemia cells and on the brush-border membrane of placental syncytiotrophoblast (Xin et al. 2012). It is well known that the annexin A2 has expressed in the adipose tissues of humans and rodents. Annexin A2 has involved in glucose homeostasis, particularly the insulin-inducible translocation of GLUT4, the vital and essential glucose transporter in adipocytes, from intracellular compartments to the cell surface. Also, Annexin A2 has been implicated in the accumulation of fatty acid (Grewal et al. 2019).

In 1985 annexin A5 was first described as a vascular anticoagulant. It is also known as Lipocortin V, Endonexin II, placental anticoagulant protein I and Thromboplastin inhibitor (Mussunoor and Murray 2008). Annexin A5 is defined as monomeric; a single-chain protein that has high-affinity binding properties to phosphatidylserine (PS) in a calcium-dependent manner (NAKAO et al. 1994). Furthermore, The timer is composed of three monomers of Annexin A5 combined in order to bound to the membrane (Schutters and Reutelingsperger 2010). The trimers tend to the assembly in a two-dimensional lattice form covering the exposed surface of the PS during trimer to trimer interactions (Olofsson et al. 1994; van Genderen et al. 2008).

The current study aimed to assess the plasma annexin A2 and annexin A5 levels post-delivery in women with a history of P-EC compared with previously healthy pregnant controls and to investigate their relationship to increase the risk of cardiovascular disease in the pre-eclamptic women in the future.

4.2 Materials and Methods

4.2.1 Subjects

Ethical approval was gained by the Southampton and South West Hampshire Research Ethics Committee (REC reference number is 05/Q1702/131). Informed consent was obtained from all participants. The research protocol did not interfere with any medical recommendations or prescription.

4.2.2 Study design

The study takes a retrospective case-control study design which includes a total of 71; P-EC (n=66) women who delivered in 2007(7), 2012(7), 2013(18), 2014(11), 2015(10) and 13 women who delivered 2016 postpartum following a pregnancy affected by early-onset P-EC and five healthy women who delivered in 2016.

4.2.3 Specimen collection

From each of the participants, a 5ml sample of venous blood was obtained using a 21-gauge needle. These were collected into vacutainer tubes containing 3.8% trisodium citrate or EDTA container tubes. The blood samples were then placed in a centrifuge at 3000 rpm for 10 minutes, at room temperature. After being immediately isolated and transferred into 250µl aliquots, the plasma samples were subsequently stored at -86°C until extraction for batch-wise analysis of the annexins A2 and A5. For each assay, a previously unthawed aliquot was used.

4.2.4 Assays

Commercially available Enzyme-Linked Immunosorbent assays were used to measure levels of ANXA2 and ANXA5 in each of the samples according to the manufacturer's instructions (Quantikine Human ELISA Kits; Lifespan Bioscience, UK). The intra-assay coefficient of variations for the measured markers annexin A2 and A5 are <10% and <6.48% respectively whereas, and the inter-assay are <12% for the annexin A2 and 5.65% for annexin A5.

4.2.5 Statistical analysis

Data were included in a database and analysed by SPSS software (version 24.0). Data normality was tested using the Shapiro-Wilk method. Results which were not normally distributed are expressed as medians and inter-quartile ranges (IQR).

4.3 **Results**

Plasma annexins A2 and A5 levels in the studied groups

4.3.1 Descriptive analysis

Levels of Annexin A2 were higher in P-EC compared with that of the control group. The converse is true for Annexin A5 where higher levels were found in the control group, relative to that found in the P-EC group. See Figure 4-1 and Figure 4-2. The plot of the mean levels of Annexin A2 and Annexin A5 for the control and P-EC groups, as shown in previous figures, gives a graphical view of the large difference between the mean levels. However, the Mann-Whitney U test suggests that there is no statistically significant difference in the plasma annexin levels between the controls and the P-EC groups ($p = 0.121 > 0.05$ for Annexin A2, and $p = 0.086 > 0.05$ for Annexin A5). The difference between the levels of A2 and A5 (A2 value – A5 value) decreased over the years. Levels of Annexin A5 were slightly higher than that of Annexin 2 for women who gave birth in more recent years, while the levels of Annexin A2 were higher in women with longer postpartum. This is true for both the control and pre-eclampsia groups.

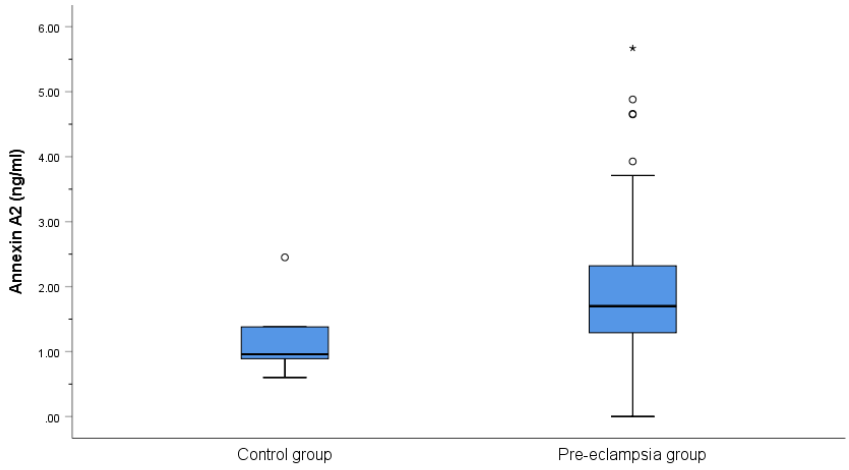


Figure 4-1: Mean plasma levels of Annexin A2 in the pre-eclampsia and control groups.

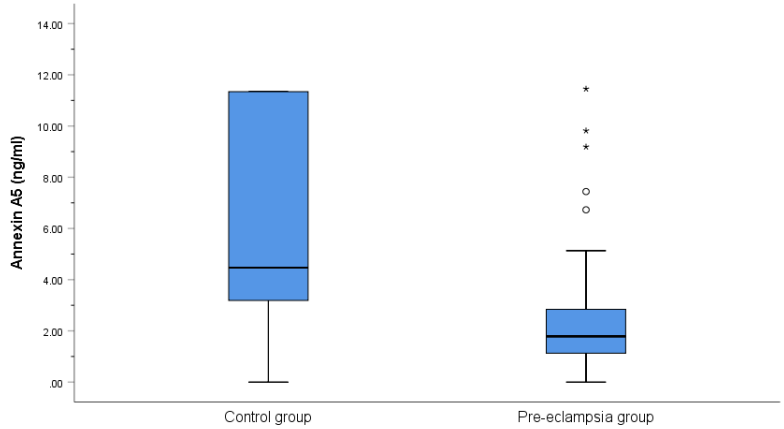


Figure 4-2: Mean plasma Annexin A5 in the studied groups.

4.3.2 To examine whether levels of ANXA2 or ANXA5 levels reduced in women who had delivered in 2007 because of the number of years since postpartum.

This analysis examined the pattern of change in the levels of Annexin A2 and Annexin A5, over the years since postpartum. The average pattern over the years is different for Annexin A2 and Annexin A5, as shown in Table 4.1. Average levels of Annexin A2 increased as the number of years since postpartum increased. Conversely, average levels of Annexin A5 appear to decrease with years post-delivery, which is very clear in the figure below. The highest levels of Annexin A2 were among women who delivered in 2007 and 2012, while 2014 was the highest average for Annexin A5. The lowest average level of Annexin A2 was in women who had their baby in 2016, but that of Annexin A5 was among women who had their baby in 2007 and 2012 (Figure 4-3, Figure 4-4 and Figure 4-5).

Table 4.1: Association between Annexin A2, Annexin A5 and year of birth.

Annexin A2	Year	Min.	1st Qu.	Median	Mean	3rd Qu.	Max.	Lower CI	Upper CI
	6	0.470	1.220	1.360	1.557	1.700	3.710	1.080	2.030
	5	0.650	1.580	1.700	1.819	2.120	3.130	1.150	2.490
	4	0.166	1.620	1.790	2.145	2.110	5.670	1.270	3.020
	3	0.002	1.270	1.440	2.804	1.810	19.570	0.546	5.060
	2	0.001	2.472	4.404	7.267	10.810	20.020	-0.734	15.300
	1	0.197	1.717	4.660	7.777	8.840	28.470	-1.290	16.800
Annexin A5	6	0.000	1.520	1.850	2.897	3.340	9.818	1.220	4.570
	5	0.610	1.540	2.190	2.067	2.720	2.950	1.450	2.680
	4	0.560	1.640	2.450	3.618	3.130	17.050	1.000	6.240
	3	0.001	1.218	1.780	3.516	3.396	16.360	1.320	5.710
	2	0.350	0.840	0.960	1.077	1.350	1.910	0.560	1.650
	1	0.700	0.795	0.970	1.163	1.230	2.420	0.611	1.710

1=2007, 2=2012, 3=2013, 4=2014, 5=2015 and 6=2016.

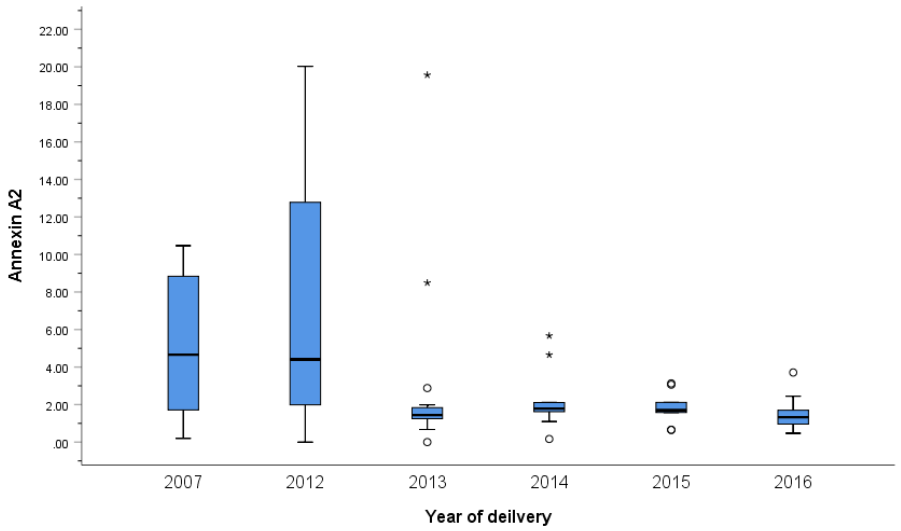


Figure 4-3: Mean plasma Annexin A2 levels in pre-eclampsia group by year of delivery.

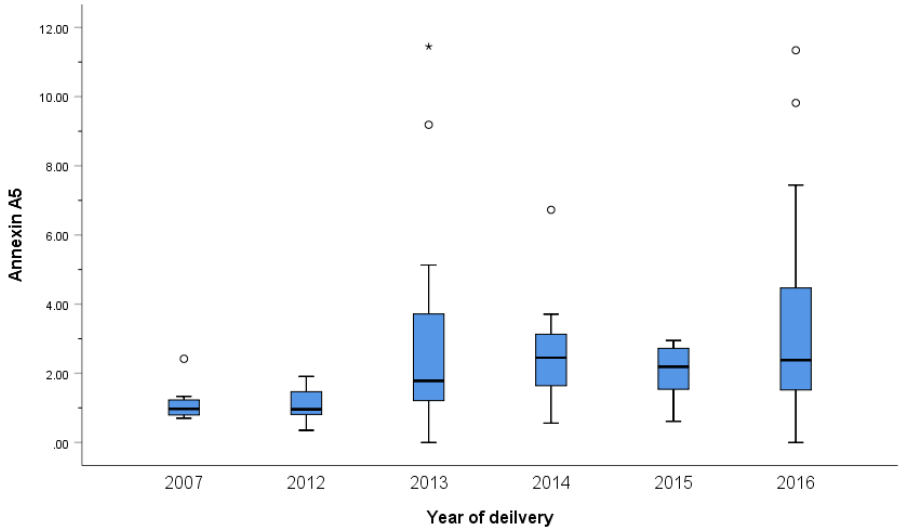


Figure 4-4 : Mean plasma Annexin A5 levels in pre-eclamptic women by year of delivery.

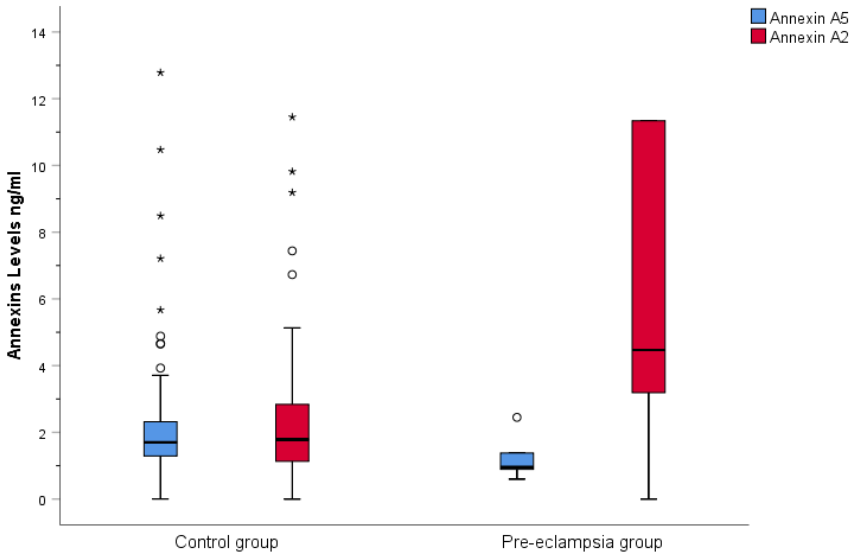


Figure 4-5: Mean plasma levels of Annexin A2 and Annexin A5 in pre-eclampsia and control groups.

4.3.3 Testing the effect of length of postpartum on levels of Annexin A2 and Annexin A5

To understand the nature and extent of the relationship between the number of years since delivering their babies and levels of Annexin A2 and Annexin A5, we used the Spearman rank correlation test because of the observed skew distribution of the datasets. Analysis indicates that there was a significant positive correlation of Annexin A2 with the number of years after delivery ($r = 0.25$, $p = 0.0393$). This suggests that as the number of postpartum years increased, levels of Annexin A2 also increased. However, there was a significantly negative relation with Annexin A5 and the number of years postpartum ($r = -0.30$, $p = 0.0148$), the longer the length of years after childbirth, the lower the levels of Annexin A5. There was no significant association of levels of Annexin A2 and Annexin A5 with either BMI or weight.

4.3.4 Modelling the difference between levels of Annexin A2 and Annexin A5 in the 66 patients with five controls and accounting for years postpartum

We used a multiple regression model to estimate the effect of years postpartum (control or P-EC) on the levels of Annexin A2 and Annexin A5. The results of the analysis indicate that the model overall is significant ($p < 0.0001$) for predicting the difference between levels of Annexin A2 and

Annexin A5. With several years postpartum and assays in the model, it can predict about 45.6% of variations in the difference between levels of Annexin A2 and Annexin A5. The model indicates that the number of years since delivery was a statistically significant predictor of differences in Annexin A2 and Annexin A5 levels (p = 0.005). The difference in the changes in levels of annexins Annexin A2, Annexin A5 between the controls and pre-eclmaptic women was not statistically significant (p-value = 0.072). For every additional year postpartum, the difference between the levels of Annexin A2 and Annexin A5 increased by 1.417.

Regression model

Model Summary					
Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Durbin-Watson
1	.456[a]	.208	.185	6.19367	2.138
a. Predictors: (Constant), Postpartum, Control 1=yes,0=no					
b. Dependent Variable: Diff2_5					

ANOVA						
Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	684.653	2	342.326	8.924	.000[b]
	Residual	2608.588	68	38.362		
	Total	3293.241	70			
a. Dependent Variable: Diff2_5						
b. Predictors: (Constant), Postpartum, Control 1=yes,0=no						

4.4 Discussion

Pre-eclampsia is an undesirable event occurring during the second half of pregnancy. Pre-eclampsia is defined as a multicausal disease, characterised by the development of hypertension and proteinuria and is considered to be one of the leading causes for maternal morbidity and mortality worldwide (Steegers et al. 2010).

Fibrinolysis is a physiological compensatory mechanism thought to be important in preventing severe bleeding during pregnancy and delivery. The fibrinolytic activity is a distinct exclusive sequential process that needs interaction between different components. Annexin A2 is

considered to be one of the key factors and plays as a crucial mediator in converting the plasminogen to plasmin. Annexin A2 serves as a surface receptor that binds both plasminogen and its activator tissue plasminogen activator (tPA), works as a co-factor for plasmin generation and localising fibrinolytic activity to the cell surface (Livak and Schmittgen 2001).

Our study demonstrates that the plasma annexin A2 levels increased in the pre-eclamptic group compared with that of the controls. On the other hand, this study is true for levels of Annexin A5, where higher levels were found in the control group relative to that found in the patients. This study did not find any statistically significant difference in levels of Annexin A2 ($p=0.121$) and Annexin A5 ($p=0.086$) between women with a history of pre-eclampsia and those with previously healthy and normal pregnancies. Our finding is in contrast to the results of (Abd El-Latif et al. 2017) where their study showed that the level of serum Annexin A2 had significantly decreased in women with pre-eclampsia, although using the same technique (ELISA) that was carried out in our study. These inconsistencies in the findings could be linked to the difference in the type of specimens collected in either the serum or plasma. Also, there was no specification in terms of the time after delivery; in our report, the blood was collected in a range of years postpartum to see whether the time since the delivery affected those two selected annexins, in women who had a history of pre-eclampsia. Surprisingly, there was an indicator that the number of years since delivery was a statistically significant predictor of differences in levels of Annexin A2 and A5 (p-value=0.005). The difference between the levels of Annexin A2 and A5 increased by 1.417, for every additional year post-delivery.

The researcher applied the statistical analysis among the pre-eclamptic participants, omitting the control group, to examine the pattern of change in the levels of Annexin A2 and Annexin A5, several years postpartum. The typical pattern over the years is different for Annexin A2 and A5. It was noticeable that average levels of Annexin A2 increased as the number of years postpartum increased. Conversely, average levels of Annexin A5 appear to reduce with the number of years post-delivery. The highest levels of Annexin A2 were among women who delivered in 2007 and 2014 for Annexin A5. The lowest average level of Annexin A2 was in women who had their baby in 2016, but that of Annexin A5 was among women who had their baby in 2007 and 2012.

Our results were consistent with those observed by (Sano et al. 2014), who investigated the placental Annexin A2 level by using immunohistochemistry and quantitative real-time PCR technique; his study was the first to demonstrate increased placental Annexin A2 mRNA expression, during the acute phase of P-EC. A study showed that annexin A2 is linked with heat shock protein 90 (Hsp90) and is up-regulated in endothelial cells of diabetic rat aorta. It leads to

an increase in the plasmin activity. Furthermore, the authors suggested that such changes in annexin A2 may be linked to clotting defects observed in diabetes (Eddy et al. 2018).

Interestingly, we found a significantly positive correlation between the levels of Annexin A2 with the number of years after delivery (rho = 0.25, p-value = 0.0393). This suggests that as the number of years postpartum increased, levels of Annexin A2 also increased. However, the level of Annexin A5 was significantly negatively associated with the number of years postpartum (rho = -0.30, p-value = 0.0148), as the number of years after childbirth increased, the lower the levels of Annexin A5. Our results are in agreement with a previous study that showed annexin A2 might increase the plasmin generation by binding to tissue plasminogen activator (tPA) and plasminogen, and repair impaired fibrinolytic activity with glucose and insulin (Liu et al. 2012). A previous study demonstrated that Annexin A2 could also alleviate the development of insulin resistance, which may be triggered by the relationship between Annexin A2, lipid metabolism, and inflammation. Since lipid metabolism and inflammation are related to cardiometabolic disease, we could draw a link between the overexpression of Annexin A2 and lipid metabolism/insulin resistance in postpartum pre-eclamptic subjects (Wang et al. 2019); these findings are consistent with the proteomic results in chapter five.

The current work showed that there is a tendency of reducing the level of Annexin A5 among the pre-eclamptic group compared to their control counterparts, this is in line with other publications reporting a decrease in the expression of annexin five through immunohistochemically in placentas from women with pre-eclampsia. Thus, leading to the status of hypercoagulability in the intervillous space, which may be associated with foetal growth restriction (FGR) and development (Ornaghi et al. 2011).

In the past years, more evidence has accumulated regarding the ability of Annexin A5 to bind with high affinity in the presence of Ca2+ to negatively charged phospholipids, such as phosphatidylserine (PS), which is expressed in the external leaflet of the trophoblast membrane (Reutelingsperger and van Heerde 1997; Gourvas et al. 2014). The cell membranes with PS exposed at the external, supply a catalytic surface for coagulation reactions, causing a high rate of prothrombinase complex formation and the activation of coagulation (Kumar et al. 1995). Consequently, Annexin A5 forms an anti-thrombotic shield around the procoagulant anionic phospholipids on the trophoblast surface, preventing the phospholipid-dependent coagulation

reactions (Matsubayashi et al. 2001). Hence, Annexin A5 has a crucial role in maintaining the blood fluidity in the placental circulation; similarly it presents itself as an attractive candidate protein, linking placental haemostatic impairment to the pathophysiology of P-EC (Peng et al. 2014).

In conclusion, we postulate that the imbalance between annexin A2 and annexin A5 levels could be used as a signal tool for the greater haemodynamic alterations and vascular damage occurred in the future for women who have experienced P-EC. Although the reduction in the level of Annexin A5 and increase levels of annexin A2 in late postdelivery pre-eclamptic women were statistically insignificant, interestingly, the levels of annexin A5 were weakly and negatively significantly correlated with the number of years postpartum.

Chapter 5 Study III

Proteomics

5.1 Introduction

Pre-eclampsia is a multisystem disorder occurring after 20 weeks of gestation with many aetiologies (Noris et al. 2005). This makes it unlikely that a unique biomarker could robustly recognise all pre-eclamptic patients. P-EC and preterm birth now represent a notable burden of adverse health. To identify new insights in the aetiology of pre-eclampsia and novel predictive biomarkers, researchers are turning towards high throughput proteomics to enable robust comparisons between the protein expression in pre-eclamptic and normotensive pregnancies. While there are multiple proteomic approaches available, mass spectrometry-based techniques remain a popular choice and are the focus of this chapter.

Proteomics offers a promising alternative to classical method based approaches for biomarkers identification of preeclampsia due to its ability to detect the whole protein complement (protein profiling). Proteomics studies the quantitative description of protein expression, and any alterations that might occur under the influence of biological changes either due to pathological condition or their treatment (Anderson and Anderson 1998). Also, this approach is used for localisation and identification of posttranslational modifications, and to assess protein-protein interactions (Wilkins et al. 1996).

Recently, the use of proteomic technologies has provided a robust view of several diseases. By focusing on the protein expression of several target samples, whether these samples have been derived from cytoplasmic cell components or secreted proteins, plasma/serum or other body fluids such as synovial, cerebrospinal and amniotic (Norwitz et al. 2005; Vascotto et al. 2007; Baillet et al. 2010). However, the collection process for these fluids may be problematic and complicated. Therefore, plasma or serum specimens are preferable as they are easy to obtain and also have a high protein content making them a valuable media for biomarker determination (Ardekani et al. 2002).

Proteomic study is, therefore, a beneficial tool for monitoring disease progression, particularly in multifactorial diseases such as pre-eclampsia due to studies showing that the most of the circulating of interest factors originally contain a protein component. This study is a pilot to look at the small number of samples to identify any protein of interest and quantify potential

biomarkers to find factors or unique proteins that could be further used as a diagnostic tool for disease detection, monitoring and treatment.

5.2 Aims of this study

1. To identify individual plasma proteins differentially expressed between pre-eclamptic and healthy women after post-pregnancy.
2. To identify clusters of differentially expressed plasma proteins in preeclampsia associated with the susceptibility to develop cardiovascular diseases in the future.

5.3 Plasma profiling

The wide dynamic range in the expression of proteins in a biological system is nearly 12 orders of magnitude (Dayarathna et al. 2008). The proteins of interest usually exist at low concentration, and their activities are masked by highly abundant proteins present in the system. It is known that albumin represents more than half of the total protein mass in human plasma, whereas the top ten proteins together make up 90% of the total protein content (Dayarathna et al. 2008). Therefore, a classic sample processing 'workflow' includes the deduction of high abundance proteins or enhancement for proteins of interest. Separation technologies include protein digestion further peptide fractionation using High-performance liquid chromatography (HPLC) or peptide enrichment, and orthogonal separation of peptides directly before MS/MS analysis (LC-MS/MS) are essential to reduce sample complexity (Qian et al. 2006).

An Immunodepletion approach is a most common technique used where high-abundance proteins have to be removed. However, this approach can lead to the concomitant removal of non-targeted protein species, and thus increase issues of specificity, reproducibility, and the capacity for meaningful quantitative analyses. Therefore, a combination of multidimensional separation with label-based methods, i.e. tandem mass spectrometry (MS/MS) have been used for this current experiment.

5.4 Ultra-Fast Speed and High Separation Performance

The speed of liquid chromatography separation becomes faster by using analytical columns with ultra-fine particle packing and also by increasing the flow rate of the mobile phase. Once the time for the actual analysis is reduced, other factors essential for the analytical cycle time need to be considered, such as the injection time, injection movement, gradient delay time and system conditioning time.

UFLC provides the key answer for these factors, most importantly reducing the total analysis cycle time, which ultimately enhances laboratory productivity. The device consists of four units:

1. Degassing unit (DGU-20A3R).
2. Solvent delivery unit (LC20AD).
3. Column ovens (CTO-20A).
4. UFLC UV-VIS detector (SPD-20A).

5.5 High Performance- Size exclusion chromatography (HP-SEC)

High Performance- Size exclusion chromatography (HP-SEC); also known as molecular sieve chromatography, separates molecules based on their size and molecular weight by filtration through a gel/column. The column or the gel consists of spherical beads containing pores of specific size distribution. Therefore, larger molecules migrate fast as they are too large to enter the pore of the column, While small molecules elute late as the move slowly. Consequently, molecules separate based on their size as they pass through the column and are eluted in order of decreasing molecular weight (MW).

Operating conditions and gel selection depend on the application and the desired resolution. Two common types of separations performed by the SEC are fractionation and desalting (or buffer exchange). When an aqueous solution is used as mobile phase, this technique is called as gel filtration chromatography (GFC-SEC), and the stationary phase tends to be hydrophilic, whereas gel permeation chromatography (GPC-SEC) uses organic solvents as the mobile phase and the stationary phase is hydrophobic (Schure and Moran 2017).

5.6 Mass spectrometry (MS)

A Mass spectrometer is an instrument that measures mass to charge ratio (m/z) values and the relative abundance of ions. Mass spectrometry-based proteomic assays have incredible breadth when it comes to protein identifications.

Proteomics studies have been categorised into two approaches:

1) analysis of the intact proteins and peptides known as the top-down approach
2) analysis of the proteolytic peptides that act as surrogate markers for the protein of interest known as the bottom-up approach.

Classically MS consists of four main parts known as :

1. Sample inlet.
2. Ion source.
3. The mass analyser.
4. Ion detection.

Each compartment will be discussed further below for more details.

5.6.1 Sample inlet

The sample is applied to the instrument via the sample inlet. Charged intact proteins are changed into gas-phase ions which are produced by the ionisation source and directed toward the mass analyser. The mass analyser then separates ions by their mass to charge ratio (*m/z*).The ion detector then quantifies the intensity of the ions at each m/z. The result is a mass spectrum which plots the intensity of the ions at each *m/z* and can be used to determine the molecular weight of the ions measured. Calculating the molecular weight of the ion from the mass spectrum allows structural information about the sample to be determined. The instrument must operate under vacuum to avoid interruption to the movement and measurement of the ions.

5.6.2 Ionisation Source

The sample must be vaporised and ionised to be measured by MS. Therefore, the ion source is that part that of the MS that is able to produce gas-phase ions so which can then be accelerated. Proteins and polypeptides are predisposed to excessive fragmentation and thermal decomposition when exposed to the high-energy ionisation methods used to ionise more stable chemical compounds. Low energy known as ‘soft’ ionisation techniques were therefore developed to ionise proteins and polypeptides (Tanaka et al. 1988; El-Aneed et al.

2009). Electrospray ionisation (ESI), and matrix-assisted laser desorption ionisation (MALDI) are the two most common techniques used in proteomics research.

5.6.2.1 Electrospray Ionisation

ESI encompasses the transfer of ions in solution into gaseous stage ions at atmospheric pressure by using charge (Quinn et al. 2012). The precise mechanism by which the peptides vaporised and ionised is not yet fully understood. ESI involves three steps (Ho et al. 2003)

- droplet formation by application of the sample to a capillary in a strong electric field
- droplet reduction by gas flow or heat
- ion formation

ESI is different from other atmospheric pressure ionisation processes since it may produce multiple-charged ions, successfully extending the mass range of the analyser to accommodate the kDa-MDa orders of the magnitude observed in proteins and their associated polypeptide fragments (Somogyi 2008). ESI is commonly coupled with HPLC; chromatography is directly combined with the MS instrument via the electrospray capillary (Ho et al. 2003). MS analysis is carried out dynamically on-line as the sample elutes from the chromatography column.

5.6.2.2 Matrix-Assisted Laser Desorption Ionisation

Hillenkamp and Karas first introduced MALDI in 1988 (Kathleen Lewis et al. 2006; Shin et al. 2010). The means for the laser desorption/ ionisation of compounds in MALDI is a matrix usually consisting of organic acid crystals that are good absorbers of laser radiation; the matrix is excited by pulses of light from a laser to the analyte molecules: a nitrogen laser at 337nm is usually used. The sample enters as a gas-phase alongside the matrix. A small amount of energy is transferred from the matrix to the sample allowing ionisation without fragmentation.

Surface-enhanced laser desorption/ ionisation (SELDI) is another technique which is a relative variation of the MALDI technique allowing selection of proteins from crude mixtures before ionisation. Proteins are applied to a solid phase surface aimed to select for proteins according to their chemical properties (Tang et al. 2004). Bound proteins are then co-crystallised with the matrix and ionised utilising the MALDI technique. Unbound proteins and undesirable

constituents (for example salts, detergents, and lipids) are removed by washing with an appropriate buffer.

5.6.3 Mass Analyser

The mass analyser separates ions by *m/z* ratio enabling detection. The mass analyser is, in large part, responsible for the performance of the mass spectrometer and the quality of the data output. The most commonly used mass analysers in proteomics are time-of-flight (TOF), quadrupole (q or Q) and ion traps (Tang et al. 2004).

The formation and operation of ions in the gaseous state are liable due to their sensitivity and short life. Mass spectrometers typically use either oil diffusion pumps or turbomolecular pumps to achieve the high vacuum required to operate the instrument. The ion source is kept at atmospheric pressure, and a continuous pressure gradient and voltage gradient from source to detector is used to help pump ions through the analyser.

5.6.3.1 Time-of-Flight Mass Analyser (TOF-MS)

Time-of-flight mass analysers measure the time that ions take to travel a set distance through an electric field (Stewart et al. 2015). The kinetic energy of an ion can be calculated using the accelerating potential and the mass and charge of the ion. As the same accelerating potential is applied to all ions, the kinetic energy of an ion is a function of both mass and charge. Consequently, ions of different mass but with the same charge travel at different velocities and so reach the detector at different times. TOF mass analysers are commonly coupled to MALDI ionisation sources.

Mass resolution can be weak due to spatial and temporal differences in the formation of the ions, and variations in flight times of ions with similar *m/z* ratios. Applying delayed extraction is necessary to avoid poor mass resolution, where ions are held in a field-free region until accelerated by the application of a voltage pulse. The use of reflections that deflect ion with high energy is commonly used to overcome the variations in flight times. The length of the flight pathway is increased proportionally to the energy of the ion and high-energy ions arrive at the detector at the same time as lower energy ions of the same *m/z*.

5.6.3.2 Quadrupole Mass Analyser

Quadrupole mass analysers consist of four parallel rods of circular or hyperbolic cross-section. Fields are recognised across pairs of parallel rods of opposite charge arranged around a central axis. Ions are introduced to the quadrupole continuously and filtered by *m/z*. The positive rods permit ions above a selected *m/z* to pass through the central axis while the negative rods allow ions beneath a selected *m/z* to pass. The arrangement of the rods in a quadrupole overlaps the mass filter regions of both positive and negative rods. This region lets merely ions between the selected *m/z* values to continue to the detector. Ions with a *m/z* outside this range collide with the rods and do not proceed through quadrupole. Scanning of the quadrupole mass analyser can be performed by altering the amplitude of current and radiofrequency at a constant ratio allowing a range of *m/z* ratios to be detected. Quadrupole mass analysers are commonly coupled to ESI sources. Ions are produced continuously by ESI, and the quadrupole can filter *m/z* of the generated ions on a continuous basis.

5.6.4 Ion Trap Mass Analyser

An ion trap mass analyser uses a magnetic field which collects a continuous stream of ions in a three-dimensional electric field trapping the ions into an orbit inside the field (Hager 2002). Ions are trapped until the space charge limit, the number of ions which can be held in the field without distorting it is reached. The *m/z* ratio of the ions is determined by application of a radio frequency voltage which causes ions to oscillate. The frequency of oscillation is a determinant of the *m/z* value of the ion. The radiofrequency voltage is increased to scan a range of *m/z* ratios. As the frequency of oscillation of the ions increases the ion becomes destabilised and is emitted from the trap. As the oscillating frequency is a function of the *m/z* value of an ion, ions with different *m/z* are ejected at different voltages and times (Aebersold and Mann 2003).

5.7 Data output

After ions generated by the ion source have been filtered and separated by *m/z values* in the mass analyser, the ions are subjected to a detector. The detector measures the numbers of ions at each *m/z*. This information is transformed into a signal and a mass spectrum produced by processing software. The relative intensity of ions detected at each *m/z* is plotted as the mass spectrum. This information then used to define the molecular weight of the ions

produced. Algorithms are used to process this data and infer structural information about the sample applied to the mass spectrometer.

5.8 Materials and Methods

5 ml samples of venous blood were collected using a 21-gauge needle, into vacutainer tubes containing Ethylenediaminetetraacetic acid (EDTA). The blood was then centrifuged at 3000 rpm for 10 min at room temperature. Plasma samples were then immediately isolated and transferred into 250ml aliquots to Eppendorf tubes, which were then kept and stored at -80°C until used for batch-wise analysis.

Serum specimens were thawed and vortex mixed for 2 min. Plasma samples were mixed in a 1:4 ratio with 6M guanidine HCl + 10% Met-OH; this condition is called isocratic where the use of a solvent that does not change the composition of the sample of interest during the run and protect the sample from protein degradation.

One of the properties of guanidine is its ability to break down the hydrogen bonds between amino acid residues. Consequently, the 3D conformation of the protein is unfolded, and the aqueous solubility of the protein is significantly elevated. After denaturation, the protein can be reduced, modified, or analysed, in a variety of procedures. Therefore, 112µl of samples and controls were mixed with 588µl of 6 M aqueous guanidine hydrochloride and were kept on dried ice.

5.8.1 First Dimension

To evaluate the technical reproducibility relative to the biological replicates, 500µL aliquots as above were subjected to SubProteome Enrichment by high-performance Size Exclusion Chromatography (SuPrE-SEC) in duplicate and handled as a stand-alone sample throughout the experimental procedure. 500 µl samples were subjected to SuPrESEC into sample load under these conditions:

- The flow rate is 1 ml/min.
- Temperature 35°C.
- The absorbance of 280°.
- Pressure between 9.2-12.5 Megapascal (MPa).
- End time: 20 minutes.

Five distinct SEC segments were selected for serial fraction collection by the UV-signal response at 280 nm so as to reduce chromatographic peak under sampling.

For example, segment one (S1) contains the high molecular weight proteins, segment two (S2) contains IgG enriched segment, segment three (S3) is known as the Albumin enriched segment, segment four (S4) is composed of low molecular weight proteins and degradation products and segment five (S5) contains small molecules such as metabolites. Collection of these samples is depending on peak and time. All the aliquots were kept at -20°C until batch analysis.

5.8.2 Reagents and Chemicals

The chemical reagents acetonitrile, methanol, acetone, triethylammonium bicarbonate (TEAB), Sodium dodecyl sulfate (SDS), Methyl methanethiosulfonate (MMT) and formic acid (HPLC grade) were obtained from Sigma Corporation (Poole, Dorset, U. K.). The ultrapure HPLC grade water, utilised for the initial peptide fractionation with high pH RP and subsequent LC-MS analysis procedures, was generated from the Barnstead water filtration system (Dubuque, IW, U. S.A.). All TMT reagents and buffers were obtained from Applied Biosystems (Warrington, Cheshire, UK.). Each 100µl of unprocessed plasma sample was mixed with 400 µL of 6 M aqueous guanidine hydrochloride.

5.8.3 Dialysis or Ultrafiltration

The proteins fractions were dialysis purified with 3.5K MWCO Slide –A-Lyzer cassettes (Thermo Fisher, Hemel Hempstead, Hertfordshire, U. K). For the first 4cycles, 10ml of ultra-purified H2O (18.2MQ) was added to 10 tubes and centrifuged at 3600g in a cold environment (4 °C) every 1.5hr intervals this step repeated four times and final step of exchange, 10ml of 0.5M ammonium bicarbonate was added to the tubes which were then centrifuged again under the same conditions. The resulting dialysates were lyophilised to dryness after discarding the precipitants, then collected from the membrane and approximately 1ml transferred into a new tube and ammonium bicarbonate added to reach a final volume of 1.5ml and left in the speed vac at room temperature overnight.

5.8.4 Protein extract

Proteins were resuspended in 0.5M Triethylamine bicarbonate (TEAB) +0.05%Sodium dodecyl sulfate (SDS) buffer at pH 8.0 -8.5 while they were kept on ice. After this step the tubes were put in Tip sonicator for 2 minutes to prepare them for quantification.

5.8.5 Reduction, alkylation and trypsin digestion

2µl of reducing agent was added to the ten tubes in the fume cupnoard and then were put in a heat block at 60°C for 1 hour. then one µl of blocking reagent was added and the speimens kept in the dark place for ten minutes. An Enzyme-substrate ratio of 1:40 is needed to calculate the amount of trypsin needed to add to the samples. Therefore 5.2 µl of trypsin was added in and left in the incubator for 16hrs at 37°C.

5.8.6 Stable isobaric Labelling

A total of 100 µg of protein content from each SuPrE-SEC segment was trypsin digested and labelled with (TMT10plex) isobaric label reagent. Vials were reconstituted with 41µl of Acetonitrile (ACN) then transferred to the samples and controls. Labels were assigned as follows: Control 1 CT1; 126, CT2; 127N, CT3; 127C, CT4; 128N, CT5; 128C and the pre-eclamptic samples assigned as PE2; 129N, PE18; 129C, PE28; 130N, PE38; 130C, PE39; 131. They were put them at room temperature for 2hrs and covered with foil as they were sensitive to oxidation. 8 µl of hydroxylamine (5%) was added to each sample to stop the reaction.

5.8.7 Peptide Pre-fractionation with Offline High-pH RP HPLC

Initial offline peptide fractionation was based on high pH Reverse phase (RP) chromatography using the Waters, XBridge C8 column (150 × 3 mm, 3.5 µm). Mobile phase (A) was composed of 2% acetonitrile and 0.05% ammonium hydroxide, and a mobile phase (B) was composed of 100% acetonitrile and 0.05% ammonium hydroxide.

5.8.8 LC−MS Analysis

The LC−nESI−MS analysis was performed with the Dionex Ultimate 3000 UHPLC system coupled with the ultra-high resolution nano ESI LTQ-Velos Pro Orbitrap Elite mass spectrometer (Thermo Scientific, Warrington, Cheshire, U. K.). Offline fractions derived from subproteomes 1, 2, 3, and four were handled as discrete proteomes and were injected sequentially by their retention time indices. Mobile phase blanks were run between subproteome injections and were verified for the absence of carry-over signal. The TMT labelled peptides were fragmented with the axial electric field-assisted higher-energy collisional dissociation (HCD) cell at the resolution setting of 15 000. The LTQ FT-Orbitrap system was externally mass calibrated every 3−4 days using the positive ion calibration solution (Thermo Pierce, Rockford, IL, U. S.A.). Ion tuning was verified on a weekly basis as recommended by the manufacturer.

5.9 MS Data processing

Processing of the acquired mass spectra was performed with the Proteome Discoverer 1.4 software Thermo protein discover 1.4 manual Fisher Scientific. Sep 15, 2017. All the spectra were searched against the UniProtKB Swiss Prot human protein (March 2018). Protein ratios were transformed to log2 ratios, and significant changes were determined by the power analysis (a <0.05, power 0.3) and by *t*-test.

5.10 KEGG Pathway analysis

Pathway analysis was mapped to KEGG pathways using DAVID Bioinformatics (free online bioinformatic resource) associated with multiple testing was corrected using the Benjamini method and an false discovery rate FDR-corrected *p*-value <0.05 was considered significant.

Here is a copy of the short communication report for the proteomics experiment findings that is to be submitted to AJOG Journal.

5.11 Plasma proteomic profiling one year postpartum of women with pre-eclampsia shows dysregulated cardiometabolic profile

Abstract

Background: Emerging epidemiological data suggest that pre-eclampsia (P-EC) is associated with long-term complications, including a two-to four-fold increased risk of cardiovascular disease. The present study aimed to examine the global plasma proteomic profile one year postpartum of women with P-EC during pregnancy, to identify which processes are dysregulated.

Methods: Non-depleted plasma collected one year postpartum from women with P-EC (n=5) and age-matched, BMI-matched women with normal pregnancy (n=5) was analysed using quantitative proteomics.

Results: Principal component analysis of all identified proteins showed that women with P-EC during pregnancy had a distinct plasma proteomic profile one-year postpartum compared to the control group. One-hundred and seventy-two proteins were differentially expressed in the P-EC vs control. Gene ontology analysis using DAVID showed that terms related to *Inflammatory | Immune response*, *Blood coagulation* and *Metabolism* were significantly enriched.

Conclusions: The identified proteins could provide insight into the long-term dysregulated cardiometabolic profile of women with P-EC.

5.11.1 Introduction

Pre-eclampsia that occurred during the first pregnancy was considered to have no long-term adverse cardiovascular effects (Chesley et al. 1976). However, emerging epidemiological data suggest that pre-eclampsia is associated with long-term complications, including a two-to four-fold increased risk of cardiovascular disease (Chen et al. 2014). More specifically, women with pre-eclampsia are at-risk for chronic hypertension (Lykke et al. 2009; Magnussen et al. 2009; Wang et al. 2013), fatal and non-fatal coronary heart disease, venous thromboembolism and stroke (Smith et al. 2001; Kestenbaum et al. 2003; Pell et al. 2004; Bellamy et al. 2007; Mongraw-Chaffin et al. 2010).

Plasma proteomics refers to the untargeted analysis of the global circulating proteome. Shotgun proteomics is becoming a very important tool in clinical research, as it can provide valuable insight into the pathophysiology of disease but also identify novel disease markers and therapeutic targets (Al-Daghri et al. 2016; Larkin et al. 2016). A recently published study by Murphy et al. (2015) examined the maternal circulating proteome six months postpartum of women with pre-eclampsia (n=12) compared to controls (n=12) using label-free quantitative proteomics (Murphy et al. 2015). This study reported the identification of 126 peptides, a low proteomic coverage, whereas the number of the respective proteins mapping to these peptides was not mentioned.

The aim of the present study was to apply a refined quantitative proteomic methodology to examine the global plasma proteomic profile one year postpartum of women with pre-eclampsia during pregnancy. An overview of the study design is presented in **Figure** 5-1.

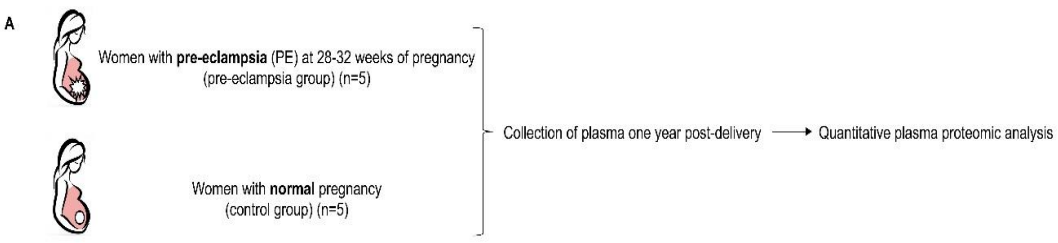


Figure 5-1 Schematic of the study design for proteomic work.

5.11.2 Materials and Methods

5.11.2.1 Clinical data analysis

Clinical data were analysed using Prism (Version 7.0a). All parameters are presented as a mean ± standard deviation. An unpaired, two-tailed Student T-test was applied to compare the baseline characteristic of participants.

5.11.2.2 Plasma proteomic analysis

The procurement and handling of plasma were in accordance with the recommendations of the Standard Operating Procedure Integration Working Group (SOPIWG). One ten-plex plasma proteomics experiment was performed. Plasma specimens were freshly thawed and vortexed for 2 minutes. For each participant, 100uL of unprocessed plasma were immediately mixed with 400uL 6M Guanidine Hydrochloride and subjected to global quantitative serum proteomic analysis using our previously published method (Al-Daghri et al. 2016).

Unprocessed raw files were submitted to Proteome Discoverer 1.4 for target-decoy search against the UniProtKB Homo Sapiens database (release date 10-Jan-2015) using SequestHT. Only reporter ion ratios from unique peptides were considered for the quantitation of the respective protein. Median normalisation and $\log_2$ transformation were performed for the reporter ion quantification ratios. A protein was considered differentially expressed between the pre-eclampsia vs control group when it's one-sample, two-sided, T-test p-value was ≤ 0.05 and mean iTRAQ $\log_2$ratio higher than $\pm$ 0.3. Only proteins identified with at least two unique peptides were further considered for bioinformatics analysis in compliance with the Paris Publication Guidelines for the analysis and documentation of peptide and protein identifications (http://www.mcponline.org/site/misc/ParisReport_Final.xhtml). All mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD009325.

5.11.2.3 Principal component analysis and bioinformatics interrogation

Principal component analysis using the reporter ion ratios of the differentially expressed proteins in P-EC vs control groups was performed using the online software tool ClustVis (https://biit.cs.ut.ee/clustvis/). DAVID (https://david.ncifcrf.gov/), STRING (https://string-db.org) and Ingenuity Pathway Analysis (IPA) (Qiagen, Hilden, Germany) bioinformatics tools were used to identify gene ontology terms, pathways and protein networks significantly enriched in the

differentially expressed proteins between P-EC and control groups. Significance was set at p-value ≤ 0.05.

5.11.3 Results

The anthropometric and clinical characteristics of the participants are presented in **Table** 5.1
The P-EC and control groups were similar with regards to age, pre-pregnancy and postpartum BMI, parity status and gestational age at delivery.
The plasma proteomic study resulted in the identification of 1,421 unique proteins (peptide FDR $p<0.05$). Principal component analysis of all identified proteins showed that women with pre-eclampsia during pregnancy had a distinct plasma proteomic profile one-year post-delivery compared to the control group (**Figure** 5-2). A volcano plot of the analysed proteome [mean iTRAQ $\log_2$ratio (P-EC/control) plotted against the minus $\log_{10}$p-value of the one-sample T-Test] is presented in (**Figure** 5-3)**.**

Table 5.1 Clinical profile of subjects studied.

Clinical parameters	P-EC (n=5)	Control (n=5)	p-value
Mean age at inclusion (yrs.)	33.95 ± 4.03	36.32 ± 5.6	0.468
Mean Maternal Height (cm)	170 ± 6.37	159.36 ± 6.0	0.013
Mean Maternal Weight (kg)	78.57 ±16.1	61.22 ± 8	0.075
BMI (kg/m²)	27.0 ± 4.88	24.2 ± 3.5	0.318

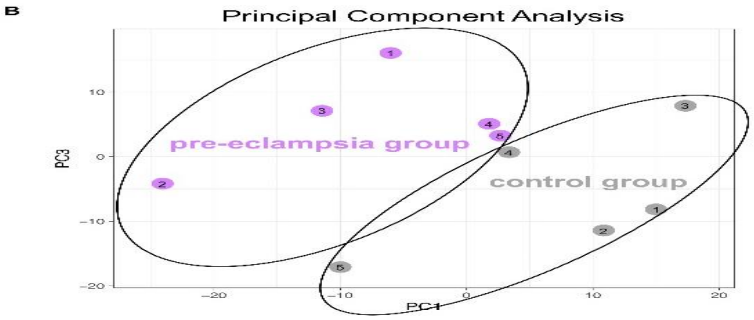


Figure 5-2 Plasma proteomic profile in P-EC and Control groups.

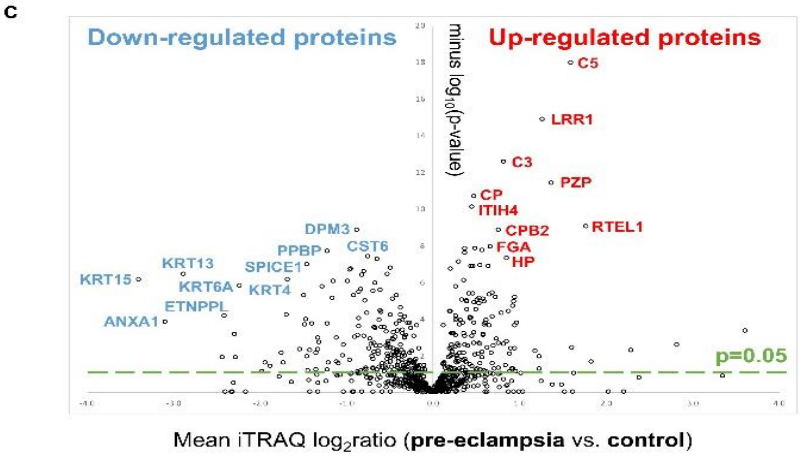


Figure 5-3 Mean iTRAQ log_2ratio (P-EC/control).

In total, 172 proteins were differentially expressed in the P-EC vs control groups. Gene ontology analysis using DAVID showed that terms related to *Blood coagulation*, *Inflammatory | Immune responses* and *Metabolism* were significantly enriched in the differentially expressed proteins (DEPs) shown in (**Figure** 5-4). DEPs mapping to these gene ontology term groups is presented in heatmap format in **Figure** 5-5, along with an IPA protein network related to cardiovascular disease. Pathway analysis with IPA showed that the intrinsic prothrombin activation pathway was significantly induced in the P-EC group compared to control (**Figure** 5-6)

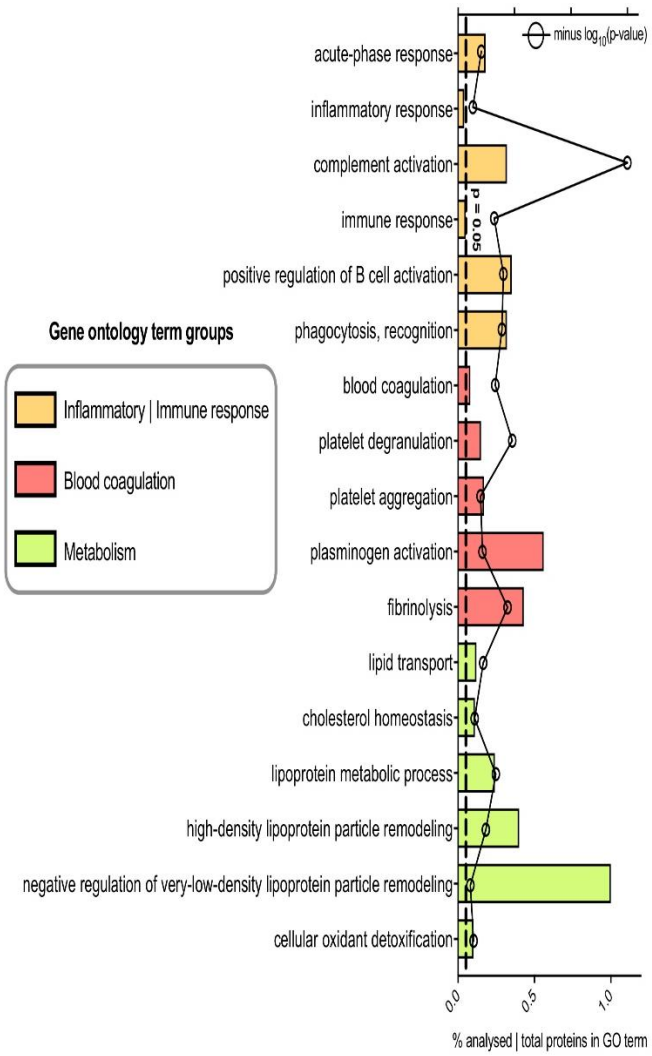


Figure 5-4 Gene ontology analysis of DEPs in P-EC vs Control.

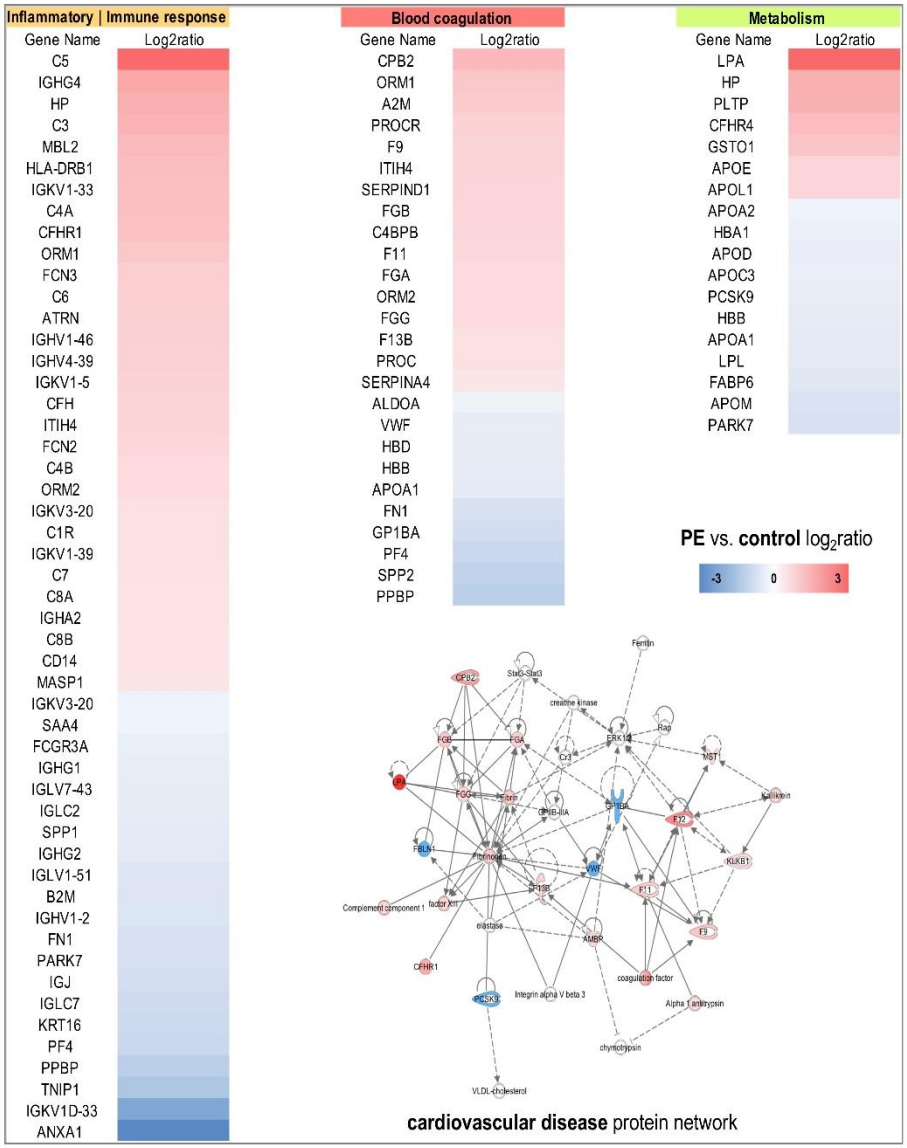


Figure 5-5 DEPs mapping and cardiovascular disease protein network.

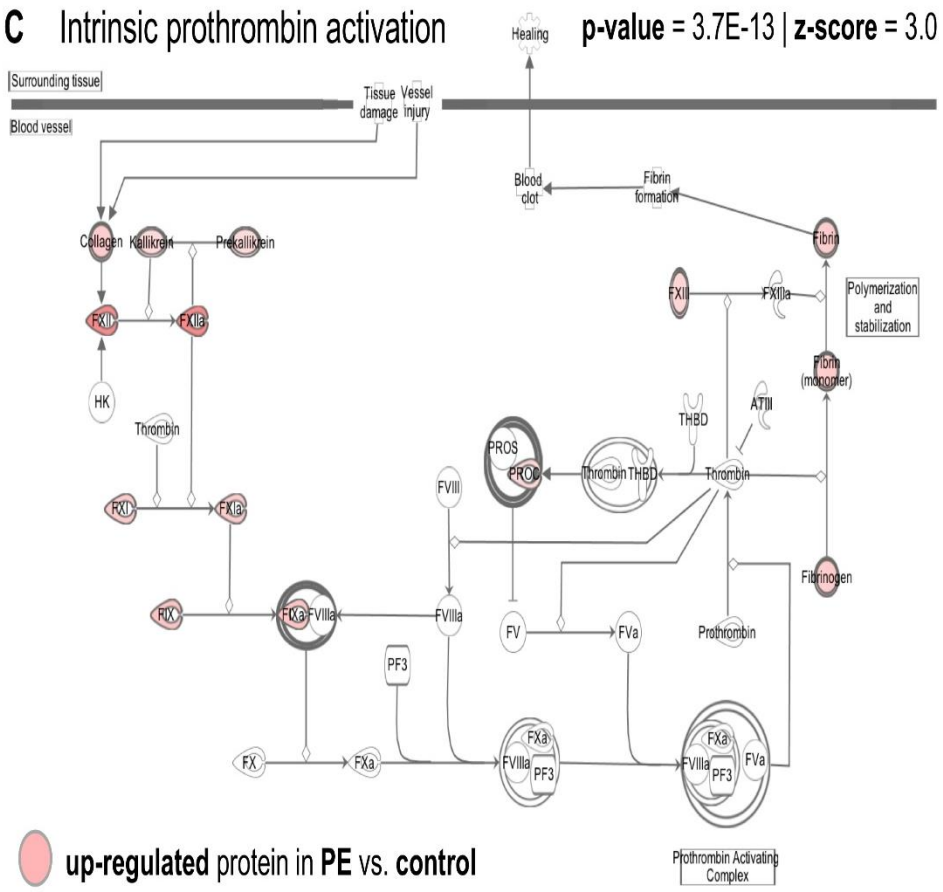


Figure 5-6 intrinsic prothrombin activation pathway.

We compared the DEPs with Exocarta, a database of mRNA/proteins enriched in exosomes, in order to identify which proteins are derived from exosomes. Of the 172 DEPs, 87 were common with the Exocarta database (http://www.exocarta.org/) (**Figure** 5-7). These proteins are presented in heatmap format in **Figure** 5-8, whereas **Figure** 5-9 shows a direct protein interaction of functional inter-relation between these proteins.

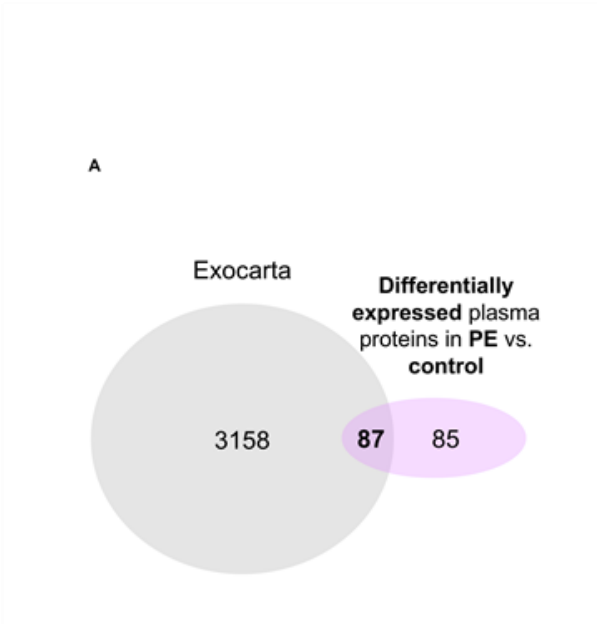


Figure 5-7 Common and unique proteins associated with comparison groups.

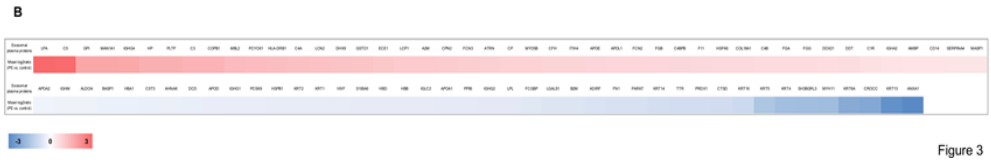


Figure 5-8 Heat map of proteins Extracted from Exocarta database.

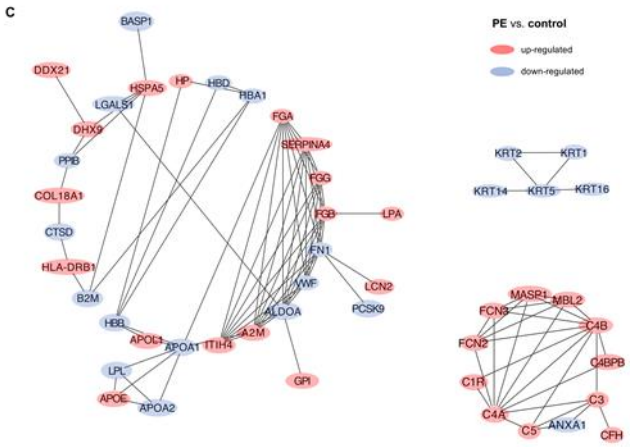


Figure 5-9 Direct protein interaction of functional inter-relation between these proteins

5.12 **Discussion**

This study reports the most comprehensive to date plasma proteomic profiling one-year postpartum of women with pre-eclampsia compared to controls. The study results show that inflammation | immune response, blood coagulation and metabolism are dysregulated processes one-year post-delivery in women with a history of pre-eclampsia.

Blood coagulation has been shown before to be induced postpartum in women with pre-eclampsia (Murphy et al. 2015), and this increased coagulation status could be causally linked to the epidemiologically observed risk for cardiovascular disease in this population group. In our study, the identification of blood coagulation as a significantly enriched gene ontology term and the induction of the intrinsic prothrombin activation pathway served as positive controls for the validity of our global plasma proteomic profiling.

Monocyte differentiation antigen CD14 (CD14) is a 55-kDa protein expressed as membrane-anchored and soluble serum protein forms. CD14 is a co-receptor for bacterial lipopolysaccharide and mediator of the inflammatory response. Furthermore, CD14 has been shown to participate in adipose tissue-related chronic inflammation and the eventual development of insulin resistance as a result of chronic inflammatory signals (Fernández-Real and Pickup 2012). Our study results of increased CD14 levels one year postpartum in women with pre-eclampsia compared to controls could reflect their increased inflammatory status and increased risk of developing insulin resistance and type 2 diabetes mellitus.

Adiponectin is a crucial cytokine secreted by adipose tissue that regulates fat metabolism and insulin sensitivity, with well-established anti-atherogenic, anti-diabetic and anti-inflammatory actions (Sargolzaei et al. 2018). Cross-sectional studies have found that adiponectin positively correlates with insulin sensitivity (Stefan et al. 2002; Stefan et al. 2013) whereas it negatively correlates with inflammatory markers (Floegel et al. 2013). Adiponectin is therefore considered to be a marker of glucose homeostasis and insulin sensitivity but does not reflect cardiovascular risk (Borges et al. 2016). Therefore, down-regulation of adiponectin in women with pre-eclampsia could also reflect an increased risk of developing insulin resistance.

Communication between cells does not only depend on secreted molecules, such as hormones and cytokines, but also on extracellular vesicles (EVs) that contain nucleic acids, proteins and lipids (Gao et al. 2017). Exosomes are a subtype of EVs featuring organotypic and organotropic characteristics. Studies have shown that EVs released by adipocytes play an essential role in the reciprocal pro-inflammatory loop between macrophages and adipocytes, thus possibly aggravating local insulin resistance (Kranendonk et al. 2014a) but also systemic insulin resistance (Kranendonk et al. 2014b). A recent study by De Silva et al. (2018) showed that exosomes from macrophages activated with

Lipopolysaccharides (LPS) affect the differentiation and insulin-mediated glucose uptake of adipocytes (De Silva et al. 2018). Exosomes are also associated with different cardiovascular pathologies, including hypertension (Gioia et al. 2015) and atherogenesis (Lener et al. 2015). More than half of the DEPs analysed in women with P-EC vs control were of exosomal origin and their role in the dysregulated cardiometabolic profile of women with P-EC one year postpartum warrants further investigation.

In conclusion, the present plasma proteomics profiling one year postpartum of women with P-EC vs controls provides insight into the dysregulated cardiometabolic profile in this population group, thus highlighting the need for long-term health monitoring.

Chapter 6

Study V

miRNA analysis in postpartum pre-eclamptic women

Abstract

Objective: Pre-eclampsia is associated with long-term complications, including a two to four fold increased risk of cardiovascular disease. MicroRNAs (miRNAs) are essential posttranscriptional regulators of gene expression. This study was performed to identify miRNA that are upregulated or downregulated in postpartum women who had a history of pre-eclampsia and whether these alterations in the levels of plasma miRNA expression could be linked to subsequent cardiovascular diseases**.** This project was based on an exploratory experiment set as a cross-sectional case-control study. Out of the 66 plasma samples that were collected during the whole project, 30 randomised plasma samples were taken from pre-eclamptic postpartum women grouped according to the incidence of pre-eclampsia at different time points in 2007, 2012, 2013, 2014, 2015 and 2016 five samples to each year and the only five plasma specimens received from healthy subjects as the control group in 2016.

Study design:

I. Discovery phase :

Post-delivery plasma samples were obtained from thirty patients with pre-eclampsia (n=30), who had pre-eclampsia at interval years starting from 2007, and then from 2012 till 2016, five women for each year and a control group (n=5) from healthy uncomplicated pregnancies in 2016. Blood specimens were collected in vacutainer tubes with Sodium citrate and Ethylenediaminetetraacetic acid (EDTA) and Next-Generation Sequencing (NGS) was carried out as a technique to identify miRNAs differentially expressed. A total of 417 miRNAs were found. However, fourteen were identified as significantly differentially expressed miRNA and annotation, with log fold change (log FC) between pre-eclampsia and the control groups.

II. Confirmation and Validation:

To confirm and validate the miRNAs were significantly differentially expressed in the previous cohort experiment, the sample size was increased to a total of 66 using plasma samples from pre-eclamptic women who delivered in 2007(7), 2012(7), 2013(18), 2014(11), 2015(10) and 13 women who delivered in 2016 following a pregnancy affected by early-onset pre-eclampsia and (n=14) from healthy women as a control group. The miRNAs transcripts previously were utilised by creating a pipeline for unsupervised classification of selected biomarkers. The validation was performed by using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay.

Results: 14 miRNAs that were significantly differentially expressed at a significance level of 0.05 (FDR) in the discovery experiment. 12 miRNAs were downregulated (hsa-miR-103a-3p, hsa-miR-190a-5p, hsa-let-7f-5p, hsa-miR-6734-5p, hsa-miR-454-3p, hsa-let-7b-5p,hsa-miR-301a-3p, hsa-miR-26a-5p, hsa-miR-4467, hsa-miR-144-5p, hsa-miR-374a-5p, hsa-miR-652-3p, hsa-miR-191-5p, hsa-miR-5010-3p, hsa-miR-324-5p, hsa-miR-664b-5p, hsa-miR-589-5p, hsa-miR-199b-5p, hsa-miR-106b-3p, hsa-miR-424-3p, hsa-let-7c-5p, hsa-let-7d-5p, hsa-miR-331-3p) and hsa-miR-122-5p, hsa-miR-885-5p were up-regulated among P-EC vs control. The plasma miR-103a-3p was significantly differentially expressed and downregulated in entire pre-eclamptic women with FC= -1.3; BH-adj P-value =0.033.

Conclusion: We believe that this study reports, for the first time, the dysregulation in plasma miRNA expression, particularly in SCN8A target gene for ion channels that might be linked to cardiac excitability and other processes pertinent to arrhythmia in postpartum preeclampsia compared to healthy women. These findings also provide novel targets for further investigation of subsequent cardiovascular events in pre-eclampsia after a long time since their delivery.

Keywords: microRNA, placenta, preeclampsia, Next Generation Sequencing**,** quantitative reverse transcription-polymerase chain reaction.

6.1 Introduction

Our knowledge about microRNAs has developed since 1993 when Rosalind Lee and his colleagues discovered that the small free-living nematode (worm) called ***Caenorhabditis Elegans*** has lin4, which is a gene well-known to control the timing of larval growth (Lee et al. 1993). This model has become recognised as a standard model organism for a great variety of genetic investigations, but is mainly useful for studying developmental biology, cell biology and neurobiology.

MicroRNAs defined as regulatory RNAs, 18–23 nucleotides long, which are involved in the transcriptional and post-transcriptional regulation of gene expression. They reportedly play a crucial role in biological pathways, including cell development, cell differentiation, regulation of the cell cycle, metabolism and apoptosis (Hu et al. 2009). A specific miRNA can have one to thousands of messenger RNA targets, establishing the potential for a small change in sequence or overall miRNA structure to have profound phenotypic effects (Friedman et al. 2009). According to miRNA repository miRBase database version 22, 2019, the human genome contains 1917 annotated hairpin precursors and 2654 mature sequences (Kozomara et al. 2018)

MicroRNAs are mainly transcribed by RNA polymerases II and III, making precursors that undergo cleavage events to make mature microRNA. RNA polymerase II is responsible for the initiation of most of the primary miRNA transcripts (pri-miRNAs) (Macfarlane and Murphy 2010). Pri-miRNAs encompass within them one or shorter sequences that form a typical thermodynamically stable hairpin structure (Perron and Provost 2008).The regulatory roles of microRNAs are completed through a RNA-induced silencing complex (RISC) (Macfarlane and Murphy 2010). MicroRNA gathers into a RISC, activating the complex to target messenger RNA (mRNA) specified by the microRNA. Several RISC assembly models have been proposed, and research continues to explore the mechanisms of RISC loading and activation (Bartel 2004). The conservative biogenesis pathway consists of two cleavage events; one is nuclear and the other is cytoplasmic. Conversely, alternative biogenesis pathways exist that differ in the number of cleavage events and enzymes responsible (Peng and Croce 2016).

Pre-eclampsia (P-EC) is a multisystem disorder occurring as a complication of pregnancy after 20 weeks of gestation and is characterised by new-onset hypertension and proteinuria. In addition, it is associated with a two-to-seven-fold increase in the risk of later cardiovascular disease development (CVD)(Bellamy et al. 2007).

Micro-RNAs have been shown to participate in cardiovascular disease pathogenesis including atherosclerosis, myocardial infarction, coronary artery disease, heart failure and cardiac arrhythmias. Generally defined, cardiac arrhythmias are a variation from the normal heart rate or rhythm. Arrhythmias are common and result in significant morbidity and mortality (Kim 2013). Ventricular arrhythmias constitute a major cause for cardiac death, particularly sudden cardiac death in the setting of myocardial infarction and heart failure (Wakili et al. 2011).

Rapid advances in understanding microRNAs have opened up new avenues of exploration in screening for any aberrant expression of the miRNA that is associated with human diseases such as cancer, gynaecological, inflammatory diseases and cardiovascular disease. These potential biomarkers may be beneficial for diagnosis, prognosis and as therapeutic targets or tools, topics which needs further investigation and validation (Santamaria and Taylor 2014). More recently, the attention of research, along with clinical focus, is shifting towards the long-term implications of P-EC on CVD (Yuan et al. 2017). In this chapter, an exploratory study was undertaken to investigate the alterations in gene expression occurring in the plasma of pre-eclamptic women postpartum in comparison to a healthy control group.

Workflow

Figure 6-1 below outlines the QIAGEN Genomic Services' pipeline for miRNA Next Generation Sequencing.

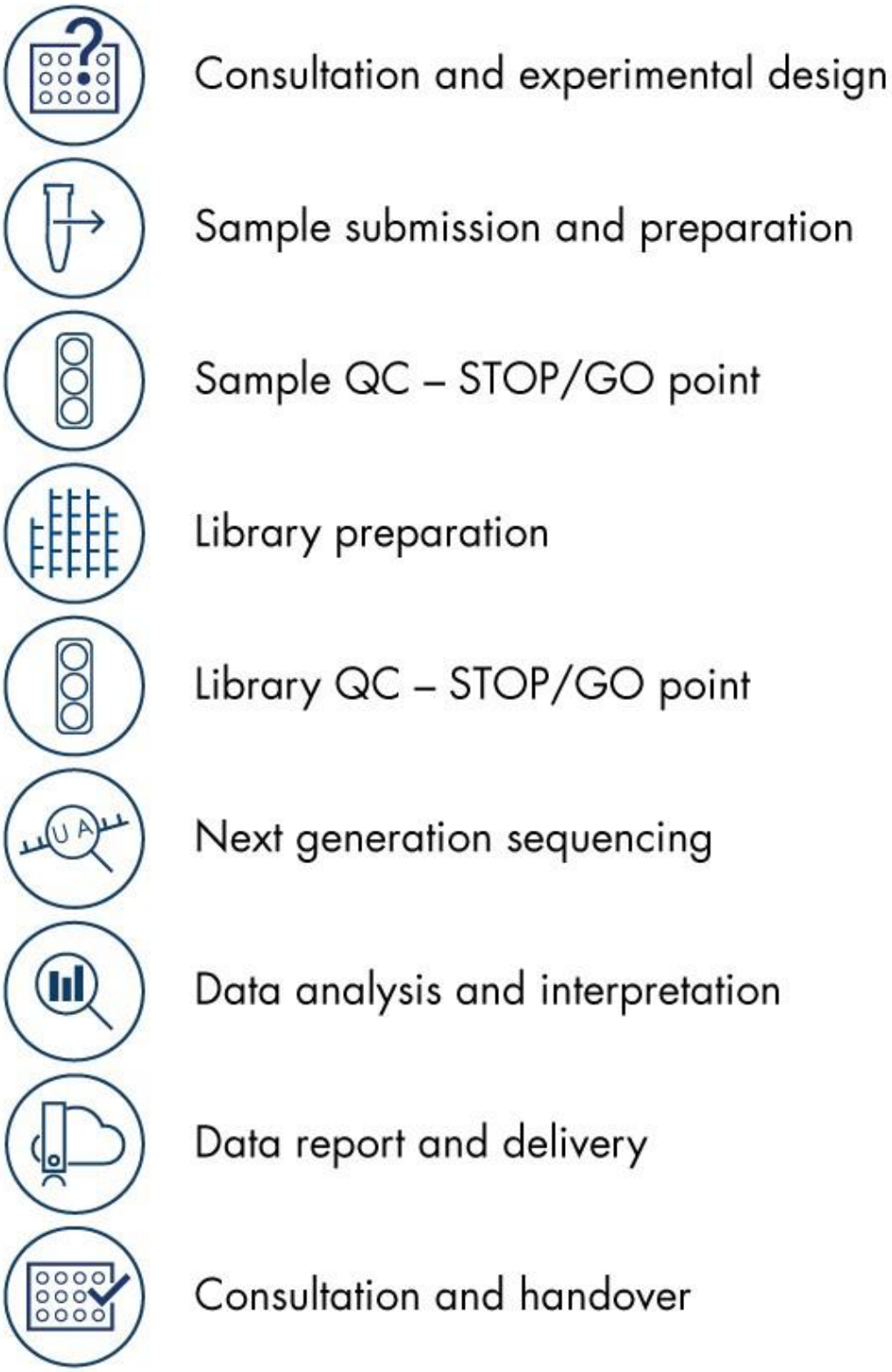


Figure 6-1: Overview of a typical miRNA NGS project with QIAGEN Genomic Services.

Experimental sample overview

30 plasma samples were collected from pre-eclamptic postpartum women grouped according to the incidence of pre-eclampsia at different time points in 2007, 2012, 2013, 2014, 2015 and 2016; five samples to each year and five plasma specimens from healthy subjects as the control group. An analysis was then performed to compare cases in the study group (P-EC) to see if there were any differences in the gene expression allowing for the effect of years since delivery. Next an analysis was carried out between the pre-eclamptic groups irrespective of the year of delivery and the control group. Table 6.1 below lists all the samples in this project and their specification groups.

Table 6.1: Sample name and sample grouping.

Sample name	Sample groups
CTRL1	Control 2016
CTRL2	Control 2016
CTRL3	Control 2016
CTRL4	Control 2016
CTRL5	Control 2016
S32	P-EC 2007
S36	P-EC 2007
S40	P-EC 2007
S41	P-EC 2007
S59	P-EC 2007
S35	P-EC 2012
S47	P-EC 2012
S48	P-EC 2012
S49	P-EC 2012
S63	P-EC 2012
S5	P-EC 2013
S8	P-EC 2013
S10	P-EC 2013
S44	P-EC 2013
S45	P-EC 2013
S6	P-EC 2014
S9	P-EC 2014
S22	P-EC 2014
S25	P-EC 2014
S43	P-EC 2014

S1	P-EC 2015
S4	P-EC 2015
S7	P-EC 2015
S42	P-EC 2015
S50	P-EC 2015
S2	P-EC 2016
S27	P-EC 2016
S30	P-EC 2016
S39	P-EC 2016
S51	P-EC 2016

6.2 Materials and Methods

6.2.1 Reference genome

Annotation of the obtained sequences was performed using the reference annotation listed below.

- **Organism: Homo_sapiens**
- **Reference genome: GRCh37**
- **Annotation reference: mirbase_20**

6.2.2 Experimental design

The experiments were performed using the following settings:

Instrument: NextSeq500

- **Average number of reads: 12 Trillion reads / per sample**
- **Number of sequencing cycles (read length): 75 nt. Single-end read (up to 46bp insert + 19bp 3' linker + 10 UMIs)**

Library preparation

- **Protocol:** QIAseq miRNA Library Prep kit
- **Platform:** Illumina

All experiments were conducted at QIAGEN Genomic Services in Germany.

QIAGEN GmbH
R&D Life Science Key Account Service
Attn. Andre Bahr / Anke Singer / Holger Wedler
Qiagen Str. 1
40724 Hilden
Germany

6.2.3 Sample preparation

RNA was isolated using the miRNeasy Serum/Plasma Kit (QIAGEN) according to the manufacturer's instructions.

6.2.4 Library preparation and Next-Generation sequencing

The miRNA library was prepared using the QIAseq miRNA Library Kit (QIAGEN). A total of 5ul total RNA was converted into miRNA NGS libraries. Adapters containing unique molecular indices (UMIs) were ligated to the RNA. Then RNA was converted to complementary DNA (cDNA). The cDNA was amplified using PCR (22 cycles), and during the PCR indices were added. After PCR, the samples were purified. Library preparation QC was performed using either Bioanalyzer 2100 (Agilent) or TapeStation 4200 (Agilent).

Based on quality of the inserts and the concentration measurements the libraries were pooled in equimolar ratios. The library pool(s) were quantified using qPCR. The library pool was then sequenced on a NextSeq500 sequencing instrument according to the manufacturer instructions (Andersen et al. 2004). Raw data was de-multiplexed, and FASTQ files for each sample were generated using the bcl2fastq software (Illumina inc.). FASTQ data were checked using the FastQC tool (Cock et al. 2010).

6.2.4.1 Trimming

Cutadapt (1.11) was used to extract information of adapter and UMI in raw reads, and output from

Cutadapt was used to remove adapter sequences and to collapse reads by UMI with an in-house script.

6.2.4.2 UMI correction

According to the experiment protocol, each raw read was expected to contain, starting from 5' end, an insert sequence, the adapter sequence, 12nt-long UMI sequence, and other ligated sequence.

Depending on the read length and insert length, not all parts were present in all reads. To correct the PCR bias with UMI information (see Figure 6-2), we processed raw reads as described below:

1. Use cutadapt on raw reads with provided adapter sequence to acquire output with information about the presence of adapter for each read.
2. Parse cutadapt output and keep only reads that fulfil all of the following requirements:
    - Reads contain adapters.
    - Insert sequence should be equal or larger than minimal insert length (default 16nt).
    - UMI sequence should be equal or longer than minimal UMI length (default 10nt).

3. Extract insert sequences from reads which do not contain full-length UMI sequence from step 2 output as partial-UMI reads.
4. Examine reads with full-length UMI in step 2 output and identify all unique insert +UMI combinations. Extract insert sequences from unique insert + UMI combinations as full-UMI reads.
5. Combine partial-UMI reads and full-UMI reads as an output of UMI correction.

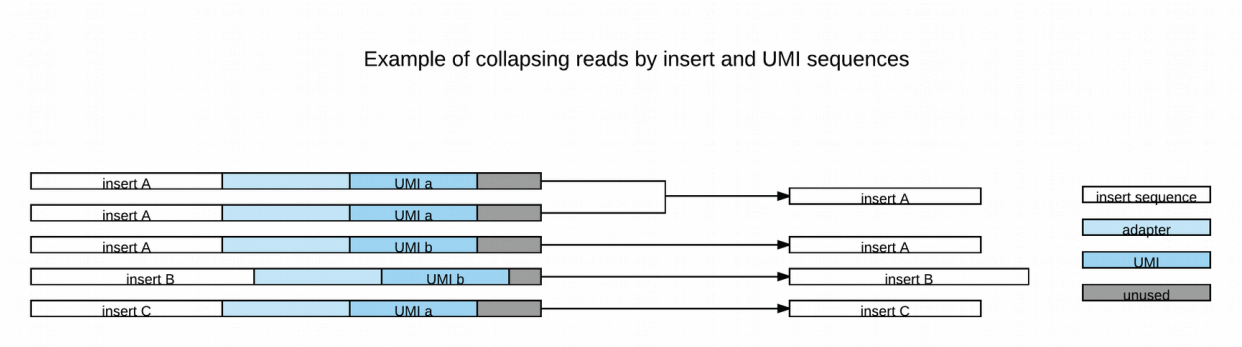


Figure 6-2: Illustration of the principle behind UMI correction.

In order to correct for amplification biases sequence containing identical insert and UMI are collapsed into a single sequence.

6.2.4.3 Mapping

Bowtie2 (2.2.2) was used for mapping the reads. The mapping criteria for aligning reads to spike-ins, abundant sequence and miRBase were the reads which had to have a perfect match to the reference sequences. For mapping to the genome, not more than one mismatch was allowed in the first 32 bases of the read. No small insertions and deletions (INDELS) were allowed in mapping.

6.3 Differential Expression

Differential expression analysis was performed using the EdgeR statistical software package (Bioconductor, http://bioconductor.org/). For normalisation, the trimmed mean of M-values method based on log-fold and absolute gene-wise changes in expression levels between samples Trimmed mean of M-values (TMM) normalization was used.

The isomiR analysis and putative miRs were done with in-house scripts (exq_ngs_mircount and exq_ngs_mirpred using the MiRPara tool)

6.3.1 Unsupervised analysis

A principal component analysis was performed with R using TMM-normalised quantifications from defined collections of samples as input. The same input was also used to generate a heatmap of expression profiles with R.

6.3.2 GO Analysis

GO analyses were performed with R package TopGO with experimentally-verified targets of significantly differentially expressed miRNAs as input. Nat. Genet, 25(1):25-9.

6.3.3 Data quality control

The following sections provide a summary of the QC results obtained for the data set. This section includes only QC of the reads themselves, whereas the subsequent section includes QC of the mapping results.

6.3.4 Average Read Quality

An overview of the average read quality is shown in Figure 6-3.
(Note that for ease of visualisation of this plot with many samples, only the average read quality of 10 samples will be displayed).

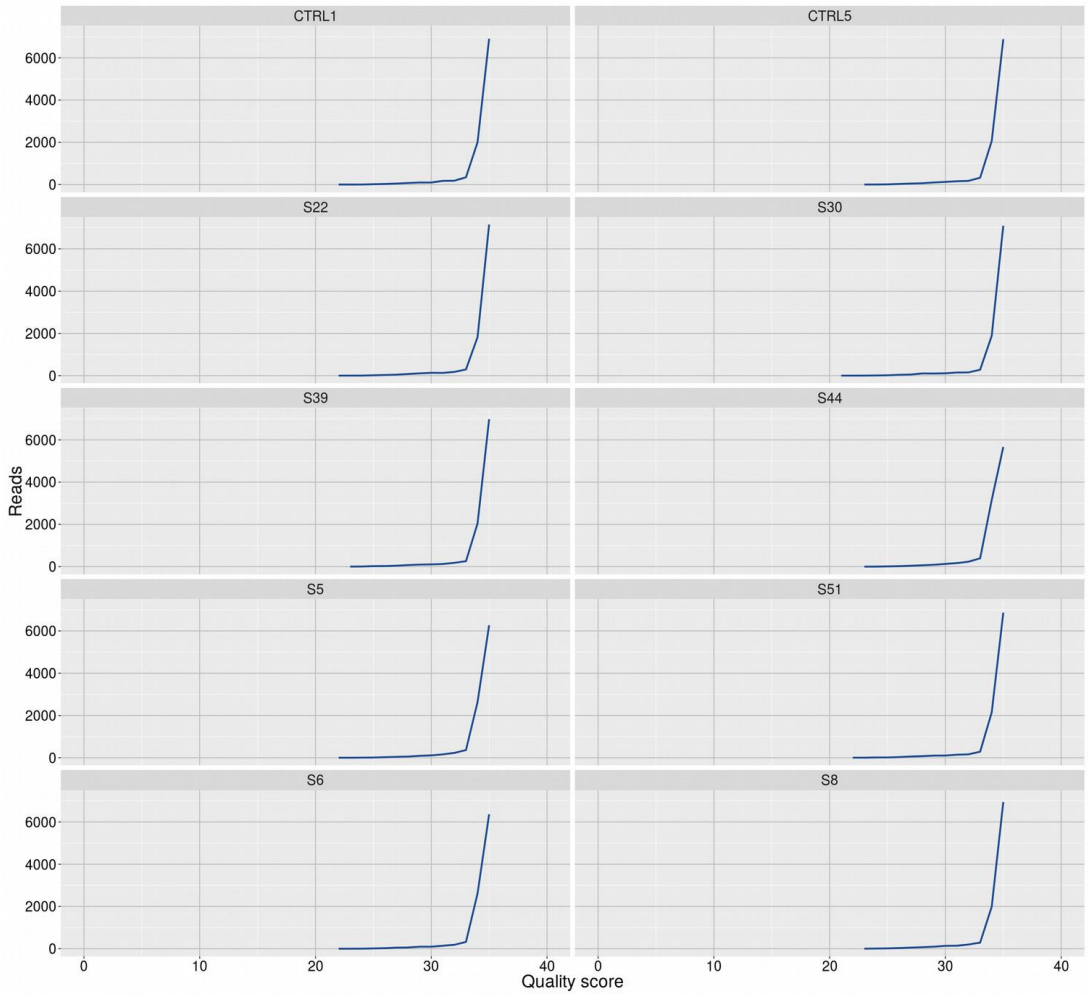


Figure 6-3: Average read quality of the UMI-corrected reads.

The average read Q-score is plotted on the x-axis and the number of reads on the y-axis. A Q-score above 30 is considered high-quality data.

6.3.4.1 Average Base Quality

An overview of the average base quality is shown in
Figure 6-4.

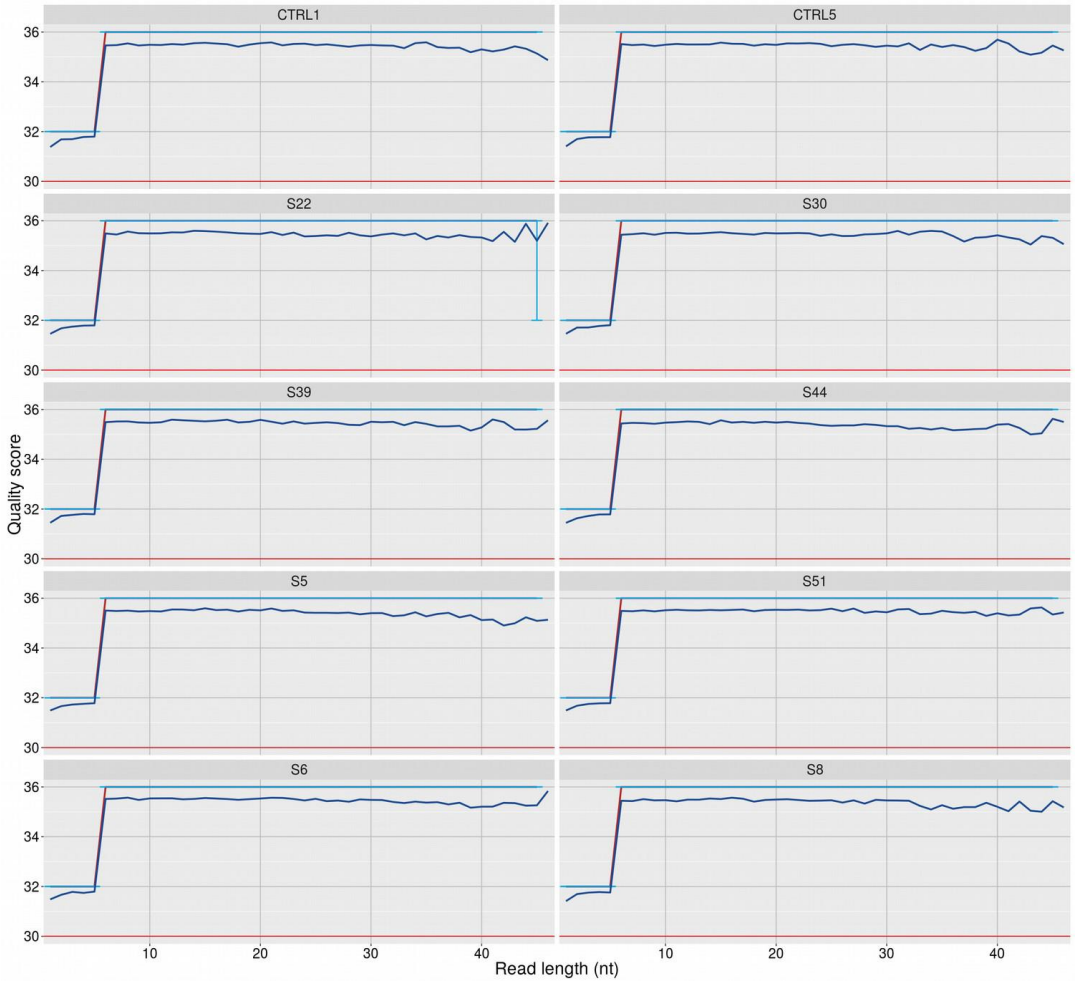


Figure 6-4: Base quality of the UMI-corrected reads.

The position in the read is plotted on the x-axis and the Q-score are plotted on the y-axis. The red line is the median value Q-score. The dark blue line is the mean value Q-score. The boxplot represents the inter-quartile range, while the whiskers represents 10% and 90% points. A Q-score above 30 (>99.9% correct) is considered high-quality data.

Adaptor Trimming

The first step of the analysis was removal of library and sequencing adapters (referred to as trimming).Trimming of adapters creates a distribution of sequences with different lengths. Figure 6-5 represents read length after filtration. Reads representing miRNAs have a length of ~18-22 nt, longer sequences of other origin have a length of ~30-50 nt (i.e. rRNA, tRNA, mRNA, and Y-RNA fragments).

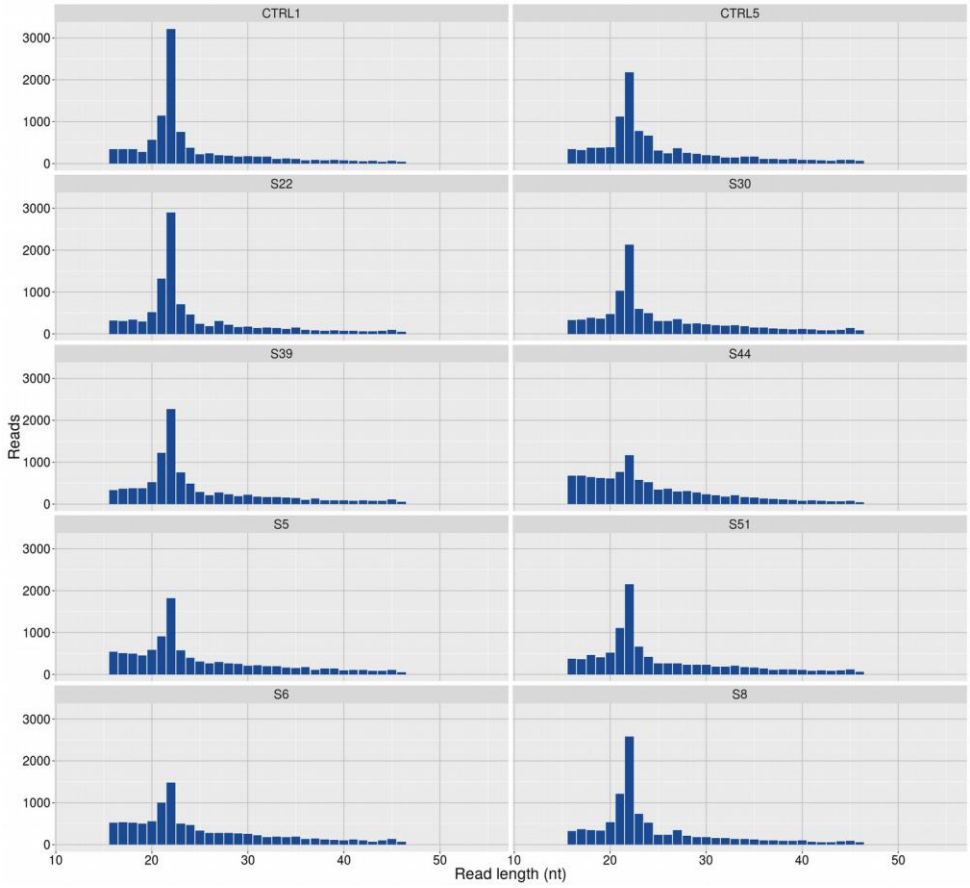


Figure 6-5: Read length distribution after filtering of the adapters.

6.4 Unique Molecular Indices

During the QIAseq miRNA Library Kit construction process, each individual miRNA molecule was tagged with a Unique Molecular Index (UMI). Following sequencing and trimming, reads were analysed for the presence of UMIs. All reads containing identical insert sequence and UMI sequence (insert-UMI pair) were collapsed into a single read. These reads were passed into the analysis pipeline. Additionally, reads containing partially UMI were also passed into the analysis pipeline.

This allowed for accurate quantification of the miRNAs by eliminating library amplification bias. For further information on UMI, see appendix D. Please note this process notably reduced the number of reads analysed, but the remaining reads were a better representation of the RNA molecules in the sample. Especially for samples with very little starting material. E.g. liquid biopsy samples.

Table 6.2: Number of reads after each step of the UMI correction process.

Sample	Raw reads	Reads with adapter	Reads after length filtering	Reads with full or partial UMI	UMI-collapsed reads
S6	13,297,622 (100%)	13,093,666 (98%)	6,590,871 (50%)	6,199,865 (47%)	2,935,844 (22%)
S30	11,492,666 (100%)	11,220,485 (98%)	7,198,626 (63%)	6,857,091 (60%)	2,381,814 (21%)
CTRL1	11,255,862 (100%)	11,067,509 (98%)	7,255,891 (64%)	6,999,266 (62%)	3,293,406 (29%)
S39	11,314,540 (100%)	11,063,931 (98%)	7,055,297 (62%)	6,750,185 (60%)	2,578,862 (23%)
CTRL5	11,351,892 (100%)	11,168,770 (98%)	6,065,933 (53%)	5,828,660 (51%)	2,450,597 (22%)
S51	11,964,486 (100%)	11,715,943 (98%)	6,663,216 (56%)	6,371,599 (53%)	2,379,659 (20%)
S8	11,539,869 (100%)	11,324,338 (98%)	7,435,563 (64%)	7,115,089 (62%)	2,973,791 (26%)
S22	10,711,944 (100%)	10,502,987 (98%)	6,890,766 (64%)	6,640,184 (62%)	2,683,296 (25%)
S44	16,281,057 (100%)	16,091,058 (99%)	8,553,489 (53%)	8,235,129 (51%)	4,484,734 (28%)
S5	12,166,438 (100%)	12,007,122 (99%)	5,070,089 (42%)	4,766,041 (39%)	2,693,734 (22%)
S2	11,618,972 (100%)	11,384,682 (98%)	6,206,202 (53%)	5,961,778 (51%)	2,246,143 (19%)
S59	11,156,939 (100%)	10,879,726 (98%)	6,303,371 (56%)	6,030,842 (54%)	1,681,785 (15%)
S45	11,320,109 (100%)	11,058,530 (98%)	7,571,582 (67%)	7,289,709 (64%)	1,806,691 (16%)
S42	11,000,866 (100%)	10,729,587 (98%)	6,067,518 (55%)	5,771,229 (52%)	2,001,182 (18%)
S43	11,754,436 (100%)	11,437,266 (97%)	7,703,131 (66%)	7,433,196 (63%)	2,292,347 (20%)
S63	11,879,802 (100%)	11,625,349 (98%)	8,764,442 (74%)	8,541,774 (72%)	3,033,811 (26%)
S32	11,979,907 (100%)	11,811,955 (99%)	6,478,717 (54%)	6,216,853 (52%)	2,492,381 (21%)
S40	11,597,230 (100%)	11,305,908 (97%)	7,927,331 (68%)	7,525,346 (65%)	2,063,133 (18%)
CTRL3	10,608,096 (100%)	10,362,630 (98%)	5,878,563 (55%)	5,640,065 (53%)	1,834,053 (17%)
S4	10,791,154 (100%)	10,524,345 (98%)	8,214,169 (76%)	7,886,192 (73%)	2,445,702 (23%)
S36	10,722,379 (100%)	10,498,942 (98%)	6,703,426 (63%)	6,453,667 (60%)	1,878,387 (18%)
S41	11,635,850 (100%)	11,341,850 (97%)	7,887,935 (68%)	7,469,971 (64%)	2,398,198 (21%)
S50	11,867,380 (100%)	11,592,407 (98%)	7,370,556 (62%)	6,973,204 (59%)	2,537,610 (21%)
S49	11,595,221 (100%)	11,316,644 (98%)	7,087,803 (61%)	6,709,280 (58%)	2,270,254 (20%)

Spike-in QC

A range of spike-ins were added to the samples prior to RNA isolation. A good correlation of counts corresponding to the spike-ins between the samples was observed. A visualisation of the spike in reads in the samples can be seen in Figure 6-6.

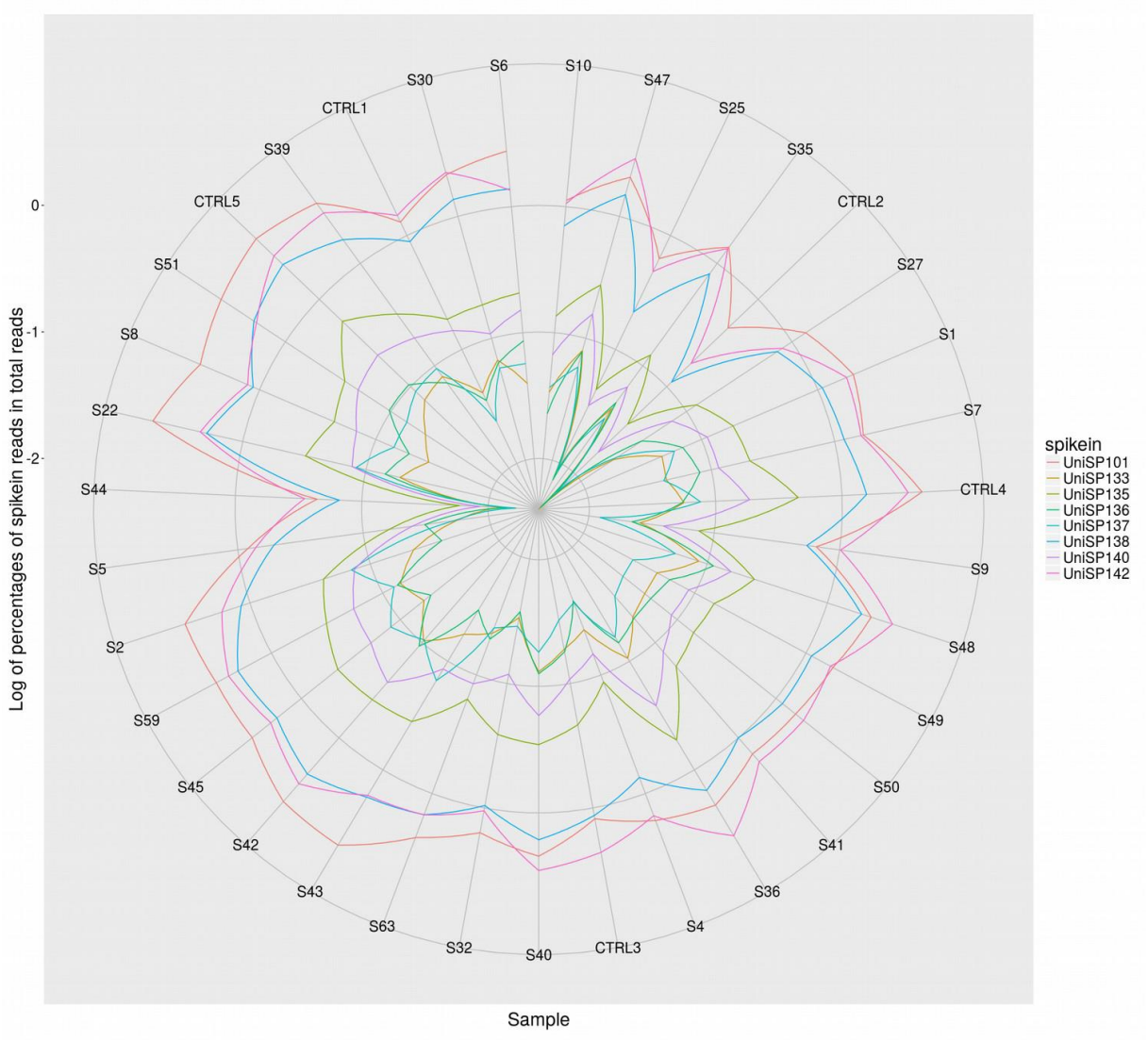


Figure 6-6: Radar plot showing a relative spike-in signal for the samples.

6.4.1 Mapping and yields

Mapping of the sequencing data was a useful quality control step in NGS data analysis as it can help to evaluate the quality of the samples. Reads were classified into the following classes:

- **Outmapped:** For example polyA and poly C homopolymers as well as abundant rRNA and mtRNA sequences
- **Unmapped reads:** no alignment to reference genome possible
- **Genome**: aligning to reference genome, but not for small RNA or miRNA
- **miRNA:** Maps to a used version of mirBase
- **SmallRNA:** Maps to smallRNA database (compiled by QIAGEN Genomic Services)
- **Predicted (pred):** There are two different kinds of predicted miRNA. Either the sequence is found in mirBase in another organism or the sequence is predicted to be miRNA.

A typical biofluids miRNA sequencing experiment yields approximately 60-90% of the reads mapping to the reference genome. 5-50% of the mappable reads are typically annotated to miRNAs. However, this is dependent on the quality of the sample and how well the reference genome is characterised, as well as the miRNA annotation in miRBase. If the sample is degraded fewer reads will be miRNA specific and more material will be degraded Y-RNA, rRNA and tRNA fragments. The following plot summarises the overall mapping results.

Figure 6-7

Sample	UMI-corrected reads	miRNA/small RNA	Genome	Outmapped	Unmapped
'CTRL1'	3,293,406	40.0%	12.2%	2.2%	39.1%
'CTRL2'	5,237,439	64.1%	8.5%	2.5%	21.8%
'CTRL3'	1,834,053	15.4%	17.4%	2.8%	57.7%
'CTRL4'	2,378,010	24.5%	15.1%	2.1%	49.4%
'CTRL5'	2,450,597	26.0%	14.4%	2.2%	47.5%
'S1'	2,393,723	25.1%	14.9%	2.3%	50.9%
'S2'	2,246,143	34.3%	13.6%	2.3%	42.1%
'S4'	2,445,702	45.4%	10.4%	1.7%	36.6%
'S5'	2,693,734	19.2%	18.3%	3.0%	54.5%
'S6'	2,935,844	12.1%	18.9%	2.9%	58.3%
'S7'	2,197,033	32.5%	12.2%	2.2%	46.2%
'S8'	2,973,791	33.8%	14.3%	2.0%	42.4%
'S9'	2,462,100	44.0%	11.4%	2.0%	37.9%
'S10'	1,975,341	20.8%	15.8%	2.2%	55.6%
'S22'	2,683,296	37.6%	13.1%	1.9%	38.7%
'S25'	3,627,625	59.1%	9.7%	1.7%	25.8%
'S27'	2,669,605	31.4%	12.1%	2.1%	48.8%
'S30'	2,381,814	21.8%	14.7%	2.1%	53.9%
'S32'	2,492,381	20.5%	15.5%	3.1%	54.7%
'S35'	2,504,924	24.9%	13.6%	2.1%	53.5%
'S36'	1,878,387	20.5%	14.9%	2.5%	53.6%
'S39'	2,578,862	26.8%	14.2%	2.2%	48.1%
'S40'	2,063,133	26.3%	12.8%	2.0%	51.0%
'S41'	2,398,198	21.1%	14.1%	2.3%	55.8%
'S42'	2,001,182	17.3%	14.9%	2.1%	56.0%
'S43'	2,292,347	35.0%	12.0%	1.9%	42.3%
'S44'	4,484,734	8.6%	22.0%	1.0%	65.5%
'S45'	1,806,691	32.5%	12.6%	1.9%	44.6%
'S47'	1,408,950	20.4%	14.0%	2.1%	55.6%
'S48'	1,826,576	22.8%	13.6%	2.0%	53.3%
'S49'	2,270,254	13.8%	15.9%	2.1%	61.7%
'S50'	2,537,610	18.6%	14.6%	2.3%	58.0%
'S51'	2,379,659	21.1%	15.2%	2.3%	52.7%
'S59'	1,681,785	21.9%	14.4%	1.9%	53.2%
'S63'	3,033,811	54.7%	7.8%	1.3%	29.2%

Table 6.3 Summary of the mapping results for each sample

The following Figure 6-7 summarises the mapping results for each sample. Each sample consists of reads that can be classified into the following categories: miRNA, small RNA, genome-mapped, out mapped or high abundance (e.g. rRNA, polyA, poly C, mtRNA) and unmapped (reads which did not align to the reference genome).The BAM files can be viewed and inspected in any standard genome viewer such as the IGV Browser (Robinson et al. 2011) and (Thorvaldsdóttir et al. 2013) downloadable from Broad Institute IGV website.

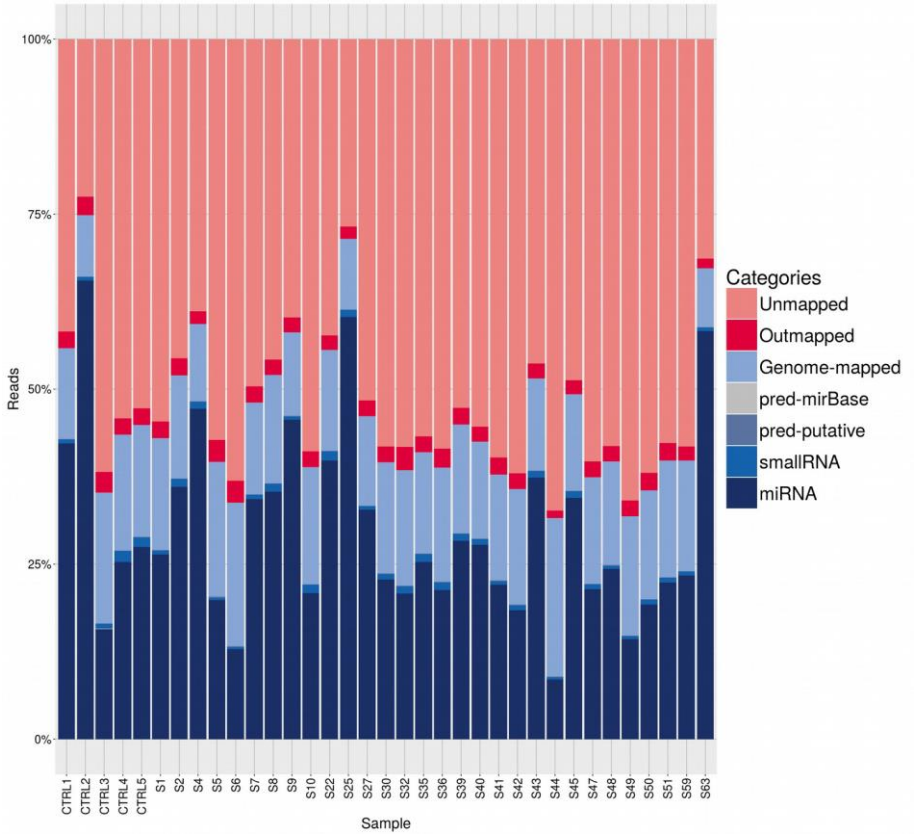


Figure 6-7: Summary of mapping results of the reads by sample.

6.5 Results

Below a summary of the principal findings for this project can be found.

Number of reads

On average 2.5 million UMI-corrected reads were obtained per sample. Figure 6-8 represents the UMI-corrected number of reads per sample. On average 2.5 million UMI-corrected reads were

obtained from each sample.

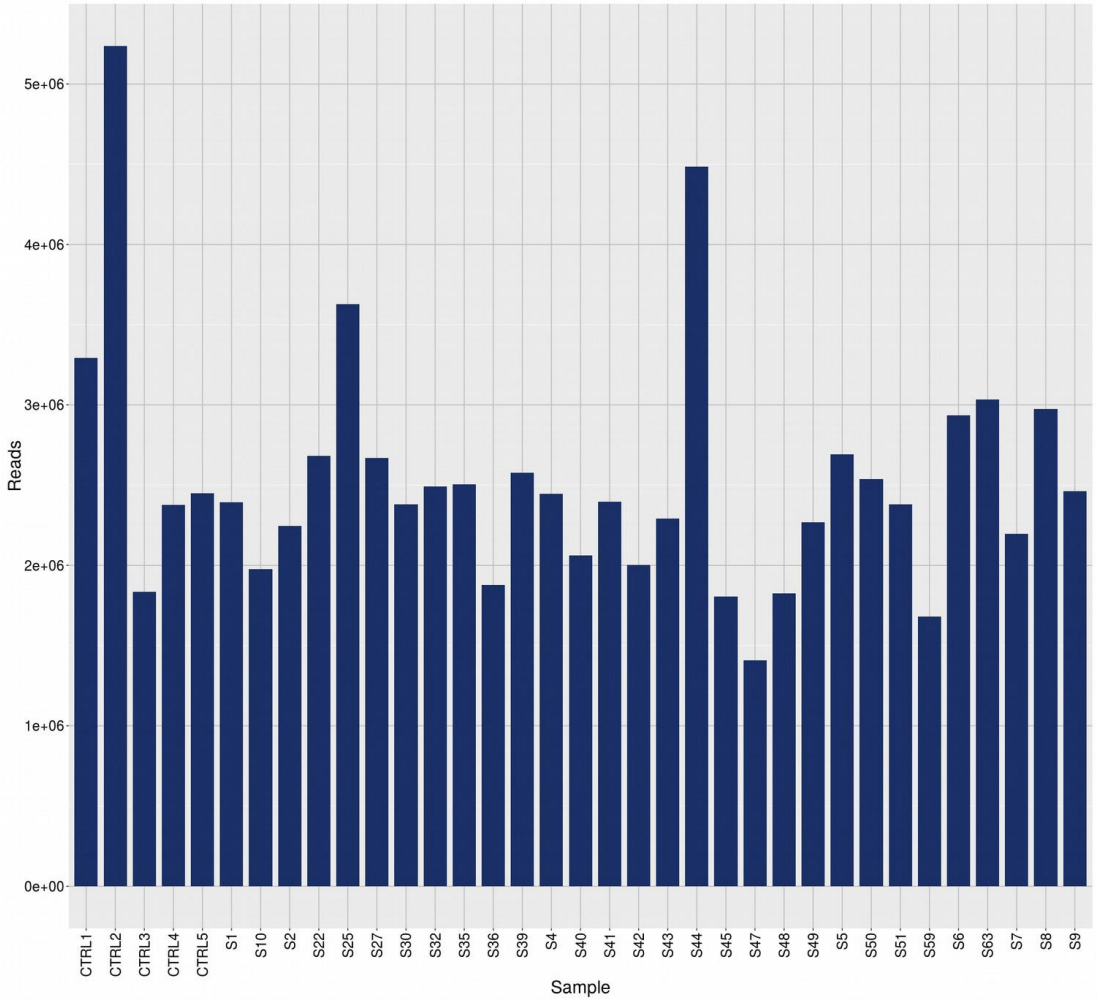


Figure 6-8: The total number of UMI-corrected reads for each sample sequenced in this study.

6.5.1 Read types of length distribution

After mapping of the data, each read was assigned to a class of RNA and the length distribution of the reads was presented in Figure 6-9 below with RNA from each class separately. The samples were expected to have a peak for miRNA around 18-23 nt. A peak around 30 nt. was also

commonly seen. This peak was likely to contain degraded RNA molecules (i.e. rRNA, tRNA and Y-RNA fragments).

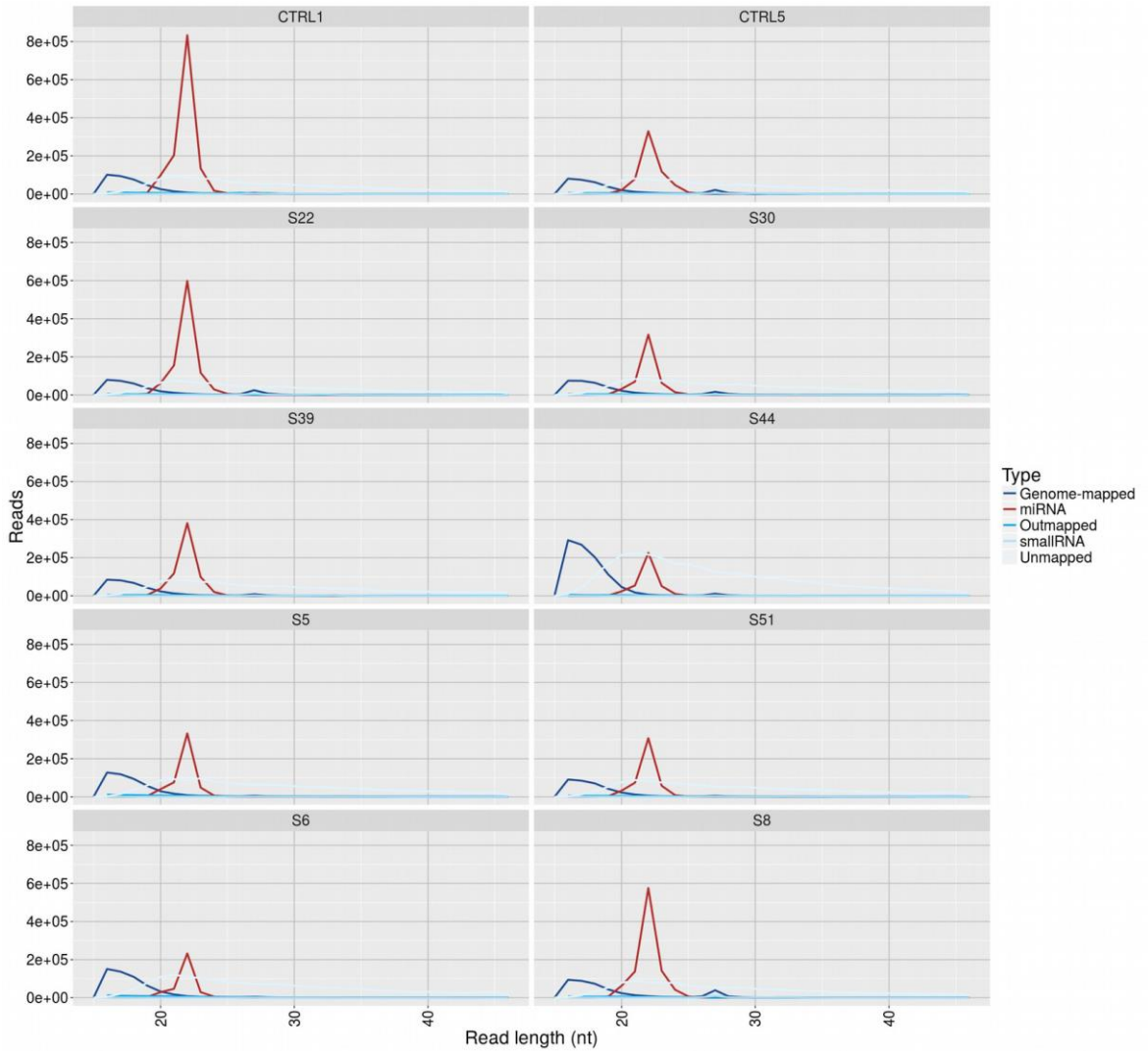


Figure 6-9 : Read length distribution for each class of RNAs.

6.5.2 Identified miRNAs (miRNA call rate)

Once mapping the data and counting to relevant entries in mirbase_20, the numbers of known miRNAs were calculated. The reliability of the identified miRNAs increases with the number of

identified fragments. When performing the statistical comparison of two groups, we include all miRNAs irrespective of how few calls have been made.

Expression levels were measured as Tags Per Million mapped reads (TPM). TPM is a unit used to measure expression in NGS experiments. The number of reads for a particular miRNA was divided by the total number of mapped reads and multiplied by 1 million (Tags per Million). See Figure 6-10 (>1TPM per sample in blue bars and >10 TPM per sample in navy blue bars).

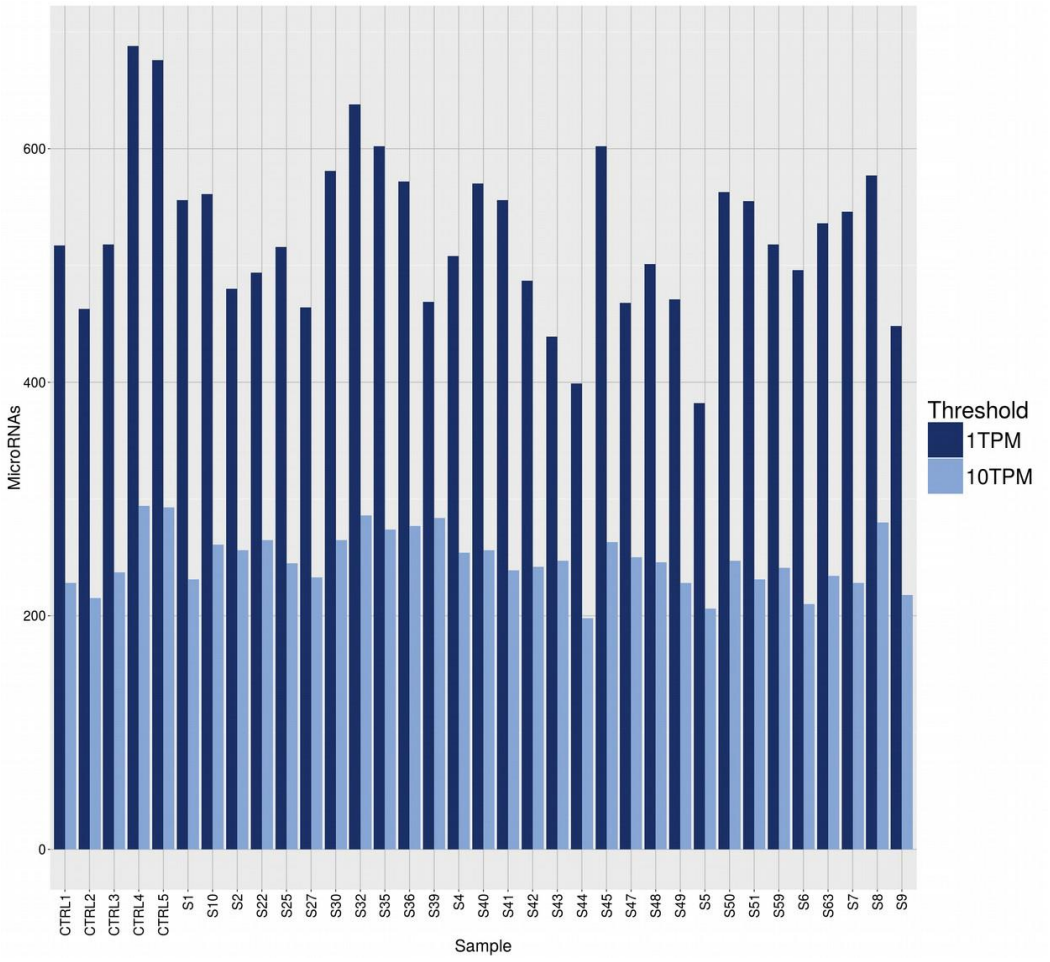


Figure 6-10: Number of identified known miRNAs with the number of counts.

Table 6.4: Summary of identified miRNAs across all samples accepted for the analysis.

miRNAs were identified according to entries in mirbase_20.

TPM Threshold	**Call rate**
Number of identified microRNAs in all samples (≥1 TPM)	252
Number of identified microRNAs in all samples (≥10 TPM)	166

6.5.3 Statistical Analysis

Data were expressed as means ± SE. Group comparisons were performed with independent Mann–Whitney U test or the Wilcoxon matched-pairs signed-rank test. Hierarchical clustering was performed to calculate principal component analysis and to generate heatmaps with the freely available online R-based tool ClustVis1 (Metsalu and Vilo, 2015). The differential expression analysis was done using TMM in the EdgeR statistical software package (Bioconductor, http://www.bioconductor.org/). Differential expression analysis on TMMs investigates the relative change in expression (i.e. counts) between different samples, and with TMM normalisation, the statistical tests will be less skewed, and the false-positive rate was reduced. Note that for each differential expression comparison, TMM was calculated, based on the subset of features and subset of samples analysed.

6.5.4 Principal Component Analysis P- EC and Control

Principal Component Analysis (PCA) is a method used to reduce the dimension of large data sets and thereby a useful way to explore the naturally arising sample clusters based on the expression profile. MicroRNA expression analysis indicated 50 out of the 252 detectable miRs were expressed in the samples. Here, by including the top 50 miRNAs that had the largest variation across all samples, an overview of how the samples cluster based on this variance is obtained. The data were normalised with the weighted trimmed mean of M-values (TMM) method (Robinson and Oshlack 2010). Then all features were filtered on “expressed in all samples” criteria and the 50

features with the highest coefficient of variation (%CV) selected for the downstream analysis as it shows in Figure 6-11. If the biological differences between the samples are pronounced, this will be a primary component of the variation. This leads to separation of samples in different regions of a PCA plot corresponding to their biology. If other factors, e.g. sample quality, inflict more variation on the samples, the samples will not cluster according to the biology. The largest component in the variation is plotted along the X-axis and the second largest is plotted on the Y-axis.

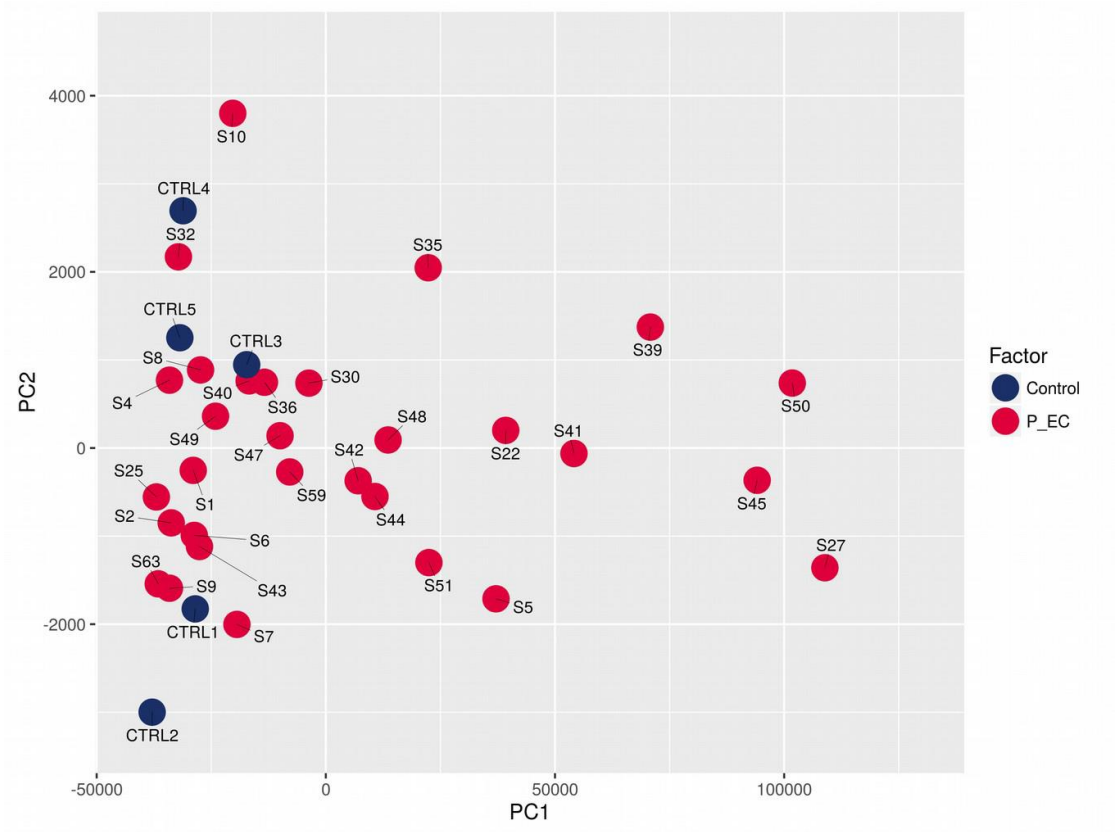


Figure 6-11: Principal component analysis (PCA) plot for P-EC and Control.

6.5.5 Heat map and unsupervised clustering P-EC and Control

The heat map diagram in Figure 6-12 shows the result of the two-way hierarchical clustering of genes and samples. It includes the 50 miRNAs expressed in the samples.

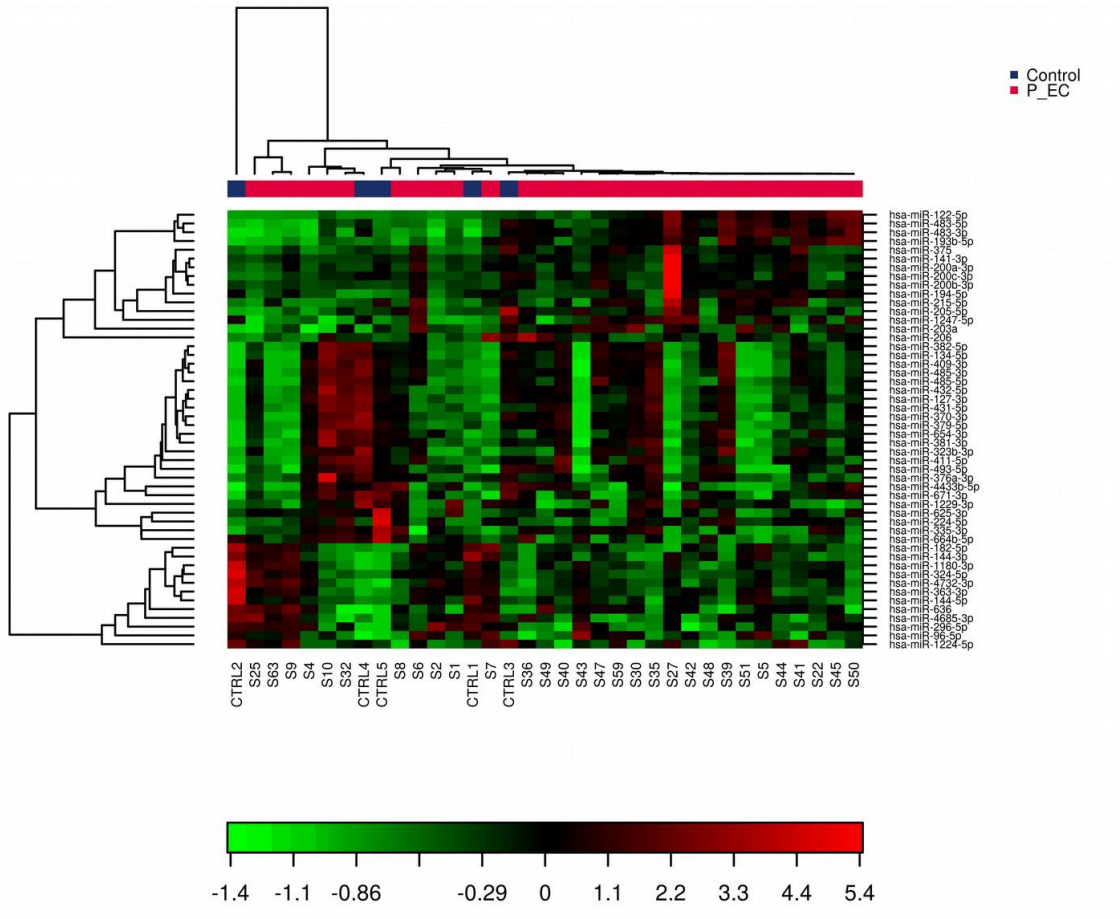


Figure 6-12: Heat map and unsupervised hierarchical clustering by sample and genes.

6.5.6 Principal Component Analysis for P-EC group and Control

There was no separation or discrimination pattern between the samples and control groups according to the year of delivery. Each circle in Figure 6-13 represents a sample.

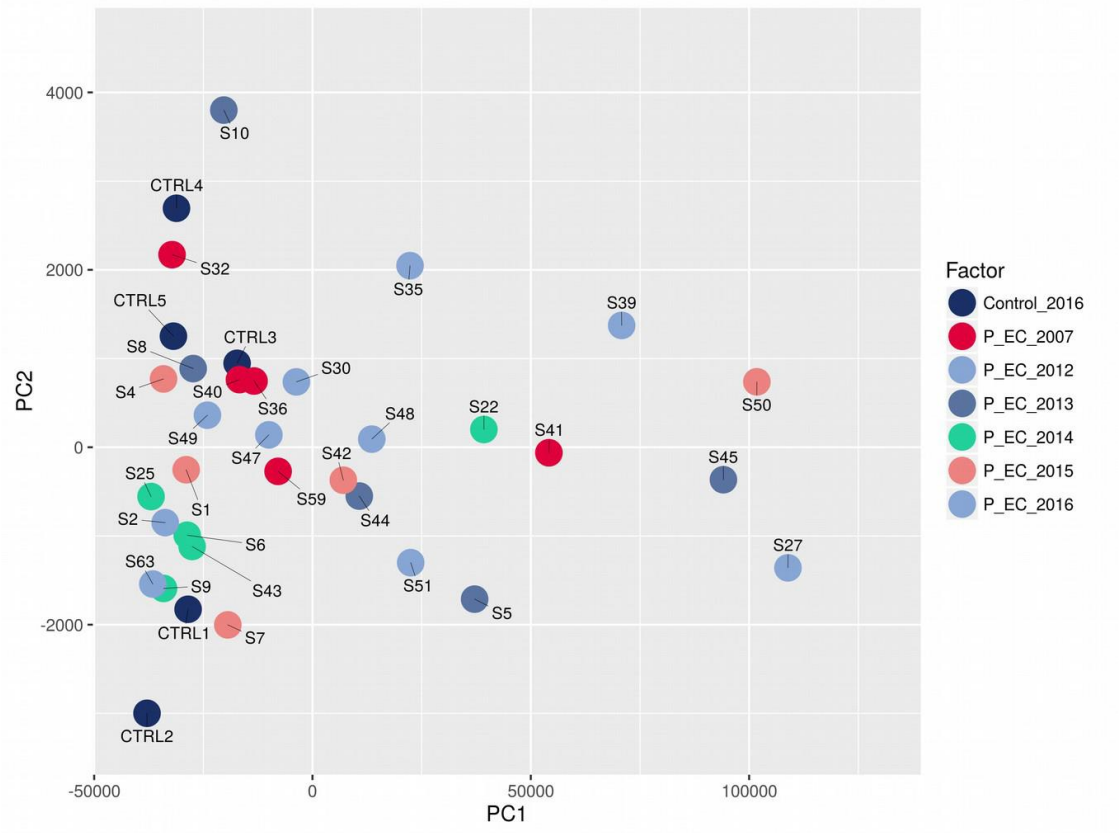


Figure 6-13: Principal component analysis (PCA) plot for each pre-eclamptic grouped time points and the control group 2016.

6.5.7 Heat map and unsupervised clustering P-EC (2007, 2012-2016) and control

A heat map was employed to demonstrate the difference in the miRs expression between pre-eclamptic groups and healthy control. The colour scale is shown below in Figure 6-14 : red represents an expression level above the mean; green represents an expression level below the mean. Data is based on samples from the P-EC 2007, P-EC 2012- 2016 groups and Control 2016.

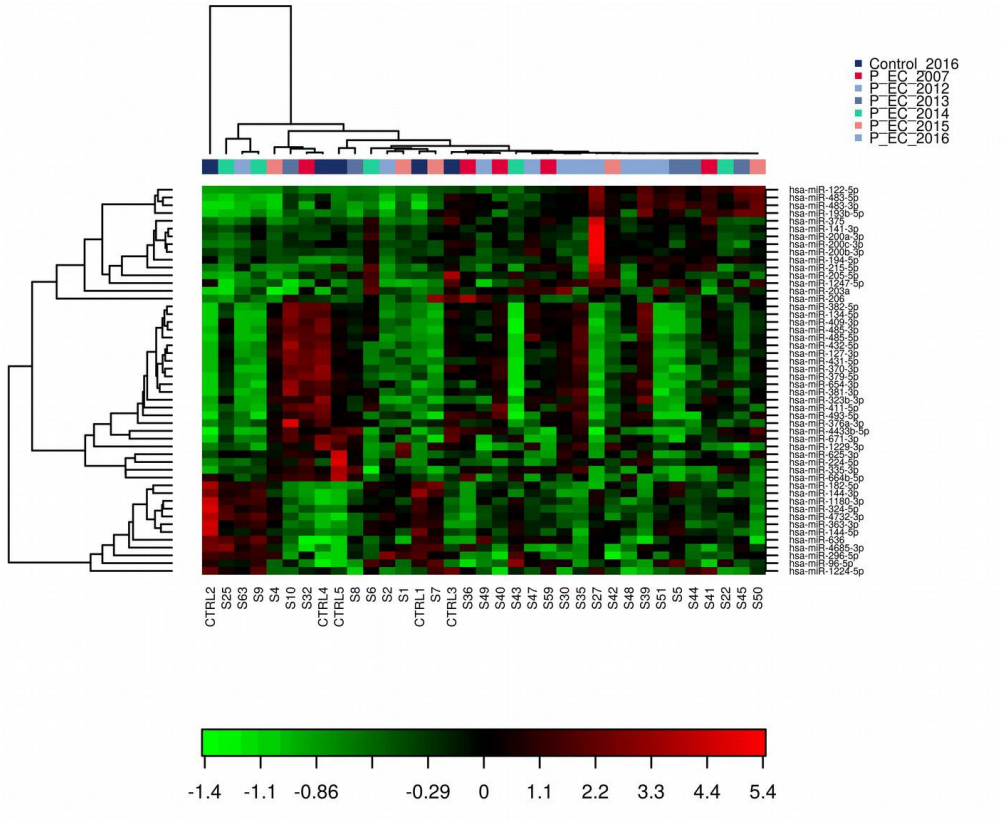


Figure 6-14: Heat map and unsupervised hierarchical clustering by sample and genes.

6.5.8 Predicted miRNAs from other organisms

We were not able to identify any sequences which are identical to those of known miRNAs in miRBase for other organisms such as mouse, rat, fly, worm, Arabidopsis.

6.5.8.1 Predicted miRNAs from prediction software

Table 6.5 below lists the top 25 predicted miRNAs sorted by UMI-corrected but non-normalised counts. The sequences reported in this table do not match any known miRNAs in miRBase, however, the structural properties of the genome in the indicated locations resemble that of known miRNAs. The miRNAs in this table are predicted based on reading count distribution and secondary structure prediction according to miRPara classification score. The location is

formatted as “chromosome: start-stop (strand)”. Count: describes the number of reads which fall onto the location of the predicted miRNA (sum for all samples).

Table 6.5: The top 25 predicted microRNAs sorted by non-normalised read counts.

Name	Location	Sequence(5’-3’)	Count
put-miR-111	16:33963845-33963868(+)	GTTGGTGGAGTGATTTGTCTGGTT	18,46
put-miR-87	5:140090932-140090959(+)	GACCCGCGGGCGCTCTCCAGTC	2,667
put-miR-70	2:6110380-6110400(+)	GCTTTGAGGACTGAAGTGGGA	2,512
put-miR-26	10:75501886-75501903(+)	CTGATAGGTCAGGTGTGG	2,377
put-miR-62	6:28574986-28575009(+)	TCAATCCCCGGCACCTCCACCA GT	1,822
put-miR-27	5:114634706-114634727(+)	TTCTGACACAGGGAGGTAGTGG	1,426
put-miR-64	6:33167378-33167398(+)	GGCTGGTCCGATGGTAGTGGG	1,327
put-miR-25	7:2102745-2102765(+)	TACACTGGGACTGAGACACGG	1,318
put-miR-123	19:9986912-9986927(+)	CGGGGATGGATTTCTG	1,185
put-miR-6	17:61706876-61706900(+)	AGATAACTGGCTTGTGGCAGCCA	1,118
put-miR-44	14:69941895-69941913(+)	TTCCCTTAGATGTTCTGGG	1,017
put-miR-8	16:78000717-78000733(+)	TTTCAAAGGCGCACTTG	819
put-miR-98	3:187427199-187427217(+)	CTCTGGCGGGCATGCCTGT	787
put-miR-86	3:172269744-172269761(+)	GCCTGTTTGAGGAATGGT	762
put-miR-113	7:18159276-18159293(+)	AAATCTGACTGTCTAATT	698
put-miR-119	19:43910379-43910404(+)	CTGAATGTCAAAGTGAAGGAATT CAA	648
put-miR-60	12:67715305-67715328(+)	TCTCTCAGTGCAGATCTTGGTGTT	641
put-miR-83	1:19256018-19256036(+)	GGGGATGAGCTGTGTGGGG	632
put-miR-90	16:61900996-61901013(-)	TCCTCAGTAGCTCAGTGG	622
put-miR-77	1:22441120-22441140(+)	CGGGGGATTAGCTCAGCTGGG	609
put-miR-100	X:84170147-84170164(+)	AGTAGAGCACTGTTTAGG	580
put-miR-46	8:19968913-19968931(+)	ATGTGGGGAGTTTGGCTGA	542
put-miR-65	8:86245822-86245840(+)	TTGCAGAATTCCAGTGAGC	536
put-miR-57	18:60645630-60645647(+)	AAGGGGTTGTGGGACAGC	523
put-miR-108	7:68527632-68527652(+)	GAAGACCCTGTTGAGTTTGAC	446

6.6 Identification of IsomiRs

IsomiR analysis was performed individually for each sample based on the occurrence of count variants for each detected miRNA. Reads were mapped to known miRNAs according to the annotation in miRBase and then investigated for the presence of different isomiRs. These variants

were identified by changes in start or stop position of occurrence of mutations within the read. The results for each sample were then merged to generate a single count file with a consistent nomenclature across the samples. Only isomiRs that were present at a level of 5% of the total reads for that miRNA were retained (Wu et al. 2011). See Figure 6-15 represents an example of differences in isomiRs in an NGS experiment.

No differential expression analysis was performed on the isomiR's, but the count file can give an impression of differences in abundance. Because of the large amount of information generated in an isomiR analysis.

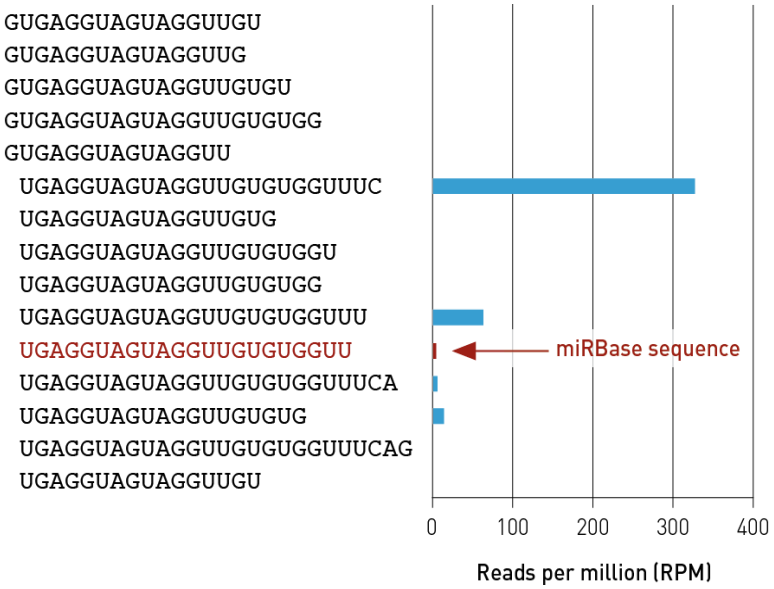


Figure 6-15: Variation in has-let-7b-5p (isomiRs).

6.7 **Normalisation**

Like TPM normalisation, TMM normalisation compensates for sample-specific effects caused by the variation in library size/sequencing depth between samples. In contrast to TPM, the TMM normalisation step also compensates potential under- and over-sampling effects by trimming and applying scaling factors that minimise log-fold changes between samples across the majority of the miRNAs (Benjamini and Hochberg 1995). If only applying TPM normalisation, differential gene expression of the samples' miRNA population will result in libraries with biases e.g. when a few highly expressed miRNAs dominate the read set in one sample type and not in the other. (In an experiment with a fixed number of sequenced reads, e.g. 10 million reads per sample, fewer

reads will “remain” to cover RNA species with constant expression levels if one or a few specific RNA species increase significantly from one biological condition to another. If uncorrected, such effects could lead to skewed analyses and apparent downregulation of RNA species which are, in fact, constantly expressed. TMM normalisation attempts to reduce the sample-to-sample effects caused by significant differences in expression levels of a subset of RNA species

The differential expression analysis is done using TMM in the Edge R statistical software package (Bioconductor, http://www.bioconductor.org/). Differential expression analysis on TMMs investigates the relative change in expression (i.e. counts) between different samples and, with TMM normalisation the statistical tests will be less skewed and the false-positive rate was reduced. Note that for each differential expression comparison, TMM was calculated, based on the subset of features and subset of samples analysed.

6.8 Differentially expressed miRNAs

The differential expression analysis step attempts to distinguish biological variation from technical variation within the experiment, assuming that this varies amongst miRNAs. P-values for significantly differentially expressed miRNAs were estimated by an exact test assuming a negative binomial distribution. The analysis carried out between pre-eclampsia in each interval time point post-delivery individually against the control group, then the pre-eclampsia group irrespective the year of delivery with the control group. There was not any miRNA significantly expressed in the study group at the delivery year from 2007-2015 vs the control individually. Interestingly, there were three miRNAs when the pre-eclamptic group in 2016 compared to the control group in 2016.

6.8.1 Comparison of P-EC in 2016 and Control 2016

Table 6.6 shows the results for the 25 most differentially expressed known microRNAs and annotation, with log fold change (log FC) between groups “P-EC 2016” and “Control 2016” raw p-values, Benjamini-Hochberg FDR corrected p-values as well as the average TMM values per group.

In total we identified 3 miRNAs that were significantly differentially expressed at a significance level of 0.05 (FDR), namely miR-885-3p, miR-122-5p and miR-885-5p (increased expression in P-EC group), hsa-miR-122 was the most abundant in pre-eclampsia samples. The plasma hsa-miR-122 level was significantly higher in the P-EC group than in the healthy group (fold change 2.51; p-

value= 0.0001; FDR=0.0126). Additionally, Figure 6-16 presents a volcano plot generated with the ten miRNAs with the lowest p-values were marked with names on the plot.

Table 6.6: miRNAs: Table of the 25 most significantly differentially expressed miRNA between groups.

Name	LogFC	P_EC_2016TMM	Control_2016 TMM	P-value	FDR
hsa-miR-885-3p	2.87	68	9	<0.0001	0.0126
hsa-miR-122-5p	2.51	75880	13309	0.0001	0.0126
hsa-miR-885-5p	3.56	50	5	0.0001	0.0126
hsa-miR-34a-5p	1.53	65	22	0.0044	0.3621
hsa-miR-664b-5p	-1.74	6	20	0.0050	0.3621
hsa-miR-6734-5p	-1.99	4	18	0.0064	0.3886
hsa-miR-141-3p	2.10	378	88	0.0138	0.5896
hsa-miR-454-3p	-1.40	238	630	0.0149	0.5896
hsa-miR-200a-3p	1.70	94	29	0.0185	0.5896
hsa-miR-3928-3p	-1.70	1	7	0.0191	0.5896
hsa-miR-301a-3p	-1.04	39	80	0.0200	0.5896
hsa-miR-365a-3p	1.35	16	6	0.0204	0.5896
hsa-miR-203a	1.30	79	33	0.0210	0.5896
hsa-let-7b-5p	-1.25	36950	88030	0.0247	0.6443
hsa-miR-29a-3p	0.90	1129	605	0.0349	0.6685
hsa-miR-642a-5p	1.70	10	3	0.0359	0.6685
hsa-miR-99a-5p	0.98	143	73	0.0369	0.6685
hsa-miR-193a-5p	0.98	330	167	0.0381	0.6685
hsa-miR-1226-3p	-1.14	8	18	0.0402	0.6685
hsa-miR-103a-3p	-0.91	4771	8944	0.0420	0.6685
hsa-miR-106a-5p	-1.33	6	15	0.0421	0.6685
hsa-miR-194-5p	1.23	722	307	0.0435	0.6685
hsa-miR-200b-3p	1.88	55	15	0.0457	0.6685
hsa-miR-483-3p	1.23	315	135	0.0462	0.6685
hsa-miR-503-5p	-1.14	22	49	0.0479	0.6685

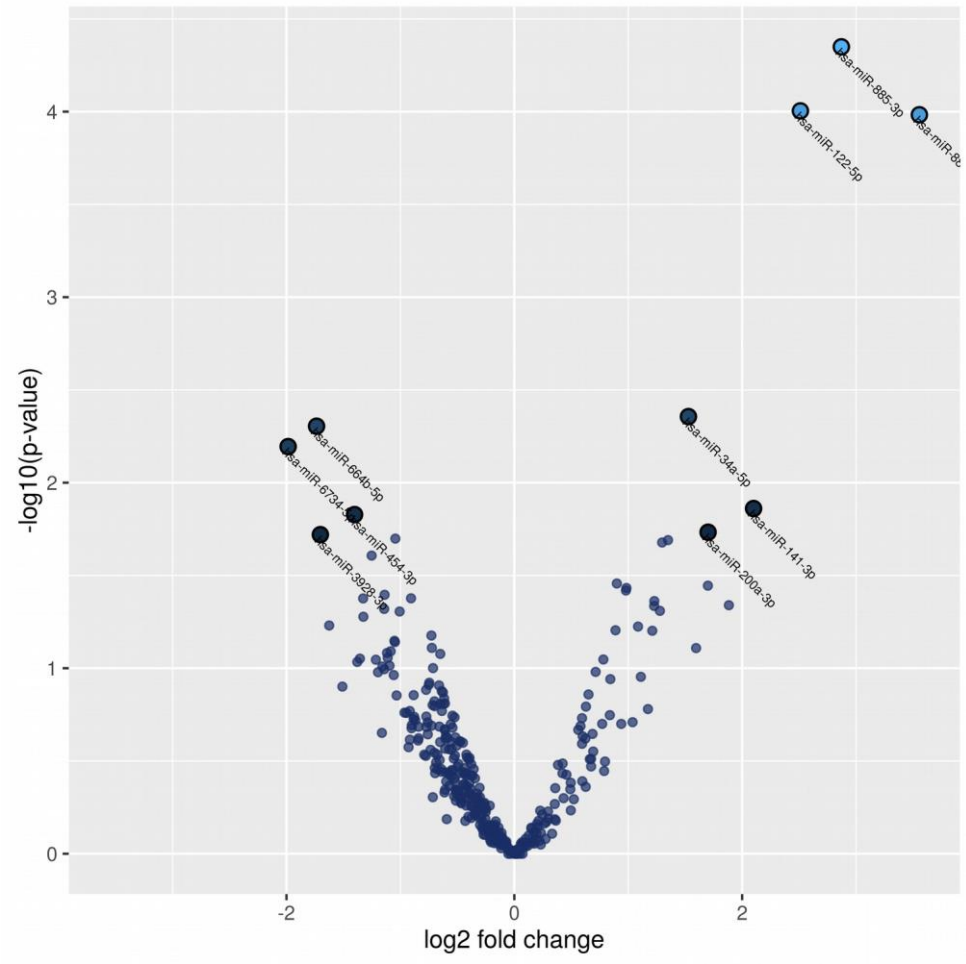


Figure 6-16: Volcano plot showing the relationship between the experimental groups "P-EC in 2016" and "Control 2016".

6.8.2 Comparison of P-EC and Control

Table 6.7 below illustrates the results for the 25 most differentially expressed known microRNAs in the entire pre-eclamptic women irrespective the year of delivery (n=30) vs the control group (n=5). In total, we identified 14 miRNAs that were significantly differentially expressed at a significance level of 0.05 (FDR). Furthermore, Figure 6-17 presents a volcano plot provided the ten miRNAs significantly downregulated with the lowest p- values are marked with names on the plot.

Table 6.7: The 25 most significantly differentially expressed miRNA between P-EC and control groups.

Name	LogFC	P-EC TMM	Control TMM	P-value	FDR
hsa-miR-103a-3p	-0.99	4683	9293	<0.0001	0.0001
hsa-miR-190a-5p	-1.21	89	205	<0.0001	0.0052
hsa-let-7f-5p	-0.67	12923	20571	0.0001	0.0052
hsa-miR-6734-5p	-1.52	6	19	0.0001	0.0052
hsa-miR-454-3p	-1.22	290	673	0.0001	0.0052
hsa-let-7b-5p	-1.07	45062	94537	0.0003	0.0177
hsa-miR-301a-3p	-0.78	47	81	0.0004	0.0241
hsa-miR-26a-5p	-0.47	7801	10783	0.0005	0.0241
hsa-miR-4467	-2.41	0	5	0.0006	0.0241
hsa-miR-144-5p	-1.27	126	302	0.0006	0.0241
hsa-miR-374a-5p	-0.85	154	279	0.0006	0.0242
hsa-miR-652-3p	-0.88	58	106	0.0012	0.0421
hsa-miR-191-5p	-0.61	6920	10567	0.0014	0.0458
hsa-miR-5010-3p	-1.36	5	15	0.0016	0.0487
hsa-miR-324-5p	-1.13	139	303	0.0019	0.0542
hsa-miR-664b-5p	-1.00	9	19	0.0037	0.0957
hsa-miR-589-5p	-0.79	21	37	0.0047	0.1140
hsa-miR-199b-5p	-0.91	16	30	0.0050	0.1140
hsa-miR-106b-3p	-0.90	425	793	0.0052	0.1140
hsa-miR-122-5p	1.86	46330	12758	0.0056	0.1160
hsa-miR-424-3p	-1.51	2	8	0.0061	0.1212
hsa-let-7c-5p	-0.55	1016	1491	0.0082	0.1503
hsa-let-7d-5p	-0.69	1733	2787	0.0083	0.1503
hsa-miR-331-3p	-0.76	17	29	0.0089	0.1519
hsa-miR-885-5p	2.35	21	4	0.0091	0.1519

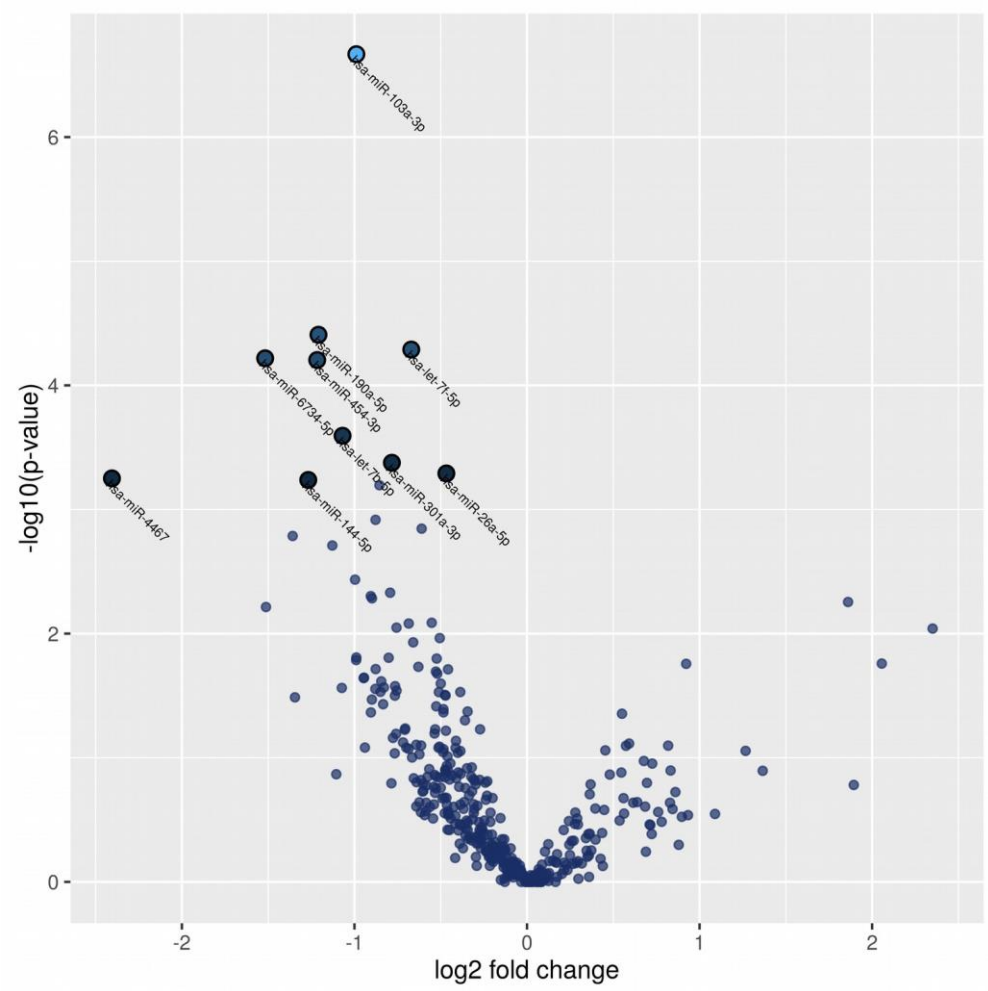


Figure 6-17: Volcano plot showing the relationship between the entire P-EC groups and Control.

6.9 NormFinder analysis

If miRNAs were found differentially expressed in the NGS analysis, these findings should be validated, e.g. by a qPCR study. Table 6.8 below shows which miRNAs were most stably expressed across all the samples and may thus be good candidates for normalisers/house-keeping genes in the qPCR validation study. Note that miRNAs with lower values of (Stability/TPM_AVG) ratio indicates good stability across all samples and could be potential normalisers for a qPCR validation study. Stability is the stability measure as calculated by Normfinder. Note that a low stability value indicates good stability. The TPM is an estimate of the abundance of the miRNA across the two groups.

Table 6.8: The 25 most stably expressed miRs across the sample set.

Name	Stability/TPM_AVG	Stability	TPM average
hsa-miR-142-5p	0.01	16.12	2029.0
hsa-let-7g-5p	0.04	82.48	2243.0
hsa-let-7d-5p	0.1	109.04	1084.0
hsa-miR-101-3p	0.1	198.78	1938.0
hsa-miR-185-5p	0.12	187.61	1615.0
hsa-miR-30e-5p	0.14	424.77	3118.0
hsa-miR-484	0.16	134.13	816.0
hsa-miR-320a	0.17	328.43	1915.0
hsa-miR-93-5p	0.2	960.94	4806.0
hsa-let-7i-5p	0.21	1991.77	9427.0
hsa-miR-26b-5p	0.21	1081.13	5035.0
hsa-let-7a-5p	0.22	4958.82	22966.0
hsa-miR-423-5p	0.23	1991.31	8657.0
hsa-miR-182-5p	0.24	185.51	772.0
hsa-miR-25-3p	0.24	1181.93	4898.0
hsa-miR-20a-5p	0.28	160.22	564.0
hsa-miR-425-5p	0.29	647.51	2223.0
hsa-miR-92a-3p	0.31	8766.5	28415.0
hsa-miR-16-5p	0.32	75511.03	234689.0
hsa-miR-21-5p	0.32	1323.5	4080.0
hsa-miR-148a-3p	0.34	696.95	2061.0
hsa-miR-103a-3p	0.36	1170.26	3232.0
hsa-let-7f-5p	0.39	3418.47	8694.0
hsa-miR-26a-5p	0.4	2101.27	5202.0
hsa-let-7b-5p	0.42	12209.8	29392.0

6.10 Gene Ontology Enrichment Analysis

Gene Ontology (GO) enrichment analysis attempts to identify biological processes that were significantly associated with differentially expressed miRNAs. Using miRSearch, we mapped the differentially expressed miRNAs identified above to their target genes and it was then possible to investigate whether specific GO terms were more likely to be associated with these miRNAs. The

GO ontology enrichment analysis expressed between the P-EC and control groups were provided in Appendix F.

Table 6.9: GO terms for the gene targets of miRNAs differentially expressed between "P-EC in 2016" and "Control 2016" and their corresponding annotation for Biological process.

GO ID	Term	P-value
GO:0034612	response to tumor necrosis factor	0.001
GO:2000036	Regulation of stem cell population maintenance	0.0027
GO:0050775	Positive regulation of dendrite morphogenesis	0.0029
GO:0031328	Positive regulation of the cellular biosynthetic process	0.0036
GO:0045935	Positive regulation of the nucleobase-containing compound metabolic process	0.0045
GO:0048332	Mesoderm morphogenesis	0.0047
GO:0007184	SMAD protein import into nucleus	0.005
GO:0060177	Regulation of angiotensin metabolic process	0.0065
GO:0045656	Negative regulation of monocyte differentiation	0.0067
GO:0090094	Metanephriccap mesenchymal cell proliferation involved in metanephros development	0.0067
GO:0050714	Positive regulation of protein secretion	0.0072
GO:0046822	Regulation of nucleocytoplasmic transport	0.0072
GO:0043603	Cellular amide metabolic process	0.0075
GO:1903533	Regulation of protein targeting	0.0077
GO:0007154	Cell communication	0.0078
GO:0072076	Nephrogenic mesenchyme development	0.0087
GO:0060389	pathway-restricted SMAD protein phosphorylation	0.01
GO:0010557	Positive regulation of macromolecule biosynthetic process	0.0103
GO:0045893	Positive regulation of transcription,DNA-templated	0.0103
GO:0051254	Positive regulation of RNA metabolic process	0.0103
GO:1902680	Positive regulation of RNA biosynthetic process	0.0103
GO:1903508	Positive regulation of nucleicacid-templated transcription	0.0103
GO:0008334	Histone mRNA metabolic process	0.011
GO:0007369	Gastrulation	0.0114
GO:0015671	Oxygen transport	0.0116

The results for the GO terms were illustrated below Figure 6-18. Nodes were coloured from red to yellow with the node with the strongest support coloured red and nodes with no significant enrichment coloured yellow. The five nodes with the most robust support were marked with rectangular nodes.

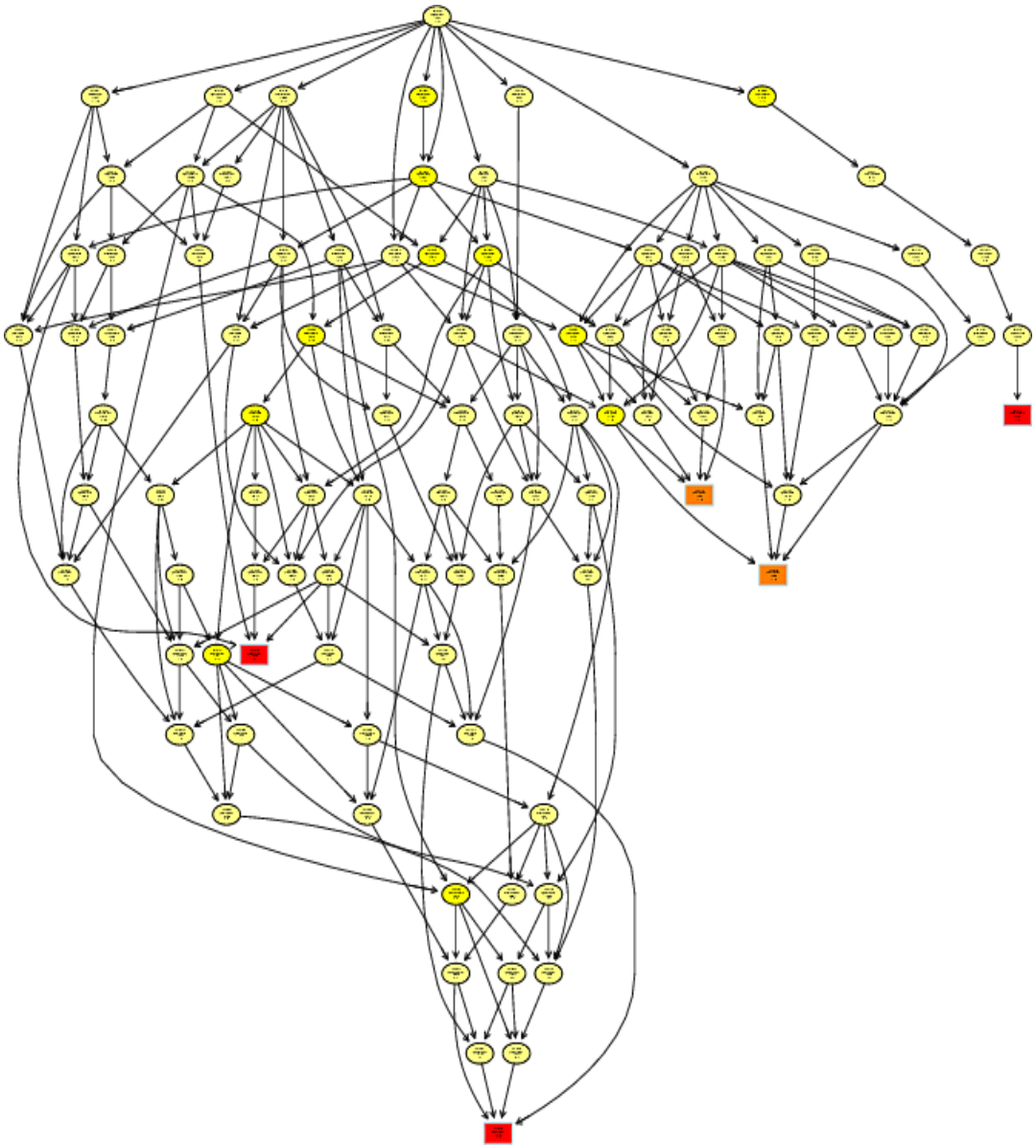


Figure 6-18: GO network generated from the GO terms predicted to be enriched for the Biological process.

6.11 Validation of the potential miRNAs in an extended set of samples and controls

All experiments were conducted by QIAGEN Genomic Services by using QIAGEN's miRCURY LNA. A multi-marker panel of 48 chosen assays customised for RT-qPCR. Thirty-seven candidate miRNAs plus five small RNA (tRNA19-GlyGCC, tRNA4-GlyCCC, tRNA9-ArgTCT and, Y_RNA.687-201) in addition to the control parameters were subjected to further validation in a total of 66 postpartum pre-eclamptic grouped according to the year of delivery; 2007 (n=7), 2012 (n=7), 2013 (n=18), 2014 (n=11), 2015 (n=10) and 2016 (n= 13) women and to increase the statistical power in

the control arm another 9 women were recruited in 2019 to reach a total number of 14 in the control group (n=5 were collected in 2016, and n=9 were collected in 2019).

6.11.1 Material and methods

All experiments were conducted by QIAGEN Genomic Services in Germany.

6.11.2 Sample preparation

Total RNA was isolated from serum/plasma using the miRNeasy Serum/Plasma Kit (QIAGEN) after the manufacturer's instructions. A total volume of 1.25 µl of UniSp2, 4 and 5 was added. RNA was eluted with 20 µl of RNase-free water.

6.11.3 miRNA real-time qPCR

2 µl RNA was reverse transcribed in 10 µl reactions using the miRCURY LNA RT Kit (QIAGEN). cDNA was diluted 50 x and assayed in 10 µl PCR reactions according to the protocol for miRCURY LNA miRNA PCR; each miRNA was assayed once by quantitative polymerase chain reaction (qPCR) on the miRNA Ready-to-Use PCR, Custom panel using miRCURY LNA SYBR Green master mix. Negative controls excluding template from the reverse transcription reaction were performed and profiled like the samples. The amplification was performed in a Light Cyclero 480 Real-Time PCR System (Roche) in 384 well plates. The amplification curves were analysed using the Roche LC software, both for determination of Cq (by the 2nd derivative method) and for melting curve analysis.

6.11.4 Data analysis

The amplification efficiency was calculated using algorithms similar to the LinReg software. All assays were inspected for distinct melting curves and the Tm was checked to be within known specifications for the assay. Furthermore assays must be detected with 5 Cq less than the negative control and with Cq<37 to be included in the data analysis. Data that did not pass these criteria were omitted from any further analysis. Cq was calculated as the 2nd derivative.

All data were normalised to the average of assays detected in all samples or alternatively to the average of custom-defined assays detected in all samples (Andersen et al. 2004).

6.11.5 Data quality control

Genomic Services has developed a rigorous and automated PCR data QC pipeline that allows us to achieve the maximum benefit of SYBR Green-based miRCURY LNA PCR assays. Each individual amplification product on PCR panels was scrutinised by:

- Melting curve analysis
- Calculation of amplification efficiency
- Comparison of Cq value to background level in the negative control sample

Data that derive from PCR reactions that fall outside our thresholds and specifications were flagged and removed. This ensures that the data analysis was performed with a consistent, high-quality data set.

6.11.6 Melting curve analysis

An additional step in the real-time PCR analysis was performed to evaluate the specificity of the amplification products by generating a melting curve for each reaction. The appearance of a single peak with the expected Tm was an indication that a single specific product was amplified during the qPCR process. PCR reactions that gave rise to multiple melting curve peaks or single peaks with a melting temperature that was inconsistent with the specifications for the corresponding assay (in-house database) were flagged and removed from the data set. Figure 6-19 illustrates the Cq values obtained for each well in a 384-well plate. The colour scale indicates the level of expression (Cq). The circles with crosses indicate assays that have more than one peak on the melting curve, or that have a Tm that deviate from the in-house database of Tm values.

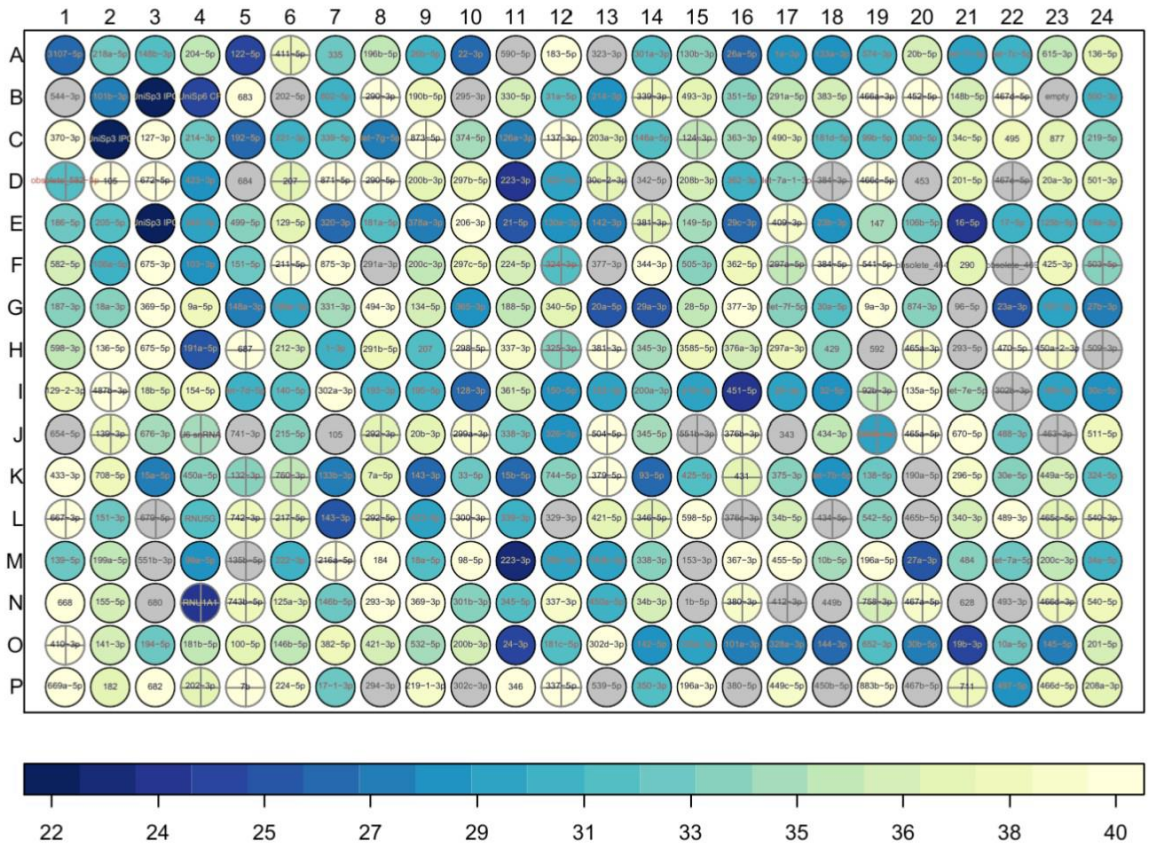


Figure 6-19: Example of Sample Submission Plot.

6.11.7 Signals from negative controls

A “no template” sample in the RT step was included as a negative control. This type of negative control was used as this was the most stringent type of control applications. It detected RNA contamination in the RT step. An assay detected 5 Cq lower than the negative control would be included in the data analysis. For assays that did not yield any signal on the negative control, the upper limit of detection was set to Cq =37. It is worth mentioning that the positive controls on the plates (DNA and RNA spike-ins) yield detectable signals as expected (far right in Figure 6-20).

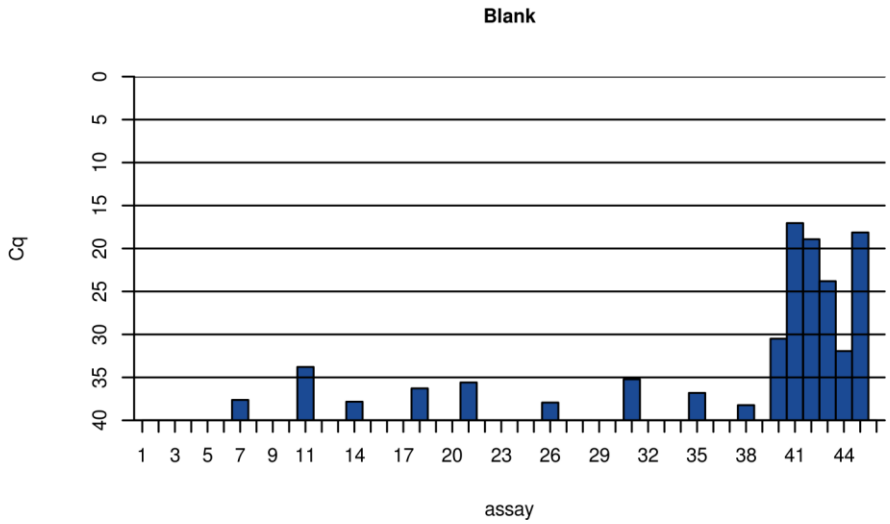


Figure 6-20: Raw Cq values for the negative control sample.

6.11.8 Normalisation using custom assays

Normalisation was performed based on the average of the custom defined assays detected in all samples. The formula used to calculate the normalised Cq values is:

Normalised Cq = custom normaliser assays mean Cq (sample 1) – assay Cq (miRNA of interest in sample 1). A higher value thus indicates that the miRNA is more abundant in the particular sample (Mestdagh et al. 2009).

6.12 Results

Demographic and clinical information of this study are summarised in Table 6.10

Table 6.10**: Demographic data for the study subjects**

Variables	Pre-eclampsia (n=66)	Control group (n=14)	P-value
Mean Age (Years)	37.97 ± 6.31	30.62 ± 2.26	0.008
Mean Height (cm)	166.7 ± 7.07	160.42 ± 6.03	0.04
Median Weight (Kg)	69.85 (50 - 183.7)	61 (50 -118)	0.029
Median BMI (kg/m²)	24.96 (17.67- 63.05)	24.22 (18.82-43.56)	0.429
Previous history of P-EC	18	None	
Family history of Hypertension	14	1	
Family history of T2DM	3	1	
Family history of MI	5	1	
Family history of DVT	1	0	
Current smokers	5	None	
Ethnicity	58 participants were White-British, 1 Pakistan, 1 Irish, 1 Bangladesh, 3 White-European, 2 Mixed	9 participants were White-British, 3 White-European, 1 Pakistan, 1 Chinese	

6.12.1 Number of detected miRNAs

The raw data all show good data quality.

Table 6.11 lists the number of assays present on the miRCURY LNA miRNA PCR panels and, the number of assays that were detected in all 79 samples.

Table 6.11: Summary of the number of miRNA PCR panels used in the validation stage.

	Number of assays
miRCURY LNA miRNA PCR Custom panel	48
Assays with signal in all samples	28
Average number of assays detected per sample	36

Figure 6-21 shows the number of miRNAs detected in samples as well as the Cq value of the global mean for each of the samples. The blue bars represent the number of miRNAs detected and the red line shows the average Cq value for the commonly expressed miRNAs. On average, 36 miRNA were detected per sample.

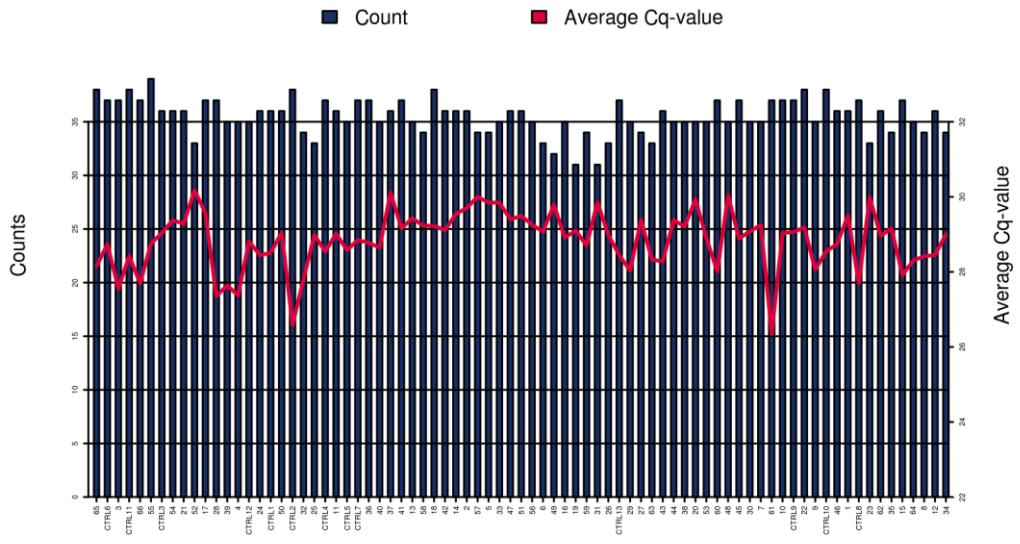


Figure 6-21: miRNA content.

6.12.2 Heat map and unsupervised clustering

The heat map diagram in Figure 6-22 shows the result of the two-way hierarchical clustering of miRNAs and samples. The clustering was performed on all samples, and on the top 28 miRNAs with highest standard deviation expressed in the pairwise comparison of the P-EC and control groups. The normalised (dCq) values have been used for the Analysis.

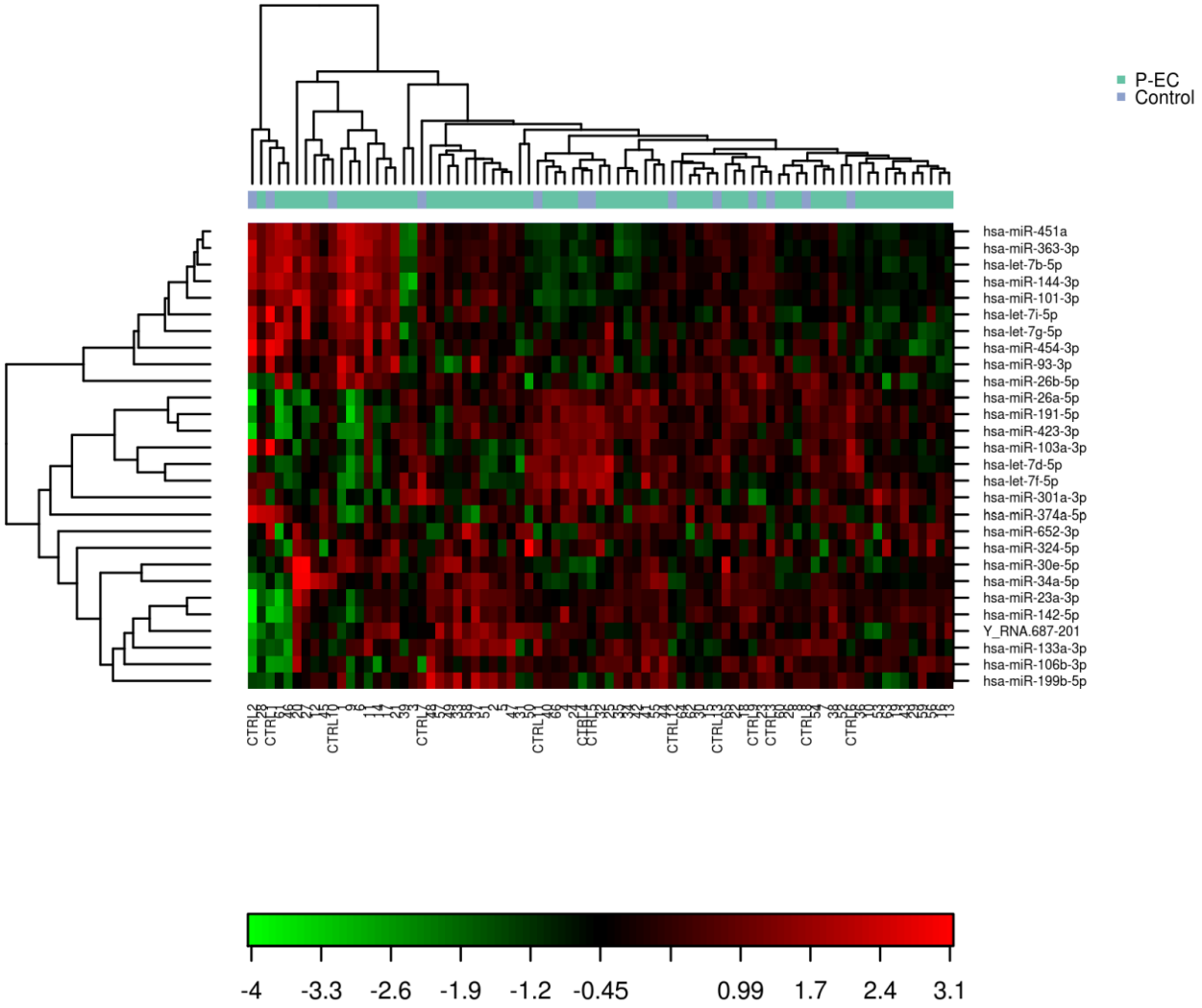


Figure 6-22: Heat map and unsupervised hierarchical clustering.

6.12.3 PCA plot

By including the top 28 miRNAs that had the largest variation across all samples, an overview of how the samples cluster based on this variance is obtained in Figure 6-23.Furthermore, clustering analysis, according to the year of delivery has performed, and there was not a pattern of separation or grouping as shown in Figure 6-24.

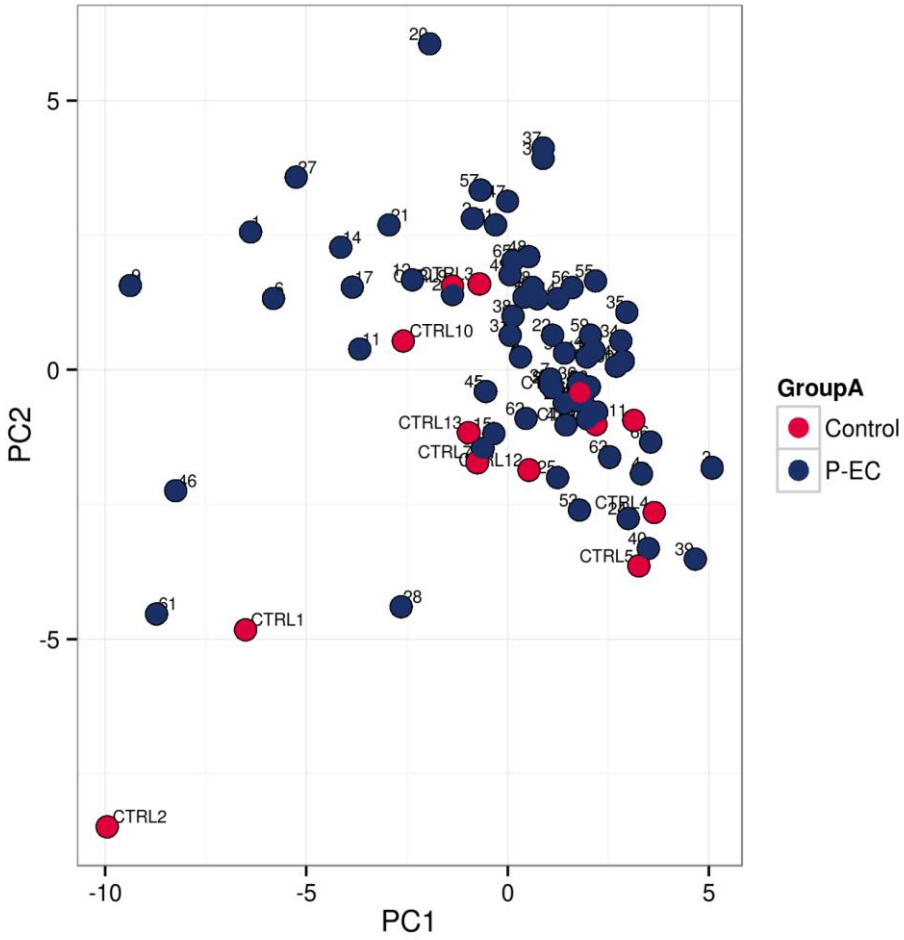


Figure 6-23: The principal component analysis is performed on all samples.

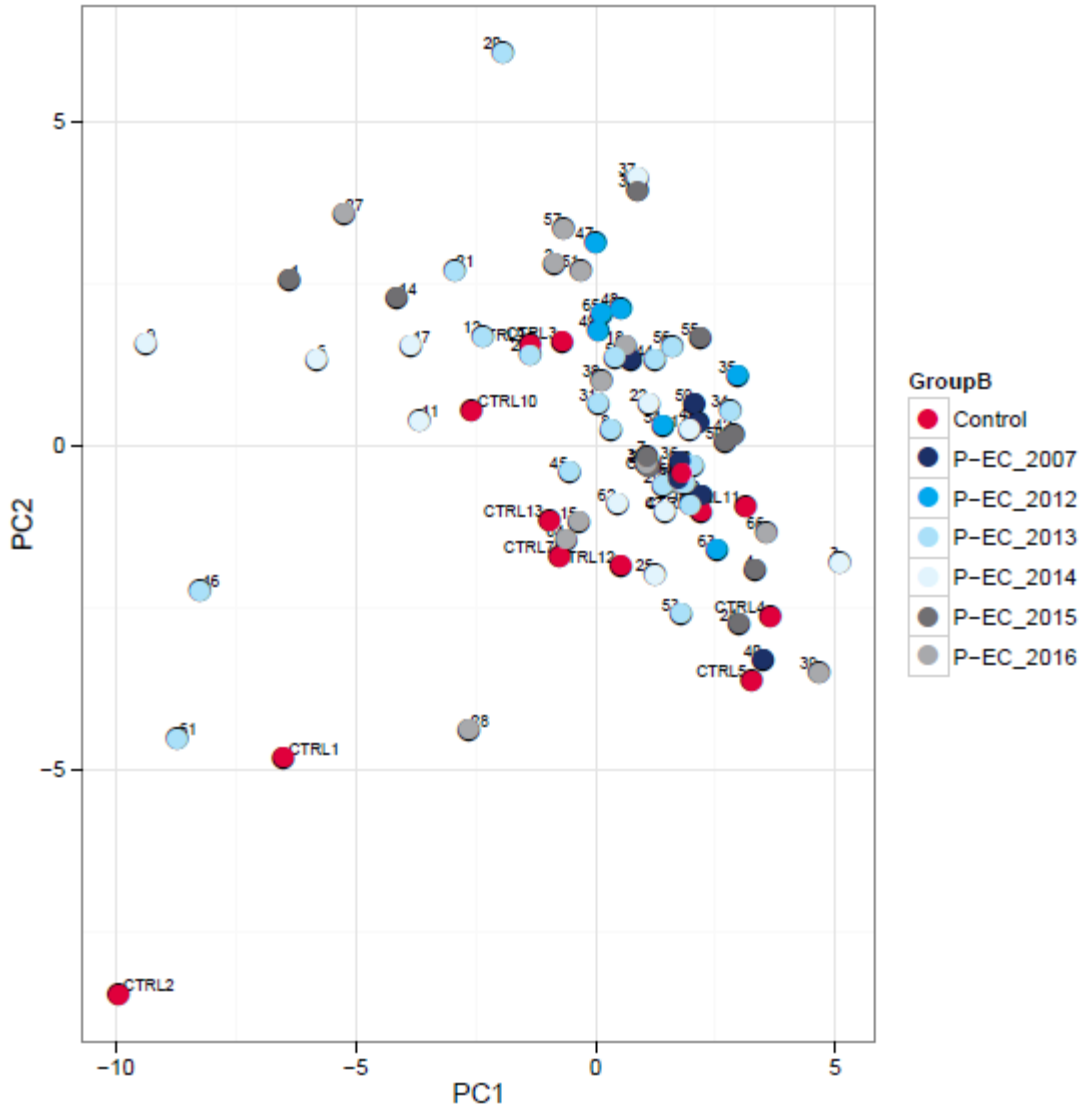


Figure 6-24 PCA for the samples grouped according to the year of delivery.

6.12.4 Differentially expressed miRNAs P-EC vs Control (Confirmation and validation RT-qPCR)

When comparing the P-EC group (n=66) to the Control group (n=14) using a t-test, 8 miRNAs were found to be differentially expressed using a cut-off of P-value < 0.05. 1 of these pass a Benjamin-Hochberg correction at a significance level of 0.05 which we recommend to do when doing panel profiling. Plasma level of hsa-miR-103a-3p was significantly lower in pre-eclampsia subjects, compared to the healthy control (Adjusted p-value =0.033). However, there were no significant differences after p-value BH adjustment for has-mir-193a-5p, hsa-miR-454-3p, has-miR-454-3p, has-miR-30e-5p,has- miR-122-5p,has- let-7f-5p, has-miR-142-5p and tRNA9-ArgTCT. Table 6.12 illustrates the top most differentially expressed miRNAs, showing the standard deviation (SD) across the groups, followed by average normalised Cq values for each group and fold change between the two groups. The last two columns show the P-value from the t-test and the Benjamin-Hochberg adjusted P-value. The miRNA names in the table links to Mir search's result pages.

Table 6.12: The individual results for the top most differentially expressed miRNAs.

miR name	SD P-EC	SD Control	Average dCqP-EC	Average dCq Control	Fold change	P-value	BH adj. P- value
hsa-miR-103a-	0.25	0.32	1.8	2.2	-1.3	0.00083	0.033
hsa-miR-193a-	1.0	0.86	-2.4	-3.2	1.8	0.0068	0.14
tRNA9-ArgTCT	0.77	0.69	-0.67	-1.2	1.5	0.020	0.17
hsa-miR-454-3p	0.55	0.67	-2.4	-1.9	-1.4	0.021	0.17
hsa-miR-30e-5p	0.42	0.35	0.88	0.60	1.2	0.021	0.17
hsa-miR-122-5p	1.6	1.3	1.9	0.98	1.9	0.029	0.19
hsa-let-7f-5p	0.60	0.58	-0.88	-0.48	-1.3	0.036	0.21
hsa-miR-142-5p	0.36	0.61	2.3	1.9	1.3	0.045	0.21

6.12.5 Volcano plot

The volcano plot was constructed by plotting the P-value on the y-axis, and the fold change (ddCq) between the two experimental groups on the x-axis so that up- and down regulations appear equidistant from the centre. Figure 6-25 shows the relation between the P-values and the ddCq. Highlighted spots are miRNAs with P-values below 0.05 after Benjamin-Hochberg correction for multiple testing

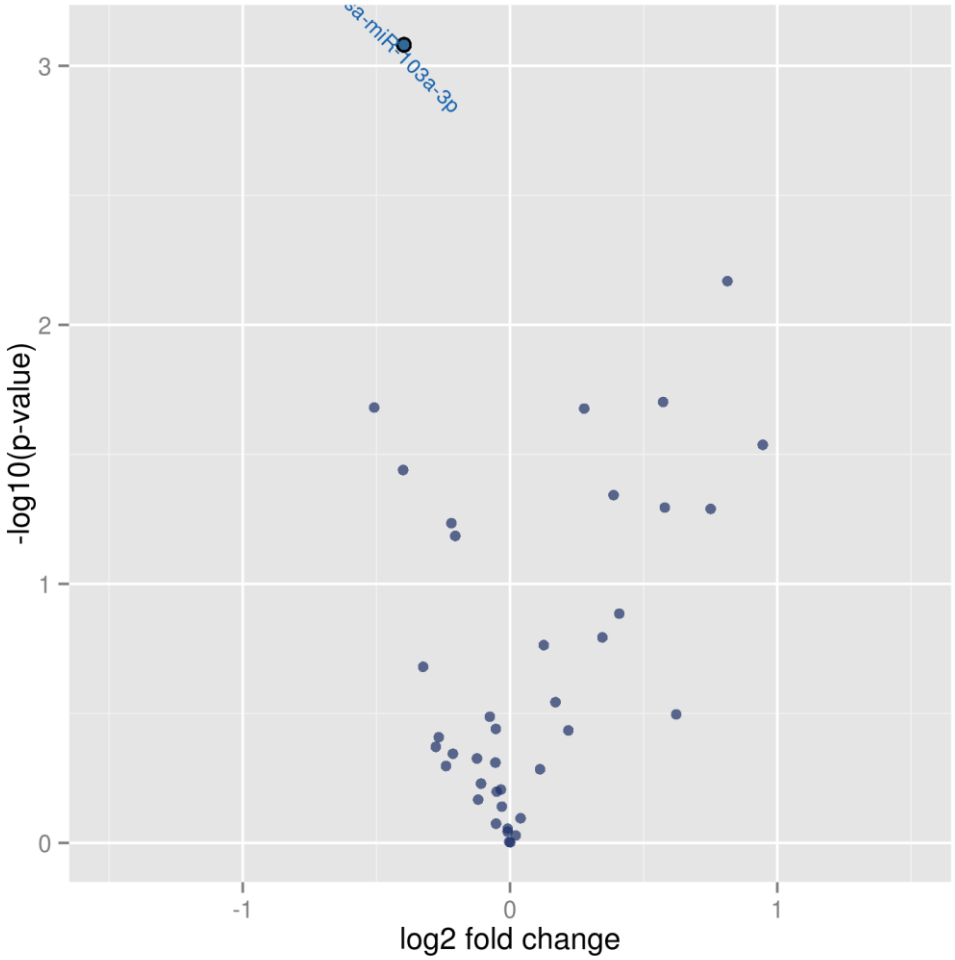


Figure 6-25: volcano plot of the top 20 miRNAs.

A preliminary assessment of the performance of the validated miRNAs in 2013 vs control and the entire pre-eclampsia group compared to the controls were conducted using receiver operator characteristic (ROC) curves and the area under the curve (AUC) as indicator of such performance. Raw CT values of significantly differentially expressed genes were used to generate individual ROC curves for selected miRNAs; miR-103a-3p in the pre-eclamptic group vs control. In addition, the three miRNAs found were significantly expressed in the pre-eclampsia group who delivered in 2013 vs the control group (miR-103a-3p, miR-7f-5p and miR-193a-5p).

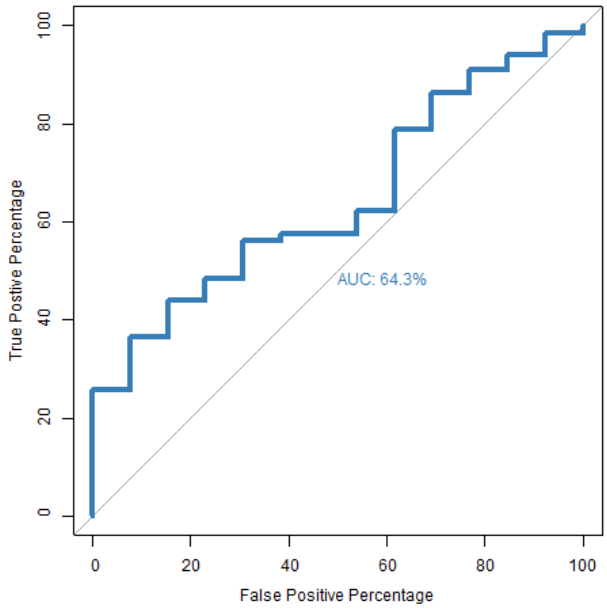


Figure 6-26 ROC miR-103a-3p in entire P-EC group vs control

Figure 6-26 shows the AUC was 64.3% for the miR-103a-3p for the entire pre-eclampsia group compared to the control.

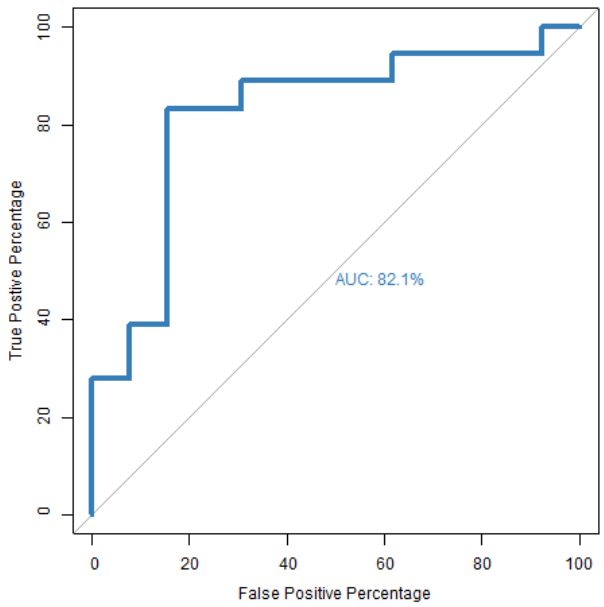


Figure 6-27 ROC curve for miR-7f-5p in P-EC 2013 vs Control groups

Figure 6-27 represents the best AUC which was achieved with miR-7f-5p (AUC=82.1%) and AUC were 65.8% and 62.4% for miR-193a-5p and miR-103a-3p, respectively.

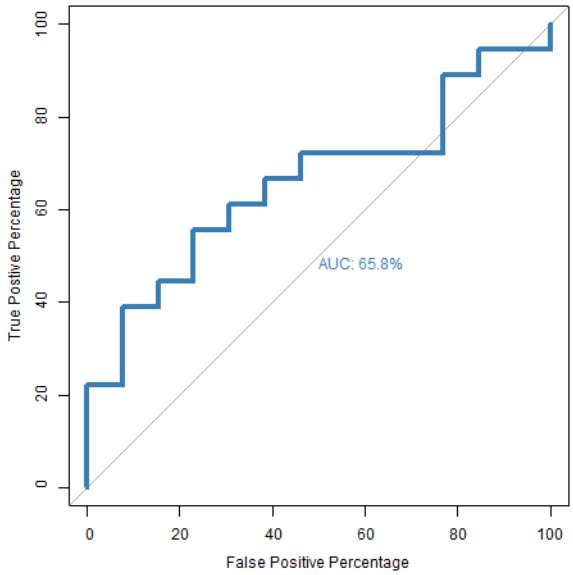


Figure 6-28 ROC curve for miR-193a-5p in P-EC 2013 vs Control groups

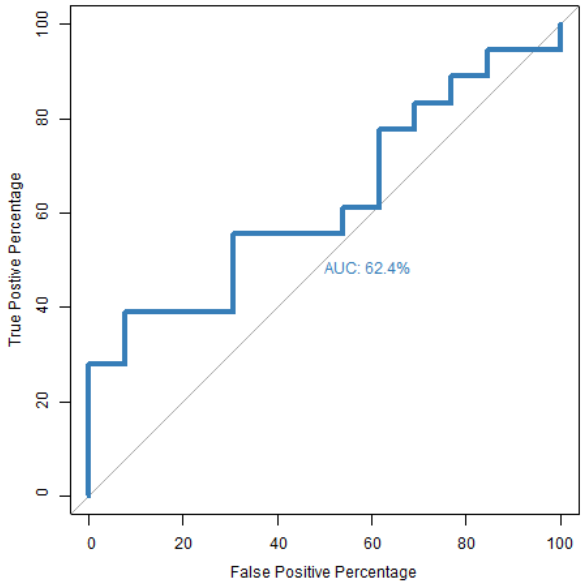


Figure 6-29 ROC miR-103a-3p P-EC 2013 vs Control groups

6.13 **Discussion**

Pre-eclampsia is the third leading cause of maternal morbidity and mortality in the world (Ghulmiyyah and Sibai 2012; Leslie and Collis 2015). However, substantial progress has been made in the diagnosis, prognosis and treatment of this condition; there is still a critical need for novel diagnostic biomarkers to decrease the incidence of this disease and to monitor and follow up those women to avoid any cardiovascular disease development later on in life. Recently, there is growing evidence that is circulating miRNAs (miRNAs) defined as endogenous, single-stranded, stable, small, non-coding RNAs could be used as novel diagnostic biomarkers for some diseases such as cancer and cardiovascular diseases (Sayed et al. 2014). Moreover, miRNAs represent potential novel therapeutic targets and non-invasive tools for use in several cardiovascular disorders.

A significant strength of this project is its robust analysis of plasma miRNA by using NGS technique as an unbiased and more sensitive approach to discover microRNAs associated with Pre-eclampsia and cardiovascular predisposing risk factors in the future (Sanders et al. 2016).

In the exploratory stage (Discovery), marked increases in the expression three miRNAs expression were found when the P-EC group who had delivery in (2016) were compared to the control group (delivery 2016), miR-885-3p, miR-122-5p and miR-885-5p were found with (p-value= <0.0001, 0.0001 and 0.0001 respectively), after adjusting the p-value according to Benjamin-Hochberg all three miRNAs FDR values equal 0.0126. Interestingly, a decrease in 12 out of 14 miRNAs expressions among the entire pre-eclamptic group (n=30) irrespective their year of delivery (down-regulated) vs the control group and two miRNAs were increased in the pre-eclampsia cohort (up-regulated).

Gene ontology analysis performed for the gene targets of miRNAs found to be differentially expressed for example, between pre-eclampsia 2016 vs control 2016 found that specific biological process categories were enriched. Processes with significantly higher in the gene target lists include response to tumour necrosis factor (p = 0.001; n= 7 genes), and regulation of stem cell population maintenance (p = 0.0027; n= 6 genes), positive regulation of dendrite morphogenesis (p = 0.0029; n= 4 genes), positive regulation of cellular biosynthetic process (p = 0.0036; n= 21 genes) and positive regulation of nucleobase-containing compound metabolic process (p =

0.0045; n= 21 genes). Therefore, an additional 36 plasma samples from pre-eclamptic women added to the previous subject arm and, eight more samples in the control group to reach in a total of 79 (P-EC= 66 and Control= 13) for confirmation and validation as the next step.

No previous work has described an experimental identification and validation of suitable endogenous miRNAs in pre-eclampsia postpartum plasma at different time points since their year of delivery. Thus, this first study aimed to identify the most differential significant microRNA expression RT-qPCR studies in pre-eclampsia. Twenty-eight miRNAs were identified in all samples, with an average of 36 miRNAs detectable per sample in the validation cohort study.

Here we focus mainly on the expression of microRNAs being previously reported to play a role in the pathogenesis of vascular inflammation, hypertension, obesity, dyslipidaemia, insulin resistance, angiogenesis, coronary artery disease and atrial fibrillation. A total of 581 genes were predicted to be targets of validated miRNA of the-miR-103a-3p using the miRTarBase and miRDB databases. The target genes included IDS, NF1, SCN8A, DICER1, NPAS3, KIF21A, MED26, AGFG1, and ANO3.A recent study was conducted by Zhang et al. (2019) revealed that the downregulation of miR-103a-3p promoted autophagy. Programmed cell death encompasses apoptosis and autophagy. Autophagy can be defined as a regulated mechanism of the cell to remove unnecessary organelles or dysfunction components (Lavandero et al. 2013). Collectively, these data suggest that the increase in autophagic flux may lead to cell death, and exacerbate cardiac diseases, including heart failure (Zhang et al. 2019).

Inhibitors of DNA binding (IDS) are define as transcription regulators containing a highly conserved helix-loop-helix domain. ID proteins, encoded by the Id gene family comprising Id1, Id2, Id3, and Id4. These Id proteins play vital roles in early development and take part in the differentiation and proliferation of cardiac progenitor cells and mature cardiomyocytes (Hu et al. 2019). There is literature in animal models supporting our results, which demonstrate a significant trend towards downregulation in ID2 genes linked to heart arrhythmia and congenital heart diseases (Moskowitz et al. 2007). Our findings provide the first evidence that such a downregulation in ID2 genes in the pre-eclampsia women compared to healthy controls could be used as a novel biomarker to assess cardiovascular risk in pre-eclamptic women later on life.

NF1 protein products generally act to modulate epithelial-mesenchymal transformation and proliferation in the developing heart by downregulating RAS activity (Tenschert et al. 1985). It has been documented in animal experiments that the absence of neurofibromin NF1 in mouse

embryo hearts results in the development of overabundant endocardial cushions as the result of hyperproliferation and lack of normal apoptosis (Zachos et al. 1997; Moustafa et al. 2011). A previous study indicated that miR-103a-3p was related to the induction of apoptosis in human glioma stem cells (Yu et al. 2017).

Interestingly, our results demonstrated the two other miRNAs in addition to the miR-103a-3p that were significantly downregulated and appear to the entire pre-eclampsia cohort in comparison to the healthy control group (FC= -1.3; BH adj P = 0.033). has-let-7f-5p and miR-193a-5p were significantly differentially expressed in women who had pre-eclampsia and delivered in 2013 in comparison to the control group (BH adj P = 0.046 and 0.047 respectively); miR-193-5p was significantly overexpressed in pre-eclampsia compared to the controls (FC 2.1; BH adj P = 0.047), This is in line with the findings stating that the miR-193 family has been implicated in various processes, especially in ischemic injury, in both human patients and animal models. Therefore, this biomarker could be used beneficially for monitoring and early prediction of CVD (Yi et al. 2017).

Let-7 family miRNAs constitute almost the fifth most abundant miRNA in the myocardium, and are predicted to regulate main cardiac conduction by targeting SCN5A. It has been shown that by affecting Na+ and Ca2+ channels, let-7f participate in arrhythmias (Bao et al. 2013). Our data showed that downregulation of let-7f-5p in pre-eclampsia subjects could be attributed to sodium or potassium ion channels that confirming that any disorder of repolarisation process in following the action potential could either be inherited or acquired due to alterations in these channels. Thus, alterations in the expression or post-translational modification of ion channels underlie the fatal arrhythmias associated with heart failure (Marbán 2002).

In summary, the current chapter has described the discovery and initial validation of a novel bio-signature for microRNAs in the plasma of postpartum women with pre-eclampsia and healthy controls women, along with satisfactory diagnostic performance in independent validation cohorts. Additionally, the significant level of miR-103a-3p in plasma provides a promising biomarker for a highly specific role, and its validation must be prioritised. This study provides the first evidence that dysregulation in the miRNAs expression occurs in the postpartum women who have had pre-eclampsia at different time points and could provide a novel biomarker tool for predicting future cardiac events. Therefore, such women might benefit from regular follow- up and lifestyle advice to provide for better outcomes.

Comprehensive statistical and bioinformatics tools have been applied to mine biologically relevant information from the QIAGEN service in Germany. This work has generated new exciting research avenues in the field of pre-eclampsia and the possibility of predictive cardiovascular biomarkers. However, further analysis of the messenger RNA, gene targets, further biological process and signalling pathways is needed to provide further insight into the underlying mechanisms. At present, no research has explicitly looked at how early identification of plasma miRNAs in women with a past history of pre-eclampsia could identify patients at risk of future cardiovascular events.

Chapter 7 General discussion and Future perspective

In this thesis, a follow-up of women with a history of P-EC postpartum revealed common and previously unknown constitutional factors that related to prior and subsequent pregnancy outcome and might predict maternal cardiovascular complications in the future. This chapter summarises the thesis, point out the limitations of the current project as well as outlines recommendations for future work.

7.1 Summary of the thesis

In our data, described in **Chapter 2**, we sought to identify inflammatory patterns characteristic of women with a history of pre-eclampsia and whether these markers could predict the development of subsequent cardiovascular disease in this group of women. It is well recognised that the regulation of the immune system has an active genetic component, which accounts for inter-individual differences in the inflammatory response after exposure to similar environmental antigens. Inflammation plays a significant role in the pathogenesis of atherosclerotic lesions of vascular walls. We propose that IL-10 functions as a vital bridge that links immunity, placental angiogenesis progression or instability of cardiovascular disease and is associated with a systemic inflammatory response. The findings showed that women with a history of P-EC had elevated levels of inflammatory cytokines and an imbalance in the haemostatic system.

Additionally, we assessed further angiogenic and anti-angiogenic makers after 6-12 months of post-delivery in women who had P-EC in **chapter 3**. Our results suggest that angiogenic, anti-angiogenic and circulating haemostatic factors are not significantly changed in women with a history of P-EC. However, we observed a weak positive association between all the measured parameters. It is unknown whether these parameters are altered even later in life, for example, 15-20 years following P-EC, which is the age at which CVD risk would normally begin to rise (Powers et al. 2005). It is known that angiogenesis is a highly regulated process involving the balance between pro- and anti-angiogenic factors. Locally, endothelial cells produce growth factors, cytokines, enzymes, receptors, adhesion molecules, and metabolic factors that regulate the angiogenic process. Thus, the interaction between immune and endothelial cells should not be neglected (Galvão et al. 2013).

Some of the plasma markers that could take place in the anticoagulation and fibrinolytic processes, as well as to explore more factors which could be linked to the deterioration of women's health after experiencing P-EC at different time interval post-delivery. The annexins

levels in pre-eclamptic women as the previous chapters were focused on the haemostatic, inflammatory and proteomic markers. **In chapter 4** the researcher assessed the plasma levels of annexins A2 and A5 demonstrating that there was an imbalance between the Annexin A2 and Annexin A5 as the late post-delivery women showed a tendency towards reduced Annexin A5 levels, conversely to the Annexin A2 levels which were higher. Such a tendency may contribute to the development of maternal hypercoagulable states and may predispose women with a history of P-EC to cardiovascular risks later in life.

The focus of this thesis-driven by advance technologies in research using proteomic studies in this project. The proteomic findings identified the most comprehensive to date plasma proteomic profiling one-year postpartum in women with P-EC compared to controls. The results were promising as they show that there is dysregulation in the inflammation, immune response, blood coagulation and metabolism processes one-year postpartum in women who had P-EC previously. Further validation carried out using the ELISA technique in order to validate firstly monocytes differentiation antigen CD14 that could reflect the inflammatory status and increased the insulin resistance and type 2 diabetes mellitus development (T2DM). Secondly, to validate the adiponectin levels as it is a marker of glucose haemostasis. Our results showed increased levels of CD14 and adiponectin in pre-eclamptic subjects compared to the healthy control group. The elevation of CD14 plasma level was considered to be a critical role in the process of atherosclerosis and complications (Xu et al. 2019). Moreover, there was an increase in the level of adiponectin in the pre-eclamptic women which could draw a link between this marker and cardiovascular events progression. It has been suggested that the adipose tissue may play an essential role in mediating this chronic inflammatory process and, subsequently, cardiovascular disease risk and therefore might not only be considered as a storage location for fat. There was a study conducted by Macheret et al. (2015) revealed that higher adiponectin levels are associated with increased risk of atrial fibrillation (AF)-related outcomes, including cardiovascular disease (CVD) and mortality. (**Chapter 5**).

Finally, **Chapter 6** another up to date technique used in the last two decades representing the most rapidly evolving tool in the research field. Recently, MicroRNAs (miRNAs) were found to play an essential role in gene regulation and therefore seem to be promising candidates involved in the pathology P-EC and the subsequent development of cardiovascular disease. During the exploratory and discovery stage 14 candidate miRNAs that were significantly and differentially expressed at a significance level of 0.05 (FDR). It has been demonstrated that miRNAs are

associated with the induction of cardiomyocyte apoptosis following ischemic injury (Di et al. 2014). In conclusion, early-onset P-EC remains an important cause of maternal and neonatal morbidity and mortality. Despite extensive research, many aspects of its pathogenesis and risk factors remain to be discovered. Important parallels between P-EC and other cardiovascular and inflammatory disorders exist and provide a fascinating field for future studies. Our promising findings demonstrate a significant and previously undiscovered cardiovascular burden in P-EC women post-delivery. The results the researcher found are promising, in particular, the proteomics and miRNAs studies, further experimental confirmation is required to consolidate these findings to be applied and provides new opportunities for preventive strategies relevant to women's health.

7.2 Limitations

There are some limitations in this research need to be highlighted. The response rates were low in patients and control arms as 600 invitation letters were sent, and the response was low i.e. 21% particularly from the control group. In addition, the small sample size creates an essential limitation of this project, and therefore, the biological insights that can be derived with confidence from this analysis are limited by a high false discovery rate. Studies with larger cohorts are likely to reduce the effects of inevitable variability between participants and yield more reliable results. However, in proteomics and miRNAs studies represent the most comprehensive plasma biomarkers described so far in P-EC postpartum studies.

Another limitation is the validity of the results and analysis of the IUGR data in study II. When comparing haematological parameters in women with and without a history of IUGR, all nine women with a history of IUGR were also formerly pre-eclamptic. The similarities in the pathogenesis of these conditions, mean that it is not unreasonable to consider that their simultaneous occurrence may have affected the data obtained (Ness and Sibai 2006; Wallner et al. 2007). Additionally, next-generation sequencing approach for miRNA was an experimentally costly process and was a new area of the researcher speciality in the last four months of the researcher PhD journey.

Preliminary confirmation of proteomic findings was carried out; however, some of the main challenges related to biomarker validation were revealed after decoding. For instance, only two candidates (CD14 and Adipo) selected from the complete plasma profile based on one iTRAQ 10-plex were verified on an independent validation cohort and the researcher worked blindly by using antibody-based assay ELISA technique. Later on, when the researcher received the decoding found out that out of 60 total samples received and tested was 17 cases, 3/17 was P-EC and 7

cases were P-EC+FGR and 7 cases who had FGR. Also, the samples were collected in a different type of anticoagulant (Lithium heparin) than the main project used, which creates inconsistency to the condition of the experiment and may mask the ELISA results for validation. Therefore, the researcher omitted these results for now and keen on the future to validate them in the same condition. The research was not able to do further lab work once the decoding received due to time and financial consideration as the sponsor covered the study for four years only. Despite the limitations mentioned above, the study findings appear promising and pave the way for the importance of piloting the results of this study.

7.3 Recommendation for future work

During the PhD journey, the researcher measured various biomarkers using different techniques ranging from basic to the most revolutionised genomics studies. Here will focus on further works need to be carried out for the future work based on the current thesis findings.

1. The researcher is going to validate the proteomics findings in independent larger cohort study to assess the levels of six selected promising markers such as CD14, adiponectin (ADP), Pregnancy zone protein (PZP), Platelet factor 4 (PF4), Annexin A1 and Complement component 5 (C5).
2. Based on our proteomic results that pointed to the presence of a tendency to develop type 2 diabetes mellitus in the subjects were affected by further pre-eclampsia studies needs to focus on the role of Insulin-like growth factor 1 (IGFBP1) (IGF-1) due to its vital role in regulating contractility, metabolism, hypertrophy, autophagy, senescence, and apoptosis in the heart.
3. Troponin test needs to be carried out at least every six months or once a year for pre-eclamptic women postnatal.
4. The researcher could not access to other information that may be related to this condition, for instance, type of blood group for foetus, mother and father/Partner, therefore, the research would recommend to include that information for next research.
5. Prophylactic measurements should be taken as there is no side effect to do them, for example, performing an electrocardiogram (ECG) and electroencephalogram (EEG) once a year.
6. For further gene validation, a transfection approach is necessary to validate the gene differentially expressed in our cohort. Transfection defines as a process of introducing nucleic acids into cells by non-viral methods. This process is typically performed by immunostaining or Western blot. However, TaqMan protein assays is an alternative

technique offer a faster, more sensitive and more quantitative alternative to these techniques.

7. We should consider that the pre-eclamptic women need long-term to follow up, therefore plan to set up a data record, and implant screening programme for this category will be beneficial. The researcher will discuss with the Ministry of Health in Libya once the researcher goes back home to avoid further health issues in the future and improve the quality of life for those women.

7.4 Publications and participation in conferences

Inflammatory and Haemostatic Changes Following Pre-eclampsia: Potential Link with Development of Subsequent Cardiovascular Events***? Gynecol Reprod Health. 2019; 3(4): 1-6.***

[DOI: GRM-19-89]

Raised plasma CD14 levels in women with a previous history of pre-eclampsia: A case-control proteomics study (Submitted to Diabetologia –Journal of the European Association for the study of Diabetes (EASD).

Participation in conferences

E-Posters

59th BSH Annual Sciebtific Meeting 1-3 April 2019 [BSH-EP-070] Glasgow
Title: Plasma proteomic profiling one year postpartum of women with pre-eclampsia shows dysregulated cardiometabolic profile.

58th BSH Annual Sciebtific Meeting 16-18 April 2018 [BSH2018-I/1276] Liverpool
Title: Plasma annexins A2 and A5 in women with a past history of pre-eclampsia.

Poster
57th Annual Scientific Meeting of the British Society for Haematology 27-29 March 2017

[BSH2017-**434] [PO-165] Brighton**

Chapter 7

Title: Do patients with a history of pre-eclampsia have elevated levels of coagulation and angiogenic markers postpartum?

36th World Congress of the ISH hosted by the British Society for Haematology 18-21 April 2016 [Reference: ISH1619FC143] Glasgow

Title: Inflammatory and Haemostatic Changes Following Pre-eclampsia: Potential Link with Development of Subsequent Cardiovascular Events?

Appendices

Appendix A Search Terms

Database	Search terms	Filter	Results
Delphis	Pre-eclampsia AND Biomarkers	English Language	2795
PubMed	Pre-eclampsia OR pre-eclamptic women AND Cytokines OR Inflammatory cytokines	None	363
Medline (OvidSP)	Pre-eclampsia or P-EC AND Haemostatic biomarkers OR Factors	None	5
Web of Sciences	Pre-eclampsia or P-EC AND Cardiovascular diseases	None	64
PubMed	Proteomics and Pre-eclampsia	Last five years	59
PubMed	miRNAs and Pre-eclampsia Or miRNAs	None	66

Appendix B Ethical Approval Letter

STA

17 November 2005

SOUTHAMPTON & SOUTH WEST HAMPSHIRE
RESEARCH ETHICS COMMITTEES (A)
1ST Floor, Regents Park Surgery
Park Street, Shirley
Southampton
Hampshire
SO16 4RJ

Tel: 023 8036 2466
023 8036 3462
Fax: 023 8036 4110

Email: GM.E.hio-au.SWHRECA@nhs.net

Dr Bashir Lwaleed
Clinical Scientist/Research Fellow
University of Southampton
Mailpoint 11, South Academic Block
Southampton General Hospital
Tremona Road
Southampton
SO16 6YD

Dear Dr Lwaleed

Full title of study: **Pre-eclampsia: A possible role of tissue factor and tissue factor pathway inhibitor in placental-microcirculation-thrombi formation?**
REC reference number: **05/Q1702/131**

Thank you for your letter of 10 November 2005, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application		28 October 2005
Investigator CV		31 October 2005
Protocol	4.1	01 September 2005
Covering Letter		01 September 2005
Letter from Sponsor		09 August 2005
Letter from Sponsor		12 August 2005
Peer Review		26 August 2005

Statistician Comments		26 August 2005
Compensation Arrangements		23 August 2005
Advertisement - Healthy Pregnant Women	4.1	01 September 2005
Advertisement - Healthy Non-Pregnant Women	4.1	01 September 2005
Participant Information Sheet - Healthy Pregnant	5	10 November 2005
Participant Information Sheet - Patients	5	10 November 2005
Participant Information Sheet - Healthy Non-Pregnant	5	10 November 2005
Participant Consent Form - Healthy Non-Pregnant	5	10 November 2005
Participant Consent Form - Patient	5	10 November 2005
Participant Consent Form - Healthy Pregnant	5	10 November 2005
Response to Request for Further Information		26 October 2005
Response to Request for Further Information		10 November 2005
Other	Sample Size tables	
Data Protection		11 August 2005

Research governance approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final research governance approval from the R&D Department for the relevant NHS care organisation.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

05/Q1702/131	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Mr Mervyn Griffiths
Chair

Email: GM.E.hio-au.SWHRECA@nhs.net

Enclosures:

Standard approval conditions, SL-AC2 for other studies
Site approval form

Copy to: Dr Peter Hooper, University of Southampton, Highfield Southampton, Hants, SO17 1BJ

Professor W Rosenberg, Director of R&D, Southampton University Hospitals NHS Trust

SF1 list of approved sites

An advisory committee to Hampshire and Isle of Wight Strategic Health Authority

Appendix C Study invitation letter

UNIVERSITY OF Southampton
School of Health Sciences
Mail point 11, Level B, South Academic Block
Southampton General Hospital
Tremona Road
Southampton
SO16 6YD

Dear

Pre-eclampsia study (Ethics ref. 05/Q1702/131)

Pre-eclampsia is a serious condition that affects some women during pregnancy. We are trying to understand some of the causes of this condition. We are working together with the University of Southampton on a research project that is looking at blood clotting factors (substances in blood that form clots and stop us bleeding after a cut). Our research will compare women who have had pre-eclampsia, and contrast the results with women who have not. We would very much appreciate your help with this project which would involve one simple blood test. No other tests or investigations are needed.

If you feel you may be able to help us or would like more information about this study, then please call Bashir Lwaleed on 02380 796559, or complete the tear-off slip below and send in the enclosed stamped, addressed envelope. Thank you for taking the time to read this letter.

Yours faithfully,

Dr. Bashir Lwaleed (Principal Investigator)

Reply slip: Pre-eclampsia study (Ethics ref. 05/Q1702/131)

I would like to hear more about this study and my contact details are:

Name: _____

Telephone number: _____

The best time to phone me is: _____

Many thanks for your time

Appendix D General Medical Questionnaire for inclusion status

CLINICAL INFORMATION

1. Identification

Name:_____ Hospital number:_____

Age:_____ Pregnancy number:_____ Height :_____ Weight:_____

Address:_____

Postcode:_____

City:_____

Ethnic group:_____

2. Clinical history

Blood pressure

Previous pre-eclampsia

Previous disease

Familiar disease

3. Anamnesis

4. Antihypertensive drugs

Other drugs:

5. Personal habits:

Smoking: ( ) Yes ( ) No Quantity: ( )

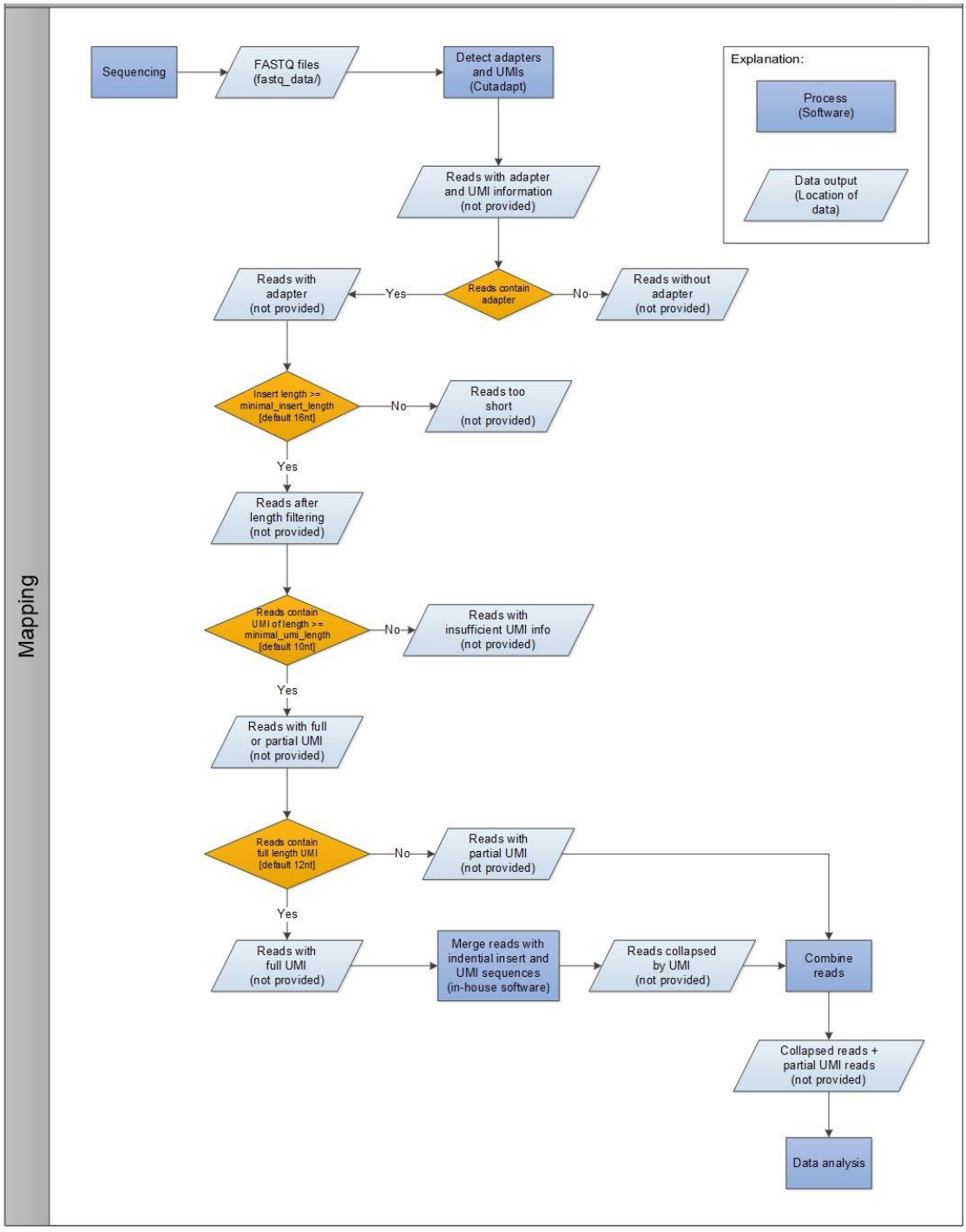
Use of alcohol: ( ) Yes ( ) No

Physical activity: ( ) Yes ( ) No Frequency: ( )

Modality:

Appendix E UMI correction process

Overview of the UMI correction process



Appendix F Gene ontology enrichment analysis for P-EC and control groups

Molecular Function		
GO ID	Term	P value
GO:0051425	PTB domain binding	0.00044
GO:0030332	cyclin binding	0.00088
GO:0035500	MH2 domain binding	0.0034
GO:0035501	MH1 domain binding	0.0034
GO:0016835	carbon-oxygen lyase activity	0.00409
GO:0015078	hydrogen ion transmembrane transporter activity	0.00588
GO:0030275	LRR domain binding	0.00685
GO:0004677	DNA-dependent protein kinase activity	0.00816
GO:0005487	nucleocytoplasmic transporter activity	0.00879
GO:0005144	interleukin-13 receptor binding	0.01093
GO:0030616	transforming growth factor beta receptor, common-partner cytoplasmic mediator activity	0.01105
GO:0019912	cyclin-dependent protein kinase activating kinase activity	0.01122
GO:0003906	DNA-(apurinic or apyrimidinic site) lyase activity	0.01149
GO:0031492	nucleosomal DNA binding	0.01149
GO:0051575	5'-deoxyribose-5-phosphate lyase activity	0.01149
GO:0016597	amino acid binding	0.01619
GO:0015293	symporter activity	0.01643
GO:0005384	manganese ion transmembrane transporter activity	0.01663
GO:0015086	cadmium ion transmembrane transporter activity	0.01663
GO:0015087	cobalt ion transmembrane transporter activity	0.01663
GO:0015094	lead ion transmembrane transporter activity	0.01663
GO:0015099	nickel cation transmembrane transporter activity	0.01663
GO:0015100	vanadium ion transmembrane transporter activity	0.01663
GO:0016151	nickel cation binding	0.01663
GO:0046870	cadmium ion binding	0.01663
GO:0004675	transmembrane receptor protein serine/threonine kinase activity	0.0191
GO:0005024	transforming growth factor beta-activated receptor activity	0.0191
GO:0015093	ferrous iron transmembrane transporter activity	0.0196
GO:0003680	AT DNA binding	0.01964
GO:0070051	fibrinogen binding	0.02156
GO:0070052	collagen V binding	0.02156
GO:0004867	serine-type endopeptidase inhibitor activity	0.02529
GO:0046966	thyroid hormone receptor binding	0.02533
GO:0015103	inorganic anion transmembrane transporter activity	0.02596
GO:0008301	DNA binding, bending	0.02753
GO:0004693	cyclin-dependent protein serine/threonine kinase activity	0.0279
GO:0097472	cyclin-dependent protein kinase activity	0.0279
GO:0019210	kinase inhibitor activity	0.03084
GO:0005515	protein binding	0.03138
GO:0051393	alpha-actinin binding	0.03381
GO:0001077	transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding	0.03392
GO:0005381	iron ion transmembrane transporter activity	0.03617
GO:0042974	retinoic acid receptor binding	0.03619

Molecular Function		
GO ID	Term	P value
GO:0004860	protein kinase inhibitor activity	0.0362
GO:0016702	oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen	0.03785
GO:0061135	endopeptidase regulator activity	0.03829
GO:0005546	phosphatidylinositol-4,5-bisphosphate binding	0.03849
GO:0004576	oligosaccharyl transferase activity	0.03873
GO:0004579	dolichyl-diphosphooligosaccharide-protein glycotransferase activity	0.03873
GO:0015295	solute:proton symporter activity	0.03965
GO:0046965	retinoid X receptor binding	0.0419
GO:0004536	deoxyribonuclease activity	0.04232
GO:0000982	transcription factor activity, RNA polymerase II core promoter proximal region sequence-specific binding	0.04246
GO:0042379	chemokine receptor binding	0.04325
GO:0004857	enzyme inhibitor activity	0.04367
GO:0004866	endopeptidase inhibitor activity	0.0448
GO:0030414	peptidase inhibitor activity	0.0448
GO:0030291	protein serine/threonine kinase inhibitor activity	0.0453
GO:0005272	sodium channel activity	0.04609
GO:0004861	cyclin-dependent protein serine/threonine kinase inhibitor activity	0.04773
GO:0016776	phosphotransferase activity, phosphate group as acceptor	0.04804
GO:0019205	nucleobase-containing compound kinase activity	0.04804
GO:0019002	GMP binding	0.04824
GO:0001078	transcriptional repressor activity, RNA polymerase II core promoter proximal region sequence-specific binding	0.0489
GO:0015081	sodium ion transmembrane transporter activity	0.04989
GO:0015294	solute:cation symporter activity	0.04989

List of References

Abd El-Latif M, Azzam H, Othman M, Warda O, El-Sharawy S and Ghoneim H (2017) Assessment of annexin A5 and annexin A2 levels as biomarkers for pre-eclampsia: A pilot study. *Pregnancy Hypertens* 8: 65-69

Achneck HE, Sileshi B, Parikh A, Milano CA, Welsby IJ and Lawson JH (2010) Pathophysiology of Bleeding and Clotting in the Cardiac Surgery Patient. *From Vascular Endothelium to Circulatory Assist Device Surface* 122(20): 2068-2077

Aebersold R and Mann M (2003) Mass spectrometry-based proteomics. *Nature* 422: 198

Ahmed R, Dunford J, Mehran R, Robson S and Kunadian V (2014) Pre-Eclampsia and Future Cardiovascular Risk Among Women. *Journal of the American College of Cardiology* 63(18): 1815-1822

Akdis M, Burgler S, Crameri R, Eiwegger T, Fujita H, Gomez E, Klunker S, Meyer N, O'Mahony L, Palomares O, Rhyner C, Ouaked N, Schaffartzik A, Van De Veen W, Zeller S, Zimmermann M and Akdis CA (2011) Interleukins, from 1 to 37, and interferon-gamma: receptors, functions, and roles in diseases. *The Journal of allergy and clinical immunology* 127(3): 701-21.e1-70

Akehurst C, Small HY, Sharafetdinova L, Forrest R, Beattie W, Brown CE, Robinson SW, McClure JD, Work LM, Carty DM, McBride MW, Freeman DJ and Delles C (2015) Differential expression of microRNA-206 and its target genes in preeclampsia. *Journal of Hypertension* 33(10): 2068-2074

Al-Daghri NM, Alokail MS, Manousopoulou A, Heinson A, Al-Attas O, Al-Saleh Y, Sabico S, Yakout S, Woelk CH, Chrousos GP and Garbis SD (2016) Sex-specific vitamin D effects on blood coagulation among overweight adults. *European journal of clinical investigation* 46(12): 1031-1040

Andersen CL, Jensen JL and Ørntoft TF (2004) Normalization of Real-Time Quantitative Reverse Transcription-PCR Data: A Model-Based Variance Estimation Approach to Identify Genes Suited for Normalization, Applied to Bladder and Colon Cancer Data Sets. *Cancer Research* 64(15): 5245

Anderson NL and Anderson NG (1998) Proteome and proteomics: new technologies, new concepts, and new words. *Electrophoresis* 19(11): 1853-61

Ardekani AM, Liotta LA and Petricoin EF (2002) Clinical potential of proteomics in the diagnosis of ovarian cancer. *Expert Review of Molecular Diagnostics* 2(4): 312-320

Arngrímsson R, Sigurõardóttir S, Frigge ML, Bjarnadóttir RI, Jónsson T, Stefánsson H, Baldursdóttir A, Einarsdóttir AS, Palsson B, Snorradóttir S, Lachmeijer AM, Nicolae D, Kong A, Bragason BT, Gulcher JR, Geirsson RT and Stefánsson K (1999) A genome-wide scan reveals a maternal susceptibility locus for pre-eclampsia on chromosome 2p13. *Human molecular genetics* 8(9): 1799-805

Bachhav AA and Waikar M (2014) Low Amniotic Fluid Index at Term as a Predictor of Adverse Perinatal Outcome. *The Journal of Obstetrics and Gynecology of India* 64(2): 120-123

Baillet A, Trocmé C, Berthier S, Arlotto M, Grange L, Chenau J, Quétant S, Sève M, Berger F, Juvin R, Morel F and Gaudin P (2010) Synovial fluid proteomic fingerprint: S100A8, S100A9 and

S100A12 proteins discriminate rheumatoid arthritis from other inflammatory joint diseases. *Rheumatology* 49(4): 671-682

Bakheit KH, Bayoumi NK, Eltom AM, Elbashir MI and Adam I (2009) Cytokines Profiles in Sudanese Women with Preeclampsia. *Hypertension in pregnancy* 28(2): 224-229

Bao M-H, Feng X, Zhang Y-W, Lou X-Y, Cheng Y and Zhou H-H (2013) Let-7 in cardiovascular diseases, heart development and cardiovascular differentiation from stem cells. *International journal of molecular sciences* 14(11): 23086-23102

Bartel DP (2004) MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell* 116(2): 281-297

Bdolah Y, Palomaki GE, Yaron Y, Bdolah-Abram T, Goldman M, Levine RJ, Sachs BP, Haddow JE and Karumanchi SA (2006) Circulating angiogenic proteins in trisomy 13. *American Journal of Obstetrics & Gynecology* 194(1): 239-245

Beigh M, Naik M, Tali I, Ara R and Malik A (2017) Prevalence of metabolic syndrome in preeclampsia. *2017* 6(10): 8

Bellamy L, Casas J-P, Hingorani AD and Williams DJ (2007) Pre-eclampsia and risk of cardiovascular disease and cancer in later life: systematic review and meta-analysis. *BMJ : British Medical Journal* 335(7627): 974-974

Benian A, Madazlı R, Aksu F, Uzun H and Aydın S (2002) Plasma and placental levels of interleukin-10, transforming growth factor-β1, and epithelial-cadherin in preeclampsia. *Obstetrics & Gynecology* 100(2): 327-331

Benjamin EJ, Blaha MJ, Chiuve SE, Cushman M, Das SR, Deo R, de Ferranti SD, Floyd J, Fornage M, Gillespie C, Isasi CR, Jiménez MC, Jordan LC, Judd SE, Lackland D, Lichtman JH, Lisabeth L, Liu S, Longenecker CT, Mackey RH, Matsushita K, Mozaffarian D, Mussolino ME, Nasir K, Neumar RW, Palaniappan L, Pandey DK, Thiagarajan RR, Reeves MJ, Ritchey M, Rodriguez CJ, Roth GA, Rosamond WD, Sasson C, Towfighi A, Tsao CW, Turner MB, Virani SS, Voeks JH, Willey JZ, Wilkins JT, Wu JH, Alger HM, Wong SS and Muntner P (2017) Heart Disease and Stroke Statistics—2017 Update: A Report From the American Heart Association. *Circulation* 135(10): e146-e603

Benjamini Y and Hochberg Y (1995) Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society: Series B (Methodological)* 57(1): 289-300

Berndt MC, Metharom P and Andrews RK (2014) Primary haemostasis: newer insights. *Haemophilia : the official journal of the World Federation of Hemophilia* 20 Suppl 4: 15-22

Bertagnolli M, Luu TM, Lewandowski AJ, Leeson P and Nuyt AM (2016) Preterm Birth and Hypertension: Is There a Link? *Current Hypertension Reports* 18(4): 28

Bode W (2006) The structure of thrombin: A janus-headed proteinase. *Semin. Thromb. Hemost.* 32: 16-31

Borges MC, Lawlor DA, de Oliveira C, White J, Horta BL and Barros AJD (2016) Role of Adiponectin in Coronary Heart Disease Risk<span hwp:id="article-title-40" class="sub-article-title">Novelty and Significance</span&gt. *Circulation research* 119(3): 491

Bremme K and Blombäck M (1996) Hemostatic abnormalities may predict chronic hypertension after preeclampsia. *Gynecologic and Obstetric Investigation* 41(1): 20-26

Butenas S, Bouchard BA, Brummel-Ziedins KE, Parhami-Seren B and Mann KG (2005) Tissue factor activity in whole blood. *Blood* 105(7): 2764-70

Cackovic M, Buhimschi CS, Zhao G, Funai EF, Norwitz ER, Kuczynski E, Lockwood CJ and Buhimschi IA (2008) Fractional excretion of tumor necrosis factor-alpha in women with severe preeclampsia. *Obstet Gynecol* 112(1): 93-100

Carey RM, Whelton PK and Committee ftAAHGW (2018) Prevention, Detection, Evaluation, and Management of High Blood Pressure in Adults: Synopsis of the 2017 American College of Cardiology/American Heart Association Hypertension Guideline. *Annals of Internal Medicine* 168(5): 351-358

Carr DB, Epplein M, Johnson CO, Easterling TR and Critchlow CW (2005) A sister's risk: Family history as a predictor of preeclampsia. *American journal of obstetrics and gynecology* 193(3, Supplement): 965-972

Cebe Suarez S, Pieren M, Cariolato L, Arn S, Hoffmann U, Bogucki A, Manlius C, Wood J and Ballmer-Hofer K (2006) A VEGF-A splice variant defective for heparan sulfate and neuropilin-1 binding shows attenuated signaling through VEGFR-2. *Cellular and molecular life sciences : CMLS* 63(17): 2067-77

Cetin I, Mandia L, Huppertz B, Burton G, Cuckle H, Gonen R, Lapaire O, Nicolaides K, Redman C, Soothill P, Spencer K, Thilaganathan B, Williams D and Meiri H (2011) Pregenesys pre-eclampsia markers consensus meeting: What do we require from markers, risk assessment and model systems to tailor preventive strategies? *Placenta* 32(SUPPL. 1): S4-S16

Chaiworapongsa T, Romero R, Espinoza J, Bujold E, Mee Kim Y, Goncalves LF, Gomez R and Edwin S (2004) Evidence supporting a role for blockade of the vascular endothelial growth factor system in the pathophysiology of preeclampsia. Young Investigator Award. *American journal of obstetrics and gynecology* 190(6): 1541-7; discussion 1547-50

Chapin JC and Hajjar KA (2015) Fibrinolysis and the control of blood coagulation. *Blood reviews* 29(1): 17-24

Chen C-P (2009) Placental Abnormalities and Preeclampsia in Trisomy 13 Pregnancies. *Taiwanese Journal of Obstetrics and Gynecology* 48(1): 3-8

Chen CW, Jaffe IZ and Karumanchi SA (2014) Pre-eclampsia and cardiovascular disease. *Cardiovascular Research* 101(4): 579-586

Chesley LC, Annitto JE and Cosgrove RA (1976) The remote prognosis of eclamptic women: Sixth periodic report. *American journal of obstetrics and gynecology* 124(5): 446-459

Cincotta RB and Brennecke SP (1998) Family history of pre-eclampsia as a predictor for pre-eclampsia in primigravidas. *International Journal of Gynecology & Obstetrics* 60(1): 23-27

Clemetson KJ (2012) Platelets and primary haemostasis. *Thromb Res* 129(3): 220-4

Cnattingius S, Reilly M, Pawitan Y and Lichtenstein P (2004) Maternal and fetal genetic factors account for most of familial aggregation of preeclampsia: a population-based Swedish cohort study. *American journal of medical genetics. Part A* 130a(4): 365-71

Cock PJA, Fields CJ, Goto N, Heuer ML and Rice PM (2010) The Sanger FASTQ file format for sequences with quality scores, and the Solexa/Illumina FASTQ variants. *Nucleic Acids Res* 38(6): 1767-1771

Conde-Agudelo A, Rosas-Bermudez A and Kafury-Goeta AC (2006) Birth spacing and risk of adverse perinatal outcomes: a meta-analysis. *Jama* 295(15): 1809-23

Conway DSG, Buggins P, Hughes E and Lip GYH (2004) Relationship of interleukin-6 and C-Reactive protein to the prothrombotic state in chronic atrial fibrillation. *Journal of the American College of Cardiology* 43(11): 2075-2082

Creutz CE, Pazoles CJ and Pollard HB (1978) Identification and purification of an adrenal medullary protein (synexin) that causes calcium-dependent aggregation of isolated chromaffin granules. *The Journal of biological chemistry* 253(8): 2858-66

Dayarathna MKDR, Hancock WS and Hincapie M (2008) A two step fractionation approach for plasma proteomics using immunodepletion of abundant proteins and multi-lectin affinity chromatography: Application to the analysis of obesity, diabetes, and hypertension diseases. *Journal of Separation Science* 31(6-7): 1156-1166

de Groot CJ, van der Mast BJ, Visser W, De Kuiper P, Weimar W and Van Besouw NM (2010) Preeclampsia is associated with increased cytotoxic T-cell capacity to paternal antigens. *American journal of obstetrics and gynecology* 203(5): 496.e1-6

De Silva N, Samblas M, Martínez JA and Milagro FI (2018) Effects of exosomes from LPS-activated macrophages on adipocyte gene expression, differentiation, and insulin-dependent glucose uptake. *Journal of Physiology and Biochemistry*

Deanfield JE, Halcox JP and Rabelink TJ (2007) Endothelial Function and Dysfunction. *Testing and Clinical Relevance* 115(10): 1285-1295

Defoort P (2005) Amniotic fluid volume. *International Congress Series* 1279: 290-294

Deitcher SR and Gardner JF (1999) PHYSIOLOGIC CHANGES IN COAGULATION AND FIBRINOLYSIS DURING NORMAL PREGNANCY. *Clinics in Liver Disease* 3(1): 83-96

Di Y, Lei Y, Yu F, Changfeng F, Song W and Xuming M (2014) MicroRNAs expression and function in cerebral ischemia reperfusion injury. *Journal of molecular neuroscience : MN* 53(2): 242-50

Donker RB, Molema G, Faas MM, Kallenberg CGM, Pampus MGv, Timmer A and Aarnoudse JG (2005) Absence of In Vivo Generalized Pro-Inflammatory Endothelial Activation in Severe, Early-Onset Preeclampsia. *Journal of the Society for Gynecologic Investigation* 12(7): 518-528

Duckitt K and Harrington D (2005) Risk factors for pre-eclampsia at antenatal booking: systematic review of controlled studies. *BMJ* 330(7491): 565

Dusse LM, Carvalho MG, Cooper AJ and Lwaleed BA (2011) Plasma factor VII: A potential marker of pre-eclampsia. *Thrombosis Research* 127(1): e15-e19

Dusse LM, Cooper AJ and Lwaleed BA (2007) *Thrombin activatable fibrinolysis inhibitor (TAFI): a role in pre-eclampsia (review)*.

Eddy AC, Bidwell GL and George EM (2018) Pro-angiogenic therapeutics for preeclampsia. *Biology of Sex Differences* 9(1): 36

El-Aneed A, Cohen A and Banoub J (2009) Mass Spectrometry, Review of the Basics: Electrospray, MALDI, and Commonly Used Mass Analyzers. *Applied Spectroscopy Reviews* 44(3): 210-230

Erez O, Romero R, Hoppensteadt D, Than NG, Fareed J, Mazaki-Tovi S, Espinoza J, Chaiworapongsa T, Kim S-S, Yoon BH, Hassan SS, Gotsch F, Friel L, Vaisbuch E and Kusanovic JP (2008) Tissue Factor and Its Natural Inhibitor in Preeclampsia and SGA. *The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstetricians* 21(12): 855-869

Esplin MS, Fausett MB, Fraser A, Kerber R, Mineau G, Carrillo J and Varner MW (2001) Paternal and Maternal Components of the Predisposition to Preeclampsia. *New England Journal of Medicine* 344(12): 867-872

Favier B, Alam A, Barron P, Bonnin J, Laboudie P, Fons P, Mandron M, Herault JP, Neufeld G, Savi P, Herbert JM and Bono F (2006) Neuropilin-2 interacts with VEGFR-2 and VEGFR-3 and promotes human endothelial cell survival and migration. *Blood* 108(4): 1243-50

Fernández-Real JM and Pickup JC (2012) Innate immunity, insulin resistance and type 2 diabetes. *Diabetologia* 55(2): 273-278

Fischer C, Mazzone M, Jonckx B and Carmeliet P (2008) FLT1 and its ligands VEGFB and PlGF: drug targets for anti-angiogenic therapy? *Nature reviews. Cancer* 8(12): 942-56

Floegel A, Stefan N, Yu Z, Mühlenbruch K, Drogan D, Joost H-G, Fritsche A, Häring H-U, Hrabě de Angelis M, Peters A, Roden M, Prehn C, Wang-Sattler R, Illig T, Schulze MB, Adamski J, Boeing H and Pischon T (2013) Identification of Serum Metabolites Associated With Risk of Type 2 Diabetes Using a Targeted Metabolomic Approach. *Diabetes* 62(2): 639

Freeman DJ, McManus F, Brown EA, Cherry L, Norrie J, Ramsay JE, Clark P, Walker ID, Sattar N and Greer IA (2004) Short- and Long-Term Changes in Plasma Inflammatory Markers Associated With Preeclampsia. *Hypertension* 44(5): 708-714

Friedman RC, Farh KK-H, Burge CB and Bartel DP (2009) Most mammalian mRNAs are conserved targets of microRNAs. *Genome Research* 19(1): 92-105

Galaviz-Hernandez C, Sosa-Macias M, Teran E, Garcia-Ortiz JE and Lazalde-Ramos BP (2019) Paternal Determinants in Preeclampsia. *Front Physiol* 9: 1870-1870

Galvão AM, Ferreira-Dias G and Skarzynski DJ (2013) Cytokines and Angiogenesis in the Corpus Luteum. *Mediators of Inflammation* 2013: 1-11

Gammill HS, Lin C and Hubel CA (2007) Endothelial progenitor cells and preeclampsia. *Frontiers in Bioscience* 12(7): 2383-2394

Gao X, Salomon C and Freeman DJ (2017) Extracellular Vesicles from Adipose Tissue-A Potential Role in Obesity and Type 2 Diabetes? *Frontiers in endocrinology* 8: 202

Gebremedhin AT, Regan AK, Malacova E, Marinovich ML, Ball S, Foo D and Pereira G (2018) Effects of interpregnancy interval on pregnancy complications: protocol for systematic review and meta-analysis. *BMJ Open* 8(8): e025008

Geisow MJ, Walker JH, Boustead C and Taylor W (1987) Annexins—New family of Ca²⁺-regulated-phospholipid binding protein. *Bioscience Reports* 7(4): 289

Gerke V and Moss SE (2002) Annexins: From Structure to Function. *Physiological Reviews* 82(2): 331-371

Ghulmiyyah L and Sibai B (2012) Maternal Mortality From Preeclampsia/Eclampsia. *Seminars in Perinatology* 36(1): 56-59

Gioia M, Vindigni G, Testa B, Raniolo S, Fasciglione GF, Coletta M and Biocca S (2015) Membrane Cholesterol Modulates LOX-1 Shedding in Endothelial Cells. *PloS one* 10(10): e0141270

Gohil R, Peck G and Sharma P (2009) The genetics of venous thromboembolism. A meta-analysis involving approximately 120,000 cases and 180,000 controls. *Thromb Haemost* 102(2): 360-70

Gourvas V, Soulitzis N, Konstantinidou A, Dalpa E, Koukoura O, Koutroulakis D, Spandidos DA and Sifakis S (2014) Reduced ANXA5 mRNA and protein expression in pregnancies complicated by preeclampsia. *Thrombosis Research* 133(3): 495-500

Granger JP, Alexander BT, Bennett WA and Khalil RA (2001) Pathophysiology of pregnancy-induced hypertension. *Am J Hypertens* 14(6 Pt 2): 178s-185s

Gray KJ, Saxena R and Karumanchi SA (2018) Genetic predisposition to preeclampsia is conferred by fetal DNA variants near FLT1, a gene involved in the regulation of angiogenesis. *American journal of obstetrics and gynecology* 218(2): 211-218

Grewal T, Enrich C, Rentero C and Buechler C (2019) Annexins in Adipose Tissue: Novel Players in Obesity. *International journal of molecular sciences* 20(14): 3449

Grozovsky R, Giannini S, Falet H and Hoffmeister KM (2015) Regulating billions of blood platelets: glycans and beyond. *Blood* 126(16): 1877-84

Grundmann M, Woywodt A, Kirsch T, Hollwitz B, Oehler K, Erdbruegger U, Haller H and Haubitz M (2008) Circulating endothelial cells: a marker of vascular damage in patients with preeclampsia. *American journal of obstetrics and gynecology* 198(3): 317.e1-317.e5

Hager JW (2002) A new linear ion trap mass spectrometer. *Rapid Communications in Mass Spectrometry* 16(6): 512-526

Hanley GE, Hutcheon JA, Kinniburgh BA and Lee L (2017) Interpregnancy Interval and Adverse Pregnancy Outcomes: An Analysis of Successive Pregnancies. *Obstetrics & Gynecology* 129(3): 408-415

Hansson GK (2005) Inflammation, Atherosclerosis, and Coronary Artery Disease. *New England Journal of Medicine* 352(16): 1685-1695

Haram K, Svendsen E and Abildgaard U (2009) The HELLP syndrome: Clinical issues and management. A Review. *BMC Pregnancy and Childbirth* 9(1): 8

Hayashi T, Morishita E, Ohtake H, Oda Y, Asakura H and Nakao S (2009) Expression of annexin II in experimental abdominal aortic aneurysms. *International Journal of Hematology* 90(3): 336-342

He S, Silveira A, Hamsten A, Blombäck M and Bremme K (1999) Haemostatic, endothelial and lipoprotein parameters and blood pressure levels in women with a history of preeclampsia. *Thrombosis and Haemostasis* 81(4): 538-542

Heart TAHBAhWAH (2017). Available from: www.thrombocyte.com/hemostasis-definition/accessed]

Hellgren M (2003) Hemostasis during normal pregnancy and puerperium. *Semin. Thromb. Hemost.* 29(2): 125-130

Herzog B, Pellet-Many C, Britton G, Hartzoulakis B and Zachary IC (2011) VEGF binding to NRP1 is essential for VEGF stimulation of endothelial cell migration, complex formation between NRP1 and VEGFR2, and signaling via FAK Tyr407 phosphorylation. *Molecular Biology of the Cell* 22(15): 2766-2776

Hladunewich M, Karumanchi SA and Lafayette R (2007) Pathophysiology of the Clinical Manifestations of Preeclampsia. *Clinical Journal of the American Society of Nephrology* 2(3): 543-549

Ho CS, Lam CWK, Chan MHM, Cheung RCK, Law LK, Lit LCW, Ng KF, Suen MWM and Tai HL (2003) Electrospray ionisation mass spectrometry: principles and clinical applications. *The Clinical Biochemist Reviews* 24(1): 3-12

Hoffman M and Pawlinski R (2014) Hemostasis: Old System, New Players, New Directions. *Thrombosis Research* 133, Supplement 1: S1-S2

Howe L, Hammer E, Badger G and Bernstein IM (2018) Effect of Pregnancy Interval on Second Pregnancy Blood Pressure Following Prior Preeclampsia. *Reproductive sciences (Thousand Oaks, Calif.)* 25(5): 727-732

Howie PW, Begg CB, Purdie DW and Prentice CR (1976) Use of coagulation tests to predict the clinical progress of pre-eclampsia. *Lancet (London, England)* 2(7981): 323-325

Hu W, Xin Y, Hu J, Sun Y and Zhao Y (2019) Inhibitor of DNA binding in heart development and cardiovascular diseases. *Cell Communication and Signaling* 17

Hu Y, Li P, Hao S, Liu L, Zhao J and Hou Y (2009) Differential expression of microRNAs in the placentae of Chinese patients with severe pre-eclampsia. *Clinical Chemistry and Laboratory Medicine.*

Hubel CA (1999) Oxidative stress in the pathogenesis of preeclampsia. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)* 222(3): 222-35

Hulstein JJJ, Van Runnard Heimel PJ, Franx A, Lenting PJ, Bruinse HW, Silence K, De Groot PG and Fijnheer R (2006) Acute activation of the endothelium results in increased levels of active von Willebrand factor in hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome. *Journal of Thrombosis and Haemostasis* 4(12): 2569-2575

Jennings LK (2009) Mechanisms of platelet activation: need for new strategies to protect against platelet-mediated atherothrombosis. *Thromb Haemost* 102(2): 248-57

Jeyabalan A, Hubel CA, Rajakumar A, McGonigal S and Gilmour C (2008) Circulating and Placental Endoglin Concentrations in Pregnancies Complicated by Intrauterine Growth Restriction and Preeclampsia. *Placenta* 29(6): 555-563

Jonsson Y, Ruber M, Matthiesen L, Berg G, Nieminen K, Sharma S, Ernerudh J and Ekerfelt C (2006) Cytokine mapping of sera from women with preeclampsia and normal pregnancies. *J Reprod Immunol* 70(1-2): 83-91

Jousilahti P, Vartiainen E, Tuomilehto J and Puska P (1999) Sex, Age, Cardiovascular Risk Factors, and Coronary Heart Disease. *A Prospective Follow-Up Study of 14 786 Middle-Aged Men and Women in Finland* 99(9): 1165-1172

Juhan-Vague I, Alessi MC and Vague P (1996) Thrombogenic and Fibrinolytic Factors and Cardiovascular Risk in Non-insulin-dependent Diabetes Mellitus. *Annals of Medicine* 28(4): 371-380

Kadyrov M, Schmitz C, Black S, Kaufmann P and Huppertz B (2003) Pre-eclampsia and Maternal Anaemia Display Reduced Apoptosis and Opposite Invasive Phenotypes of Extravillous Trophoblast. *Placenta* 24(5): 540-548

Karami M, Jenabi E and Fereidooni B (2018) The association of placenta previa and assisted reproductive techniques: a meta-analysis. *The Journal of Maternal-Fetal & Neonatal Medicine* 31(14): 1940-1947

Kathleen Lewis J, Wei J and Siuzdak G (2006) *Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry in Peptide and Protein Analysis*.

Kathpalia SK, Arora D, Sandhu N and Sinha P (2018) Ectopic pregnancy: Review of 80 cases. *Medical Journal Armed Forces India* 74(2): 172-176

Kawamura H, Li X, Goishi K, van Meeteren LA, Jakobsson L, Cebe-Suarez S, Shimizu A, Edholm D, Ballmer-Hofer K, Kjellen L, Klagsbrun M and Claesson-Welsh L (2008) Neuropilin-1 in regulation of VEGF-induced activation of p38MAPK and endothelial cell organization. *Blood* 112(9): 3638-49

Kenny LC and Kell DB (2018) Immunological Tolerance, Pregnancy, and Preeclampsia: The Roles of Semen Microbes and the Father†. *Frontiers in Medicine* 4(239)

Kestenbaum B, Seliger SL, Easterling TR, Gillen DL, Critchlow CW, Stehman-Breen CO and Schwartz SM (2003) Cardiovascular and thromboembolic events following hypertensive pregnancy. *American Journal of Kidney Diseases* 42(5): 982-989

Kim GH (2013) MicroRNA regulation of cardiac conduction and arrhythmias. *Transl Res* 161(5): 381-392

Kintiraki E and Goulis DG (2018) Gestational Diabetes Mellitus: multi-disciplinary treatment approaches. *Metabolism*

Klonoff-Cohen HS, Savitz DA, Cefalo RC and McCann MF (1989) An epidemiologic study of contraception and preeclampsia. *Jama* 262(22): 3143-7

Koga K, Osuga Y, Yoshino O, Hirota Y, Ruimeng X, Hirata T, Takeda S, Yano T, Tsutsumi O and Taketani Y (2003) Elevated serum soluble vascular endothelial growth factor receptor 1 (sVEGFR-1) levels in women with preeclampsia. *The Journal of clinical endocrinology and metabolism* 88(5): 2348-51

Kozomara A, Birgaoanu M and Griffiths-Jones S (2018) miRBase: from microRNA sequences to function. *Nucleic Acids Res* 47(D1): D155-D162

Kranendonk ME, Visseren FL, van Balkom BW, Nolte-'t Hoen EN, van Herwaarden JA, de Jager W, Schipper HS, Brenkman AB, Verhaar MC, Wauben MH and Kalkhoven E (2014a) Human adipocyte extracellular vesicles in reciprocal signaling between adipocytes and macrophages. *Obesity (Silver Spring, Md.)* 22(5): 1296-308

Kranendonk ME, Visseren FL, van Herwaarden JA, Nolte-'t Hoen EN, de Jager W, Wauben MH and Kalkhoven E (2014b) Effect of extracellular vesicles of human adipose tissue on insulin signaling in liver and muscle cells. *Obesity (Silver Spring, Md.)* 22(10): 2216-23

Krauss T, Kuhn W, Lakoma C and Augustin HG (1997) Circulating endothelial cell adhesion molecules as diagnostic markers for the early identification of pregnant women at risk for development of preeclampsia. *American journal of obstetrics and gynecology* 177(2): 443-9

Kukk E, Lymboussaki A, Taira S, Kaipainen A, Jeltsch M, Joukov V and Alitalo K (1996) VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. *Development (Cambridge, England)* 122(12): 3829-37

Kumar A, Begum N, Prasad S, Agarwal S and Sharma S (2013) IL-10, TNF-α & IFN-γ: potential early biomarkers for preeclampsia. *Cellular immunology* 283(1-2): 70-74

Kumar R, Beguin S and Hemker HC (1995) The effect of fibrin clots and clot-bound thrombin on the development of platelet procoagulant activity. *Thrombosis and Haemostasis* 74(3): 962-968

Kvehaugen AS, Dechend R, Ramstad HB, Troisi R, Fugelseth D and Staff AC (2011) Endothelial Function and Circulating Biomarkers Are Disturbed in Women and Children After Preeclampsia. *Hypertension* 58(1): 63-69

Kwiatkowski S, Kwiatkowska E, Rzepka R, Torbe A and Dolegowska B (2016) Ischemic placental syndrome – prediction and new disease monitoring. *The Journal of Maternal-Fetal & Neonatal Medicine* 29(12): 2033-2039

Lachmeijer AM, Arngrímsson R, Bastiaans EJ, Frigge ML, Pals G, Sigurdardóttir S, Stéfansson H, Pálsson B, Nicolae D, Kong A, Aarnoudse JG, Gulcher JR, Dekker GA, ten Kate LP and Stéfansson K (2001) A genome-wide scan for preeclampsia in the Netherlands. *European journal of human genetics : EJHG* 9(10): 758-64

Lachmeijer AMA, Dekker GA, Pals G, Aarnoudse JG, ten Kate LP and Arngrímsson R (2002) Searching for preeclampsia genes: the current position. *European Journal of Obstetrics & Gynecology and Reproductive Biology* 105(2): 94-113

Larkin SE, Johnston HE, Jackson TR, Jamieson DG, Roumeliotis TI, Mockridge CI, Michael A, Manousopoulou A, Papachristou EK, Brown MD, Clarke NW, Pandha H, Aukim-Hastie CL, Cragg MS, Garbis SD and Townsend PA (2016) Detection of candidate biomarkers of prostate cancer progression in serum: a depletion-free 3D LC/MS quantitative proteomics pilot study. *British journal of cancer* 115(9): 1078-1086

Lavandero S, Troncoso R, Rothermel BA, Martinet W, Sadoshima J and Hill JA (2013) Cardiovascular autophagy. *Autophagy* 9(10): 1455-1466

Lee RC, Feinbaum RL and Ambros V (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 75(5): 843-854

Lener T, Gimona M, Aigner L, Börger V, Buzas E, Camussi G, Chaput N, Chatterjee D, Court FA, Portillo HAd, O'Driscoll L, Fais S, Falcon-Perez JM, Felderhoff-Mueser U, Fraile L, Gho YS, Görgens A, Gupta RC, Hendrix A, Hermann DM, Hill AF, Hochberg F, Horn PA, Kleijn Dd, Kordelas L, Kramer BW, Krämer-Albers E-M, Laner-Plamberger S, Laitinen S, Leonardi T, Lorenowicz MJ, Lim SK, Lötvall J, Maguire CA, Marcilla A, Nazarenko I, Ochiya T, Patel T, Pedersen S, Pocsfalvi G, Pluchino S, Quesenberry P, Reischl IG, Rivera FJ, Sanzenbacher R, Schallmoser K, Slaper-Cortenbach I, Strunk D, Tonn T, Vader P, Balkom BWMv, Wauben M, Andaloussi SE, Théry C, Rohde E and Giebel B (2015) Applying extracellular vesicles based therapeutics in clinical trials – an ISEV position paper. *Journal of Extracellular Vesicles* 4(1): 30087

Leslie D and Collis R (2015) Hypertension in pregnancy. *BJA Education* 16(1): 33-37

Levine RJ, England LJ, Yu KF, Schisterman EF, Maynard SE, Epstein FH, Sukhatme VP, Karumanchi SA, Lim KH, Sachs BP, Qian C, Thadhani R and Sibai BM (2004) Circulating Angiogenic Factors and the Risk of Preeclampsia. *New England Journal of Medicine* 350(7): 672-683

Levine RJ, Hauth JC, Curet LB, Sibai BM, Catalano PM, Morris CD, DerSimonian R, Esterlitz JR, Raymond EG, Bild DE, Clemens JD, Cutler JA, Ewell MG, Friedman SA, Goldenberg RL, Jacobson S-L, Joffe GM, Klebanoff MA and Petrulis AS (1997) Trial of Calcium to Prevent Preeclampsia. *New England Journal of Medicine* 337(2): 69-77

Levine RJ and Karumanchi SA (2005) Circulating angiogenic factors in preeclampsia. *Clinical Obstetrics & Gynecology* 48(2): 372-386

Levine RJ, Lam C, Qian C, Yu KF, Maynard SE, Sachs BP, Sibai BM, Epstein FH, Romero R, Thadhani R and Karumanchi SA (2006) Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. *NEW ENGLAND JOURNAL OF MEDICINE* 355(10): 992-1005

Liberman AC, Refojo D and Arzt E (2003) Cytokine signaling/transcription factor cross-talk in T cell activation and Th1-Th2 differentiation. *Arch Immunol Ther Exp (Warsz)* 51(6): 351-65

Lie RT, Rasmussen S, Brunborg H, Gjessing HK, Lie-Nielsen E and Irgens LM (1998) Fetal and maternal contributions to risk of pre-eclampsia: population based study. *BMJ (Clinical research ed.)* 316(7141): 1343-1347

Lin J, Liu H, Wu DD, Hu HT, Wang HH, Zhou CL, Liu XM, Chen XJ, Sheng JZ and Huang HF (2019) Long interpregnancy interval and adverse perinatal outcomes: A retrospective cohort study. *Science China. Life sciences*

Lippi G and Favaloro EJ (2016) Allergy and Venous Thromboembolism: A Casual or Causative Association. *Semin Thromb Hemost* 42(01): 063-068

Liu B, Xu Y, Voss C, Qiu F-h, Zhao M-z, Liu Y-d, Nie J and Wang Z-l (2012) Altered Protein Expression in Gestational Diabetes Mellitus Placentas Provides Insight into Insulin Resistance and Coagulation/Fibrinolysis Pathways. *PloS one* 7(9): e44701

Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif.)* 25(4): 402-8

Luft FC (2006) Soluble endoglin (sEng) joins the soluble fms-like tyrosine kinase (sFlt) receptor as a pre-eclampsia molecule*Comment on Venkatesha S, Toporsian M, Lam C et al. Soluble endoglin contributes to the pathogenesis of preeclampsia. Nat Med 2006; 12. *Nephrology Dialysis Transplantation* 21(11): 3052

Lwaleed BA, Breish MO, Birch BR, Chowdhary AP, Saad RA, Perigo O, Kazmi RS, Dusse LM and Cooper AJ (2014) Tissue factor and tissue factor pathway inhibitor in women with a past history of preeclampsia: implication for a hypercoagulable state postpregnancy. *Blood Coagulation & Fibrinolysis* 25(7): 671-674

Lyall F (2005) Priming and remodelling of human placental bed spiral arteries during pregnancy – A Review. *Placenta* 26, Supplement: S31-S36

Lyall F, Greer IA, Boswell F and Fleming R (1997) Suppression of serum vascular endothelial growth factor immunoreactivity in normal pregnancy and in pre-eclampsia. *BJOG: An International Journal of Obstetrics & Gynaecology* 104(2): 223-228

Lykke JA, Langhoff-Roos J, Sibai BM, Funai EF, Triche EW and Paidas MJ (2009) Hypertensive Pregnancy Disorders and Subsequent Cardiovascular Morbidity and Type 2 Diabetes Mellitus in the Mother. *Hypertension* 53(6): 944

Macfarlane L-A and Murphy PR (2010) MicroRNA: Biogenesis, Function and Role in Cancer. *Curr Genomics* 11(7): 537-561

Macheret F, Bartz TM, Djousse L, Ix JH, Mukamal KJ, Zieman SJ, Siscovick DS, Tracy RP, Heckbert SR, Psaty BM and Kizer JR (2015) Higher circulating adiponectin levels are associated with increased risk of atrial fibrillation in older adults. *Heart* 101(17): 1368-1374

Magnussen EB, Vatten LJ, Smith GD and Romundstad PR (2009) Hypertensive disorders in pregnancy and subsequently measured cardiovascular risk factors. *Obstet Gynecol* 114(5): 961-70

Marbán E (2002) Cardiac channelopathies. *Nature* 415(6868): 213-218

Martin U, Davies C, Hayavi S, Hartland A and Dunne F (1999) Is normal pregnancy atherogenic? *Clinical science* 96(4): 421-5

Martineau MG, Raker C, Dixon PH, Chambers J, Machirori M, King NM, Hooks ML, Manoharan R, Chen K, Powrie R and Williamson C (2015) The Metabolic Profile of Intrahepatic Cholestasis of Pregnancy Is Associated With Impaired Glucose Tolerance, Dyslipidemia, and Increased Fetal Growth. *Diabetes Care* 38(2): 243

Matsubayashi H, Arai T, Izumi SI, Sugi T, McIntyre JA and Makino T (2001) Anti-annexin V antibodies in patients with early pregnancy loss or implantation failures. *Fertility and Sterility* 76(4): 694-699

Matsuzaki J, Tsuji T, Imazeki I, Ikeda H and Nishimura T (2005) Immunosteroid as a regulator for Th1/Th2 balance: Its possible role in autoimmune diseases. *Autoimmunity* 38(5): 369-375

Maynard SE and Karumanchi SA (2011) Angiogenic Factors and Preeclampsia. *Seminars in Nephrology* 31(1): 33-46

Maynard SE, Min J-Y, Merchan J, Lim K-H, Li J, Mondal S, Libermann TA, Morgan JP, Sellke FW, Stillman IE, Epstein FH, Sukhatme VP and Karumanchi SA (2003) Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *Journal of Clinical Investigation* 111(5): 649-658

McGinnis R, Steinthorsdottir V, Williams NO, Thorleifsson G, Shooter S, Hjartardottir S, Bumpstead S, Stefansdottir L, Hildyard L, Sigurdsson JK, Kemp JP, Silva GB, Thomsen LCV, Jääskeläinen T, Kajantie E, Chappell S, Kalsheker N, Moffett A, Hiby S, Lee WK, Padmanabhan S, Simpson NAB, Dolby VA, Staines-Urias E, Engel SM, Haugan A, Trogstad L, Svyatova G, Zakhidova N, Najmutdinova D, Dominiczak AF, Gjessing HK, Casas JP, Dudbridge F, Walker JJ, Pipkin FB, Thorsteinsdottir U, Geirsson RT, Lawlor DA, Iversen AC, Magnus P, Laivuori H, Stefansson K and Morgan L (2017) Variants in the fetal genome near FLT1 are associated with risk of preeclampsia. *Nat Genet* 49(8): 1255-1260

Mestdagh P, Van Vlierberghe P, De Weer A, Muth D, Westermann F, Speleman F and Vandesompele J (2009) A novel and universal method for microRNA RT-qPCR data normalization. *Genome biology* 10(6): R64-R64

Mogren I, Högberg U, Winkvist A and Stenlund H (1999) Familial occurrence of preeclampsia. *Epidemiology (Cambridge, Mass.)* 10(5): 518-22

Moldenhauer JS, Stanek J, Warshak C, Khoury J and Sibai B (2003) The frequency and severity of placental findings in women with preeclampsia are gestational age dependent. *American journal of obstetrics and gynecology* 189(4): 1173-1177

Molvarec A, Szarka A, Walentin S, Beko G, Karadi I, Prohaszka Z and Rigo J, Jr. (2011) Serum leptin levels in relation to circulating cytokines, chemokines, adhesion molecules and angiogenic factors in normal pregnancy and preeclampsia. *Reproductive biology and endocrinology : RB&E* 9: 124

Mongraw-Chaffin ML, Cirillo PM and Cohn BA (2010) Preeclampsia and cardiovascular disease death: prospective evidence from the child health and development studies cohort. *Hypertension* 56(1): 166-71

Moses EK, Lade JA, Guo G, Wilton AN, Grehan M, Freed K, Borg A, Terwilliger JD, North R, Cooper DW and Brennecke SP (2000) A genome scan in families from Australia and New Zealand confirms the presence of a maternal susceptibility locus for pre-eclampsia, on chromosome 2. *American journal of human genetics* 67(6): 1581-5

Moskowitz IPG, Kim JB, Moore ML, Wolf CM, Peterson MA, Shendure J, Nobrega MA, Yokota Y, Berul C, Izumo S, Seidman JG and Seidman CE (2007) A Molecular Pathway Including Id2, Tbx5, and Nkx2-5 Required for Cardiac Conduction System Development. *Cell* 129(7): 1365-1376

Moustafa S, Patton DJ and Connelly MS (2011) Unusual cardiac presentation in neurofibromatosis type 1. *European Heart Journal - Cardiovascular Imaging* 12(12): 972-972

Murphy MS, Bytautiene E, Saade G and Smith GN (2015) Alterations to the maternal circulating proteome after preeclampsia. *American journal of obstetrics and gynecology* 213(6): 853.e1-9

Mussunoor S and Murray G (2008) The role of annexins in tumour development and progression. *The Journal of Pathology* 216(2): 131-140

Muszbek L, Bereczky Z, Bagoly Z, Komáromi I and Katona É (2011) Factor XIII: A Coagulation Factor With Multiple Plasmatic and Cellular Functions. *Physiological Reviews* 91(3): 931-972

NAKAO H, WATANABE M and MAKI M (1994) A new function of calphobindin I (annexin V). *European Journal of Biochemistry* 223(3): 901-908

Ness RB and Roberts JM (1996) Heterogeneous causes constituting the single syndrome of preeclampsia: a hypothesis and its implications. *American journal of obstetrics and gynecology* 175(5): 1365-70

Ness RB and Sibai BM (2006) Shared and disparate components of the pathophysiologies of fetal growth restriction and preeclampsia. *American journal of obstetrics and gynecology* 195(1): 40-49

Noori M, Donald AE, Angelakopoulou A, Hingorani AD and Williams DJ (2010) Prospective Study of Placental Angiogenic Factors and Maternal Vascular Function Before and After Preeclampsia and Gestational Hypertension. *Circulation* 122(5): 478-487

Noris M, Perico N and Remuzzi G (2005) Mechanisms of Disease: pre-eclampsia. *Nat Clin Pract Neph* 1(2): 98-114

Norwitz ER, Tsen LC, Park JS, Fitzpatrick PA, Dorfman DM, Saade GR, Buhimschi CS and Buhimschi IA (2005) Discriminatory proteomic biomarker analysis identifies free hemoglobin in the

cerebrospinal fluid of women with severe preeclampsia. *American journal of obstetrics and gynecology* 193(3, Supplement): 957-964

O'Brien M (2012) The Reciprocal Relationship Between Inflammation and Coagulation. *Topics in Companion Animal Medicine* 27(2): 46-52

O'Donnell CJ and Elosua R (2008) Cardiovascular Risk Factors. Insights From Framingham Heart Study. *Revista Española de Cardiología (English Edition)* 61(03): 299-310

ØIan P, OmsjØ I, Maltau JM and ØSterud B (1985) Increased sensitivity to thromboplastin synthesis in blood monocytes from pre-eclamptic patients. *BJOG: An International Journal of Obstetrics & Gynaecology* 92(5): 511-517

Olofsson A, Mallouh V and Brisson A (1994) Two-Dimensional Structure of Membrane-Bound Annexin V at 8 Å Resolution. *Journal of Structural Biology* 113(3): 199-205

Olsson A-K, Dimberg A, Kreuger J and Claesson-Welsh L (2006) VEGF receptor signalling ? in control of vascular function. *Nat Rev Mol Cell Biol* 7(5): 359-371

Ornaghi S, Vergani P, Urban G, Giardini V, Moltrasio F and Leone BE (2011) Immunohistochemical expression of Annexin A5 in preeclamptic placentas. *Placenta* 32(3): 264-268

Ostendorf T, De Vriese AS and Floege J (2007) Renal side effects of anti-VEGF therapy in man: a new test system*. *Nephrology Dialysis Transplantation* 22(10): 2778-2780

Padmavati R (2016) Metabolic syndrome, serious mental illnesses & lifestyle. *The Indian Journal of Medical Research* 143(4): 395-397

Parker SE, Jick SS and Werler MM (2016) Intrauterine device use and the risk of pre-eclampsia: a case-control study. *BJOG : an international journal of obstetrics and gynaecology* 123(5): 788-795

Pell JP, Smith GC and Walsh D (2004) Pregnancy complications and subsequent maternal cerebrovascular events: a retrospective cohort study of 119,668 births. *American journal of epidemiology* 159(4): 336-42

Peng B, Guo C, Guan H, Liu S and Sun M-Z (2014) Annexin A5 as a potential marker in tumors. *Clinica Chimica Acta* 427: 42-48

Peng Y and Croce CM (2016) The role of MicroRNAs in human cancer. *Signal Transduction And Targeted Therapy* 1: 15004

Perron MP and Provost P (2008) Protein interactions and complexes in human microRNA biogenesis and function. *Frontiers in bioscience : a journal and virtual library* 13: 2537-47

Petäjä J (2011) Inflammation and coagulation. An overview. *Thrombosis Research* 127: S34-S37

Petla L, Chikkala R, Ratnakar K, Kodati V and Sritharan V (2013) Biomarkers for the management of pre-eclampsia in pregnant women. *Indian Journal of Medical Research* 138(1): 60-67

Pijnenborg R, Anthony J, Davey DA, Rees A, Tiltman A, Vercruysse L and Van Assche A (1991) Placental bed spiral arteries in the hypertensive disorders of pregnancy. *BJOG: An International Journal of Obstetrics & Gynaecology* 98(7): 648-655

Pineles BL, Romero R, Montenegro D, Tarca AL, Han YM, Kim YM, Draghici S, Espinoza J, Kusanovic JP, Mittal P, Hassan SS and Kim CJ (2007) Distinct subsets of microRNAs are expressed differentially in the human placentas of patients with preeclampsia. *American journal of obstetrics and gynecology* 196(3): 261.e1-261.e6

Pocock G and Richards CD (2006) *Human Physiology: The basis of medicine*. OUP Oxford

Poston L (2006) Endothelial dysfunction in pre-eclampsia. *Pharmacological reports : PR* 58 Suppl: 69-74

Powers RW, Roberts JM, Cooper KM, Gallaher MJ, Frank MP, Harger GF and Ness RB (2005) Maternal serum soluble fms-like tyrosine kinase 1 concentrations are not increased in early pregnancy and decrease more slowly postpartum in women who develop preeclampsia. *American journal of obstetrics and gynecology* 193(1): 185-191

Pratt A, Da Silva Costa F, Borg AJ, Kalionis B, Keogh R and Murthi P (2015) Placenta-derived angiogenic proteins and their contribution to the pathogenesis of preeclampsia. *Angiogenesis* 18(2): 115-123

Qian W-J, Jacobs JM, Liu T, Camp DG and Smith RD (2006) Advances and Challenges in Liquid Chromatography-Mass Spectrometry Based Proteomic Profiling for Clinical Applications. *Molecular & cellular proteomics : MCP* 5(10): 1727-1744

Quinn KD, Cruickshank CI and Wood TD (2012) Ultra High-Mass Resolution Paper Spray by Fourier Transform Ion Cyclotron Resonance Mass Spectrometry. *International Journal of Analytical Chemistry* 2012: 6

Rafeeinia A, Tabandeh A, Khajeniazi S and Marjani A (2014) Metabolic Syndrome in Preeclampsia Women in Gorgan. *The Open Biochemistry Journal* 8: 94-99

Rana S, Karumanchi SA, Levine RJ, Venkatesha S, Rauh-Hain JA, Tamez H and Thadhani R (2007) Sequential changes in antiangiogenic factors in early pregnancy and risk of developing preeclampsia. *Hypertension* 50(1): 137-42

Red-Horse K, Zhou Y, Genbacev O, Prakobphol A, Foulk R, McMaster M and Fisher SJ (2004) Trophoblast differentiation during embryo implantation and formation of the maternal-fetal interface. *The Journal of Clinical Investigation* 114(6): 744-754

Redman CW and Sargent IL (2005) Latest advances in understanding preeclampsia. *Science (New York, N.Y.)* 308(5728): 1592-4

Redman CW and Sargent IL (2007) Microparticles and immunomodulation in pregnancy and pre-eclampsia. *J Reprod Immunol* 76(1-2): 61-7

Redman CWG (2014) Diagnostic and predictive accuracy of placental growth factor in suspected pre-eclampsia. *Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health* 4: 241

Redman CWG and Jefferies M (1988) REVISED DEFINITION OF PRE-ECLAMPSIA. *The Lancet* 331(8589): 809-812

Reutelingsperger CPM and van Heerde WL (1997) Annexin V, the regulator of phosphatidylserine-catalyzed inflammation and coagulation during apoptosis. *Cellular and Molecular Life Sciences CMLS* 53(6): 527-532

Roberts DJ and Post MD (2008) The placenta in pre-eclampsia and intrauterine growth restriction. *Journal of clinical pathology* 61(12): 1254-60

Roberts JM and Cooper DW (2001) Pathogenesis and genetics of pre-eclampsia. *Lancet (London, England)* 357(9249): 53-6

Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G and Mesirov JP (2011) Integrative genomics viewer. *Nature Biotechnology* 29: 24

Robinson MD and Oshlack A (2010) A scaling normalization method for differential expression analysis of RNA-seq data. *Genome biology* 11(3): R25-R25

Rodie VA, Freeman DJ, Sattar N and Greer IA (2004) Pre-eclampsia and cardiovascular disease: metabolic syndrome of pregnancy? *Atherosclerosis* 175(2): 189-202

Romero R, Nien JK, Espinoza J, Kusanovic JP, Gotsch F, Erez O, Edwin S, Mazaki-Tovi S, Chaiworapongsa T, Todem D, Fu W, Chung H, Gomez R, Levine RJ and Karumanchi SA (2008) A longitudinal study of angiogenic (placental growth factor) and anti-angiogenic (soluble endoglin and soluble vascular endothelial growth factor receptor-1) factors in normal pregnancy and patients destined to develop preeclampsia and deliver a small for gestational age neonate. *Journal of Maternal-Fetal and Neonatal Medicine* 21(1): 9-23

Rousseau A, Favier R and Van Dreden P (2009) Elevated circulating soluble thrombomodulin activity, tissue factor activity and circulating procoagulant phospholipids: New and useful markers for pre-eclampsia? *European Journal of Obstetrics & Gynecology and Reproductive Biology* 146(1): 46-49

Rowe T (2014) Placenta prævia. *Journal of Obstetrics and Gynaecology Canada* 36: 669-670

Sanders KA, Benton MC, Lea RA, Maltby VE, Agland S, Griffin N, Scott RJ, Tajouri L and Lechner-Scott J (2016) Next-generation sequencing reveals broad down-regulation of microRNAs in secondary progressive multiple sclerosis CD4+ T cells. *Clin Epigenetics* 8(1): 87-87

Sandset PM, Hellgren M, Uvebrandt M and Bergström H (1989) Extrinsic coagulation pathway inhibitor and heparin cofactor II during normal and hypertensive pregnancy. *Thrombosis Research* 55(5): 665-670

Sanghavi M and Rutherford JD (2014) Cardiovascular Physiology of Pregnancy. *Circulation* 130(12): 1003-1008

Sano M, Matsumoto M, Terada H, Wang H, Kurihara Y, Wada N, Yamamoto H, Kira Y, Tachibana D and Koyama M (2014) Increased annexin A2 expression in the placenta of women with acute worsening of preeclampsia. *Osaka city medical journal* 60(2): 87-93

Santamaria X and Taylor H (2014) MicroRNA and gynecological reproductive diseases. *Fertility and Sterility* 101(6): 1545-1551

Sargolzaei J, Chamani E, Kazemi T, Fallah S and Soori H (2018) The role of adiponectin and adipolin as anti-inflammatory adipokines in the formation of macrophage foam cells and their association with cardiovascular diseases. *Clinical Biochemistry* 54: 1-10

Sayed ASM, Xia K, Salma U, Yang T and Peng J (2014) Diagnosis, Prognosis and Therapeutic Role of Circulating miRNAs in Cardiovascular Diseases. *Heart, Lung and Circulation* 23(6): 503-510

Schure MR and Moran RE (2017) Size exclusion chromatography with superficially porous particles. *Journal of Chromatography A* 1480: 11-19

Schutters K and Reutelingsperger C (2010) Phosphatidylserine targeting for diagnosis and treatment of human diseases. *Apoptosis* 15(9): 1072-1082

Sharma A, Satyam A and Sharma JB (2007) Leptin, IL-10 and Inflammatory Markers (TNF-α, IL-6 and IL-8) in Pre-Eclamptic, Normotensive Pregnant and Healthy Non-Pregnant Women. *American Journal of Reproductive Immunology* 58(1): 21-30

Shin JS, Choi MY, Longtine MS and Nelson DM (2010) Vitamin D effects on pregnancy and the placenta. *Placenta* 31(12): 1027-1034

Shukla GC, Singh J and Barik S (2011) MicroRNAs: Processing, Maturation, Target Recognition and Regulatory Functions. *Mol Cell Pharmacol* 3(3): 83-92

Sibai BM, Caritis SN, Thom E, Klebanoff M, McNellis D, Rocco L, Paul RH, Romero R, Witter F, Rosen M, Depp R, Health tNIoC and Units HDNoM-FM (1993) Prevention of Preeclampsia with Low-Dose Aspirin in Healthy, Nulliparous Pregnant Women. *New England Journal of Medicine* 329(17): 1213-1218

Sibai BM, Taslimi MM, El-Nazer A, Amon E, Mabie BC and Ryan GM (1986) Maternal-perinatal outcome associated with the syndrome of hemolysis, elevated liver enzymes, and low platelets in severe preeclampsia-eclampsia. *American journal of obstetrics and gynecology* 155(3): 501-507

Sikkema JM, van Rijn BB, Franx A, Bruinse HW, de Roos R, Stroes ES and van Faassen EE (2001) Placental superoxide is increased in pre-eclampsia. *Placenta* 22(4): 304-8

Silasi M, Rana S, Powe C, Cohen B, Lim KH, Zsengeller ZK, Karumanchi SA and Stillman IE (2011) Placental expression of angiogenic factors in Trisomy 13. *American journal of obstetrics and gynecology* 204(6): 546.e1-4

Skjærven R, Vatten LJ, Wilcox AJ, Rønning T, Irgens LM and Lie RT (2005) Recurrence of pre-eclampsia across generations: exploring fetal and maternal genetic components in a population based cohort. *BMJ* 331(7521): 877

Smith GCS, Pell JP and Walsh D (2001) Pregnancy complications and maternal risk of ischaemic heart disease: a retrospective cohort study of 129 290 births. *The Lancet* 357(9273): 2002-2006

Somogyi Á (2008) Chapter 6 - Mass spectrometry instrumentation and techniques A2 - Vékey, Károly IN: Telekes A and Vertes A (eds) *Medical Applications of Mass Spectrometry*. Amsterdam: Elsevier 93-140

Sower LE, Froelich CJ, Carney DH, Fenton JW and Klimpel GR (1995) Thrombin induces IL-6 production in fibroblasts and epithelial cells. Evidence for the involvement of the seven-transmembrane domain (STD) receptor for alpha-thrombin. *The Journal of Immunology* 155(2): 895-901

Steegers EAP, Von Dadelszen P, Duvekot JJ and Pijnenborg R (2010) Pre-eclampsia. *The Lancet* 376(9741): 631-644

Stefan N, Häring H-U, Hu FB and Schulze MB (2013) Metabolically healthy obesity: epidemiology, mechanisms, and clinical implications. *The Lancet Diabetes & Endocrinology* 1(2): 152-162

Stefan N, Vozarova B, Funahashi T, Matsuzawa Y, Weyer C, Lindsay RS, Youngren JF, Havel PJ, Pratley RE, Bogardus C and Tataranni PA (2002) Plasma adiponectin concentration is associated with skeletal muscle insulin receptor tyrosine phosphorylation, and low plasma concentration precedes a decrease in whole-body insulin sensitivity in humans. *Diabetes* 51(6): 1884-8

Stewart D, Dhungana S, Clark R, Pathmasiri W, McRitchie S and Sumner S (2015) Chapter 4 - Omics Technologies Used in Systems Biology A2 - Fry, Rebecca C *Systems Biology in Toxicology and Environmental Health*. Boston: Academic Press 57-83

Sucak GT, Acar K, Sucak A, Kirazli S and Haznedar R (2006) Increased global fibrinolytic capacity as a clue for activated fibrinolysis in pre-eclampsia. *Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis* 17(5): 347-52

Sutherland MR, Bertagnolli M, Lukaszewski M-A, Huyard F, Yzydorczyk C, Luu TM and Nuyt AM (2014) Preterm Birth and Hypertension Risk. *Hypertension* 63(1): 12

Swieringa F, Spronk HMH, Heemskerk JWM and van der Meijden PEJ (2018) Integrating platelet and coagulation activation in fibrin clot formation. *Res Pract Thromb Haemost* 2(3): 450-460

Szarka A, Rigó J, Lázár L, Bekő G and Molvarec A (2010) Circulating cytokines, chemokines and adhesion molecules in normal pregnancy and preeclampsia determined by multiplex suspension array. *BMC Immunology* 11: 59-59

Szymonowicz W and Yu VY (1987) Severe pre-eclampsia and infants of very low birth weight. *Arch Dis Child* 62(7): 712-716

Tanaka K, Waki H, Ido Y, Akita S, Yoshida Y, Yoshida T and Matsuo T (1988) Protein and polymer analyses up to m/z 100 000 by laser ionization time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry* 2(8): 151-153

Tang N, Tornatore P and Weinberger SR (2004) Current developments in SELDI affinity technology. *Mass Spectrometry Reviews* 23(1): 34-44

Teasdale F (1985) Histomorphometry of the human placenta in maternal preeclampsia. *American journal of obstetrics and gynecology* 152(1): 25-31

Tenschert W, Holdener EE, Haertel MM, Senn H and Vetter W (1985) Secondary hypertension and neurofibromatosis: Bilateral renal artery stenosis and coarctation of the abdominal aorta. *Klinische Wochenschrift* 63(13): 593-596

Thadhani R, Mutter WP, Wolf M, Levine RJ, Taylor RN, Sukhatme VP, Ecker J and Karumanchi SA (2004) First trimester placental growth factor and soluble fms-like tyrosine kinase 1 and risk for preeclampsia. *The Journal of clinical endocrinology and metabolism* 89(2): 770-5

Thorvaldsdóttir H, Robinson JT and Mesirov JP (2013) Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Brief Bioinform* 14(2): 178-192

Tjoa ML, Khankin EV, Rana S and Karumanchi SA (2010) Angiogenic factors and preeclampsia *Placental Bed Disorders: Basic Science and its Translation to Obstetrics*. 229-242

Tranquilli AL, Dekker G, Magee L, Roberts J, Sibai BM, Steyn W, Zeeman GG and Brown MA (2014) The classification, diagnosis and management of the hypertensive disorders of pregnancy: A revised statement from the ISSHP. *Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health* 4(2): 97-104

Trott DW and Harrison DG (2014) The immune system in hypertension. *Advances in Physiology Education* 38(1): 20-24

Tubbergen P, Lachmeijer AMA, Althuisius SM, Vlak MEJ, van Geijn HP and Dekker GA (1999) Change in paternity: a risk factor for preeclampsia in multiparous women? *Journal of Reproductive Immunology* 45(1): 81-88

Tuohy JF and James DK (1992) Pre-eclampsia and trisomy 13. *British journal of obstetrics and gynaecology* 99(11): 891-4

van der Poll T, de Jonge E and Levi M (2001) Regulatory role of cytokines in disseminated intravascular coagulation. *Semin Thromb Hemost* 27(6): 639-51

van Dijk M, Mulders J, Poutsma A, Könst AA, Lachmeijer AM, Dekker GA, Blankenstein MA and Oudejans CB (2005) Maternal segregation of the Dutch preeclampsia locus at 10q22 with a new member of the winged helix gene family. *Nat Genet* 37(5): 514-9

van Eijsden M, Smits LJ, van der Wal MF and Bonsel GJ (2008) Association between short interpregnancy intervals and term birth weight: the role of folate depletion. *The American journal of clinical nutrition* 88(1): 147-53

van Engeland M, Nieland LJ, Ramaekers FC, Schutte B and Reutelingsperger CP (1998) Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* 31(1): 1-9

van Genderen HO, Kenis H, Hofstra L, Narula J and Reutelingsperger CPM (2008) Extracellular annexin A5: Functions of phosphatidylserine-binding and two-dimensional crystallization. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1783(6): 953-963

van Rijn BB, Bruinse HW, Veerbeek JH, Post Uiterweer ED, Koenen SV, van der Bom JG, Rijkers GT, Roest M and Franx A (2016) Postpartum Circulating Markers of Inflammation and the Systemic Acute-Phase Response After Early-Onset Preeclampsia. *Hypertension* 67(2): 404-14

Vascotto C, Salzano AM, D'Ambrosio C, Fruscalzo A, Marchesoni D, di loreto C, Scaloni A, Tell G and Quadrifoglio F (2007) *Oxidized Transthyretin in Amniotic Fluid as an Early Marker of Preeclampsia*.

Venkatesha S, Lam C, Hanai JI, Mammoto T, Bdolah Y, Lim KH, Yuan HT, Libermann TA, Stillman IE, Epstein FH, Sellke FW, Sukhatme VP, Karumanchi SA, Toporsian M, Letarte M, Kim YM, Romero R, Roberts D and D'Amore PA (2006) Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nature Medicine* 12(6): 642-649

Visser N, van Rijn BB, Rijkers GT, Franx A and Bruinse HW (2007) Inflammatory changes in preeclampsia: current understanding of the maternal innate and adaptive immune response. *Obstetrical & gynecological survey* 62(3): 191-201

von Dadelszen P, Magee LA and Roberts JM (2003) Subclassification of Preeclampsia. *Hypertension in pregnancy* 22(2): 143-148

Wakili R, Voigt N, Kaab S, Dobrev D and Nattel S (2011) Recent advances in the molecular pathophysiology of atrial fibrillation. *J Clin Invest* 121(8): 2955-68

Walker JJ (2000) Pre-eclampsia. *The Lancet* 356(9237): 1260-1265

Walker JJ (2007) Ectopic pregnancy. *Clinical Obstetrics and Gynecology* 50(1): 89-99

Walker JJ (2011) Inflammation and preeclampsia. *Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health* 1(1): 43-47

Wallner W, Sengenberger R, Strick R, Strissel Pamela L, Meurer B, Beckmann Matthias W and Schlembach D (2007) Angiogenic growth factors in maternal and fetal serum in pregnancies complicated by intrauterine growth restriction. *Clinical science* 112(1): 51-57

Wang IK, Muo C-H, Chang Y-C, Liang C-C, Chang C-T, Lin S-Y, Yen T-H, Chuang F-R, Chen P-C, Huang C-C, Wen C-P, Sung F-C and Morisky DE (2013) Association between hypertensive disorders during pregnancy and end-stage renal disease: a population-based study. *CMAJ : Canadian Medical Association Journal* 185(3): 207-213

Wang Y, Cheng Y-S, Yin X-Q, Yu G and Jia B-L (2019) Anxa2 gene silencing attenuates obesity-induced insulin resistance by suppressing the NF-κB signaling pathway. *American Journal of Physiology-Cell Physiology* 316(2): C223-C234

WikstrÖM A-K, Larsson A, Eriksson UJ, Nash P and Olovsson M (2008) Early postpartum changes in circulating pro- and anti-angiogenic factors in early-onset and late-onset pre-eclampsia. *Acta Obstetricia et Gynecologica Scandinavica* 87(2): 146-153

Wilkins MR, Sanchez JC, Gooley AA, Appel RD, Humphery-Smith I, Hochstrasser DF and Williams KL (1996) Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. *Biotechnology & genetic engineering reviews* 13: 19-50

Wolf M, Hubel CA, Lam C, Sampson M, Ecker JL, Ness RB, Rajakumar A, Daftary A, Shakir AS, Seely EW, Roberts JM, Sukhatme VP, Karumanchi SA and Thadhani R (2004) Preeclampsia and future cardiovascular disease: potential role of altered angiogenesis and insulin resistance. *The Journal of clinical endocrinology and metabolism* 89(12): 6239-43

Woolard J, Bevan Hs, Harper SJ and Bates DO (2009) Molecular Diversity of VEGF-A as a Regulator of Its Biological Activity. *Microcirculation* 16(7): 572-592

Wu Y, Wei B, Liu H, Li T and Rayner S (2011) MiRPara: a SVM-based software tool for prediction of most probable microRNA coding regions in genome scale sequences. *BMC Bioinformatics* 12(1): 107

Xin H, Zhang Y, Wang H and Sun S (2012) Alterations of profibrinolytic receptor annexin A2 in pre-eclampsia: a possible role in placental thrombin formation. *Thromb Res* 129(5): 563-7

Xu J-J, Liu K-Q, Ying Z-M, Zhu X-W, Xu X-J, Zhao P-P, Bai W-Y, Qiu M-C, Zhang X-W and Zheng H-F (2019) Effect of CD14 polymorphisms on the risk of cardiovascular disease: evidence from a meta-analysis. *Lipids in Health and Disease* 18(1): 74

Yang J, Hartmann KE, Savitz DA, Herring AH, Dole N, Olshan AF and Thorp JM, Jr. (2004) Vaginal bleeding during pregnancy and preterm birth. *American journal of epidemiology* 160(2): 118-25

Yi F, Shang Y, Li B, Dai S, Wu W, Cheng L and Wang X (2017) MicroRNA-193-5p modulates angiogenesis through IGF2 in type 2 diabetic cardiomyopathy. *Biochemical and Biophysical Research Communications* 491(4): 876-882

Yu M, Xue Y, Zheng J, Liu X, Yu H, Liu L, Li Z and Liu Y (2017) Linc00152 promotes malignant progression of glioma stem cells by regulating miR-103a-3p/FEZF1/CDC25A pathway. *Molecular cancer* 16(1): 110

Yuan L-Q, de Jesus Perez V, Liao X-B, Kr, #xf3, l M and Yeh C-H (2017) MicroRNA and Cardiovascular Disease 2016. *BioMed Research International* 2017: 2

Zachos M, Parkin PC, Babyn PS and Chait P (1997) Neurofibromatosis type 1 vasculopathy associated with lower limb hypoplasia. *Pediatrics* 100(3): 395-397

Zeng Z, Liu F and Li S (2017) Metabolic Adaptations in Pregnancy: A Review. *Annals of Nutrition and Metabolism* 70(1): 59-65

Zhang C, Zhang C, Wang H, Qi Y, Kan Y and Ge Z (2019) Effects of miR103a3p on the autophagy and apoptosis of cardiomyocytes by regulating Atg5. *International journal of molecular medicine* 43(5): 1951-1960

Zhao W, Qiao J, Zhang Q, Zhao Y and Chen Q (2010) Levels of antiangiogenic factors in preeclamptic pregnancies. *Growth Factors* 28(4): 293-298

Zhou Y, Damsky CH and Fisher SJ (1997) Preeclampsia is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype. One cause of defective endovascular invasion in this syndrome? *The Journal of Clinical Investigation* 99(9): 2152-2164

Zhu B-P and Le T (2003) Effect of Interpregnancy Interval on Infant Low Birth Weight: A Retrospective Cohort Study Using the Michigan Maternally Linked Birth Database. *Maternal and Child Health Journal* 7(3): 169-178

Zoet GA, Benschop L, Boersma E, Budde RPJ, Fauser BCJM, Graaf Yvd, Groot CJMd, Maas AHEM, Lennep JERv, Steegers EAP, Visseren FL, Rijn BBv, Velthuis BK, Franx A, Appelman YE, Baart SJ, Brouwers L, Cannegieter SC, Dam V, Eijkemans MCJ, Ferrari MD, Gunning MN, Hoek A, Koffijberg E, Koster MPH, Kruit M, Lagerwij GR, Lambalk CB, Laven JS, Linstra K, Lugt Avd, Brink AMvd, Meun C, Middeldorp S, Moons KGM, Roos-Hesselink JW, Scheres LJJ, Steegers-Theunissen RPM, Terwindt GM and Wermer MJH (2018) Prevalence of Subclinical Coronary Artery Disease Assessed by Coronary Computed Tomography Angiography in 45- to 55-Year-Old Women With a History of Preeclampsia. *Circulation* 137(8): 877-879