The diagnostic and prognostic role of proteomics and metabolomics in POLYCYSTIC OVARY SYNDROME

By

GULAFSHANA HAFEEZ KHAN
MBBS, FCPS, FRCOG

A thesis submitted to the University of Nottingham for the degree of

DOCTOR OF PHILOSOPHY
(PhD)

School of Medicine

July 2016

I dedicate this work to Baba, Babo, Rashid, Zarmala and Zarmina.

COPYRIGHT STATEMENT

This copy of the thesis has been supplied on the condition that anyone who consults it is understood to recognise that its copyright rests with the author and that no quotation from the thesis and no information derived from it may be published without the author's prior written consent.

GULAFSHANA HAFEEZ KHAN

The diagnostic and prognostic role of proteomics and metabolomics in Polycystic Ovarian Syndrome

ABSTRACT

Polycystic ovarian syndrome (PCOS) is a common endocrine disorder, affecting up to 10% -26% of women of reproductive age. Diagnostic criteria is somewhat controversial but ever evolving.

PCOS is characterized by an adverse metabolic profile (dyslipidaemia, diabetes, obesity), increased susceptibility to develop pregnancy complications like miscarriage, preterm labour, pre-eclampsia (PE) and cardiovascular disease (CVD) later in life. Predicting which women with PCOS will go on to develop PE and other major long-term health implications, however remains a challenge in routine clinical practice.

We embarked on a journey to explore whether proteomic, and metabolomics studies would unravel the pathophysiology and help us understand the diagnostic criteria and prognostic features of Polysystic ovarian syndrome.

The studies in this thesis aimed to address some of these challenges through the identification of a proteomic and metabolomic biomarker fingerprint unique to PCOS that could aid diagnosis.This might help improve our understanding of it's aetiology and if associated with PE, form the basis of future validation studies to aid the prediction of which women with PCOS were more likely to develop PE.

The null hypothesis was that women with PCOS did not have a proteomic and metabolomic and genomic profile unique to PCOS and PE. That, computational analysis would not reveal a single unifying pathway which could improve the diagnosis of PCOS and predict which women with PCOS would develop long term health complications and PE.

First, a systematic review was performed to identify proteomic biomarkers similarly expressed in women with PE and PCOS.This was to help the subsequent synthesis of these results with primary metabolomics data and obtain a comprehensive overview of novel pathways potentially linking PCOS with PE. One hundred

and ninety two proteomic biomarkers for PE were cross-referenced with a publicly available PCOS proteomic biomarker database to determine if any were also differentially expressed in PCOS. Five biomarkers were found to be differentially expressed in women with PE and with PCOS compared to controls. These included transferrin, fibrinogen alpha, beta and gamma chain variants and kininogen-1 which were raised and annexin 2 and peroxiredoxin 2 were decreased in both women with PCOS and with PE. In PE these biomarkers were found in serum, plasma and placenta respectively whereas in PCOS the biomarkers identified were in serum, follicular fluid, ovarian and omental biopsy respectively.

Next, a cross sectional study involving 59 women with PCOS and 61 controls was undertaken to identify oxylipin biomarkers (metabolomics biomarkers markers of cardiovascular disease and PE) unique to PCOS. Fasting blood samples were collected and serum extracted after centrifugation for 10 minutes. Samples were stored at -80 degrees centigrade for later analysis. Solid Phase Extraction, Liquid Chromatograph Mass Spectrometry was used for analyzing the samples, which were tested for 20 oxylipins in a targeted manner. Weight, BMI and waist: hip ratio were all higher in PCOS cohort compared to the controls. The results found that of the 19 Oxylipins analysed in the plasma samples, 14,15 dihydroxyeicosatrienoic acid (DHET) AND 19 Hydroxyeicosatetraenoic acid (HETE) were found to be significantly raised in PCOS women compared to the controls ($p=<0.05$). When adjustments were made for BMI and waist: hip ratio, 14,15 DHET was found to be still significant but on the other hand, 19 HETE was only significantly high in PCOS group when adjusted for waist: hip ratio only.

Finally, a computer based network analysis of lipids was performed. A software was used to see whether there were any common pathways shared by PE and PCOS identified from the published metabolomic studies.

A literature search was performed on metabolomics (lipid) biomarkers in women with PE compared with controls. I also carried out the same exercise in the only published study on metabolomic lipid biomarkers expressed differently in women with PCOS compared with controls. Data was then inputted into a novel lipidomic (metabolomic lipid biomarkers) network containing all lipid reactions that are found in public databases to identify smaller regional networks and key genes that potentially played a pivotal role in the mechanisms of PCOS and PE. The network analysis on lipids in PCOS found 3 key genes (CHPT1,

PTDSS1 and PLD1).The network analysis of lipids in PE,revealed five genes (CPT1, CPT2, ACOT2, PTDSS1 and CHPT1).

Two genes (PTDSS1, CHPT1) were comparable in both the PE and PCOS lipids.However, PTDSS1 was the only gene similarly over expressed in both PCOS and PE.

I was not able to identify a single unifying pathway from the list of biomarkers and genes identified in my study. My null hypothesis was therefore not rejected. The proteomic biomarkers common to PCOS and PE are involved in a range of diverse molecular pathways including immunoregulation/inflammation, fibrinolysis,iron transport and blood coagulation.The oxylipins differentially expressed in PCOS are involved in cardio-protection, vasoconstriction, renal function, vascular tone and arterial pressure, inflammation, oxidative stress, diabetes, impaired insulin signalling, platelet aggregation and generation of thrombin. Finally, the two genes (PTDSS1, CHPT1) identified in both the PE and PCOS lipid network analyses are associated with Lenz-Majewski hyperostotic dwarfism, glycerophospholipid and cholesterol metabolism, kidney ischemia-reperfusion injury and the formation and maintenance of vesicular membranes.

As far as I know, this is the first study that has investigated the links between PCOS and PE using this unique combination of "omic" approaches complemented by computational analysis of a novel lipid network to identify novel genes and pathways linking PCOS and PE approach.

More mechanistic studies are however required to investigate the inter-relationships between the proteomic biomarkers, oxylipins and genes identified from my lipidomic network analysis to improve our understanding of the mechanisms linking PCOS and PE. The diverse mechanistic pathways identified in my study may however reflect the complexity of both PCOS and PE, and it may well be that a range of factors (molecular, lifestyle, environmental and intra-uterine factors) all interact to result in both phenotypes. Future research would ideally require large collaborations so that the methodology and interpretation of data is consistent.

ACKNOWLEDGEMENTS

I thank ALLAH for giving me the opportunity to undertake this work and complete it.

I don't have words to express my gratitude and respect for my supervisor Mr William Atiomo, whose constant guidance, encouragement and belief in me made me accomplish this valuable piece of work. His support helped me through all the phases of research. His collaboration through PCOS group in University of Nottingham facilitated various aspects of the study.

Professor D.Barrett, Dr Raheela Khan and their student Katrin Sanders for their help and support in undertaking the analysis of plasma for oxylipins.

Dr Robert Layfield in providing me his expert opinion in the systematic review on proteomics.

Dr Zeina Haoula who had started the study and directed me through the initial phases of the study process.

Mr Nick Raine Fenning and his team, Miriam and Luckaz (Nurture) who taught me to do 3-D transvaginal ultrasound and also guided me to use sono- AVC software.

Dr Charlie Hodgman and his PhD student Ferran Casbas, collaborated and used lipodomic pathway analysis using bioinformatics to find connectivity between PCOS and Pre-eclampsia.

Prof.Thornton, who always kept me on my toes.

Nick Bullimore, Division manager for assisting and helping at every step of the way.

Dr Andrea Venn, who made doing statistical analysis so easy
(Well it seemed that way at the time).

I am also thankful to the lab staff at clinical sciences that were always there when the centrifuge refused to budge.

I thank all the patients, staff and colleagues who participated in the study and without whom this study would not have been possible.

I am indebted to my parents, siblings, friends especially Shazia Sanaan and most of all my teachers who have always been very encouraging.

I am grateful to all that I have not mentioned here.

AUTHORS DECLARATION

I declare that I conducted this study under the direct supervision of Mr William Atiomo, Mr Nick Raine Fenning and co-researchers.
Dr Zeina Haoula prepared the study protocol and the study was registred with the Research ethics committee (REC) with reference no: 10/H0408/6 under the advice of Mr William Atiomo with the University of Nottingham as sponsor.

Following research ethics approval, The recruitment commenced with the support of nurses and medical staff in the Nottingham Diagnostic and Treatment Centre, Clinic 3 at the Queen's Medical Centre, Nottingham and the staff and colleagues at Queens Medical centre, Nottingham.
I was responsible for obtaining all the data and blood samples from the participants. Blood was sent for biochemical analysis to the laboratory at Queens Medical Centre, Nottingham.

I prepared the plasma samples and they were stored in the Clinical Sciences (CS) Building Laboratory that held a licence compliant with the UK the Human Tissue Authority Regulatory Act.

I provided the plasma samples in a full batch for storage in the School of Pharmacy, University of Nottingham after an agreement with Professor Barrett.

Professor Barrett in the School of Pharmacy, University of Nottingham, and his PhD student Katrin Sanders were responsible for the Oxylipin analysis of the plasma samples.

I analysed the data with the help of medical statisticians in the School of Medicine, Dr Andrea Venn, University of Nottingham, my supervisor and other researchers.

I did a systematic review of proteomic biomarkers in Pre- eclampsia and compared them with the proteomic biomarkers database for PCOS. The systematic review did show that five biomarkers were similarly expressed in both Pre-eclampsia and PCOS. This proved the previously published link between the two conditions.

I did further systematic review of the metabolomics in Pre-eclampsia and the lipids found in that review were linked with the lipodomic study in PCOS.This was facilitated by Dr Charlie Hodgman and his PhD student Ferran Casbas.Bioinformatics was used to find the link between PCOS and Pre-eclampsia.

PUBLICATIONS AND PRESENTATIONS

Shafiee MN, **Khan G**, Ariffin R, Abu J, Chapman C, Deen S, Nunns D, Barrett DA, Seedhouse C, Atiomo W. **Preventing endometrial cancer risk in polycystic ovarian syndrome (PCOS) women: could metformin help?** Gynecol Oncol. 2014 Jan; 132 (1): 248-53.doi: 10.1016/j.ygyno.2013.10.028. Epub 2013 Oct 30. Review**.**

Overlap of proteomic Biomarkers between PCOS and PE, database integration.15th April 2014.Biomarkers and Clinical Research. St Hilda's College. Oxford.

Gulafshana Hafeez Khan, Nicolas Galazis, Nikolina Docheva, Robert Layfield, William Atiomo, **Overlap of proteomic Biomarkers between Pre eclampsia and PCOS: a systematic review and biomarker database integration.** Hum. Reprod. (2014) doi: 10.1093/humrep/deu268

Oxylipins: Can we predict pregnancy complications and CVS disease in PCOS. Oral Presentation at 16th World Congress on Human Reproduction, Berlin.18-21st March 2015.

Uncovering disease Pathways from Lipodomic Profiles.Poster presentation at 5th Lipidomic Meeting, 2-4th September 2015.Swansea University UK.F.Casbas, **G Khan**, W.Atiomo, D.Barrett, C.Hodgman, University of Nottingham.UK.

Metabolomic study to improve the diagnosis of PCOS and predict long-term health implications. Poster Presentation at the research forum. University of Nottingham.26th June 2013.

Metabolomic fingerprinting in PCOS.Presentation at Sue Watson Postgraduate presentation prize event, held at University of Nottingham.22nd MAY 2014.

LIST OF ABBREVIATIONS

AA: Arachidonic acid

AEPCOS: Androgen Excess and PCOS Society
AES: Androgen Excess Society
AMH: Anti-Mullerian hormone
ASRM: American Society for Reproductive Medicine

ATP: Adenosine triphosphate
BMI: Body mass index

°C: Degree Celsius

CAD: Coronary artery disease

CI: Confidence interval
COC: Combine oral contraceptives
CoMet: metabolomics data analysis
COX: Cycloxygenase
CYP 450: Cytochrome P450
CHD: Coronary heart disease
CVD: Cardiovascular disease
DENND1A: DENN domain-containing protein 1A
DHEAS: Dehydro epiandrosterone sulphate
DHET: Dihydroxy eicosatrienoic acid
DM: Diabetes mellitus
DNA: Deoxyribonucleic acid
3DUSS: Three dimension ultrasound scan
EET: Epoxyeicosatrienoic acid
ESHRE: European Society for Human Reproduction and Embryology

E2: Estradiol

EC: Endometrial cancer

ELISA: Enzyme-linked immunosorbant assay

ESE: European Society of Endrocrinology
FA: Fatty acid
FAI: Free androgen index
FBS: Fasting blood sugar
FIGO: International Federation of Gynaecology and Obstetrics
FSH: Follicle-stimulating hormone
FSHR: Follicle-stimulating hormone receptor
GDM: Gestational diabetes mellitus
GIR: Glucose infusion rate
GLUT4: Glucose transporter type 4
GnRH: Gonadotrophin-releasing hormone
GWA: Genome- wide association
GWAS: Genome wide association studies
HCG: Human chorionic gonadotrophic
HDL: High density lipoprotein

HOD: Hydroxy- octa deadienoates
HOMA-IR: Homeostatic model assessment insulin resistance
HPLC: High performance liquid chromatography
IBM: International business machines
IGF1: Insulin-like growth factor-1
IGFBP1: Insulin-like growth factor binding protein-1
IGT: Impaired glucose tolerance
INSR: Insulin receptor
IR: Insulin resistance
IRP: Insulin regulated protein
IRS1: Insulin receptor substrate-1
IVF: In-vitro fertilization
Kg/m_2: kilogram per meter square
LA: Left atrium
LC: Liquid chromatography
LC-MS: LC mass spectrometry
LDL: Low density lipoprotein
LH: Luteinising hormone
LHCGR: Luteinising hormone-choriogonadotropin receptor
LOX: Lipoxygenase
LV: Left ventricular
LVEF: Left ventricular ejection fraction
Lyso PE: Lysophosphatidylethanolamine
LysoPC: Lysophosphatidylcholines
m/z: Mass-to-charge ratio
ml: millilitres
mm: millimetre
MMR: Mismatch repair
mRNA: Messenger ribonucleic acid
ng/mL: Nano gram per millilitre
NHS: National Health Service
NICHD: National Institute of Child Health and Human Development
NIH: National Institute of Health
NMR: Nuclear magnetic resonance
OR: Odds ratio
P: Progesterone
PC: Phosphotidylcholine
PCOS: Polycystic ovary syndrome
PCR: Polymerase chain reaction
PE: Pre eclampsia
PLS-DA: Partial least – squares discriminant analysis
pmol/l: picomole per liter
PPAR: Periperoxisome proliferator-activated receptor
PUFA: Polyunsaturated fatty acid.
QC: Quality control
RCOG: Royal College of Obstetricians and Gynaecologists
RPM: Revolutions per minute
SD: Standard deviation
SHBG: Sex hormone binding globulin
SGA: Small for gestational age

SPE: Solid phase extraction
SPSS: Statistical package for the social sciences
T2 DM: Type 2 Diabetes mellitus
THADA: Thyroid adenoma associated protein gene
TNF: Tumour necrosis factor
UK: United Kingdom
US: United states.

WC: Waist circumference

WHO: World Health Organisation
WHR: Waist-hip-ratio

WISE: Women's ischemia syndrome evaluation study

LIST OF TABLES AND FIGURES

Table 1: Phenotypes of PCOS according to the NIH 1990 and Rotterdam 2003 Criteria

Table 2: Summary of management options of PCOS related infertility.

Table 3: Studies included in the literature search (Metabolomic and PCOS)

Table 4: The main characteristics of each study with proteomics differencially expressed in patients with the Pre-eclampsia compared to normotensive pregnant individuals

Table 5: Methodological Quality Assessment using Quadomics tool (Part 1,Part 2)

Table 6: Patients and controls demographics and p values

Table 7: Oxylipins in PCOS compared with controls

Table 8: Oxylipins in PCOS compared with controls, adjusted for waist:hip and BMI

Table 9: List of metabolomics in PE

Table 10: List of lipids expressed significantly different in controls and patients for PCOS

Table 11: Node- contribution scores

Table 12: Genes expression in PCOS

Table 13: List of lipids expressed significantly in controls and PE

Table 14:Genes expression in PE

Figure 1: PCOS: changing women's health paradigm
Figure 2: Insulin Resistance and its impact
Figure 3: Insulin Resistance, hyperinsulinemia –hyperandrogenemia cycle in PCOS
Figure 4: Bioinformatics
Figure 5: Metabolomic: the apogee of the omics trilogy
Figure 6:Flow chart of Literature search of Metabolomic in PCOS
Figure 7: Arachidonic Acid cascade
Figure 8: Impact of circulating estified Eicosanoids and other oxylipins on Endothelial function
Figure 9: Flow chart showing selection of studies included in the systematic review
Figure 10: Arachidonic acid cascade
Figure 11: Flow chart of the literature search(Metabolomics and Pre eclampsia)
Figure 12: Diagramatic transformation of phosphatidylcholine to lysophosphatidyl choline
Figure 13: Lipodomic network
Figure 14: A network of study
Figure 15: Metabolic pathway for the pair CPT1 and CPT2 in acyl carnitines
Figure 16: Sub network generated from the PE metabolites of interest

Table of contents

Title page……1
Dedication……2
Copyright statement……3
Abstarct……4
Acknowledgement……7
Author's declaration……9
Publications and Presentations……10
List of abbreviations……11
List of tables and figures……14
List of contents……16
1.Introduction and Literature Review……21
1.1 Defination and overview……21

1.2 Literature Review
1.2.1Daignosis of PCOS and Historical overview……26
1.2.2 Evolution of and challenges with the diagnosis of PCOS……28
1.2.3Three-D Ultrasound in the diagnosis of PCOS……31

1.3 Overview of the Pathogenesis of PCOS……33
1.3.1 Hyperandrogenism in PCOS……36
1.3.2 Hyperinsulinemia and Insulin resistance(IR) in PCOS……37
1.3.3 Genetics……39

1.4 PCOS and Pregnancy Complications
1.4.1 Miscarraige……..43
1.4.2 Gestational Diabetes……44
1.4.3 Pre Eclampsia(PE)……..45
1.4.4 Pre Term Labour(PTL)……46

1.5 Long term Health implications in PCOS……46
1.5.1 Diabetes……..47
1.5.2 Cardiovascular disease……50
1.5.3 PCOS and Cancers……53

1.6 Management of PCOS……55

1.7 Overall summary of the current challenges in PCOS……59

1.7.1 Potential role of new biomarkers……59
1.7.2 New diagnostic biomarkers and potential role in PCOS……59
1.7.3 Data analysis /Bioinformatics in biomarker research……60

1.7.4 Metabolomic /Metabonomic markers……63

1.7.5 Historical overview of metabolomics……63
1.7.6 Metabolomic assay……64
i.Analytical tools……64
a.Nuclear magnetic resonance spectroscopy(NMR)……..64
b.Mass Spectrometry(MS)……66
ii.Analytical strategies……67

1.7.7 Application of metabolomics in Medicine……67
1.7.8 PCOS and metabolomics……68

1.8 Oxylipins and potential role in PCOS……74

1.8.1 Oxylipins and atherosclerosis……75
1.8.2 Oxylipins and Insulin production……77
1.8.3 Oxylipins and PE……77
1.8.4 Assessing Oxylipins……78
1.8.5 Conclusion……79

2.1 Aims and Objectives of Thesis……80
2.2 Organisation of thesis……81

3 Methods
3.1.1 Research design……82
3.1.2 Ethics approval……82
3.1.3 Systematic review and proteomic biomarker database integration
a.Studies eligible for review……83
b.Data extraction……83
c.Main characteristics of the PE studies……83
d.Methodological Quality Assessment(QUADOMICS)……84
e.PCOS proteomics database……84
3.1.4 Searching for PE biomarkers in the PCOS biomarkers database……85
3.1.5 Collection of primary data on oxylipins in PCOS……85
a.Inclusion criteria……86
b.Exclusion criteria……87
c. Clinical intervention……87
d. 3-D Transvaginal scan……88
3.1.6 Metabolomic assays……89
i.Analytical tools……89

ii.Analytical strategies……90

3.1.7 Statistics……92
3.1.8 Network analysis of lipids……92
3.1.9 Summary……93

4 Overlap of proteomics biomarkers between women with PE AND PCOS:A systematic review and biomarker database integration

4.1.1 Introduction……94
4.1.2 Methods……96
4.1.3 Results……97
4.1.4 Discussion……120
4.1.5 Conclusion……125

5 Oxylipin biomarkers in POCS

5.1.1 Introduction……126
5.1.2 Arachidonic acid derivatives and PE……130
5.1.3 Oxylipins and PCOS……130
5.1.4 Methods……131
5.1.5 Results……132
5.1.6 Discussion……135
5.1.7 Conclusion……141

6 Network analysis of lipid metabolites differentially expressed in PE and PCOS from metabolomics studies
6.1.1 Introduction……143
6.1.2 Systematic review on lipid biomarkers of PE and PCOS using metabolomics approaches……144
6.1.3 Network Analysis……158

6.1.4 PCOS network analysis……163
6.1.5 PE network analysis……163
6.1.6 Discussion……171
6.1.7 Conclusion……173

7.Summary and conclusion……175
8 Suggestions for future research……180
References……182
Appendices……202

CHAPTER 1

Introduction and Literature Review

1.1 Definition and Overview :

Polycystic ovarian syndrome (PCOS) is the most common endocrine disorder of women, with a prevalence between 6% and 10% based on the U.S National Institute of Health (NIH) criteria and as high as 15% when the broader Rotterdam criteria are applied (Norman 2005). The aetiology of PCOS remains obscure and presents major challenges. Fundamental features include oligomenorrhea /anovulation and hyperandrogenism. Endocrine changes include raised testosterone, free androgens, increased luteinising hormone/follicular hormone ratio and insulin resistance. Ultrasound features of increased ovarian volume, and presence of 12 or more follicles measuring 2-9 mm in diameter have been an integral component of the diagnostic criteria as defined by the Rotterdam consensus (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, Fertility Sterility 2004). These currently agreed diagnostic criteria for the diagnosis of PCOS (Rotterdam 2003), was based on majority opinion of a panel of experts against a background of the heterogeneous way women with PCOS may present, presenting a further challenge of consistency in diagnosis.

PCOS is also now recognised as more than a reproductive system disorder.Although majority clinicians remain focused on the

menstrual problems, hirsuitism and infertility caused by PCOS; lately there has been more drive to consider it as a metabolic syndrome.It is known to cause pregnancy complications such as pre-eclampsia and major long-term health implications including, type 2 diabetes, cardiovascular disease (CVD) and endometrial cancer as shown in Fig 1. (Boomsma 2006) (Hardiman 2003).

Since the last century cardiovascular disease has been shown to be linked with PCOS and various studies have shown a direct or indirect correlation. This risk of CVD can be to a degree autonomous of high BMI (DE GROOT 2010). In a large retrospective cohort study, conducted that included 2301 women with PCOS, it was shown that there was a high incidence of type 2 DM, myocardial infarction and angina which was age specific (Mani 2013). These findings have a huge clinical consequence. Although there is still controversy as to how PCOS intrinsically increases the risk of cardiovascular events, this study emphasises the fact that an aggregation of metabolic risk factors in PCOS need close scrutiny. Early life style changes may help to reduce these risks. Predicting which women with PCOS will go on to develop pre-eclampsia and these major long-term health implications however remains a challenge for routine clinical practice.

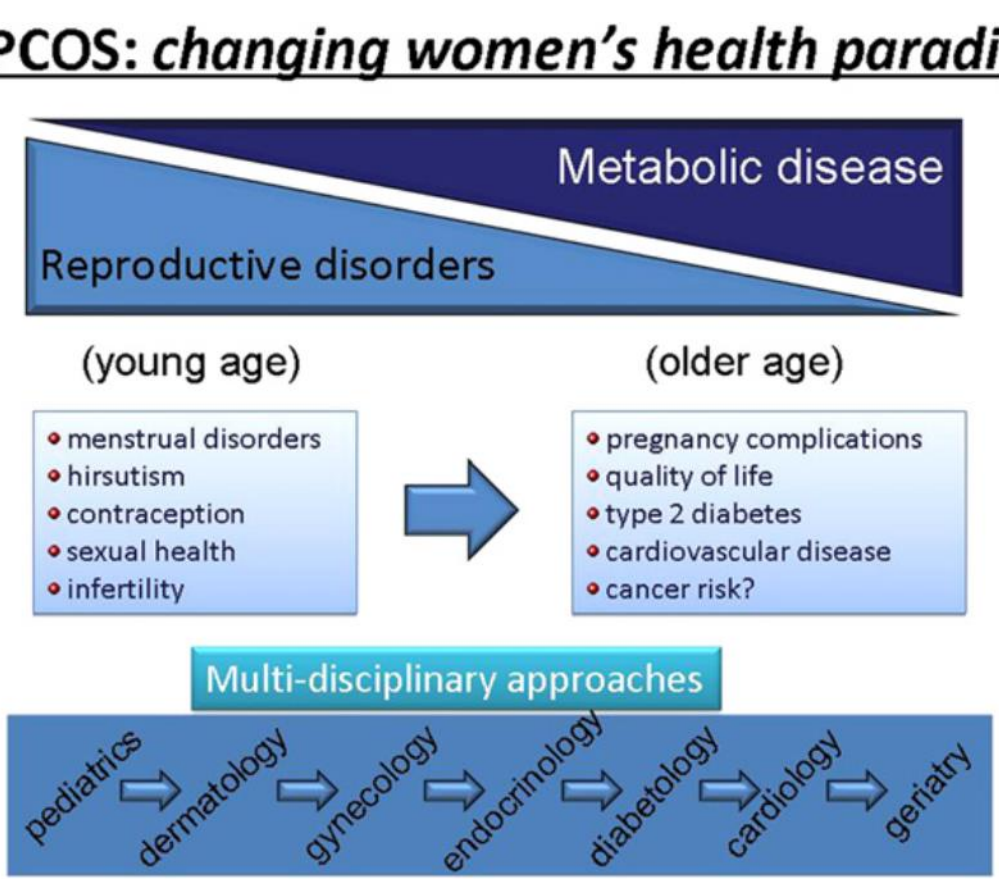


Figure 1:PCOS: changing women's health paradigm.

Molecular and Cellular Endocrinology Volume 373, Issues 1–2, 5 July 2013.

Treatment of PCOS is also now mainly directed at the key presenting complaint rather than the underlying pathophysiology, as this remains unclear. Although women are encouraged to lose weight to improve insulin resistance which is thought to be the factor underpining infertility and hirsuitism in PCOS, not all women with PCOS are able to loose weight successfully (Hoeger 2001).Predicting which women with PCOS will respond to treatment for infertility with clomiphene and hirsuitism with the combined oral contraceptive for example, also remains a challenge.

The studies in this thesis aimed to address some of these challenges through the identification of a unique proteomic and metabolomic biomarkesr finger print unique to PCOS. This could

aid diagnosis, improve our understanding of its aetiology and if associated with pre-eclampsia, form the basis of future validation studies to aid the prediction of which women with PCOS were more likely to develop pre-eclampsia.

Pre-eclampsia (PE) is a pregnancy specific condition of unknown aetiology that affects 4 million women /year worldwide. It is more common in first pregnancy and affects 5% of women. It is a leading cause of maternal and fetal morbidity and mortality. Pre-eclampsia is diagnosed in the presence of high blood pressure and proteinuria. It has healthcare implications in later life with an increased risk of hypertension, cardiovascular disease and type 2 diabetes (Sibai 2005) and (Bellamy 2007).

Prior to the studies in this thesis, it had been shown in various meta-analysis that the risk of developing pregnancy induced hypertension and pre -eclampsia in women with PCOS is more prevalent, with an odds ratio of 4.23,95% CI (2.77-6.46) times more than controls (Kjerulff 2011). Early prediction of PE and understanding the pathophysiology of the disease and it's link to PCOS might help in the prevention of the PE and its potential life threatening consequences. There have been however, no studies aiming to identify factors that predict which women with PCOS will go on to develop PE and no study has been able to predict which factors increase cardiovascular morbidity.
However, advances in the field of 'OMICS' such as genomics, proteomics and metabolomics might offer the opportunity to unravel the mystery surrounding the diagnosis of PCOS and help predict which women with PCOS will go on to develop PE and suffer from increased cardiovascular morbidity. These techniques have been applied elsewhere in medicine as well in obstetrics and gynaecology (Seli 2010), (Kedia 2016). They used a single

experiment to identify genes, proteins and metabolites involved in pathological/disease states through the application of novel molecular biology techniques.

Recent developments have suggested that understanding changes in metabolite profiles would confer a high degree of predictive accuracy in terms of understanding the fundamental mechanisms resulting in perturbations of the metabolic state as often found in PCOS. Metabolomics involves the establishment of relationships between phenotype and a metabolic signature, which are key aspects of biological function. These approaches have been applied to the identification of serum/plasma metabolic markers involved in obesity, diabetes and vascular disease using animal models or in humans (Gulston 2007). Different kinds of metabolite profiling techniques using nuclear magnetic resonance spectroscopy, mass spectrometry and ultra-performance liquid chromatography have been employed to generate global metabolic profiles. Scientific information derived from these techniques offer the potential to provide accurate and clinically useful prognostic/diagnostic capability for the management of major chronic diseases.

Prior to the commencement of this thesis, there has been one pilot study carried out to evaluate the use of metabolomic markers in women with PCOS and to demonstrate whether women with PCOS had a unique metabolomic profile that might distinguish them from controls. This could be used as a diagnostic criteria and also help in understanding the pathophysiology of the syndrome. In the study, 12 women with PCOS and 10 healthy women were chosen as controls for the study. Initial visual inspection of the NMR data showed only very subtle differences between PCOS and control group but data analysis in the form of partial least-squares discriminant analysis (PLS-DA) and non-

parametric univariate analysis, did show some robust differences.Levels of citrulline, lipid (CH2–CH2–C¼C), arginine, lysine, ornithine, proline, glutamate, acetone, citrate and histidine were significantly decreased in PCOS compared with controls (Atiomo 2012).

1.2 **Literature Review**

1.2.1Diagnosis of PCOS and Historical overview:

It was in 1935 that Stein and Levanthal coined the term Polycystic Ovaries that was originally described by Chereau and Rokitansky in 1844 to be associated with amenorrhea (Stein IF, Leventhal 1935),(Chereau 1844)and (Rokitansky 1844). The heterogeneity of the syndrome was evident even at the time of its inception where seven women were described to have the syndrome. Three out of these seven women were obese, four hirsuite, one obese and one had acne.

So far, the two main criteria that have been proposed for the diagnosis are by National Institute of Health (NIH)/National Institute of Child Health and Human Development (NICHD) criteria proposed in April 1990 (Zawadzki 1992) and the European Society for Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM) proposed in May 2003 (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group2004).

The diagnostic criteria proposed by NIH in 1990 required the presence of hyperandrogenism and /or hyperandrogenemia and chronic anovulation as features of PCOS in the absence of other endocrine causes of chronic anovulation and hyperandrogenemia e.g. thyroid disorders, androgen secreting tumours, hyperprolactinaemia and adrenal hyperplasia. The ultrasound

features of polycystic ovaries were not made part of the diagnostic criteria due to its low specificity (Balen 2003).

An International conference in Rotterdam in 2003 revised the diagnostic criteria and ESHRE/ASRM expanded on the previous NIH proposed criteria and proposed that two of the following three features should be present to diagnose PCOS after the exclusion of related disorders.

1) Oligo- or anovulation.

2) Clinical and/or biochemical signs of hyperandrogenism.

3) Polycystic ovaries on ultrasound.

Medical science usually defines a syndrome as a "collection of symptoms and signs associated with any morbid process (Stedman's Medical Dictionary, 2000).

Fundamentally, for the different phenotypes to be part of the same syndrome, they should have a common theme. If different phenotypes of PCOS depict the same morbidity (e.g. insulin resistance and hyperinsulinism) these different phenotypes should be considered to suggest the same syndrome.

The Androgen Excess Society (AES) task force, which was formed to determine what phenotypes suggested PCOS based on available data adopted this latter approach. It was concluded by the AES task force that PCOS was defined by all the phenotypes that indicated an increased risk of insulin resistance and the morbidities associated with it.

In 2006, the Androgen Excess and PCOS society (AE/PCOS) advocated that PCOS should be considered a syndrome of androgen excess and proposed that the criteria should include a combination of hyperandrogenism with one of the following:

1) Oligo or anovulation.

2) Polycystic ovaries on ultrasound.

This criterion was supported based on the fact that the risk for metabolic morbidity in PCOS is raised in the presence of clinical or biochemical evidence of hyperandrogenism (Azziz 2006).

1.2.2 Evolution of and challenges with the diagnosis of PCOS:

PCOS phenotypes vary according to the diagnostic criteria followed, but most clinicians follow the Rotterdam criteria for diagnosis.Regardless of the inconsistency of the diagnostic criteria adopted, the main clinical features associated with the syndrome, although not pathognomonic, are menstrual disorders, anovulatory infertility, and signs of hyperandrogenism. These challenges require a novel approach to diagnosis as intended in this thesis.

The combination of hyperandrogenism and chronic anovulation was the basis for defining PCOS and formed the diagnostic criteria agreed in the International workshop on PCOS held at the National Institute of Health (NIH) in 1990 and ovarian morphology was not part of these diagnostic parameters (Zawadzki 1992).It was in 1980's that the images of polycystic ovaries were highlighted on high definition ultrasound and it became apparent that polycystic ovaries were commonly associated with hyperandrogenism even in the absence of amenorrhea (Adams 1986),(Conway 1989). It then became apparent that the clinical expression of PCOS was much broader than that defined by the 1990 NIH criteria and in 2003 (Rotterdam ESHRE/ASRM, 2004), ovarian imaging by ultrasound became an important component in the diagnosis mainly because

ovarian morphology was part of the original description of PCOS. Polycystic ovaries were defined as an ovary containing 12 or more immature follicles measuring 2-9 mm in diameter and /or increased ovarian volume (>10ml) (Chang 2004).

The Rotterdam criteria is more inclusive than NIH criteria and has generated debate as it created two new phenotypes:

1. Hyperandrogenic, ovulatory women with PCO

   And

2. Oligo-ovulatory polycystic ovaries but without hyperandrogenism.

Although both the NIH 1990 and Rotterdam 2003 agree on two phenotypes (A&B), two further phenotypes were added (C&D) (table 1) when Rotterdam criteria was adapted.

Features Phenotypes	A	B	C	D
Ovulatory dysfunction	X	X	X	
Hirsuitism and/or hyperandrogenemia	X	X		X
Polycystic ovaries	X			X
PCOS by NIH 1990	√	√		
PCOS by Rotterdam 2003	√	√	√	√

X, Features included in phenotypes; √, phenotypes included in definitions.
Assuming exclusion of other androgen excess or related disorders.
Table 1**:** Phenotypes of PCOS according to the NIH 1990 and Rotterdam 2003 criteria
J Clin Endocrinol Metab, March 2006, 91(3): 781–785 Azziz • NIH 1990 *vs.* Rotterdam 2003 Criteria for PCOS

In a review by Aziz et al in 2006, comparing NIH and Rotterdam criteria, it was argued that the two new phenotypes proposed by Rotterdam criteria i.e. ovulatory women with PCO and hyperandrogenism and anovulatory women with PCO could have a negative impact. The Rotterdam group had clarified that the

criteria was possibly not suitable for studies that were focused on clinical outcomes and over the years, this appears to be happening. It was also argued that the Rotterdam criteria increased the heterogenicity of the phenotypes, and its use potentially limited the ability of the genetic and molecular studies to uncover a conjoint principal abnormality in PCOS. Finally, it was felt that, adopting the Rotterdam criteria indicated to patients and clinicians that women with the new PCOS phenotypes were at an increased risk of developing cardio vascular disease and metabolic consequences despite the fact that there was no data for that at the time.

A task force was therefore appointed by the Androgen Excess Society (AES) (an international organization) to view all data concerning the phenotype of PCOS (Azziz 2006).It looked at the available published and peer-reviewed data and concluded at the end of a year-long process that the diagnostic criteria for PCOS, should incorporate the original 1990 National Institutes of Health criteria and also take into consideration the Rotterdam consensus statement and therefore the diagnostic criteria should include all three of the following:

1. Hirsuitism and /or hyperandrogenemia.
2. Oligo-anovulation and /or polycystic ovaries.
3. Exclusion of androgen excess or related disorders.

A principal conclusion was that PCOS should be first considered a disorder of androgen excess or hyperandrogenism. It also concluded that the absence of clinical or biochemical hyperandrogenism which has not been treated, in women less than 40yrs of age, makes a diagnosis of PCOS less certain, regardless of the presence of ovulatory or menstrual dysfunction or the presence of polycystic ovaries.The task force also accepted

the fact that the definition of this syndrome would evolve over time to incorporate new research findings (Ricardo Azziz 2006). True to the statement above, another suggestion has since been put forward by Androgen Excess Society, which proposes that in the absence of either oligo-ovulation or hyperandrogenism, the number of follicles in the ovary or Anti–Mullerian Hormone (AMH) could be used as a surrogate for hyperandrogenism or oligo-anovulation (Dewailly 2011).

Whether hyperandrogenism is an essential criterion remains debatable and moreover there has been little clarification over the normal ranges of hormones in normal women, due partly to the quality of testosterone immunoassays (Bart 2007) again reflecting the complexity of PCOS and a need for novel research approaches as offered in my thesis.

1.2.3 Three-Dimensional Ultrasound Scan in the diagnosis of PCOS:

Three- dimensional (3D) ultrasound imaging is a modality that permits improved spatial awareness, true volumetric calculation and quantitative assessment of the vascularity within a defined volume of tissue. The high degree of accuracy and reproducibility of 3D measurement of ovarian and follicular volume has been used in an attempt to improve the diagnosis of PCOS (Wu, 1998). 3D ultrasonic reconstruction provides stereo-shape images as well as accurate volume measurement by analyzing stored data that has been acquired over a period of several seconds without causing discomfort to the patient (Kyei- Mensah, 1996). 3D ultrasonography permits the measurement of stromal volume through the calculation and subtraction of total follicular volume from total ovarian volume. It permits visualisation of the coronal

plane of the pelvis and hence more accurate measurement of the total ovarian volume and stromal volume. The precision and high degree of reproducibility of ovarian volume obtained using this technique have been reported and compared with conventional two-dimensional (2D) ultrasound volume measurements (Kyei-Mensah, 1996).

The traditional morphologic changes in PCO on ultrasonographic studies include increased ovarian size, excessive number of small follicles, and increased volume of ovarian stroma.They were considered important but did not form a part of the Rotterdam diagnostic criteria, probably because of its inability to standardize measurements (Balen et al 2003). Several groups have applied 3D sonography to assess ovarian volume and vascularity, which were found to be increased in PCOS (Jarvela 2002).

3D USS however provides a new method for the objective quantitative assessment of all of these parameters as well as blood flow within the ovary as a whole (Raine-Fenning 2003b; Raine-Fenning 2004a). These result obtained are more subjective and reproducible. A number of groups have tried to define the 3D ultrasonographic features of a polycystic ovary, with variable results (Mensah 1998), (Dolz 1999), (Jarvela 2002), (Pan 2002), (Ng 2005). These studies have confirmed that the inherent features of a PCO include more astral follicles and a larger ovarian volume (Lam 2007). Lam et al (2007) also reported that women with PCOS had ovaries with a larger stromal volume and a more marked blood supply, but their echogenicity was analogous to normal ovaries. Any differences in vascularity between different groups were only evident with 3D ultrasound and were not identified through the application of pulsed wave Doppler (Lam 2007).

The Rotterdam criteria for PCO with either 12 or more follicles or an increased ovarian volume of >10 cm3 were derived from the literature with ultrasound descriptions based on 2D Ultrasonography (Pache 1992), (Vocke-Hell and Schmid 2001), (Jonard 2003). The Rotterdam guidelines did represent a credible step towards standardization by defining uniform diagnostic criteria for PCOS and support the objective role of ultrasound in defining the appearance of the PCO (Balen 2003). However, with the development of 3D ultrasonography and more advanced and sophisticated technology it can be argued that the current ultrasound criteria suggested by the Rotterdam consensus are still too open and that the PCO is more complex ultrasonographically.

With the evolution of PCOS, the spectrum and implications have been better understood, but the poorly defined margins have created confusion amongst gynaecologists and endocrinologists. As previously highlighted, there is a need for research looking at the epidemiology, pathophysiology and treatment of PCOS but this will need a robust definition for the diagnosis (Barth 2005) including clarification of the precise ultrasound criteria (2D or 3D). These challenges again suggest a need for novel research approaches as offered in my thesis.

1.3 Overview of the Pathogenesis of PCOS

The pathophysiology of PCOS is thought to be multi -factorial having both genetic and environmental components (Kosova 2013). Although PCOS can have extra-ovarian aspects, ovarian dysfunction is thought to be the central key factor (Balen 2004). Family studies have shown that PCOS is significantly more prevalent among family members than in the general population suggesting a genetic component to the pathogenesis of PCOS.

Among first-degree female relatives of patients with PCOS, 35% of premenopausal mothers and 40% of sisters were affected by the disorder (Miller 1998).These rates were significantly higher than the 6–7% observed in the general population. Other than PCOS itself, increased androgens, insulin secretion, and insulin resistance as often found in PCOs also appear to be under significant genetic control, as established by family studies (Lunde 1989),(Hague 1988),(Ferriman 1979).

Although several genes have been chosen as potential candidates, most of these studies were performed in small cohorts and confounded by several biases. Thus, no particular gene is universally recognized as importantly contributing to PCOS risk. Several environmental factors probably modulate the clinical expression of genetic predisposition. In particular, obesity, insulin resistance, prenatal androgen excess during early gestation, birth weight, precocious pubarche, dietary intake, lifestyle, and stress may act on the genetic substrate (Sanches de Melo 2015).

Obesity is the most important environmental factor contributing directly to insulin resistance and hyperinsulinemia. Low birth weight modifies the relationship between insulin resistance and PCOS and seems to increase the risk of developing PCOS. Glucocorticoid excess might be a reason for the higher risk of PCOS and the accompanying clinical-metabolic disorders demonstrated by small-for-gestational-age (SGA) infants who present compensatory growth (Anderson Sanches de Melo 2015). Diet composition, eating disorders, and psychological stress seem to be related to the syndrome, even if no causal link has been clearly demonstrated.

Insulin is produced in the pancreas by the β cells and has an effect on multiple organs in the body including reproductive system. The recognition of the role of insulin resistance with compensatory hyperinsulinemia in the pathogenesis of the PCOS, has contributed to an improved understanding of the syndrome and its associated short and long-term complications.

The molecular mechanisms of insulin resistance in PCOS differ from those in other common insulin-resistant states, such as obesity and type 2 DM (Pasquali 1982), (Shoupe 1983), (Chang 1983).Despite the compensatory hyperinsulinemia that accompanies insulin resistance in non-diabetic individuals, insulin-mediated glucose uptake remains subnormal. Interestingly, insulin resistance occurring in PCOS patients is not evident in all tissues, but it seems to have a tissue peculiarity (Kandarakis 2008). Specifically, in skeletal and adipose tissue, insulin resistance lead to a decreased glucose uptake and increased lipolysis, respectively. Insulin stimulation however causes the ovaries to produce androgens, encourages ovarian growth and causes cyst formation as a result of inhibiting apoptosis of the follicles (Poretsky 1999),(Baillargeon and Nestler 2006).

Insulin resistance in PCOS has led to some academics labelling PCOS a metabolic syndrome mainly characterized by causing insulin resistance (Dunaif 1997). The long-term impact of PCOS-associated metabolic disorders remains poorly studied and the evidence indicates that not all PCOS phenotypes have similar metabolic risk.In women with PCOS,there is a proven risk of developing type 2 diabetes but an increased risk of cardiovascular disease and endometrial cancer is still debated (Fauser 2012). In the context of this heterogeneity, it important that research (as

intended in this thesis) is designed to improve the stratification of these women in accordance with their adverse metabolic profile.

1.3.1 Hyperandrogenism in PCOS

Biochemical hyperandrogenism forms an integral part of the criteria required to diagnose PCOS. The majority of evidence suggests that the excess androgen in PCOS is ovarian in origin and this occurs as a result of abnormal steroidogenesis (Rosenfield 1990). Specifically, it is thought that women with PCOS have ovarian hyper responsiveness to gonadotrophins. Theca cells to LH, producing excess androgens and granulose cells to FSH producing large amounts of oestrogens.

The causes of hyperandrogenism are however complex as it has also been found that plasma inhibin levels are increased PCOS, which has a direct stimulatory effect on androgen production and as a result causes a relatively low level of FSH compared with LH in anovulatory women with PCOS, as inhibin reduces FSH production (Anderson 1998). Up to 70% of women with PCOS demonstrate insulin resistance and the resultant high levels of Insulin and Insulin like growth factor 1(IGF 1) act as 'co-gonadotrophins and increase the production of androgens by theca cells of the ovary directly and also act indirectly by inhibiting SHGB (Prevelic 1990),(Hughes 2006).

Adrenal androgen production in PCOS has also been shown to be increased. Dysfunction of adrenocortical axis is associated with PCOS in 50% of the cases. The exact aetiology is not known but it could be a manifestation of extra adrenal factors that regulate the androgen production in the adrenals and it has been argued that this can only be possible because of ovarian factors

influencing the adrenals (Suhail 2004). This excess of adrenal androgens can be detected by the raised levels of dehydroepiandrosterone sulphate (DHEAS). This is present in 20-30% of women with PCOS. The adrenal cortex is also responsible for producing 25% of the circulating testosterone (T) (Bulent 2007).

Enhanced steroidogenesis in PCOS has also been shown to occur through 17-hydoxylation and increased but ineffective activity of 17, 20 lyase. The activity of both of these is regulated by the enzyme cytochrome P450c 17. The activity of P450c17 mRNA in ovaries and adrenal glands is stimulated by the insulin:IGF system. It is thought that the over activity of this enzyme may be the contributing factor in the causation of hyperandrogenism in PCOS (Rosenfield 1998).

The complexity of hyperandrogenemia in PCOS therefore strengthens the case for further research into novel mechanistic pathways that may provide a single unifying hypothesis for hyperandrogenism in PCOS. Metabolomic technologies as applied in this thesis offer this opportunity.

1.3.2 Hyperinsulinemia and insulin resistance in PCOS

Insulin resistance is defined as a compromised biological response to endogeneous and exogeneous insulin manifesting disturbed metabolic and mitogenic activities (Consensus Development Conference on Insulin Resistance 1998). Insulin resistance in humans is measured with the euglycemic glucose clamp technique. In the experiment a required dose of insulin is administered and euglycemia is maintained by glucose infusion, the rate of infusion is adjusted by regularly checking arterial blood glucose. Peripheral sensitivity to insulin is measured by the

amount of glucose infused compared to the peripheral uptake of the glucose by the tissues (DeFronzo 1979)(Bergman 1989).
In women with PCOS, a key finding is reduced insulin sensitivity at the receptor level (Dunaif, 1997). The insulin receptor function was studied in the receptors from cultured skin fibroblasts. It was confirmed that insulin resistance in PCOS is secondary to post binding defect in insulin receptor signalling. The study also found that in 50% of PCOS women in the study the abnormality was an enzyme that regulates the insulin receptor tyrosine kinase. This defective enzyme is speculated to be responsible for altering P450c17 activity that causes hyperandrogenism (Dunaif 1995).

This reduced insulin sensitivity is present in both obese and non-obese PCOS women compared to age and weight matched women with normal ovaries (Dunaif 1989).Insulin resistance in PCOS can have a deleterious effect on glucose metabolism increasing the risk of type 2 diabetes and it has been associated with worsening hyperandrogenism in obese women (Dunaif 1988).Insulin resistance in PCOS is also aggravated by obesity especially when it is centrally distributed as commonly found in PCOS (Dunaif 1992). Central obesity is more likely to be risk factor for developing increased insulin resistance, type 2 diabetes, dyslipidaemia, hypertension and left ventricular hypertrophy (Lord 2002).

Other mechanisms apart from reduced insulin sensitivity at the receptor level have however been suggested as probably leading to insulin resistance in PCOS. This includes post-binding defect. The action of insulin is mediated through a protein tyrosine kinase receptor. Tyrosine auto-phosphorylation increases the insulin receptor tyrosine kinase activity. On the contrary, serine phosphorylation inhibits it. In 50% of the PCOS women the

potential cause for insulin resistance is serine phosphorylation (Dunaif 1995).It is postulated that this defect in the action of insulin is limited to glucose metabolism. The same mechanism is also believed to be responsible for affecting P450c17 activity that results in hyperandrogenism (Zhang 1995).

The action of insulin and IGF-1 was assessed in cultured skin fibroblasts by Dunaif and Book (1999), it was suggested that the defect in the action of insulin in PCOS is selective, in that it only affects the metabolic pathways. This defect is shared by IGF-1. As a result it's been proposed that both insulin and IGF-1 stimulate glycogen synthesis. There is also evidence that insulin promotes ovarian androgen production in PCOS, but the exact mechanism is unclear. Finally, it has been postulated that insulin cross reacts with IGF-1 receptor on the ovarian thecal cells and that this might be the mechanism of insulin induced hyperandrogenism (Bergh 1993).

This complexity in the potential aetiology of insulin resistance in PCOS and its interaction with steroidogenesis (including hyperandrogenemia), obesity and the insulin-like growth factor axis (IGF axis) again highlights the need for research aiming to identify novel mechanistic pathways that may shed some more light on this complexity.

1.3.3 Genetics

There is evidence of a genetic component to the aetiology of PCOS but due to a lack of large cohort studies, only a few studies have obtained any conclusive results. This may be because PCOS has different phenotypes and affected genes might be different. This evidence comes from studies showing familial clustering of PCOS (Ehrmann 2005) (Legro 1998) and a high incidence of inheritance, but it's not clear whether the mode of inheritance is

dominant or multigenetic (Vink 2006),(Kahsar-Miller 1998), (Franks 2004).

Genome-wide association (GWA) studies have been very useful in other complex medical disorders and have been recently used in PCOS. There have been two such (GWAS) published studies from China (Chen 2011), (Shi 2012). The study (GWAS2) by Shi et.al.looked at a new cohort of 1,510 PCOS and 2,016 controls of Han Chinese ancestry and followed it up with identified signals from the previous (GWAS1) study by Chen et al. This GWAS2 study confirmed three previously reported genetic loci associated with PCOS on chromosome, 2p16.3 (rs13405728), 2p21 (rs13429458, rs12478601) and 9q33.3 (rs10818854,rs2479106).The associated linkage disequilibrium (LD) blocks comprised of the susceptibility genes LHCGR, THADA and DENND1A, respectively (Chen 2011), which were partly established in some recent studies in European cohorts (Lerchbaum 2011),(Eriksen 2012),(Goodarzi 2012),(Welt 2012) as mentioned below.

The LHCGR gene which encodes for luteinising hormone /choriogonadotropin receptor is located on chromosome 2p16.3.This belongs to G –protein coupled receptors (Atger 1995) and this gene is present in ovaries, testis and other gonads (Rahman 2009). The THADA (Thyroid adenoma-associated gene) has been mapped to chromosome 2p21. Zeggini et al in a GWAS stated that THADA is a novel type 2 diabetes associated gene affecting pancreatic beta cell function (Zeggini 2008). Chromosome 9q33.3 carries DENNA1A gene and it is also differentially expressed in normal and cancer cell.

In PCOS women of European descent, studies (Welt 2012) have identified DENN domain-containing protein 1A (DENNDIA) gene, a

novel gene identified through GWAS as a potential PCOS susceptibility gene. This DENND1A is implicated in aetiology of PCOS for the first time (Kosova 2013). Welt et al (2012), did a case control study in women of European descent with the objective to test whether PCOS risk variants identified in Hans Chinese women bestow risk for PCOS in women of European descent. The results showed that two powerful linked Han Chinese PCOS risk variants were replicated in the European women. They were present on chromosome 9q33.3 ,rs109861 and rs10818854 with odds ratios of 1.68(p_0.00033) and 1.53(p_0.0019) respectively. The rs10986105 was associated with the phenotypic features of PCOS and may provide clues to the aetiology of the syndrome. It is associated with hyperandrogenism even in women without PCOS. The difference between the Chinese and this study by Welt et al was that Rotterdam criteria were used to diagnose PCOS in the Chinese study, while Welt et al used NIH criteria.

Other studies investigating genetic mechanisms associated with PCOS include a study from Denmark (Erikson 2012) that set out to investigate whether three single nucleotide polymorphisms (SNPs), identified in the Han Chinese women could be replicated in Caucasian women. The genes explored were rs13405728 (LHCGR gene), rs13429458 (THADA gene) and rs2479106 (DENND1A gene). It was concluded that DENND1A gene allele was associated with a decreased predisposition to PCOS.

Mutharasan et al also did a study on 905 women with PCOS (NIH criteria) and 956 controls of European ancestry. The main aim of the study was to establish the part the LHCGR and FSHR gene played in the aetiology of PCOS in European women. The results of the study showed a strong association between FSHR and rs

1922476(p_0.0053). The markers of the FSHR gene were associated with FSH levels in PCOS women.

Other genetic studies by Welt et al, Erikson et al, Goodarzi et al and Lerchbaum et al on Caucasian population failed to reproduce the findings of the Chinese GWAS studies. It is thought that this was because they only explored the Chinese PCOS susceptibility SNP in Caucasians. However, when fine mapping of the chromosome 2p16.3 was done in a study by Mutharasan et al it was found that Chinese PCOS susceptibility locus was also found in a European ancestry.

A recent study by Saxena et al also found that the variant rs2268361, which is found near the FSHR gene, influences FSH levels. This gene is found to be significant in both Han Chinese and European cohorts which points to the fact that disruption in the FSH may be the key to the pathogenesis of PCOS (Saxena 2015).

There are however likely to be many potential routes by which women develop PCOS, as there is a strong possibility of an interaction between several genes and environmental factors. Integrating the results of genomic, proteomic and metabolomic studies are being currently applied in the pursuit to find definitive diagnostic criteria. This may help us understand the pathophysiology of PCOS and improve the understanding of these interactions (Insenser 2013) (Yang 2015). I have attempted to do this, in this thesis by focusing on the relationship between PCOS and PE, integrating proteomic and metabolomics data.

1.4 PCOS and Pregnancy Complications

Beyond the issues outlined above including hyperandrogenism, menstrual abnormalities and infertility which women with PCOS experience, they may also be more likely to suffer some early and late pregnancy complications.These include miscarriage, gestational diabetes, pre-eclampsia and preterm labour. The precise risk of developing these complications is however uncertain and how best to predict which women with PCOS will develop these complications is unknown.

1.4 .1Miscarriage

Early pregnancy loss happens in about 15% of clinically proven pregnancies and the true percentage may be higher if all pregnancies (non-clinical) are taken into account. Recurrent miscarriage is defined as three consecutive spontaneous pregnancy losses. Approximately 1% of women suffer from recurrent pregnancy loss (Weinberg 1988). Recurrent miscarriage has been associated with several factors, the most common being chromosomal abnormalities (Li 1998),(Warburton 1964), (Wramsby 1987), anatomical abnormalities, anti-phospholipid syndrome and PCOS. Some less clear associations have been found with infections, environmental and endocrine factors. In about half of recurrent miscarriages, the aetiology is unknown.

In one of the earliest studies linking PCOS with recurrent miscarriage, 82% of women with recurrent miscarriage had polycystic ovaries (Sagle 1988) on ultrasound. One potential explanation was that recurrent miscarriage in PCOS was linked to the raised concentration of luteinising hormone (LH) found in PCOS women when compared to controls (Fauser 1991). This finding (raised LH) has been reported in 40-60% of women with PCOS and an elevation of LH is known to be associated with failed

conception and augmented risk of miscarriage (Balen 1997). A randomised controlled trial in which LH levels were suppressed in women with PCOS however did not improve pregnancy rates in PCOS women with recurrent miscarriage.This again called the hypothesis into question, reflecting the complexity of PCOS (Clifford 1996).

1.4.2 Gestational Diabetes

Gestational diabetes (GDM) is one of the most common conditions affecting pregnancy. It poses a major health problem and affects 0.4-7.3% of all pregnancies (Reece 2009). GDM affected pregnancies are at an increased risk of having preterm labour, pre-eclampsia, fetal macrosomia and its associated complications of prolong labour, obstructed labour, shoulder dystocia. Long term complications include childhood obesity and diabetes for the children and almost 50% of women with GDM develop type 2 diabetes mellitus in later life (Catalano 2003), (Reece 2009), (Nolan 2011). There is some degree of insulin resistance present during pregnancy and this happens so that the fetus gets enough nutrition. As a result, insulin production increases in the mother in addition to the increased levels of glucose and free fatty acids. It is thought that women with PCOS are at an increased risk of developing GDM, due to insulin resistance with the increased obesity in PCOS also playing a part (Chu 2007),(Boomsma 2006), (Kjerulff 2011).

1.4.3 Pre Eclampsia

A meta-analysis published in 2011, showed that pregnant women which are known to have PCOS were also four times more likely to develop pre-eclampsia as compared to controls (Kjeruff 2011). This review by Kjeruff et al included 23 published papers. These included 2544 patients with PCOS compared with 89,848 without

PCOS. The patients with PCOS had higher rates of GDM with an odds ratio of 2.82 (95% CI, 1.93-4.10). The odds ratio for gestational hypertension was 4.07 (95% CI, 2.75-6.02) and pre-eclampsia 4.23 (95% CI, 2.77-6.46). The meta-analysis confirmed previous associations of pregnancy complications in women with PCOS and further updated the odds ratios for the complications. Although, there were higher rates of other pregnancy complications only gestational diabetes, pregnancy-induced hypertension, pre-eclampsia, preterm delivery and small for gestational age were found to be statistically significant. Another significant finding from this meta- analysis was that the ORs for pregnancy induced hypertension and pre- eclampsia was higher when compared to a previous study in 2006 (Boomsma 2006).

It has also been shown that women with PCOS have decreased levels of insulin like growth factor binding globulin -1, which might be a contributing factor towards developing pre-eclampsia and growth abnormalities (Jensen 2003).

Although the association between PCOS and pre-eclampsia has been documented, the underlying pathophysiological mechanisms involved are not completely understood. It is however possible that raised androgen levels, hyperinsulinemia and subsequent diabetic and hypertensive susceptibilities in PCOS may act as co-factors (Troisi 2003). Among the various implicating factors, defective placental vasculature appears to be central to the disease (Duckitt 2005).

1.4.4 Pre Term Labour

A systematic review by Galazis et al showed that pregnant women with PCOS were at least 2 times more likely to give birth prematurely (i.e. before the 37th of gestation) compared to

controls (Galazis 2013).The pathophysiological mechanisms involved in preterm birth in women with PCOS are not completely understood but it might be possible that the associated raised estrone levels, hyperinsulinemia and the subsequent diabetic and hypertensive predispositions may act as co-factors. Preterm birth, defined as birth before the 37th week of gestation, is responsible for 75% of all neonatal deaths and over half the neurological handicap in children (Galazis 2013).Currently, there is insufficient evidence to establish causation and to establish screening for patients for these complications, solely based on PCOS diagnosis.

There is therefore, a need for research studies to explore the molecular mechanisms underpinning the link between PCOS and these pregnancy complications including pre-eclampsia as proposed in this thesis. This could facilitate screening in women with PCOS for pregnancy complications specially pre-eclampsia, which could minimize the occurrence of maternal and fetal morbidity/mortality associated with pre-eclampsia in pregnant women with PCOS.

1.5 Long Term Health Implications in PCOS

Women with PCOS have also been found to have an increased risk of impaired glucose tolerance, type II diabetes and sleep apnoea than that observed in the general population (Randeva 2012). They also exhibit an adverse cardiovascular risk profile, characteristic of the cardio-metabolic syndrome as suggested by a higher reported incidence of hypertension, dyslipidaemia, visceral obesity, insulin resistance and hyperinsulinemia and an increased risk of endometrial cancer.

1.5.1Diabetes

Women with PCOS show various abnormalities in the action of insulin. This is characterised by increased production of insulin as shown in fasting and postprandial insulin levels in these women due to peripheral insulin resistance. A systematic review and meta-analysis by Moran et al showed that women with PCOS were four times more likely to develop T2DM compared with BMI-matched controls (Moran 2010). Insulin resistance is proposed as a key pathophysiological feature of PCOS contributing to the development of not only diabetes but is also implicated in the pathogenesis of hyperandrogenemia in PCOS.

Stepto et al (2013) in a cross sectional study which included 20 over weight and 20 lean PCOS (Rotterdam criteria) women and 14 overweighed and 19 lean controls measured insulin resistance (IR) after stopping the use of insulin sensitizers and oral contraceptives for three months. This study, using gold standard euglycemic clamp technique, demonstrated that women with PCOS were more insulin resistant than controls irrespective of their BMI. After overnight fast the patients were given high carbohydrate diet. After taking fasting venous blood, insulin infusion was started for 120 min to maintain a blood glucose level of 5mmol/lit. The Glucose infusion rate (GIR) was calculated in the last 30 min and was expressed as glucose (mgs) per body surface area/min. Insulin resistance was diagnosed using WHO criteria for non PCOS individuals which is glucose uptake less than the lowest quartile for background population under hyperinsulinemic, euglycemic conditions (Grundy 2004).

The study showed the prevalence of IR was 75% in lean PCOS and 95% in obese PCOS compared to 65% in obese controls. It was also shown in the study that lean PCOS women showed

higher IR compared to their lean control counterparts backing up the hypothesis that there is an intrinsic defect in PCOS that causes IR.

This study demonstrated that 70% of lean women with PCOS who did not meet the NIH criteria were still IR compared to BMI matched controls. Sub-group analysis in the study confirmed that women who had PCO and irregular menstrual cycle but no hyperandrogenism, were still insulin resistant.

It is now clearer that PCOS is associated with significant insulin resistance and this may be due to factors extrinsic to insulin receptor like serine/threonine kinase signalling defect. Hyperinsulinemia can also result from decreased insulin clearance and this is receptor mediated. Acquired decrease in the receptor number or function may present in insulin resistance (Dunaif 1997).It is thought that insulin resistance results in compensatory hyperinsulinemia in PCOS, and excess insulin causes exaggerated effects on less traditional target tissues. These tissues include ovarian theca cells, which leads to increased androgen production. High levels of insulin in the portal circulation cause reduction in SHBG that in turn increases the levels of free androgens in addition to direct increase in the level of LH mediated androgen production.

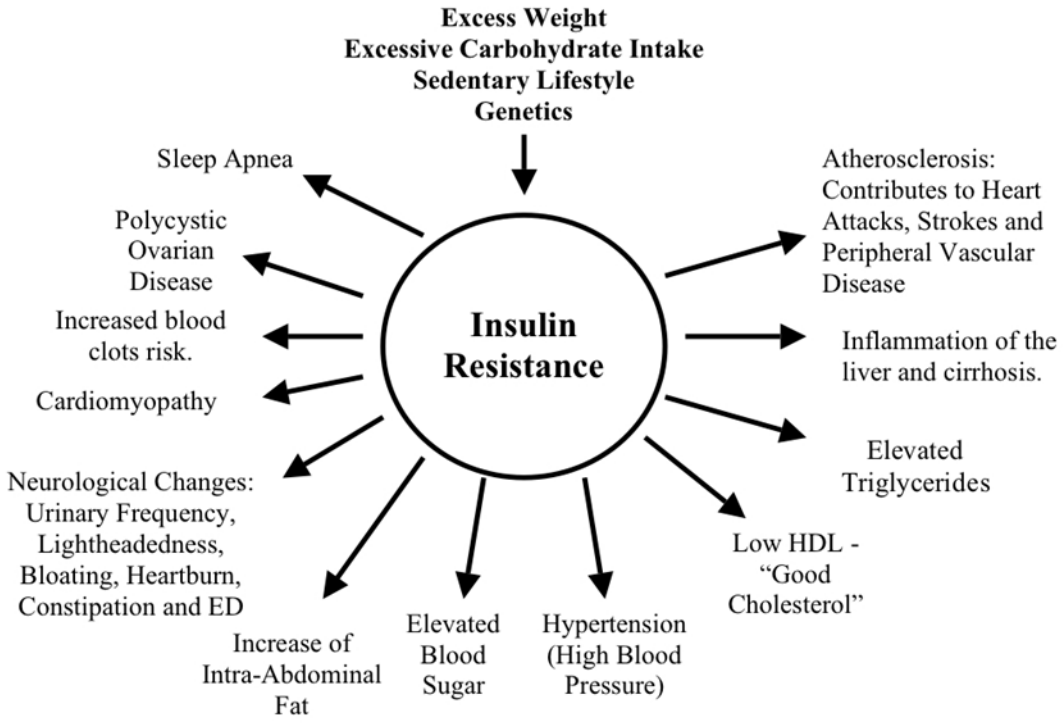


Figure: 2 Insulin Resistance and its Impact.

In summary PCOS is known to cause reproductive disturbances in the form of menstrual irregularities, hyperandrogenism, infertility and increased pregnancy complications, psychological consequences like depression, anxiety and impaired quality of life. Women with PCOS have metabolic sequelea like increased risk factors for impaired glucose (IGT) tolerance type 2 Diabetes mellitus (DM2) and cardiovascular disease (CVD) (Azziz 2006), (Boomsma 2006),(Himelein 2006).Insulin resistance is suggested to be the fundamental factor contributing to the pathophysiology of both reproductive and metabolic disturbances in PCOS.

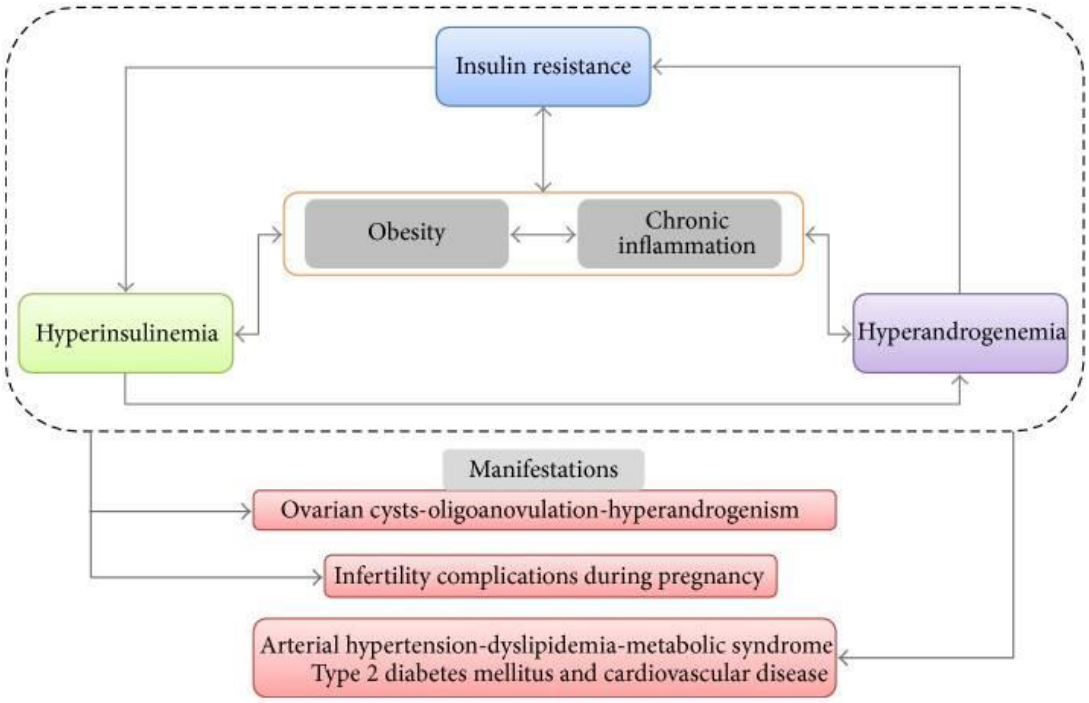


Figure 3:

The insulin resistance-hyperinsulinemia-hyperandrogenemia cycle in Polycystic Ovary Syndrome. PCOS is dominated by three major endocrine disruptions: insulin resistance, hyperinsulinemia, and hyperandrogenemia. The cycle leads to the manifestations of PCOS and infertility, complications during pregnancy, and chronic cardio metabolic co morbidities.

Joselyn Rojas, The Role of Metformin in Metabolic Disturbances during Pregnancy: Polycystic Ovary Syndrome and Gestational Diabetes Mellitus, Int J Reprod Med. 2014:797681.

1.5.2 Cardiovascular disease

Cardiovascular disease remains one of the principal cause of death in women. Although women with PCOS are thought to have an increased lifetime risk of CVD, the exact level of risk is uncertain. Evidence in support for an increased risk comes from studies showing that biomarkers associated with an increased risk of CV disease such as C-reactive protein, homocysteine and tumour necrosis factor (TNF-a) were shown to be increased in women with PCOS (Toulis 2011). A meta-analysis also showed a twofold increase in risk of arterial disease irrespective of BMI in PCOS compared to the general population (De Groot 2011). Another study looked at a large (984 women) bi-racial cohort of women who were diagnosed to have PCOS using the NIH criteria, women with PCOS had a large left ventricular (LV) mass index and larger left atrial (LA) diameter when compared to the reference group in the study (Wang 2012). These findings were independent of the typical cardiovascular risk factors.

Further evidence in support for increased cardiovascular risk in PCOS comes from the Women's Ischemia Syndrome Evaluation Study (WISE), which found increased cardiovascular events and deaths in postmenopausal women who were known to have PCOS (Leslee 2008). WISE was a four-centre study; analysis was done in 390 postmenopausal women according to clinical features of PCOS, which were enrolled in the National Institute of Health-National Heart, Lung, and Blood Institute (NIH-NHLBI). Women with clinical features of PCOS were more frequently diabetic ($P<0.0001$), had metabolic syndrome ($P<0.0001$), had more angiographic coronary artery disease (CAD) ($p<0.04$), when compared to women without clinical characteristics of PCOS. It was concluded that identifying postmenopausal women with

history of PCOS should be evaluated for risk factors of CAD and cardio- vascular events.

A prospective study by Caren G et al looked at a cohort of 82,439 women aged 20-35 years and followed them up for 14years for cardiovascular events. When women who reported regular menstrual cycles were compared, with women who reported irregular menstrual cycle, the risk of non- fatal or fatal coronary heart disease (CHD) was significantly higher with age- adjusted relative risk (RR), 1.25 and 1.67 respectively in women who reported irregular menstrual cycles. The increase risk of coronary heart disease remained significant after adjustment for body mass index and several other cofounding factors like cigarette smoking, parity, oral contraceptive use or family history of cardiovascular disease (Caren 2013). The only flaw in the study was that it was presumed that these women with menstrual irregularities had PCOS although there was no data on either clinical or biochemical hyperandrogenism or ultrasound of the ovaries.

A prospective 21 year controlled follow up study was conducted in a University hospital in Sweden which also provided further evidence of increased cardiovascular risk in PCOS. It included 35 PCOS and 120 age-matched controls. The study reported a higher prevalence of hypertension ($p<0.008$) and raised levels of triglycerides ($p<0.012$) in women with PCOS compared to controls. Myocardial infarction (MI), stroke, diabetes, mortality prevalence was however similar in both PCOS and controls when matched for BMI (Schmidt 2011). The limitation of the study was its small numbers that might have prevented the smaller differences in morbidity and mortality rates.

Studies on the association between PCOS and echocardiographic parameters have shown mixed results. In one study, PCOS was associated with an increased left atrial (LA) diameter and left ventricular (LV) mass, as well as a decreased LV ejection fraction (LVEF), compared with age and body mass index (BMI)-matched controls (Wang 2012). On the other hand, another study showed that PCOS women and matched controls had similar echocardiographic measures of cardiac structure and function, including LV and LA diameters, ejection fraction, deceleration time, and isovolumetric relaxation time (Tekin 2009).PCOS patients in the Dallas Heart Study were also not found to have a higher rate of coronary artery calcification or abdominal aortic plaque (Chang 2011).

In managing women with PCOS, clinical guidelines recommend that they should be counselled to make adjustments in their lifestyle to reduce cardiovascular risk including measures to prevent obesity, stop smoking. The Royal College of Obstetricians and Gynaecologists guidelines also recommends that the women diagnosed with PCOS should be screened for cardiovascular risk factors at the initial diagnosis (RCOG Green top guideline no.33). The uncertainty about the exact level of cardiovascular risk in women with PCOS including predicting which ones will actually go on to develop cardiovascular disease requires more research. Novel techniques such as those applied in the studies in my thesis could potentially address this challenge.

1.5.3 PCOS and Cancers

It's been acknowledged for decades that the occurrence of amenorrhoea, oligomenorrhea in PCOS resulting in unopposed oestrogen exposure in pre-menopausal women can lead to endometrial hyperplasia and carcinoma (Speert 1949). A meta-

analysis by Chittenden in 2009 looked at eight studies and concluded that the odds ratio (OR) of developing endometrial cancer in women with PCOS was 2.70, 95% CI of 1.00-7.29 (Chittenden 2009). In another systematic review by Haoula et al (2012), which focused on only endometrial cancer, a 2.89 fold increased risk of endometrial cancer in women with PCOS was observed in a meta-analysis (Haoula 2012). It is however, unclear whether this increased risk is due to PCOS per se or due to other confounding factors like obesity, diabetes.

A Danish study looked at a large cohort of 12,070 PCOS women identified from the Danish National Patient Registry and were followed until 2012 using the Danish Cancer Registry. The results showed that women with PCOS were four times more at risk of developing endometrial cancer, whereas the risk for breast and ovarian cancer was similar to general population. It was however found in this study that there was also an increased risk of cancers of the kidneys, colon and brain but this would require further studies (Mathilde 2015).

Breast cancer is the most common cancer in women and affects 1 in 8 women, 80% occurring after the age of 50 years (Cancer Research UK 2012). No link has been established between PCOS and breast cancer and a systematic review by Chittenden BG (2009) found a RR of 0.88, 95% CI 0.44-1.77 for breast cancer. This was further reinforced by the recent meta-analysis by Barry et al, which included 11 observational studies (5 studies on endometrial cancer and 3 each on ovarian and breast cancer). As previously known the risk of endometrial cancer was found to be significantly increased ($p<0.008$), but the risk of ovarian and breast cancer was not significantly increased ($p<0.11$) ($p<0.78$) respectively. An important finding in the study was that when

studies that included women aged >54 years were excluded from the analysis, the risk for endometrial cancer in women with PCOS increased further ($p<0.00001$). The risk for developing ovarian cancer became significant ($p<0.03$), while the risk for breast cancer remained insignificant (Barry 2014).Barry et al included three studies on ovarian cancer (Schildkraut 1996), (Olsen CM 2008), (Bodmer 2011).

A few studies have looked at the likelihood of a possible association of ovarian cancer with PCOS with conflicting results. Olsen et al tested the hypothesis that androgen plays a role in the proliferation of ovarian epithelium thus causing ovarian cancer. They did a population- based case -control study and looked at 1276 cases of invasive epithelial ovarian cancers and 315 borderline malignant tumours. Selected women self- reported history of PCOS. This study concluded that there was no evidence that history of PCOS, acne, hirsuitism was associated with ovarian cancer overall. There was however, some association of borderline serous tumours with PCOS (OR 2.6; 95% CI 1.0-6.1).

Bodmer at el looked at the incidence of ovarian cancer in women using long-term metformin in a large epidemiological study and concluded that long-term use of metformin is associated with reduced risk of ovarian cancer (OR 0.61, 95% CI 0.30–1.25). They further did a univariate analysis of the potential cofounders including PCOS and found that PCOS was associated with a tendency towards an increased risk of ovarian cancer (OR 1.63 95% CI 0.65–4.08). In clinical practice, women with PCOS are not provided increased surveillance for breast or ovarian cancer.

The uncertainty about the exact mechanisms of endometrial, ovarian and breast cancer risk in PCOS requires further research.

1.6 Management of PCOS

The presentation of PCOS is varied and so is its management. PCOS manifests as short and long term complications and its management should be tailored to presenting complaints and ought to be customized. Management must concentrate on both short and long term, reproductive, metabolic and psychological aspects of PCOS.

It is vital to deal with the psychological aspect of the syndrome as that might play a vital role in managing other features of PCOS. This would be helpful in willingness to change and sustainability of lifestyle interventions as well as improvement in QOL (quality of life) (Teede 2011).

The treatment depends on the presenting complaints, if the patient with PCOS presents with menstrual irregularity, then the option would be to start her on hormonal treatment to make the menstrual cycle regular. In those presenting with infertility, the management would include life style modifications including advice on strategies to enhance weight loss and the use of ovulation induction agents either by using Clomiphene citrate or Letrozole (Aromatse inhibitor).Legro et al conducted a multicentre double-blind controlled trial, which tested Letrozole with clomiphene for infertility in women with PCOS. Letrozole showed a 44% chance of a live birth compared to clomiphene. The multiple pregnancy rates were 3.9% with Letrozole vs 7.4% with clomiphene. However, for Letrozole to be used as first line treatment option for ovulation induction replication of these results in another trial would be obligatory (Legro 2014).

Summary of recommendations for the management of infertility in oligoanovulatory women with PCOS	
Management of early complications	
Lifestyle modification (Diet and physical activity)	First-line nonpharmacologic approach to treat obese/overweight oligoanovulatory PCOS patients
Clomiphene citrate	First-line treatment of anovulatory infertility in women with PCOS
Letrozole	Potential first-line treatment of anovulatory infertility but it is still off-label drug not recommended in clinical practice
Metformin	Treatment of choice as clomiphene citrate sensitizing and as adjuvant therapy to prevent OHSS in PCOS women undergoing IVF cycle, but it is still off-label drug
Laparoscopic ovarian drilling	Indicated in well-selected cases of oligoanovulatory PCOS women who need laparoscopic assessment of the pelvis
Gonadotropins	Considered last treatment option in PCOS population for high costs, high risk of multiple pregnancies, and OHSS

Table: 2 Summary of management options of PCOS related infertility.

Stefano Palomba et al, Complications and challenges associated with polycystic ovary syndrome: current perspectives. International Journal of Women's Health, 31 July 2015.

Abbreviations: IVF, in vitro fertilization; OHSS, ovarian hyper stimulation syndrome; PCOS, polycystic ovary syndrome.

It is a well-known fact that differences exist between endocrinologists and gynaecologists in defining, diagnosing, and managing PCOS (Cussons 2005). A survey was done on behalf of The European society of endocrinology (ESE) in 2015.It was a mailed questionnaire sent to clinicians across Europe to assess the practice adopted by specialists in diagnosing and managing PCOS. The Response rate was 13.3% amongst members of ESE. Majority that responded were endocrinologists. Menstrual irregularity was the main diagnostic criteria amongst the respondents as well as majority took hirsuitism and biochemical hyperandrogenism into consideration when diagnosing PCOS. It appeared that majority were following NIH criteria when it came to diagnosing PCOS.

The most common treatment options used were Metformin (33%), life style modifications (25%) and oral contraceptives (22%). Infertility was treated by using clomiphene citrate alone or in combination with metformin in 6 and 23% respectively. Only 6% were using other methods for induction of ovulation (Conway 2014). The endocrine society guidelines also suggest different treatment options based on the clinical presentation. The combined oral contraception (COC) is recommended as first line management of menstrual irregularity and hirsuitism. The guidelines recommend Metofrmin as second line treatment modality in women who cannot take COC but does not recommend it in hirsuitism (Legro 2013) (Martin 2008).

There is still debate, about whether PCOS is a metabolic disease or it’s just an endocrine disorder, resulting in often a disagreement about its treatment. Those who favour the school of thought that PCOS is a metabolic disorder advocate the use of Metformin for the complaints from metabolic attributes and also

to treat hyperandrogenism although conspicuously the ESE guidelines do not recommend its use for hyperandrogenism. Many clinicians use Metformin for infertility as well (Legro 2015).

1.7 Overall summary of the current challenges in PCOS

1.7.1 Potential role for new biomarkers

In summary, the preceding paragraphs highlight the fact that PCOS is a complex condition that is associated with considerable morbidity. The pathophysiology is however multifactorial and the diagnostic criteria vaguely defined. Clinical management and the prediction of which women will go on to develop complications in pregnancy or its associated long term health problems such as diabetes, endometrial cancer and cardiovascular disease could be improved. Novel molecular biology techniques evaluating genomic, proteomic and metabolomics biomarkers offer the opportunity to address these challenges.

1.7.2New Diagnostic biomarker and potential role in PCOS

The National institute of Health (NIH) has defined biomarkers as biological characteristics that are objectively measured and evaluated as an indicator of biological processes, pathological processes or pharmacologic responses to a therapeutic intervention (Biomarkers Definitions Working Group 2001). A key role of a biomarker in clinical practice is its capability to assess the response to medical treatment of a clinical condition. They can also be used as clinical end-points both in clinical practice and in a research setting. The use of biomarkers can be in the form of measuring proteins, DNA or a metabolite in blood and this is less costly and less invasive. Biomarkers can be categorised into diagnostic, prognostic and screening biomarkers. The biomarkers

used for screening purpose attract most attention as they can predict the onset of disease (Gerszten 2008).

These technologies form the omics flow, which describes the cascade of biological information, from the genome to the transcriptome, then, the proteome and ending in the metabolome. Transcriptomics and proteomics are useful in the studying the effect of drugs on gene expression, however little information is gathered regarding the global response to disease and desirable or undesirable drug effects, since they both ignore the dynamic metabolic status of the organism (Nicholson 1999). On other hand, metabonomics enables the understanding of the completion of biological processes as it is capable of providing biological end-point markers of the cellular processes that occur as a result of the disease, drug exposure or altered gene function (Lindon 2004). Major advantages of metabolomics are the size of the metabolome relative to the genome or proteome and the fact that it provides a view of the existing biochemical phenotype. As such, metabolomics is fast becoming an important discovery tool for new diagnostic and prognostic biomarkers(Milburn and Lawton 2013).

1.7.3 Data analysis / Bioinformatics in biomarker research

The whole discovery process involves search, verification, interpretation and validation of the biomarkers. Bioinformatics play a fundamental part in the biomarker discovery process, bridging the gap between initial discovery phases and bio-analysis. The pre- requisites are setting up a study, sample collection, preparation, separation and MS analysis. Data mining process is performed to convert the raw data into a format, which can be easy to process for targeted analysis as shown in the figure below.

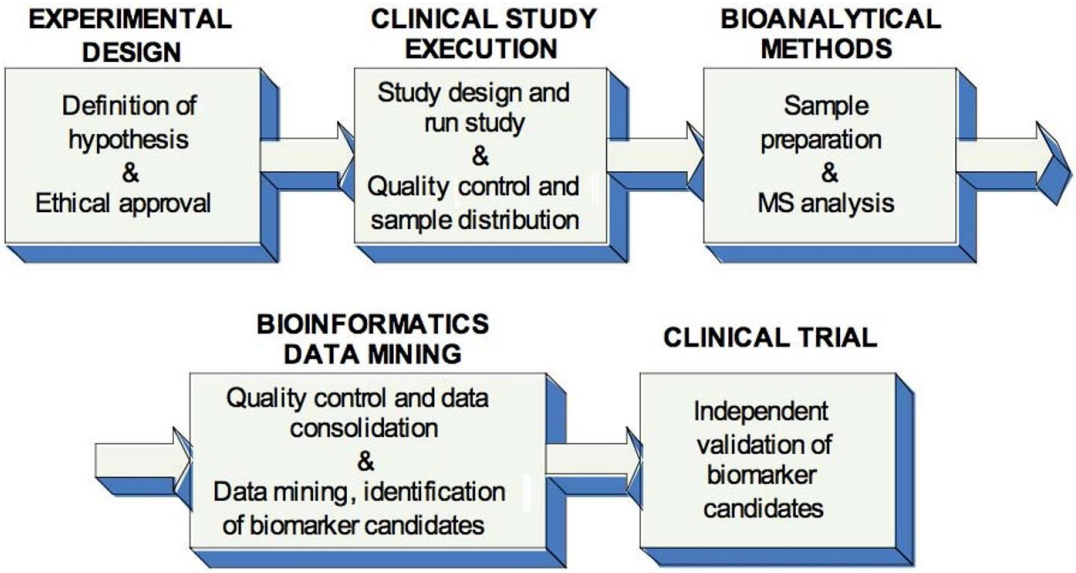


Figure: 4 Bioinformatics

It is thus concluded that the emerging role of bioinformatics is an essential tool in translational research, which can be applied clinically by novel profiling techniques, creating databases, data mining and biostatistics. This helps in rapid biomarker and drug discovery that in turn would help in managing patient's health and also help reduce the cost of drug development (Baumgartner 2011).

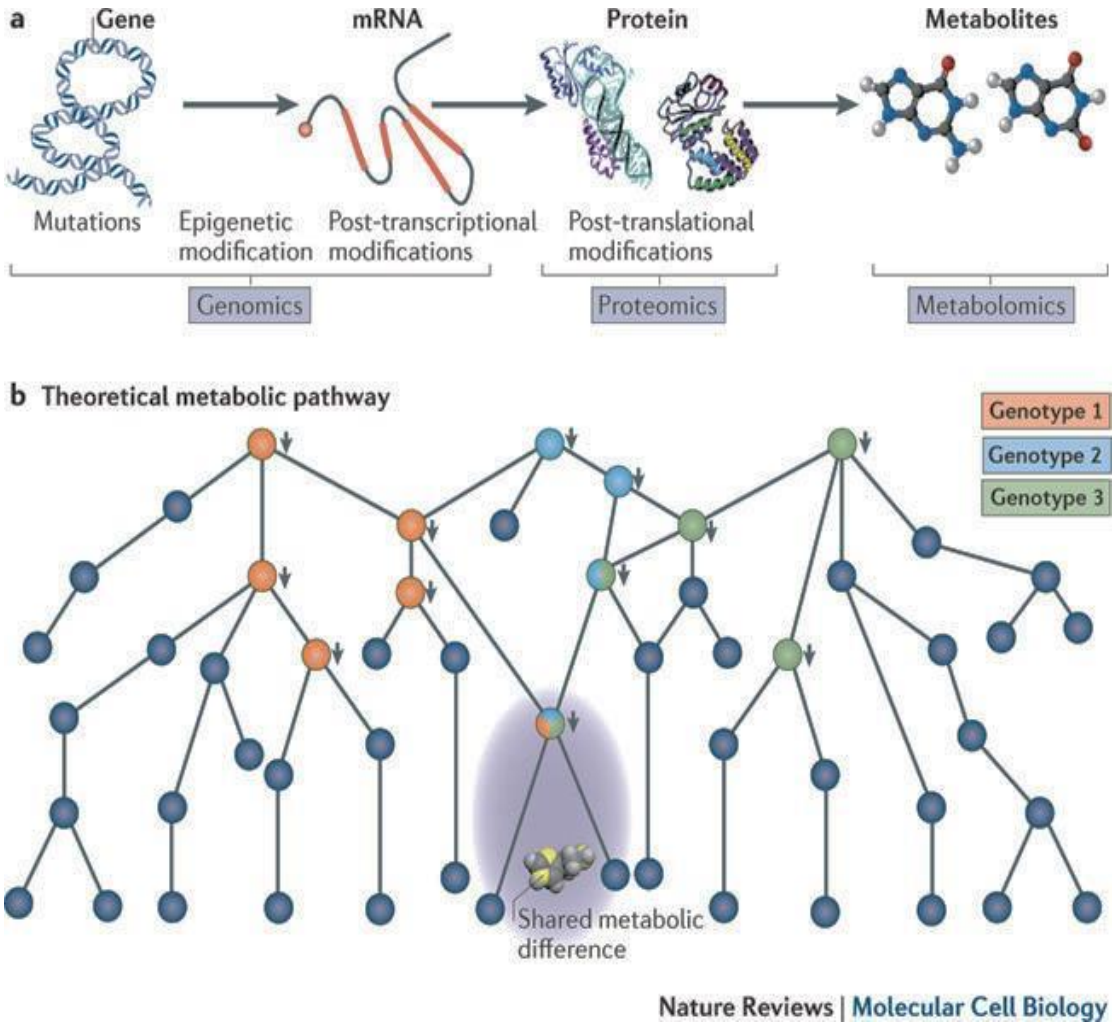


Figure 5: Metabolomics: the apogee of the omics trilogy,
GaryJ.Patti, Oscar Yanes & Siuzdak, Nature Reviews Molecular Cell Biology.2012: 13,263-269

1.7.4 Metabolomic/metabonomic markers

Small molecules that are the intermediates of the metabolism are called metabolites. It is becoming increasingly apparent that these small molecules which can be amino acids, lipids and sugars play a far more important role in all aspects of disease aetiology and disease treatment. The quantitative complement of all metabolites is defined as the metabolome. The metabolome reveals what actually occurs in the body, whereas genetics and proteomics only suggest what might happen (Schmidt 2004). Knowledge of the metabolic differences between patients and healthy controls can aid the discovery of biomarkers for diagnosis and the evaluation of therapy and can also provide insights into the underlying pathology, which in turn can be helpful for advances of new treatments (Sun 2012).Metabolomics could therefore address some of the challenges of PCOS highlighted previously.

1.7.5 Historical overview of metabolomics

Small molecules have been studied in relation to human health for centuries and can be dated back to 2000-1500 B.C. Chinese doctors have been known to use ants to analyse glucose. In 300 B.C. Greeks realised that it was vital to scrutinize body fluids in order to diagnose diseases. In 1970's Gas Chromatography (GC) was used to analyse chemical components in a urine sample and it was revealed that it contained important information about human health (Pauling 1970), but the knowledge at that time was limited.The 20th century saw the development of mass spectrometry that included different separation techniques and this made it possible to detect small molecules (Oresic 2009). An important milestone was in 2001 when first draft sequence of the

human genome was published. This was followed by a rapid increase in the development of various 'omic' applications (Venter 2001).Despite the important technological advances, global metabolome cannot be measured using a single ''array'' measurement, e.g. as in measurement of gene expression. Multiple analytical platforms are therefore still needed to increase the coverage of the metabolome.

1.7.6 Metabolomic assay

i. Analytical tools:

Various analytical tools are used for metabolomic research such as Nuclear magnetic resonance (NMR) spectroscopy and Mass Spectrometry (MS) due to the complex nature of the metabolome and the assorted properties of metabolites, and no single analytical platform can be applied to detect all metabolites in a biological sample.

a. Nuclear magnetic resonance (NMR) spectroscopy

High-resolution proton nuclear magnetic resonance (NMR) spectroscopy is a favourite as it uses minimal sample processing, is non-invasive and low cost (Lindon 2007). NMR is a technique used to determine the structure of the organic compounds.

NMR spectroscopy is a high resolution, rapid, quantitative, non-invasive, perturbing technique that provides detailed information on solution-state molecular structures, based on atom-centred nuclear interactions and properties.

NMR is a robust and reliable technique for metabolomic applications in which high analytical reproducibility is paramount (Dumas 2006). Another main advantage of NMR is the simplicity of sample preparation (Griffin 2003). It allows the detection of a wide range of structurally diverse metabolites simultaneously, providing a metabolic 'snapshot' at a particular time point. Usually, the whole spectral information is used for chemometric

analysis, but specific NMR pulse sequences can be employed to select subsets of metabolites, if necessary. On the other hand, using no selection or extraction of metabolites up-front to investigate all possible variables is advantageous in metabolomics studies in which no prior information about metabolites is known. It also gives advantage in investigating metabolites in diseases, where it still remains to be established whether several metabolites are linked to factors of disease.

NMR is the only detection technique that does not rely on separation of the analytes, and the sample can thus be recovered for further analyses. All kinds of small molecule metabolites can be measured simultaneously in this sense.

NMR is close to being a universal detector. In summary, these technical advances have now extended the coverage of metabolomics approaches to enable the use of these methods in clinical research.

The main principle involved in NMR, is that most nuclei have a spin and all nuclei are electrically charged. When external magnetic field is applied, there is energy transfer between the base energy to the higher energy level. The wavelength at which this energy transfer happens corresponds to the radio frequencies. Energy is emitted once the spin returns to the base level. The signal obtained which matches this energy transfer is measured and NMR spectrum of the nucleus is analysed. It is a straightforward and largely automated and non-destructive process, so samples can be used for further analysis. High resolution NMR provides an ideal mechanism for the profiling of metabolites within bio- fluids or tissue extracts.

b. Mass Spectrometry (MS):

This is the art of displaying the spectrum of the masses of the molecules comprising a sample of material.It is used for determining the elemental composition of a sample.Mass spectrometry works by ionizing chemical compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge ratios.In a typical MS procedure: a sample (which may be solid, liquid, or gas) is ionized, the ions are then accelerated so they all have the same kinetic energy, the ions are then deflected by magnetic field according to their masses, the beam of ions is detected electrically and finally the elements or molecules are identified by correlating known masses by the identified masses (Sparkman 2000).

Due to its high sensitivity and wide range of covered metabolites, MS has become the technique of choice in many metabolomics studies. Its utility lies in its wide dynamic range, reproducible quantitative analysis, and the ability to analyze bio -fluids with extreme molecular complexity (Lee 2010).MS is significantly more sensitive than NMR and generally requires less material than that utilized in an NMR experiment. It is a truly 'high-throughput' platform. However, MS generally requires chemical derivatization and pre-separation steps in order to select individual metabolites for analysis, since different types of metabolites cannot be analysed simultaneously. Pre-separation is usually achieved by coupling MS to liquid or gas chromatography. Other disadvantages of MS compared with NMR spectroscopy are that it suffers from variable detection responses.

MS can be used to analyse biological samples either by direct injection or following chromatographic separation. MS-based technologies are playing a central role in metabolomics research

and are widely available to facilitate successful metabolomics studies.

ii. Analytical strategies:

The analytical strategies for metabolomics can be broadly divided into ‘‘non-targeted’’ and targeted methods.

The targeted methods are in principle, similar to those available in a typical clinical chemistry laboratory, where a limited number of metabolites are known to be relevant before the experiment is designed (e.g. potential biomarkers), and absolute quantification of the metabolites is performed in a targeted approach. The non-targeted approach also called metabolomic-profiling aims to cover the metabolome as broadly as possible. The major challenge in this approach is to identify and quantify metabolomics and to process the data

1.7.7 Application of metabolomics in Medicine

In humans, metabolomics has principally been applied to define biomarkers related to prognosis or diagnosis of a disease or toxicity/efficacy of therapeutic interventions and to provide greater pathophysiological understanding of disease or drug toxicity/efficacy. Metabolomics have been productively applied to clinical conditions including inborn errors of metabolism, cardio-vascular disease and cancer studies resulting in the discovery of novel biomarkers for the diagnosis, assessment of severity and provision of prognostic factors in these conditions.

One of the first applications of a metabolomic marker in clinical practice was to diagnose inborn errors of metabolism (Arn 2007). Early pioneering work by Følling in the 1930s provided the discovery of phenylketonuria, as reviewed again (Centerwall and Centerwall 1961), and applications have now been used worldwide to screen populations. A high profile discovery was, metabolomic biomarkers in prostate cancer showing quite

significant differences between benign prostatic tumours and cancers (Sreekumar 2009).These findings offered promise in women with PCOS.

1.7.8 PCOS and Metabolomics

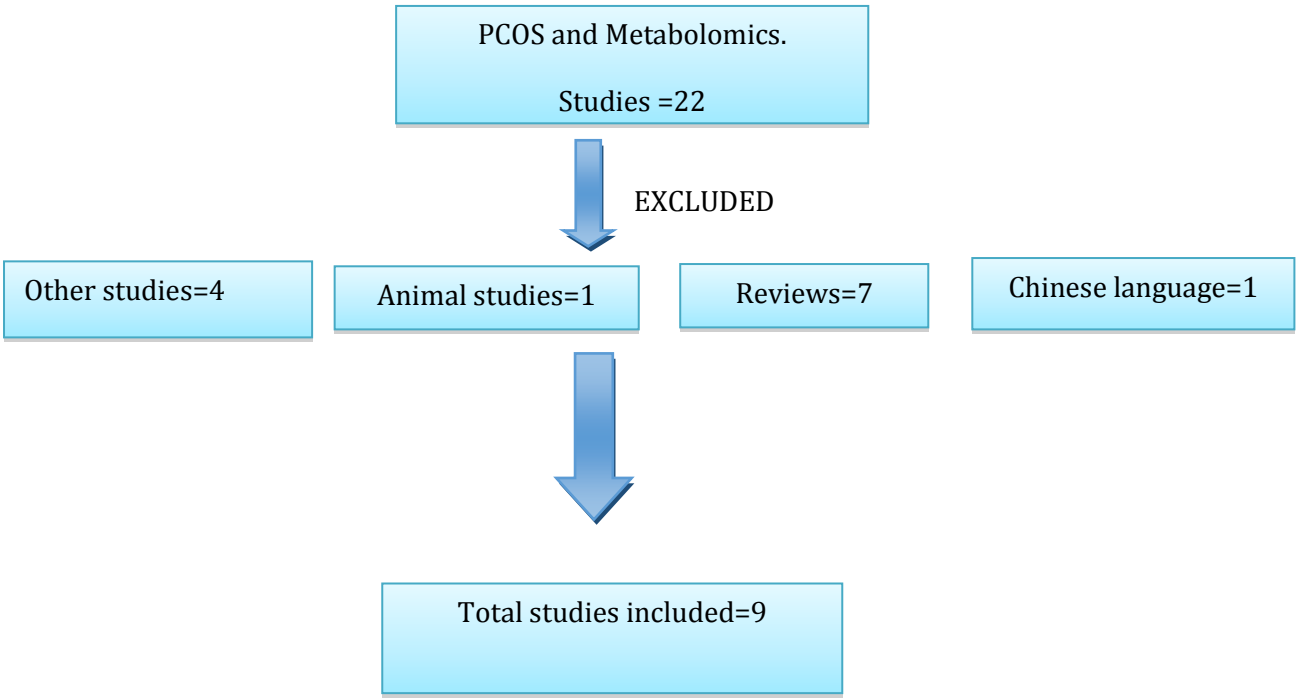


Figure 6: Flow chart of the literature search (Metabolomics and PCOS)

In a l iterature review, relevant studies were identified from searching Pubmed, Medline and Web of Science databases using the term" Polycystic ovarian syndrome" and "Metabolomics" "PCOS and metabolomics" and "Metabonomics". It included all the studies until December 2015. In total, 22 studies were identified. Seven were excluded as they were reviews, one was an animal

study and one study was in Chinese language so excluded. Four studies were excluded as one looked at the metabolomics in women with PCOS on medication, one looked at treatment aspect, one at day 3 embryo and the other two looked at metabolomics in follicular fluid to look at the quality of the oocyte.

Author (year)	Country	Biological Sample	Sample size (n)		Definition
			Controls	PCOS	
Atiomo (2012)	UK	Plasma	10	12	Rotterdam
Escobar (2012)	Spain	Plasma	39	36	NIH
Sun (2012)	China	Plasma	36	34	Rotterdam
Zhao (2012)	China	Plasma	48	217	Rotterdam
Zhao (2014)	China	Plasma	54 49	77 60	Rotterdam
Wang (2014)	China	Urine	15	22	Rotterdam
Dong (2015)	China	?	15	20	? (Full text not available)
Haoula (2015)	UK	Plasma	40	40	Rotterdam
Wigham (2014)	USA	Serum	10	10	Rotterdam

Table 3: Studies included in the literature search (Metabolomics and PCOS)

In a pilot study undertaken by our group looking at metabolomic markers in plasma of PCOS women and controls using hydrogen -1, nuclear magnetic resonance (H NMR) spectroscopy (Atiomo

2012), the results on visual inspection did not show any difference but when data was analysed using partial least-squares discriminant analysis (PLS-DA) and non- parametric univariate analysis there were differences between PCOS and controls. The results showed that citrulline, lipid (CH2-CH2-C1/4C), arginine, lysine, ornithine, proline, glutamate, acetone, citrate and histidine were decreased in PCOS. Low levels of amino acids have been previously linked with Type 2 diabetes (Mihalik 2012). The authors concluded that if these amino acids were validated in a larger cohort they might develop into biomarkers for detecting group of PCOS women who are more likely to progress to Type 2 diabetes but acknowledged that that these were preliminary results and needed to be validated (Atiomo 2012).

Another cross sectional study also using NMR based techniques also found clear metabolic differences between PCOS women and controls. Marked differences were observed ($p<0.05$) in the levels of amino acids (leucine, isoleucine, methionine, glutamine and arginine), citrate, choline and glycerophosphocholone /phosphocholine (GPC/PC), and an increase in the levels of lactate, dimethylamine (DMA), creatine and N-acetyl glycoprotein were noted in women with PCOS compared with controls. These metabolic differences were more marked in PCOS women with obesity, metabolic syndrome and hyperandrogenism. The high level of glycoprotein suggests that PCOS is an inflammatory process. The authors concluded that this study demonstrated that metabonomics was a powerful tool in the study of PCOS that would help in the pathogenesis of the condition as well as exploring new methods for diagnosis and treatment (Sun 2012).

Other studies as outlined in the table above have also found differences in metabolites between women with PCOS and

controls. A study by Zhao Yue et al studied 217 women with PCOS and 48 controls. It was concluded from the study that the levels of lactate, long chain fatty acids, triglycerides and very low density lipoproteins were elevated in women with PCOS, while glucose and high-density lipoprotein (HDL) concentrations were reduced in PCOS patients as compared with controls. Additionally, the levels of alanine, valine, serine, threonine, ornithine, phenylalanine, tyrosine and tryptophan were generally increased, whereas the levels of glycine and proline were significantly reduced in PCOS samples compared to controls. It concluded that high levels of valine and leucine, and low levels of glycine in PCOS plasma could contribute to insulin sensitivity and could be considered as the potential biomarkers for long-term risk assessment of diabetes mellitus (Zhao 2012).

A further study looked at the metabolomic biomarkers to assess the response of the PCOS patients to treatment and found that after 30 months of receiving low-dose flutamide, metformin and pioglitazone in combination with an estro-progestagen, the levels of oxidized LDL particles in serum were decreased as well as the downstream oxidation products of LDL were also decreased. This study showed that metabolomics could be useful in monitoring the effects of treatment as well (Vinaixa 2011).

More recently, five more studies have been published, three from China and one each from UK and US. Zhao Xinjie et al in 2014 published results from a study carried out in two phases, discovery and validation phase, and concluded that there was significant increase in the levels of unsaturated free fatty acids, sulphated-steroids, glycated-aminoacids showing abnormalities in the lipid and androgen metabolism. It was noted that there was a decrease in the levels of lysophosphatidylcholines and

lysophosphatidylethanolamines. A different group of women with PCOS and controls was then tested for five metabolites in the validation phase of the study which included free fatty acids, dihydrotestosterone sulphate, glycated phenylalanine and showed significant differences between the groups (Zhao X 2014). The validation sample included 49 controls and 60 PCOS women.

Whigham et al from USA only had a small cohort of women and they tested exhaled breath, urinary nitrogen and metabolites in the serum and concluded that after analysis of all three there were subtle but significant differences between PCOS and controls in lipid, glucose and amino acid metabolism (Whigham 2014).

Our group looked at plasma samples form 40 PCOS women and 40 controls and tested for hydrophobic lipids. Enhanced levels were noted in the plasma triglycerides, sphingomyelins in PCOS when compared with samples from controls in the luteal phase of the menstrual cycle, while the levels of lysophosphatidylcholines and phosphatidylethanolamines were decreased in the PCOS cohort. These may be useful in differentiating PCOS from controls if tested in the luteal phase of the menstrual cycle (Haoula 2015). These findings are somewhat similar to Zhao X et al.

Wang et al tested urine for metabolites, 59 metabolites were identified but of significance were two novel ones, testosterone-glucoronide and 11α hydroxyprogesterone. These two potential biomarkers correlate to the metabolites catalysed by ovarian cytochrome P450c17α.The study was done on a small sample and the authors suggested larger sample size for validation of the findings in their study (Wang 2015).

Finally, another study from China in 2015 tested for metabolites in 15 controls and 20 PCOS women who were further sub-grouped into insulin resistant (IR) and non- insulin resistant. A total of 36 metabolites were discovered which were significantly different between PCOS and controls. Substantial increases in the levels of saturated and unsaturated fatty acids (myristic acid, linoleic acid, 9-/13-HODE, etc.), fatty amides (palmitic amide, oleamide), dehydroepiandrosterone sulfate, l-glutamic acid, azelaic acid, l-glyceric acid, pyroglutamic acid, and decreases in the levels of lysophosphatidylethanolamine, lysophosphatidylcholine, uridine, and l-carnitine were found in PCOS patients compared with controls.

In summary, most of the studies have come to the same conclusion that it is mainly the lipids and amino acid metabolites that are differentially expressed in PCOS compared to controls. Metabolomic studies are always flawed by limitations, because of a number of factors that include the different diagnostic criteria. Up till now all the published studies used Rotterdam criteria for diagnosing PCOS apart from one study that used NIH criteria (Escobar 2012). Using variable diagnostic criteria can cause diagnosis bias and may not depict the number of actual cases included in the study. The other limitation is small sample sizes and different techniques used for analysing the samples. Discoveries from metabolomic studies in PCOS still have to establish their worth as biomarkers in PCOS and its accompanying metabolic co-morbidities, emphasizing the need for more studies including the study in my thesis.

1.8 Oxylipins and potential role in PCOS

Oxylipins are somewhat new metabolites, which have been associated with pre-eclampsia and cardiovascular diseases (both conditions associated with PCOS). Prior to the study in my thesis, there had been no studies done on oxylipins in PCOS

Eicosanoids, or oxygenated 20-carbon polyunsaturated fatty acids (PUFAs), are oxylipins that can be derived from cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome p450 (CYP), or through the interaction of reactive oxygen species with arachidonic acid (AA). Collectively, over 50 species of oxygenated fatty acids (FAs) derived from AA are known.

In addition to well-known AA metabolites, other PUFAs can also be oxygenated to produce the octadecanoid products of linoleic acid (LA), α-linolenic acid, and γ-linolenic acid; the eicosanoid products of eicosapentaenoic acid (EPA) and dihomo-γ-linolenic acid; and the docosanoid products of docosapentaenoic acid (n-6 DPA) and docosahexaenoic acid (DHA). These oxygenated PUFAs add to AA-derived eicosanoids and are collectively organized into a super class termed *oxylipins*, which better describes the in- vivo oxygenated lipid matrix. Oxylipins act locally in autocrine or paracrine modes, yet other roles for these highly bioactive molecules remain largely unexplored. We already know that eicosanoids in the prostanoid cascade are esterified in the cell lipids such as phospholipids and triglycerides. The understanding of these reservoirs is in infancy.

Oxylipins are esterified with lipoprotein lipids and have an important effect on endothelial function. These oxylipins are released by the action of one of the lipases and thus exert their inflammatory effect on the endothelial cells. Esterified oxylipins

are also known to be present in leukocytes, platelets and erythrocyte membranes.

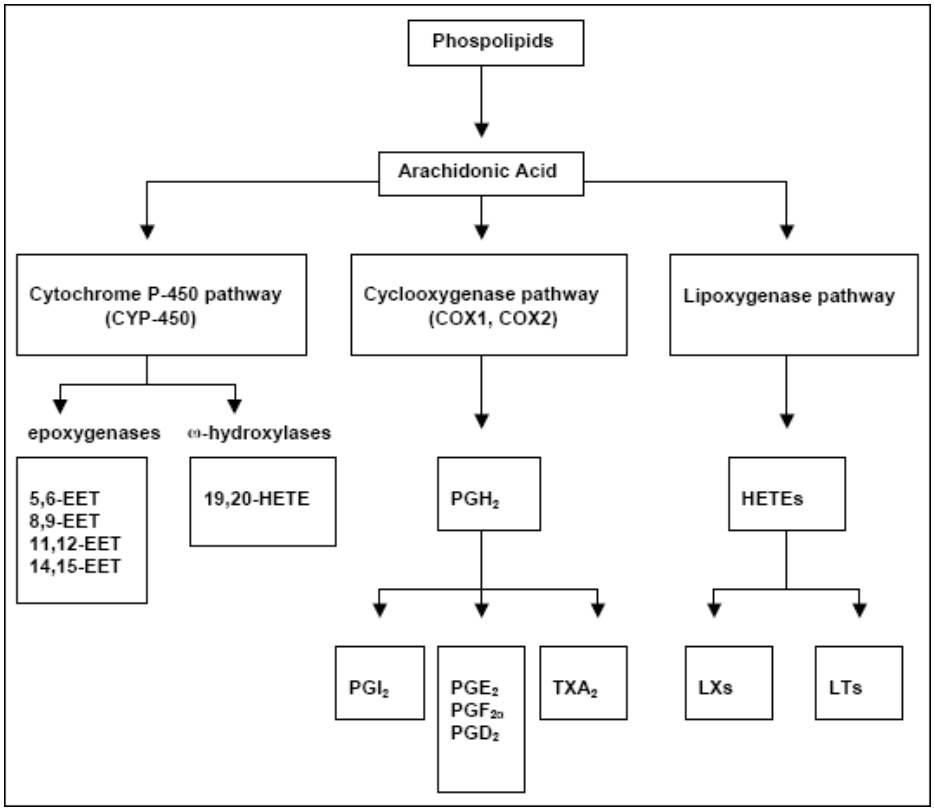


Figure 7: Arachidonic Acid cascade

Platelets contain a wide variety of arachidonic acid (AA) derived oxylipins including 12 HETE, 20-HETE and many EET isomers.The action of oxylipins may be exercised by either lipolytic effect of lipases and the release of an array of anti- inflammatory and pro-inflammatory oxylipins, or the local production of oxylipins in the cells that causes an increased production of a specific metabolite.

1.8.1OXYLIPINS AND ATHEROSCLEROSIS

Oxylipins have potent and often opposing effects on smooth muscle including blood vessels and myometrium. Platelets are also affected as previously outlined. Some of the eicosanoids such as leukotrienes B4 and C4 are known to be pro- inflammatory,

whereas resolvins and protectins have anti- inflammatory actions and are also involved in healing after tissue damage.
Eicosanoids, or oxygenated 20-carbon polyunsaturated fatty acids (PUFAs), are however thought to play key roles in the progression of atherosclerosis. The epoxyeicosatrienoic acids (EETs) derived from arachidonic acid (AA) are known to have anti-inflammatory and cardio-protective actions (Spector 2007).The anti-atherogenic effect of EET's is exercised through nuclear factor – KB, vanilloid receptor, peroxisome proliferator–activated receptors (PPAR), and cyclic adenosine monophosphate (Ullu 2008), (Fang 2006). The vasoactive and inflammatory effect of oxylipins on the vascular endothelium is facilitated by lipolysis of the anti- inflammatory esterified oxylipins (EETs). This action is mediated by the action of lipase on the lipoproteins. This leads to the formation of hydroxy- octadeadienoates (HODEs). HODEs cause endothelial dysfunction that can lead to vascular disease.

It is clear therefore, that normal chemistry of eicosanoids is disrupted in atherosclerosis, thus favouring inflammation, arterial smooth muscle hyperplasia and thrombosis leading to acute athero-thrombotic events.It is however thought that the associated cardiovascular risks and atherosclerosis is caused by polymorphisms in the genes regulating the formation and metabolism of epoxide (Atherosclerosis Risk in Communities (ARIC) study ((Lee 2006).A 3.5 fold increase was noted in the hazard rate ratio for coronary heart disease when a polymorphism was observed in the soluble epoxide hydrolase (sEH) gene (EPHX2).

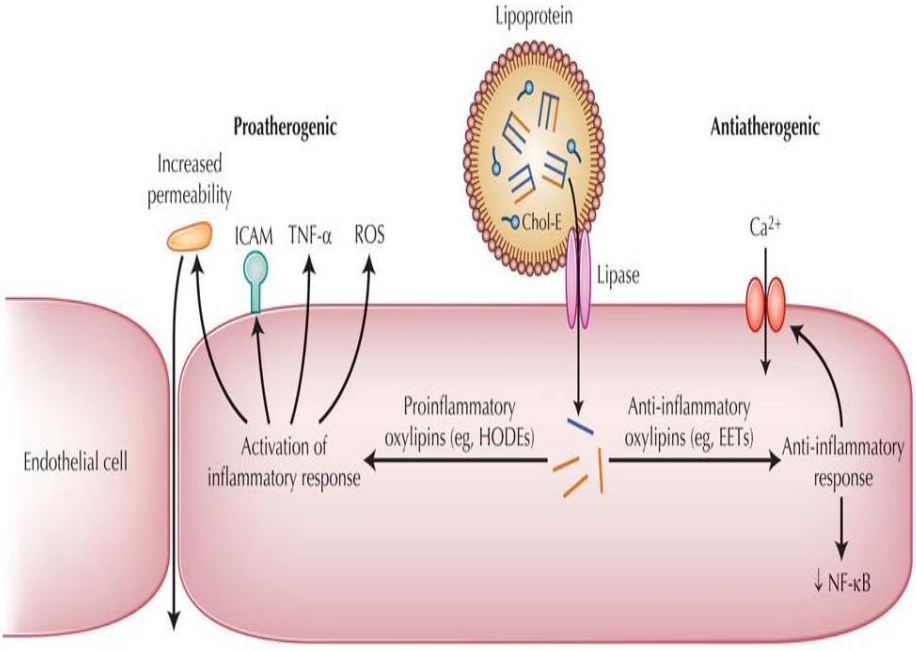


Figure 8: "Impact of Circulating Esterified Eicosanoids and Other Oxylipins on Endothelial Function." Shearer, C. G, Newman, J. W
Current Atherosclerosis Reports November 2009, 11:403

1.8.2 Oxylipins and its effect on insulin production

Insulin resistance as outlined above is a feature in PCOS. There have been a few studies looking at the role of EETs on insulin production. A few reported close collaboration between the sEH (soluble epoxide hydrolase) gene (G860A) polymorphism and insulin resistance (IR) in type 2 diabetes (Ohtoshi 2005).

In addition to EETs, DHET (dihydroxyeicosatrienoic acids) which results from the hydroxylation of EETs, are thought to play a vital role in the pathogenesis of IR in diabetes (Mustafa 2009).

1.8.3 Oxylipins and PE:

Pre- eclampsia (PE) is a hypertensive/proteinuric disorder of pregnancy often accompanied by reduced placental perfusion and fetal growth restriction.

It was in early 1970's that the possible role of prostanoid eicosanoids was postulated in causing pre eclampsia.

Imbalance between vasoconstrictor, platelet aggregator thromboxane and prostacycline, which are a vasodilator and platelet aggregation inhibitor are implicated in the pathogenesis of Pre- eclampsia.

Little is known about the non- prostanoid eicosanoids in the pathogenesis of Pre- eclampsia.

Eicosanoids or oxylipins are produced by the oxidation of arachidonic acid (AA) by various enzymes.The most important enzyme is cytochrome P-450(CYP4500).There have been indentification of oxlipins in term placentae, fetal membrabes and myometrium(Pearson 2009).

The role of oxylipins have been extensively researched in cardiovascular diseases, renal diseases.Their role in pre eclampsia has been researched but not as widely as other conditions.

1.8.4 Assessing Oxylipin:

As outlined above, there are variable sources of oxylipins, their production is through different pathways and they act on different receptors but they eventually act a cellular and tissue level resulting in a pro- inflammatory or anti- inflammatory effect. One approach that has been suggested for is to "phenotype" the action of oxylipin profiles on biomarkers of interest. This can be achieved through application of dimension reduction techniques such as principal components analysis. Simple characteristic markers can be correlated to these complex matrices using approaches such as those recommended by Lemley (2007). This "oxylipin- omics" approach may be shown to be more informative than focusing on one metabolite, one pathway at a time, a situation that does not occur in vivo (Gregory 2009).

One of the aims of my thesis was to use this approach of phenotypying the action of oxylipin profiles in the investigation of the role of oxylipins in women with PCOS.

1.8.5 Conclusion

This chapter has provided an overview of the current diagnosis, pathophysiology, management and long term health implications of PCOS. The current challenges with management and prediction of the long-term health implications were outlined.The potential role of novel molecular biology techniques evaluating genomic, proteomic and metabolomics biomarkers that offered the opportunity to address these challenges were outlined with a focus on metabolomics and oxylipins (the techniques used in my thesis). The next chapter outlined the detailed methods used in my thesis.

CHAPTER 2

2.1 Aims and objectives of Thesis

The aim of this thesis was to investigate the role of proteomics and metabolomics to identify more robust diagnostic and prognostic factors that would help in the understanding and management of PCOS, the associated pregnancy complications (PE) and long-term health implications of the syndrome, with a specific focus on PE and cardiovascular disease. This aim was addressed by the following objectives.

1. Identify proteomics biomarkers similarly expressed in women with pre-eclampsia and PCOS to aid the subsequent synthesis of these results with primary metabolomics data and obtain a comprehensive overview of novel pathways potentially linking PCOS with PE.
2. Identify oxylipin biomarkers (metabolomics markers of cardiovascular disease and pre-eclampsia) unique to Polycystic Ovary Syndrome.
3. Perform a network analysis of lipids differentially expressed in Pre-Eclampsia and Polycystic Ovary Syndrome from Metabolomic studies to identify novel pathways implicated in the link between PCOS and PE.

The **null hypothesis** was that women with PCOS did not have a proteomic or metabolomic profile unique to PCOS and PE and that computational analysis would not reveal a single unifying pathway which would form the basis for future prospective studies aiming to improve the diagnosis of PCOS and predict which women with PCOS would develop pre-eclampsia.

2.2 Organisation of thesis

Chapter 2 of this thesis provides a more detailed review of the literature relevant to the thesis, chapter 3 describes the methods used, chapter 4 outlines the results of study investigating the overlap of proteomic biomarkers between women with pre-eclampsia and PCOS, Chapter 5, the results of a study investigating oxylipin biomarkers in PCOS, Chapter 6, a computerized network analysis of lipids differentially expressed in women with PE and PCOS, and then in chapter 7, with an overall data synthesis, of the studies presented in chapters 4 to 6 and an overall discussion and conclusion is provided.

The thesis ends on chapter 8 which touches on suggestions and ideas for future research.

CHAPTER 3

3.0Methods

3.1.1Research design

A systematic review of the literature was conducted for proteomic biomarkers identified in PE and the biomarkers identified integrated with a database of proteomic biomarkers identified in PCOS to identify biomarkers common to PE and PCOS.Next, primary data on oxylipin biomarkers (metabolomic markers denoting increased cardiovascular risk) was collected in a cross-sectional single-centre study at the University hospital department of Obstetrics and Gynaecology at Queens Medical Centre Hospital, Nottingham. Following this, a computer based metabolomics network analysis was performed using a computer program to identify novel metabolomic networks common to PE and PCOS after identify metabolomic lipid biomarkers identified separately in women with PCOS and women with PE from a literature review.

3.1.2 Research Ethics Approvals

A new Integrated Research Application System (IRAS) application form was submitted in accordance with the regulation of the University of Nottingham and under their guidance and their approval to the Nottingham research Ethics Comittee, reference number: 10/H0408/69 in June 2010.

Separate research ethics approvals were not obtained for the systematic review and biomarker database integration for proteomic biomarkers common to PE and PCOS and the pathway analysis of metabolomic lipid biomarkers common to PCOS and PE, as these did not require direct patient/participant contact.

3.1.3. Systematic review and proteomic biomarker database integration.

a. Studies Eligible for Review:

MEDLINE (1966- December 2013), EMBASE (1980- December 2013) and Cochrane (1993- December 2013), ISI web of knowledge (v4.2) databases were searched using the terms "proteomics", "proteomic", "pre-eclampsia", "pre-eclamptic toxemia, "proteomic biomarker", and "polycystic ovary syndrome" without any restrictions.

The search generated 58 articles. Review articles, studies that did not use proteomic techniques or studies that did not compare PE with a normotensive (control) group were excluded.Moreover, studies involving animals only, studies presenting protein m/z values only rather than protein identifications, or thosestudies that compared different proteomic approaches were further excluded, leaving 16 primary studies eligible for this review

b. Data Extraction:

The original PDFs of studies obtained from the search were located through direct online links to the files from the search results. A manual search of references from all the studies was also conducted to identify any other potentially relevant studies. The search criterion ended in December 2013. The searches were independently conducted by myself and a collaborator, a final year medical student (ND), who volunteered in return for co-authorship on publications.

c. Main Characteristics of the PE Studies:

The selected studies were screened and specific study characteristics were recorded. These included: type of study design, number of participants (N), type of proteomic technique used and the precise nature of the sample analyzed in each study (whether serum, urine etc). Finally, a list of proteins identified to

have been expressed differently in women with PE versus controls (normal pregnancy) was created. These parameters are presented in Table (4). To improve accuracy, screening of the studies was independently performed by myself and the volunteer medical student (ND).

d. Methodological Quality Assessment (QUADOMICS):

The methodological quality of primary studies applying proteomics in women with PE was determined using the QUADOMICS Tool, an adaptation of QUADAS (a quality assessment tool for use in systematic reviews of the diagnostic accuracy studies) that takes into account the particular challenges encountered using "omics" based techniques (Whiting 2003),(Blanca 2008).

The QUADOMICS tool is a version of QUADAS applied to the special class of '-omics'-based diagnostic research. The tool adds new items that assess quality issues which are particular to this research, and which may augment the application of '-omics'-based discoveries to clinical \and public health practice.

It has 16 criterias and is scored from the item present(Y), not present (N), high quality (HQ), low quality (LQ) and not applicable (NA).

The methodologies of the studies which achieved 12/16 or more on the QUODOMICS Tool were classified as high quality (HQ) whereas those which scored 11/16 or less were classified as low quality (LQ). The methodological quality assessment was also performed independently by myself and the volunteer medical student (ND).

e. The PCOS Proteomics Biomarkers Database:

The database on PCOS proteomic biomarkers had been previously published and validated (Atiomo WU 2009).

A further literature search was however performed on MEDLINE (1966– December 2013), EMBASE (1980– December 2013) and the ISI web of knowledge (v4.2) databases using the following search terms "polycystic ovary syndrome" and "proteic", "proteomics", "proteomic biomarker" or "proteomics biomarker" without any limits/restrictions. All relevant studies published since the database was last updated in Feb 2011 were reviewed. One relevant study has since been published but the updated PCOS database already contained the listed biomarkers found in the paper. Data on the original and updated database have been previously published and validated (Atiomo 2009), (Galazis 2013).

3.1.4 Searching for PE biomarkers in the PCOS Biomarker Database

Proteomic biomarkers for PE were compared to the updated database of proteomic biomarkers for PCOS. Where overlaps were found, a note was made of the original tissue in women with PCOS where these biomarkers had been identified and of protein function.

3.1.5 Collection of primary data on oxylipin biomarkers in Polycystic Ovary Syndrome

Patient selection and recruitment:

Women attending the gynaecology /endocrine clinic and the fertility clinics at Nottingham University Hospitals NHS Trust, who were known to suffer with PCOS were recruited over an 18 month period.

Controls were women who volunteered to participate who had regular 21-35 day menstrual cycles, no signs of PCOS on scan and no evidence of hyperandrogenism, and who were not using any form of hormonal contraception.

An informed written consent was obtained from the participants. Due to lack of funding for interpreting services, women who needed an interpreter were not included in the study.

The following eligibility criteria were applied to select subjects for the study:

a. INCLUSION CRITERIA

1. PCOS

-Women between ages of 18-40

-Definition of PCOS (Rotterdam criteria):

PCOS was defined as, women with two or more of the following in the absence of other endocrine causes of oligo-and/or anovulation.

1. Oligo-and/or anovulation
2. Clinical and/or biochemical signs of hyperandrogenism
3. Polycystic ovaries morphology on ultrasound scans, defined as the presence of 12 or more follicles in each ovary (with one ovary is sufficient for diagnosis) measuring 2-9 mm in diameter, and/or increased ovarian volume (> 10 ml).

2. Controls

Consenting age and weight-matched women without PCOS who had regular 21-35 day menstrual cycles were used as controls.

Specific inclusion criteria included the following

1. Age between 18-40.
2. Regular 21-35 day menstrual cycles.
3. No Ultrasound evidence of PCO
4. No evidence of hyperandrogenism.
5. Not using hormonal contraception.
6. Rule out other endocrine disorders.

B .EXCLUSION CRITERIA

Exclusion Criteria for both groups:

- Thyroid disease
- Current pregnancy
- Delivery or miscarriage occurring within the preceding 3 months
- Recent surgery (within 3 months)
- History of myocardial infarction
- Use of aspirin or heparin
- Sex steroid therapy
- A history of haematological disease, malignancy or liver disease
- Hyperprolactinaemia
- A positive synacthen test following raised 17-hydroxyprogesterone levels
- A history of thrombosis
- Recent Viral Illness
- Women that could not speak the English language (no funding available for interpreters)

c. Clinical Intervention:

Women with PCOS were invited once in their cycle for a fasting blood venepuncture and 3D US scanning. Women without PCOS were also invited once in their cycle in the follicular phase (day 3-5) of the menstrual cycle for fasting blood venepuncture and 3D US scanning. Women were asked about their cycles and confirmed the date for their last menstrual period. I took the required measurements in a clinic at Nottingham Queens Medical Centre Hospital and carried out the following; a clinical Interview, baseline measurements of Blood Pressure, Height, Weight, Waist and Hip Circumference, venepuncture for endocrine assays and metabolomic assay. Fasting blood samples were collected and

whole blood was allowed to clot at room temperature, and the plasma was collected by centrifugation at 4000 rpm for 10 min at 4^{o}C, aliquoted into smaller fractions and stored at -80^{o}C until required.

Samples were stored in the Clinical Sciences, department of Human Reproduction (Queens Medical Centre) and Boots building (Department of Pharmacy), University of Nottingham. They were stored in a minus 80 freezer. The access to the department is controlled via swipe card entry. Samples were labelled with the study number only. The paper record of the patient's details and study number were stored in a separate part of the department.
Only the author was responsible for the samples. The author, her supervisors and the laboratory assistant and manager had access to the samples.

d. 3-D Transvaginal Ultrasound:

I then arranged for women to attend for 3-D ultrasound scans on a separate day.These scans were performed in a standard manner with the subject in a supine position with knees flexed and hips abducted. The settings used for every scan and every patient was kept constant and was recorded. Measurements included endometrial thickness, follicular count, size and blood flow to the ovaries. The three- dimensional ultrasound machine software (Automated Volume Count, SonoAVC) was used. As it could identify follicles and automatically calculate their volumes. It then converts the measured volumes to diameters by using the sphere formula.

3.1.6 Metabolomic assays

a. Overall principles of metabolomics

i. Analytical tools:

Various analytical tools are used for metabolomic research such as Nuclear magnetic resonance (NMR) spectroscopy and Mass Spectrometry (MS) due to the complex nature of the metabolome and the assorted properties of metabolites, and no single analytical platform can be applied to detect all metabolites in a biological sample.

Although it has many advantages, the sensitivity of NMR is relatively poor compared with Mass Spectrometry (MS) methods, and concentrations of potential biomarkers may be undetectable (Zhang 2012).

Due to its high sensitivity and wide range of covered metabolites, MS has become the technique of choice in many metabolomics studies. Its utility lies in its wide dynamic range, reproducible quantitative analysis, and the ability to analyze bio fluids with extreme molecular complexity (Lee 2010). MS is significantly more sensitive than NMR and generally requires less material than that utilized in an NMR experiment. It is a truly 'high-throughput' platform. However, MS generally requires chemical derivatization and pre-separation steps in order to select individual metabolites for analysis, since different types of metabolites cannot be analysed simultaneously. Pre-separation is usually achieved by coupling MS to liquid or gas chromatography. Other disadvantages of MS compared with NMR spectroscopy are that it suffers from variable detection responses.

MS can be used to analyse biological samples either by direct injection or following chromatographic separation. MS-based technologies are playing a central role in metabolomics research

and are widely available to facilitate successful metabolomics studies.

ii. Analytical strategies:

The analytical strategies for metabolomics can be broadly divided into ‘‘non-targeted’’ and targeted methods.

The non-targeted approach also called metabolomic-profiling aims to cover the metabolome as broadly as possible. The major challenge in this approach is to identify and quantify metabolomics and to process the data. The targeted methods are, in principle, similar to those available in a typical clinical chemistry laboratory, where a limited number of metabolites are known to be relevant before the experiment is designed (e.g. potential biomarkers), and absolute quantification of the metabolites is performed in a targeted approach.

Analytical method used for the study experiment:

Liquid chromatography Mass Spectrometry (LCMS) was used for analyzing the samples. My colleague Dr Katrin Sanders analysed the samples. I helped her with the sample preparation for analysis.

In a typical MS procedure, a sample (which may be solid, liquid, or gas) is ionized, the ions are then accelerated so they all have the same kinetic energy, the ions are then deflected by magnetic field according to their masses, the beam of ions is detected electrically finally, the elements or molecules are identified by correlating known masses by the identified masses (Sparkman 2000).

Targeted metabolomics of oxylipin biomarkers

For my study, the targeted method metabolomics of oxylipin biomarkers was used.

Fasting blood samples were collected from 59 women with PCOS and 61 controls. The plasma was extracted after centrifugation

for 10 minutes. The samples were stored at -80 degrees centigrade until analysed.

Initial Solid Phase Extraction (SPE) was first performed. The plasma samples were allowed to thaw on ice. Next, a 13mlsx5 time of H2O was added to the sample. 400mls of plasma was put in the eppendorf tubes and 40 µl of the internal standard was added to each sample. The precipitation solvent was made up by adding 0.5µl of 0.5% BT and 900µl of Ethanol to each sample. The samples were then vortexed in a multiplex vortexter for 15 min and then centrifuged @15000g for 10 minutes. After centrifugation the supernatant was mixed with3mls of H2O and was vortexed.

Following this, for each sample the SPE columns were pre conditioned according to the manufacturer's instructions. The prepared samples were then put in the columns and run through without pressure. Each column was washed with 10mls of H2O and then 5mls of 25% ethanol. 5mls of Ethyl acetate was then added to each column and was run through without pressure and analytes were eluted from the column. Solvent was evaporated and analytes were reconstituted by adding 100µlof 70% ethanol and vortexed for 20 seconds. The contents were then transferred to appropriately labelled sample vials.Phenomene starta-XL 100µm, polymeric sorbent. IC mix was made as; 10µl of each 15-HETE-d8, PGF2α-d8, AA-d8, and PGD2-d4 in a vial.
Analytes were eluted from the column using 5 mL ethyl acetate and the solvent was evaporated. The residue was reconstituted in 100 µL of 70% ethanol. 20 µL of each sample were injected into a Shimadzu HPLC ABI 4000 QTRAP system. An ACE 3 C18 150x2.1mm column with 3 µm particle size was used. Mobile phase

A consisted of H_2O and 0.02 % formic acid, mobile phase B was acetonitrile: methanol (4:1) with 0.02 % formic acid.

3.1.7 Statistics:

Continuous data that were normally distributed were summarized using means and standard deviations (SD). Where the continuous data were not normally distributed these were presented with medians and quartiles. Difference between PCOS and control was analyzed using t-test or non-parametric tests. Statistical significance was shown when the p-value <0.05 (2-sided).

3.1.8 Network analysis of lipids differentially expressed in Pre-Eclampsia and Polycystic Ovary Syndrome from Metabolomic Studies

Analysis of pathways have become a fundamental component of 'omic' experiments. The aim is to biologically interpret the results. The limitations are that most of the time both genes and metabolomics are involved in numerous pathways, thus making it tedious to study several pathways to underpin the connections between them.

On the other hand, if a network is built where genes/metabolites are unique nodes, then nodes from multiple pathways can be connected together. Use of such networks enables the user to easily connect pathways and helps in studying the data

For this aspect of the study, two resources were developed with the support of Professor Charlie Hodgman (Professor of Bioinformatics at the University of Nottingham) and his PhD student Ferran Casbas. The first was a metabolic network, called "*Cytoscape"* that contained all the metabolic reactions for the

different family of lipids. The second was a system for converting generic lipid assignments into lists of specific metabolites as defined in the (https://www.nottingham.ac.uk/~stxfc1/Part1.php).Querying this network with the set of lipids whose levels are statistically significantly different in patients suffering a certain disease, it is possible to extract scored sub-networks and genes that correspond to disease pathways. This approach was used with a published lipodomic dataset from patients with Polycystic Ovary Syndrome and patients with pre-eclampsia. More details about the derivation of the lipodomic datasets and the metabolomics network are provided in chapter 6.

3.1.9 Summary

In this chapter, I have outlined the overall methods used for my study which included a systematic review and proteomic biomarker database integration identify biomarkers common to PE and PCOS, a cross sectional study comparing oxylipin biomarkers in women with PCOS and controls and a metabolomic network analysis of lipodomic datasets identified separately in women with PCOS and women with PE from a literature review. The next chapter outlines the results of the systematic review and proteomic biomarker database integration identify biomarkers common to PE and PCOS.

CHAPTER 4

Overlap of proteomics biomarkers between women with pre-eclampsia and PCOS: A systematic review and biomarker database integration

4.1.1 Introduction

Prior to this study, a systematic review showed that pregnant women with PCOS were at least 4 times more likely to develop pre-eclampsia (PE) compared to controls (Kjerulff 2011). Although the link between PCOS and PE have been documented, the pathophysiological mechanisms involved are not completely understood; however it is possible that the associated raised androgen levels, hyperinsulinemia and the subsequent diabetic and hypertensive predispositions in PCOS act as co-factors (Troisi 2003). Among the various implicating factors, defective placental vasculature appears to be central to the disease (Duckitt 2005). There was however insufficient evidence to establish causation and to establish screening for patients for these complications, solely based on PCOS diagnosis. Research studies into the molecular mechanisms underpinning the link between PCOS and PE are therefore required. This offers the potential of facilitating screening in women with PCOS for PE, which can minimize the occurrence of maternal and fetal morbidity/mortality associated with PE in pregnant women with PCOS. Proteomic Biomarker discovery programmes offers the opportunity to address this need, especially if data is synthesized with data derived from other high throughput data generating platforms such as genomics and metabolomics (pan-omics).

Same principals apply in other complex diseases like multiple sclerosis (MS) where pan-omics (genomics, proteomics and metabolomics) have provided a list of possible MS biomarkers, but these have not been validated and as a result they are not useful clinically (Tumani 2009), (Villoslada 2010). The dearth of validation is a common issue in biomarkers studies of complex diseases. There is always a need for validation of such representative biomarkers as well as integration of information offered by these biomarkers. It means that systems biology can be very useful for biomarker discovery, especially if the data generated from "omic" studies can be integrated to understand the underlying disease pathophysiology arriving at the possible explanation of physiology. This integration would also detect how all these biomarkers are shared across different levels of biological complexity genes, molecule, cells, tissues) and to the clinical phenotype (Baranzini 2006).

PE is pregnancy-induced hypertension in association with proteinuria (>0.3 g in 24 hours) with or without oedema (RCOG, 2010). Virtually any organ system may be affected. The incidence of severe PE is about 5/1,000 maternities and is a major cause of poor pregnancy outcomes, including maternal and fetal mortality and severe obstetric morbidity (RCOG 2010). PE is associated with fetal growth restriction, low birth weight, preterm delivery and respiratory distress syndrome (RCOG 2010). Pregnant women who are at high risk of developing PE can be identified in the early antenatal period from a comprehensive history enquiring about risk factors including among others: previous history or family history of PE, age, BMI as well as other co-morbidities such as hypertension, renal disease and diabetes (Duckitt 2005). PE is still the second most common cause of maternal mortality as reported by the confidential enquiry into

maternal and child health (CEMACH) for the triennium of 2006-2008 (CMACE 2011). The exact pathophysiological mechanism in PE is still unknown.

In a literature review of MEDLINE (1966- December 2013), EMBASE (1980- December 2013), ISI web of knowledge (v4.2) and Cochrane (1993- December 2013) databases combining the terms: "proteomics", "proteomic", "preeclampsia", and "PCOS" or "polycystic ovary syndrome"; no studies were identified where proteomic biomarkers for PE had been specifically investigated in women with PCOS. However, there were numerous studies where proteomic techniques had been used in the study of PE and studies where proteomic approaches had been applied to women with PCOS.

The aim of this present study was therefore to systematically review research undertaken using proteomic methodologies for the identification of proteomic biomarkers in PE and consider whether any could represent candidate biomarkers for the detection of PE risk in women with PCOS. This was achieved by performing comparison of PE biomarkers against an updated previously published database of all proteomic biomarkers identified so far in women with PCOS (Atiomo 2009).Any biomarkers common to both conditions could be investigated in further studies to understand the mechanisms linking PE with PCOS.

4.1.2 Methods

These have been outlined in chapter 3. Patient contact was not involved in this study hence Institutional Review Board (IRB) approval was not necessary.

4.1.3 Results

i.*Proteomic studies of PE*

Figure 9 demonstrates the selection process of the primary studies where proteomic methodologies were used for the identification of biomarkers of PE. The search conducted yielded 58 articles. Studies were excluded if they were review articles, did not use proteomic techniques or if they did not compare PE with a normotensive pregnant (control) group. Moreover, studies involving animals only, those presenting protein m/z values only rather than protein identifications, or those simply comparing different proteomic approaches were further excluded leaving 16 primary studies eligible for this review (Epiney M, Buhimschi IA, Park JS, Johnstone ED, Gharesi-Fard B, Blumenstein M, Watanabe H, Blankley RT, Liu C, Rasanen J, Jin H, Vascotto C, Myers JE, Centlow M, Kolla V, Blumenstein M).

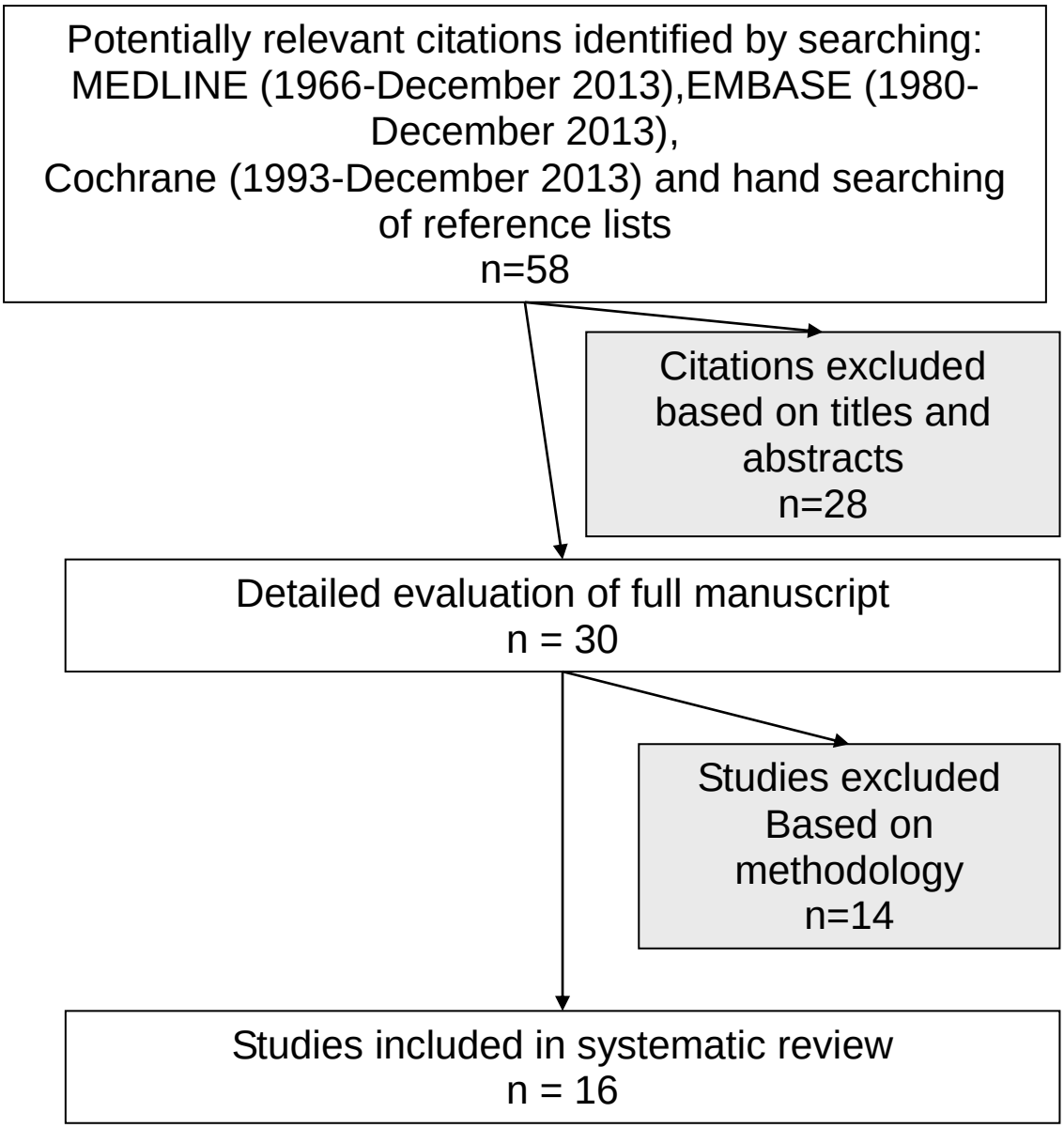


Figure 9: Flow chart showing selection of studies included in the systematic review.

There were a total of 1423 patients and controls in all the selected studies and 192 different proteomic biomarkers for PE were identified. Six studies investigated placental tissue (Epiney, Buhimschi, Johnstone, Gharesi-Fard , Jin H, Centlow), with one of which also assessing urine (Buhimschi).Two studies used amniotic fluid (Park, Vascotto), five used plasma (Blankley, Liu C, Myers,Kolla,Blumenstein) and finally, three used serum samples

(Blumenstein, Watanabe, Rasanen). These are summarized in Table 4:

Study	Study design	Population		Selection Criteria		Proteins Identified	Change Vs Normal (↑/↓)	Sample Site	Technique Used
		N	Mean Age ± SD & Age Range	Inclusion	Exclusion				
Epiney et al.	Cross sectional	Control n= 6	33.2 ±2.6	Normotensive pregnant patients.	NA	FN1 protein	Decreased	Placenta	LC-ESI-MS/MS
		PE n= 4	38.8 ±2.3	Systolic blood pressure level≥160mmHg or a diastolic blood pressure level≥110mmHg on two occasions and proteinuria ≥3+ on a urine stick or ≥5 g ina 24-hour urine specimen (ACOG practice, 2002).		Apha-actinin-4	Decreased		
						Actin	Increased		
						Transgelin-2	Decreased		
						Pregnancy-specific beta-1-glycoprotein 3	Increased		
						Choriogonadotropin subunit beta	Increased		
						Pregnancy-specific beta-1-glycoprotein 2	Increased		
						Protein Si00-A11	Increased		
						Pregnancy specific beta-1-glycoprotein 4	Decreased		
						Phosphatidylethanolamine-binding protein 1	Increased		
						Beta-2-microglobulin	Decreased		
						Coagulation factor XIII A chain	Decreased		

Epiney et al. cont…						Follistatin-related protein 1	Decreased		
						Malate dehydrogenase	Decreased		
						Annexin A2	Increased		
						Thioredoxin domain-containing protein 4	Decreased		
						Serotransferrin	Decreased		
						C9orf88 variant protein (Fragment)	Decreased		
						Cystatin-M	Increased		
						Polypyrimidine tract-binding protein 1	Decreased		
						40S ribosomal protein S5	Decreased		
						Calnexin	Increased		
						Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform			
Buhimschi et al.	*Exploratory phase*	38 =PE 21=control	NA	Severe Pre Eclampsia(spe)	NA	21-aa C-terminus fragment of SERPINA-1	Increased	Urine+ Placenta	SELDI TOF-MS

cont…	Challenge phase	206(Cross sectional cohort) 19(Longitudnal cohort)		Low risk of pre eclampsia(n=4) High risk of pre eclampsia(n=15)		24-aa N-terminus fragment of SERPINA-1	Increased		Q-TOF-MS
Park et al.	Cross Sectional	PE N=18 Chronic Hypertension N=7 Control N=16 Control N=6	32.5 ±4.7 33.0 ±4.5 31.6 ±3.0	Blood pressure ≥140/90 after 20 wks and proteinuria. Blood pressure ≥140/90before pregnancy or after 20 wks No evidence of high blood pressure or proteinuria No evidence of high blood pressure or proteinuria.	Multiple pregnancy Evidence of intra-uterine infection.	Proapolipoprotein A-I Apolipoprotein A-I SBBI42	Increased Not different Increased	Amniotic fluid	SELDI TOF-MS WESTREN BLOT
Johnstone et al.	Cross sectional	PE N=6	NA	Blood pressure ≥140/90 after 20 wks and proteinuria.	Smokers Diabetes IUGR Medication other than antihypertensives.	a-2-HS-glycoprotein Glutathione S-transferase Peroxiredoxin 6 Aldose reductase Heat shock protein 60 β-tubulin Heat shock protein 70 Proteasome, a subunit Ezrin Protein disulfide isomerise	Increased Decreased Decreased Decreased Decreased Decreased Decreased Decreased Decreased Decreased	Placenta	LC/MS /MS Western blot

Johnstone et al. Cont...						Phosphoglycerate mutase 1	Decreased		
						Triosephosphate isomerase	Decreased		
Gharesi et al.	Cross sectional	Normal n=5	24.1 ±3	No evidence of high blood pressure or proteinuria.	Multigravida.	Heat shock protein gp96 precursor	Decreased	Placenta	MALDI TOF/TOF
		Severe PE N=5	24.3 ±2.4	Blood pressure, 160 mmHg or higher systolic or 110 mmHg or higher diastolic on two occasions at least 6 h apart,and new onset of proteinuria, 500 mg or more of protein in a 24 h urine collection or 3+ or greater on urine dipstick testing of two random urine samples collected at least 4 h apart .	Vaginal birth.	Chloride intracellular channel 3	Increased		
						Chain A of enoyl-coenzyme A hydratase	Decreased		
						Chain A, crystal structure of human Apolipoprotein A-I	Decreased		
						Protein disulfide isomerise	Increased		
						Cu/Zn-superoxide dismutase	Increased		
						Actin, gamma 1 propeptide	Decreased		
						Peroxiredoxin 3, isoform CRA-a	Decreased		

Gharesi et al Cont..						HSPA8 (Hsc 70)	Decreased		
						Peroxiredoxin 2 isoform a	Decreased		
						Chain A, Transthyretin	Decreased		
Blumenstein et al.	Retrospective cross sectional	Normal n=57	29.7	Plasma obtained at 20±1 wk gestation from the SCOPE biobank. No evidence of high blood pressure or proteinuria.	NA	Fibronectin 1 isoform 3 preproprotein	Increased	Serum	LC-MS/MS Western Blot
		Pre eclampsia(appropriate growth for gestation) N=27	30.0			Fibrinogen, β chain preproprotein	Increased		
		Pre eclampsia(Small for gestation age) N=12	30.3	Blood pressure ≥140/90 after 20 wks on two occasions 4hrs apart and combined with either proteinuria or multi organ complication. Customized birth weight less than tenth centile for gestational age.		Clusterin isoform 1	Increased		
						TTR	Increased		
						Apolipoprotein A-I precursor	Increased		
						Hemopexin	Increased		
						Transferrin	Increased		

Watanabe et al.	Cross sectional	Normal pregnant women n=80 Pre – eclampsia n=80	30.3 ±4.3 30.6 ±5.3	No evidence of high blood pressure or proteinuria Diagnosis of preeclampsia was based on the criteria of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy.		Fibrinogen gamma chain Alpha-1-antichymotrypsin	Increased Increased	Serum	2-Dimentional electrophoresis MALDI-TOF-MS Western Blot
Blankley et al.	Cross sectional	Normal pregnant n=23 Pre eclampsia n=23	NA	No evidence of high blood pressure or proteinuria. PE was diagnosed using standard definitions from the International Society for the Study of Hypertension in Pregnancy.	NA NA	Clusterin Apolipoprotein B Inter-alpha inhibitor H1 Inter-alpha inhibitor H2 Inter-alpha inhibitor H3 Complement C6 Complement C7 PAPP-A Vitamin K-dependent protein Z Complement C1s Sex hormone-binding globulin	Increased Increased Increased Increased Increased Increased Increased Increased Increased Increased	Plasma	MALDI TOF/TOF

Blankley et al.cont…						Clusterin	Increased		
						Coagulation factor X	Increased		
						Coagulation factor V	Increased		
						Insulin-like growth factor binding protein	Increased		
						complex acid labile chain precursor (ALS)	Increased		
						Pregnancy-specific B-1 glycoprotein 11	Increased		
						Pregnancy-specific B-1 glycoprotein 1	Increased		
						Vitamin D binding protein	Decreased		
						Serum amyloid P-component	Increased		
						Complement C2	Increased		
						Pregnancy-specific glycoprotein 9	Increased		
						Paraoxonase 1	Increased		
						Peroxiredoxin-2	Increased		
						Carboxypeptidase N catalytic chain	Increased		
						Vitamin D binding protein	Decreased		
						Alpha-2-macroglobulin	Decreased		

Blankley et al. Cont….						Vitronectin precursor	Decreased		
						Afamin precursor (Alpha-albumin)	Decreased		
						Fibronectin 1	Decreased		
						Trypsin-1	Decreased		
						Extracellular matrix protein 1	Decreased		
						Complement C1q	Decreased		
						Plasma protease C1 inhibitor	Decreased		
						Fetuin-A	Decreased		
						Zinc finger protein	Decreased		
						Complement C 4B	Decreased		
Liu et al	Cross sectional	Normal pregnant n=5 Severe Pre eclampsia n=5	28.2 ±1.8 30.3 ±2.4	No evidence of high blood pressure or proteinuria. Severe preeclampsia was defined as increased blood pressure (≥160 mmHg systolic or ≥ 110 mmHg diastolic on ≥ 2 occasions at least 6h apart) that occurred after 20 weeks of gestation in women with preciously normal blood pressure,	NA	Serpin peptidase inhibitor, clade A, member 1	Increased	Plasma	LC MS/MS
						Alpha-2-HS-glycoprotein	Increased		
						AMBP protein	Increased		
						Apolipoprotein E	Increased		

Liu et al… cont….				accompanied with *proteinuria* (≥5 g/24h) or proteinuria of 2+ or more by *dipstick* measurement.		Apolipoprotein H	Increased		
						Ceruloplasmin	Increased		
						Chorionic somatomammotropin hormone	Increased		
						Clusterin	Increased		
						Coagulation factor X	Increased		
						Complement C1q subcomponent subunit A	Increased		
						Complement C1q subcomponent subunit C	Increased		
						Complement component C8 beta chain	Increased		
						Complement component C9	Increased		
						Complement factor H	Increased		
						Complement factor H-related 1	Increased		
Liu et al Cont…						Complement factor properdin	Increased		
						Fibrinogen alpha chain	Increased		
						Fibrinogen beta chain	Increased		
						Fibrinogen gamma chain	Increased		
						Fibronectin	Increased		
						Fibulin-1	Increased		

						Galectin-3-binding protein	Increased		
						Hyaluronan-binding protein 2	Increased		
						Kininogen-1	Increased		
						Lysozyme C	Increased		
						Serpin peptidase inhibitor, clade F, member 1	Increased		
						Plasminogen	Increased		
						Pregnancy-specific beta-1-glycoprotein 3	Increased		
						Pregnancy-specific beta-1-glycoprotein 4	Increased		
						Vitamin D-binding protein	Increased		
						Vitronectin	Increased		
						Alpha-2-macroglobulin	Decreased		
						Angiogenin	Decreased		
						Serpin peptidase inhibitor, clade C, member 1	Decreased		
						Apolipoprotein A-II	Decreased		
						Apolipoprotein B-100	Decreased		
						Apolipoprotein-L1	Decreased		
						C4b-binding protein alpha chain	Decreased		

Liu et al Cont…						Complement factor H-related protein 1	Decreased		
						Glutathione peroxidase 3	Decreased		
						Hemoglobin subunit alpha	Decreased		
						Hemoglobin subunit zeta	Decreased		
						Leucine-rich repeat-containing protein 6	Decreased		
						Mannan-binding lectin serine protease 2	Decreased		
						Plasma retinol-binding protein 4	Decreased		
						Platelet factor 4	Decreased		
						Pregnancy zone protein	Decreased		
						Serum amyloid A2 protein	Decreased		
						Serum amyloid A-4 protein	Decreased		
						Serum amyloid P-component	Decreased		
						Transthyretin	Decreased		

Rasanen et al	Prospective Cross sectional	Total =267 Clinical Cohort:118 Mild PET N=30 Severe PET N= 30 Normotensive N =58 Preclinical cohort: N= 149 Mild PE N=30 Severe PE N=40 Normotensive N=79 Severe PE N=8	28.3 27.8 28.8	Working Group Criteria on high blood pressure in pregnancy	NA	Matrix metalloprtoteinase-9 Fibronectin Pappalysin-2 Choriogonadotropin-β Apolipoprotein C III Cystatin C sFlt-1 Endoglin Pre clinical cohort Complement factor D Vascular cell adhesion protein 1 Beta -2-microglobulin Cystatin-C Pappalysin Pregnancy zone protein Pregnancy-specific beta-a-gycoprotein Chorionic somatomammotrophin hormone Lipopolysaccharide-binding protein	Decreased Increased Increased Increased Increased Increased Increased Increased Increased Increased Increased Increased Increased Increased Decreased Decreased	Serum	2D-LC-MS/MS

Ranasen et al Cont..... .						Actin Plastin 2 Vinculin Apolipoprotein B-100 Matrix metalloproteinase Receptor-tyrosin-protein phosphate gamma Vascular endothelial growth factor receptor 3 Profiling-1 SHE domain-binding glutamic acid-rich-like protein 3 Tropomyosin alpha-4chain Transgelin-2 Filamin-A Talin-1	Decreased Decreased Decreased Decreased Decreased Decreased Decreased Decreased Decreased Decreased Decreased Decreased Decreased		

Jin et al.	Cross sectional	Control n= 8 PE n=8	NA	Normotensive pregnant women Hypertension was defined as a blood pressure >140 mm/Hg (systolic) or >90 mm/Hg (diastolic) on at least two occasions and at least 4-6 h apart after the 20th week of gestation in women known to be normotensive beforehand.	NA	Heat shock 27 kDa protein 1 78 kDa glucose-regulated protein precursor. Titin Prohibitin Calnexin Annexin A1 NADH-ubiquinone oxidoreductase 24 kDa Chloride intracellular channel protein 3 Smooth muscle and non-muscle myosin alkali light chain isoform 1 Actin alpha 1 skeletal muscle protein Keratin 10 Centrosome pr Not identified	Increased Increased Decreased Increased Decreased Increased Increased Increased Increased Increased Increased Decreased	Placenta	LC–MS/MS

Vascotto et al.	Cross sectional	Controls N=5 Preeclampsia N=5		Normotensive and Women diagnosed with preeclampsia	Women with pregestational diseases and pregnancy complications	Transthyretin	Increased	Amniotic fluid	MALDI-TOF-MS
Myers et al.	Cross sectional	*Taining set* Controls N= 200 PE N=100 *Validation set* Controls N=250 PE N=50	35 year 35years 26.8 (6.4) 26.6 (6.0)	Normotensive patients. Preeclampsia = hypertension associated with proteinuria. Hypertension was defined as a blood pressure >140 mm/Hg (systolic) or >90 mm/Hg (diastolic) on at least two occasions and at least 4-6 h apart after the 20th week of gestation in women known to be normotensive beforehand. Proteinuria= renal excretion of at least 300 mg of proteins in a 24 h urine sample	NA	IGFALS, MCAM,	Increased Decreased	Plasma	MS/SRM/ELISA
Centlow et al	Cross sectional	Control N=30 PE N=30	28.9 (5.3) 29.7 (5.5)	Preeclampsia was defined as systolic blood pressure ≥ 140 mm Hg or diastolic blood pressure ≥ 90 mm Hg, or both, on 2 occasions 4 hours apart after 20 weeks ' gestation but before the onset of	Patients with other systemic diseases.	Apolipoprotein 1 Tropomyosin -3	Increased Decreased	Placenta	MALDI–TOF MS/PAGE2/ Western blot

Centlow et al Cont…				labor, or postpartum, with either proteinuria (24-hour urinary protein ≥ 300 mg or spot urine protein:creatinine ratio ≥30 mg/mmol creatinine or urine dipstick protein ++) or any multisystem complication of preeclampsia.					
Kolla et al	Case Control/retrospective	Control N=6 PE N=6	35.1 35.1	No evidence or previous history of pre eclampsia Preeclampsia= blood pressure above 140/90mmHg and proteinuria above 0.3 g/L or rise in blood pressure above 20mmHg from the first trimester of pregnancy.	NA	Fibrinogen Fragment D Clusterin isoform 2 Apolipoprotein A-I Fibronectin Angiotensinogen Galectin 3 binding Plasminogen Transferrin C4 beta binding Hemopexin	Increased Increased Increased Increased Increased Incraesed Increased Increased Increased Increased	Plasma	MALDI-TOF/TOF iTRAQ

Blumenstein Prakash et al	Longitudinal study	Control N=6 PE N=6	30.7 (2.9) 31.7 (1.8)	Healthy pregnancy outcome Preeclampsia was defined as systolic blood pressure (BP) ≥140 mm Hg and/or diastolic BP ≥90 mm Hg on 2 or more occasions after 20 weeks' gestation but prior to the onset of labor, or postpartum systolic BP ≥140 mmHg and/or diastolic BP ≥90 mm Hg postpartum on at least 2 occasions 4 hours apart, combined with either proteinuria (spot protein to creatinine ratio ≥30 mg/mmol, or 24-hour urinary protein ≥0.3 g/24 h, or dipstick proteinuria ≥2+) or any multiorgan complication.	NA	Vitronectin 75kd Vitronectin 65kd a-1-antichymotrypsin (SERPINA3) Kininogen-1	Increased Decreased Decreased Decreased	Plasma	DIGE Western Blot LC-MS/MS analysis on a QSTAR XL ESI-qTOF

Table 4: The main characteristics of each study with proteomics differencially expressed in patients with the Pre-eclampsia compared to normotensive pregnant individuals

Index:

2D-GE/2DE= 2D (gel) electrophoresis, 2D-LC= 2D liquid chromatography, DIGE= differential gel electrophoresis, ELISAs= enzyme-linked immunosorbent assays, iTRAQ= isobaric tags for absolute and relative quantitation, LC-MS/MS=Liquid Chromatography – Tandem Mass Spectrometry, MALDI-TOF= Matrix-assisted laser desorption time-of-flight, MS= mass spectrometry, N= Number of participants, PE= Pre-eclampsia, Q TOF= quadrupole time of flight, SD= Standard deviation, SDS-PAGE=Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis, SELDI TOF= Surface-enhanced laser desorption ionization time-of-flight, SRM= Selective reaction monitoring

Various proteomic techniques were used in the 16 studies with SELDI-TOF (Surface-Enhanced Laser Desorption Ionization Time-Of-Flight) Mass Spectrometry, MALDI-TOF (Matrix-Assisted Laser Desorption Time-Of-Flight) Mass Spectrometry, and LC-MS/MS (Liquid Chromatography – Tandem Mass Spectrometry) being the most commonly applied (Table 4).

ii. *Assessing the quality of the relevant studies*

Ten out of the 16 studies were HQ fulfilling 12 or more of the 16 QUADOMICS criteria (Epiney, Buhimschi, Park, Blumenstein, Watanabe , Vascotto , Myers ,Centlow , Kolla , Blumenstein M). The remaining 6 studies were LQ achieving less than 12 out of the 16 quality criteria (Johnstone, Gharesi-Fard, Blankley, Liu C, Rasanen, Jin H).

Quality Criteria	Epiney et al., 2011 (16)	Buhimschi et al., 2008 (17)	Park et al., 2008 (18)	Johnstone et al., 2011 (19)	Gharesi-Fard et al., 2010 (20)	Blun et a (21)
1	N	Y	Y	N	N	N
2	N	Y	Y	N	N	Y
3	Y	Y	Y	Y	Y	Y
4	Y	Y	Y	N	N	Y
5	Y	Y	Y	Y	Y	Y
6	Y	Y	Y	Y	Y	Y
7	Y	Y	Y	Y	Y	Y
8	Y	N	Y	Y	Y	Y
9	Y	N	Y	Y	Y	Y
10	Y	Y	Y	Y	Y	Y
11	Y	Y	Y	Y	Y	Y
12	N/A	Y	N	N	N	N
13	Y	Y	Y	Y	Y	Y
14	Y	Y	Y	Y	N	Y
15	Y	Y	N	N/A	N	Y
16	Y	Y	N	N	N	N
TOTAL	**13**	**14**	**13**	**10**	**9**	**13**

Table 5: Methodological Quality Assessment using the QUADOMICS Tool (Part1)

Quality Criteria	Rasanen et al., 2010 (25)	Jin et al., 2008 (26)	Vascotto et al., 2007 (27)	Myers et al, 2013 (28)	Centlow et al., 2009 (29)	Kolla et al, 2011 (30)	Blumenstein, Prakash et al 2009 (31)
1	N	N	Y	Y	Y	N	N
2	Y	N	N	Y	N	Y	Y
3	N	N	Y	Y	Y	Y	Y
4	Y	N	Y	Y	Y	Y	Y
5	Y	Y	Y	Y	Y	Y	Y
6	N/A	Y	N/A	N	Y	Y	Y
7	Y	Y	Y	Y	Y	Y	Y
8	Y	Y	Y	Y	Y	Y	Y
9	Y	Y	Y	Y	Y	Y	Y
10	Y	Y	Y	Y	Y	Y	Y
11	N	Y	Y	Y	Y	N	Y
12	N	N	N	Y	N	N	N
13	Y	Y	Y	Y	Y	Y	N
14	Y	N	Y	Y	Y	Y	Y
15	N	N/A	Y	N	Y	Y	Y
16	N	N	N/A	Y	Y	Y	Y
TOTAL	**9**	**8**	**12**	**14**	**14**	**13**	**13**

Table 5: Methodological Quality Assessment using the QUADOMICS Tool (Part2)

Cross referencing proteomic biomarkers identified in primary studies of PE in database of proteomic biomarkers for PCOS:

The 192 proteomic biomarkers for PE were cross referenced with the PCOS data base to determine if any were also differentially expressed in PCOS. Five biomarkers were found to be differentially expressed in women with PE and with PCOS compared to controls. Transferrin, fibrinogen alpha, beta and

gamma chain variants and kininogen-1 were raised and annexin2 and peroxiredoxin 2 were decreased in both women with PCOS and with PE. In PE these biomarkers were found in serum, plasma and placenta respectively where as in PCOS the biomarkers identified were in serum, follicular fluid, ovarian and omental biopsy respectively.

4.1.4 Discussion

This study, for the first time, identified a panel of five proteomic biomarkers which were similarly differentially expressed in women with PE and in women with PCOS. These include transferin, fibrinogen-alpha, beta and gamma chain variants, kininogen-1, annexin 2 and peroxiredoxin 2. These are interesting findings but they will need to be validated, and further studies will need to investigate how these proteins interact with each other and other proteins, genes and metabolites. These five proteomic biomarkers might explain the link between PCOS and PE. Notably a consistent and emerging theme from studies applying proteomic approaches in PCOS is the potential role of immunoregulation/inflammation and antioxidants in the pathogenesis of the condition. These two pathways have also been implicated in PE which is associated with PCOS (Redman 2011) ,(Szarka 2010), (Tousoulis 2008),(Yun 2012).

Annexin A2 was down regulated in both patients with PE and PCOS, although in PCOS it was in ovarian biopsy and in PE it was in the placental biopsy. Annexin A2 is the main physiological receptor for plasminogen on the extracellular surface of endothelial cells (Gugliucci 2002). It causes fibrinolysis by accelerating tissue plasminogen activator (tPA) (Kang 1999) at the endothelial leve. The possibility that Annexin A2 plays a role in insulin-stimulated glucose transporter isoform 4 (GLUT4)

translocation and glucose transport, was tested in the experiment by Huang et al .The study done by Huang et al confirmed the role of Annexin A2 in glucose transport(Huang 2004).

This was further confirmed in an experiment by Liu B et al.They tested the proteomic differences in the placental villi from women with Gestational diabetes mellitus (GDM) and normal pregnant women (Liu 2012).

Annexin A2 was found to be differentially expressed in the study and may play a part in insulin resistance in GDM (Lui 2012).

The down-regulation observed in PE tilts the coagulation/fibrinolysis balance towards enhanced coagulation and thrombosis (Gugliucci 2002), (Kang 1999),(Huang 2004), (Lennon 2003), (Ma X 2007).

I therefore hypothesise that the down-regulation of annexin A2 found in some women with PCOS could be a strong marker for the development of PE in this group.

Annexin A2 and fibrinogen alpha, beta and gamma chains are central in regulating fibrinolysis and thrombosis and their altered expression might represent changes in permeability of the peripheral vessels, and vasculature of the various tissues including ovaries, causing fibrinolysis and abnormal fibrogenesis and thrombosis in PCOS. I speculate that the impaired expression of these proteins may account for the early pregnancy complications such as miscarriage and could impinge upon the cardiovascular system in PCOS patients due to hypofibrinolysis and thrombophilia (Gugliucci 2002).

Transferrin was found to be upregulated in sera of women with PE and PCOS. It is an important β-globulin responsible for transporting iron to various tissues and promoting cell growth and development (Kevin 1983). Transferrin also plays a crucial role in

pregnancy where its expression in the villous syncytiotrophoblasts is significant in women with PE compared to those with normal pregnancies and the reason for this significant expression in the placentae of pregnancies complicated by either gestational diabetes or PE could be because of developing or existing fetal stress (Kralova 2008).Transferrin in high concentrations can inhibit FSH to interact with its receptors on the granulose cells and this can affect the maturation of oocytes by decreasing the levels of cAMP. (Kawano 1995). Transferrin is a recognized stress/acute phase response molecule.

Several studies have demonstrated that increase in iron stores and transferrin can cause insulin resistance by increasing lipolysis in adipocytes.This would result in release of free fatty acids resulting in impaired glucose uptake in muscle ,increase hepatic intake of glucose which in turn lead to insulin resistance and type 2 diabetes mellitus(Rumberger 2004),(Allan 2006).

Its increase in both women with PCOS and PE could be explained on the basis of the inflammatory component of the two conditions.

Kininogen-1 was found to be upregulated in both women with PE and PCOS in plasma and omental biopsy respectively. Kininogens play an important role in blood coagulation by helping to position optimally pre-kallikrein and factor XI next to factor XII and inhibiting the thrombin- and plasmin-induced aggregation of thrombocytes. Moreover, they are a mediator of inflammation and cause increase in vascular permeability, stimulation of nociceptors, and release of other mediators of inflammation (Wong 2013). These mechanisms have been implicated in the pathogenesis of both PE and PCOS (Redman 2011), (Gugliucci 2002), (Cubedo 2013).

The literature suggests that inflammation is linked with insulin resistance.

A study looked at obese and healthy individuals and checked their fibrinogen levels in addition to plasma and blood viscosity and Insulin resistance.The study concluded that the levels of fibrinogen and insulin resistance were higher in obese patients (Sola 2007).

Peroxiredoxin 2 was found to be down-regulated in both PE and PCOS (Kevin 1983). Considering the important role of peroxiredoxin in protection against H_2O_2-induced cell damage and apoptosis, down-regulation in pre-eclamptic placentas, emphasizes the role of oxidative stress as an important factor in the development of PE (Cubedo 2013). Furthermore, studies have recently suggested that reactive oxygen species induce oxidative stress that stimulates androgen-producing steroidogenic enzymes leading to the hyperandrogenism observed in women with PCOS (Burton 2004).

The disparate accuracy and precision of the various quantitative and semi-quantitative proteomic techniques could pose a challenge with a combined assessment of the results.This is however, an issue with all systematic reviews and meta-analyses, which could be affected by clinical heterogeneity. This was the reason I chose to report differential protein expression as either simply up or down regulated, which is consistent with previously, published systematic reviews of proteomic biomarkers (Baek 2010).In addition, in practice, using inflammatory factors as biomarkers for disease states represents a challenge, as inflammation is associated with a multitude of other pathological

conditions.However, this is a limitation that applies to all biomarker studies of complex diseases (Ling 2011). I do not propose at this stage that the biomarkers identified in our study are used as definitive biomarkers of PE and PCOS rather that our results inform further mechanistic and validation studies that can be used to better understand the pathophysiological mechanisms linking PCOS and PE for example by synthesizing these results with genomic and primary metabolomics data generated in a similar was to obtain a comprehensive overview of pathways potentially linking PCOS with PE.

Although proteomic and other "omic" technologies offer a great potential for the generation of new insights into disease etiology, concerns have been expressed about the relatively slow pace at which research findings have been translated into clinical care (Peral 2009).In addition, proteomic techniques have limited ability in detecting low-abundance proteins, some of which may have diagnostic potential. There has been a call for greater focus on data integration from primary proteomic studies in order to improve translation of research findings and prospective validation (Hojlund 2008).The sample sizes and number of biomarkers identified following these studies runs the risk of false positives and this is a limitation of all biomarker studies (Solomon 2006).

These issues again emphasize the need for collaboration, data synthesis and integration (as in this review) in order to identify a shortlist of replicated biomarkers which can be validated in subsequent hypothesis-driven research.I therefore see great value in informing the scientific community about these research findings at this stage as in the area of "omic" research, data sharing and collaboration is vital for progress.

4.1.5 Conclusion

In conclusion, by integrating data from proteomic studies in PE with data from proteomic studies in PCOS, I have for the first time identified a panel of five biomarkers of PE which are common to women with PCOS including transferin, fibrinogen alpha, beta and gamma chain variants, kininogen-1, annexin 2 and peroxiredoxin 2. If validated, these biomarkers could provide a useful framework on which the knowledge base in this area could be developed. In the next chapter, data generated from primary research of oxylipin biomarkers (targeted metabolomics biomarkers of increased cardiovascular risk) are presented.

Chapter 5

Oxylipin biomarkers in Polycystic Ovary Syndrome

5.1.1 Introduction

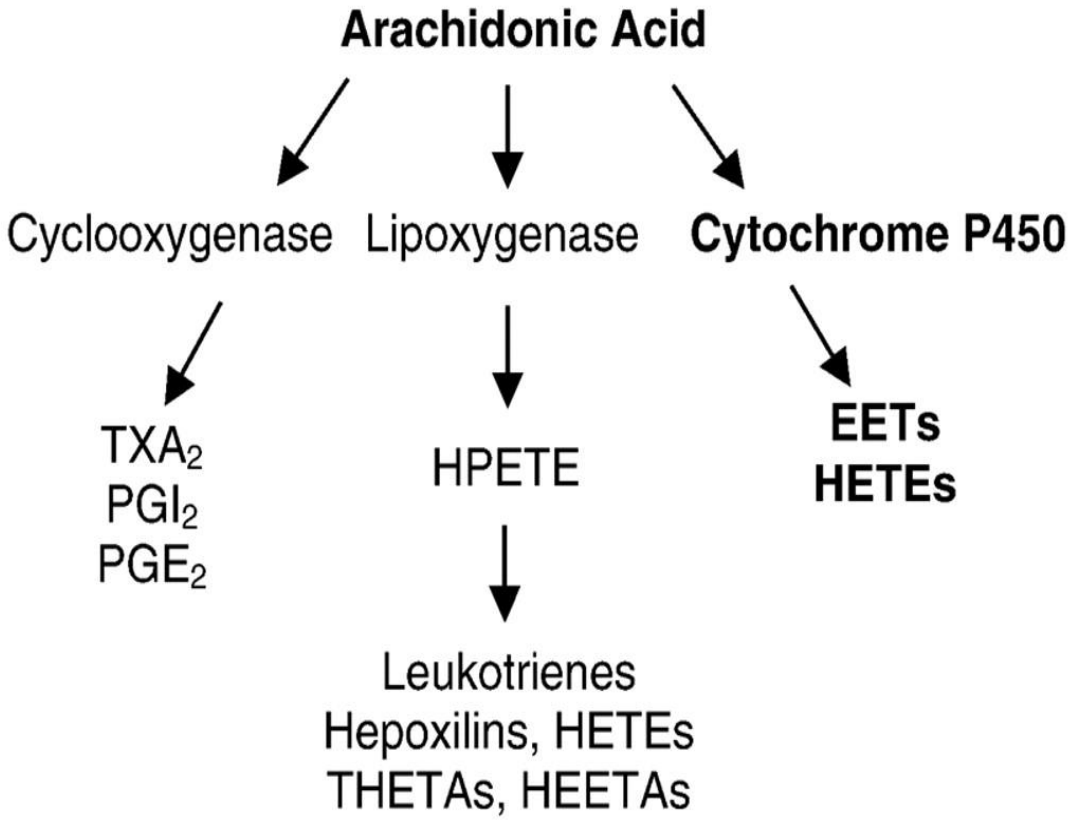
The last chapter outlined the results of a study that identified proteomic markers similarly expressed in PCOS and PE.
This chapter outlines the results of a study measuring prostaglandin-derived products (oxylipins) associated with similar pathways (inflammation, oxidative stress, vascular haemostasis and cardiovascular disease) in women with PCOS using a targeted metabolomic approach.As highlighted in the introduction, this was to aid a planned subsequent overall computational analysis. This would potentially reveal a single unifying pathway which would form the basis for future prospective studies aiming to improve the diagnosis of PCOS, and also predict which women with PCOS would develop pre-eclampsia and cardiovascular disease.

PROSTAGLANDINS
Prostaglandins were first discovered as early as 1930's by Kurzok and Lieb, Goldblatt and Von Euler named them "Prostaglandins" as they were under the impression that they were derived from prostate gland. It was not until 1982, that Bergstorm, Vane and Samuelson won a Nobel Prize for revelation of the "Arachidonic cascade". In the 80's the epoxygenase pathway was discovered and their functions were explored.
Arachidonic acid derived eicosanoids, or oxygenated 20-carbon polyunsaturated fatty acids (PUFAs), are thought to play key regulatory roles in most parts of the human body. Arachidonic acid is derived from the membrane phospholipid and then through various enzyme actions produces, a variety of lipid

mediators. They can be derived from cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome p450 (CYP), or through the interaction of reactive oxygen species with arachidonic acid (AA) (Figure 10). Collectively, over 50 species of oxygenated fatty acids (FAs) or oxylipins are known to be derived from AA. Arachidonic acid derivatives or oxylipins are well known for their involvement in vascular haemostasis, platelet aggregation and protection of gastric mucosa. Recently eicosanoids have been discovered to play a major role in inflammation, cancer, rheumatoid arthritis, asthma and other autoimmune disorders.

EET's:

The cytochrome p450 (CYP) enzyme complex (which also plays a role in steroidogenesis in PCOS), converts Arachidonic acid into four epoxyeicosatrienoic acid (EET) isomers which are: 5,6 EET, 8,9 EET, 11,12 EET and 14,15 EET (Capdevila et al., 1990). Each EET can be further metabolized to dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH), resulting in the generation of four corresponding DHET regioisomers. They all function in an autocrine and paracrine intermediaries. Sometimes their actions can be discounted in experiments as EETs are labile in cultured cells and their actions are subdued where there is production of H2O2 (Larson 2008). EETs are produced in the endothelium and exert their action through activation of Ca2+ and K+ channels. This results in vasodilatation and this endothelium-derived hyperpolarizing factor (EDHF) of EETs is seen in coronary and renal vessels that in turn causes a fall in blood pressure.



COX= cyclooxygenase
CYP= cytochrome P450
EET= epoxyeicosatrienoic acid
HEETA= hydroxyepoxyeicosatrienoic acid
HETE= hydroxyeicosatetraenoic acid
HPETE= hydroperoxyeicosatetraenoic acid
LOX= lipoxygenase
PG= prostaglandin
THETA= trihydroxyeicosatrienoic acid
TX= thromboxane

Figure: 10 Arachidonic acid cascade

In many studies EETs have been found to have a beneficial effect on heart, kidneys and nervous system (Seubert 2007),

(Flemming 2008), (Imig 2005), (Chiamvimonvat 2007). Soluble epoxide hydrolase (sEH) facilitates conversion of EETs to dihydroxyeicostarienoic acids (DHET) and these weaken various functions of EETs (Campbell 1999), (Capdevila 2000), (Roman 2002). Recently there has been a focus on evaluating the role of inhibiting sEH that would result in enhancing the positive effects of EETs (Arthur 2007).

OXYLIPINS AND ATHEROSCLEROSIS:

Oxylipins have effective but at times divergent effects on smooth muscle that includes blood vessels and myometrium. Platelet aggregation is also affected. They play an integral role in endothelial inflammation and subsequent atherosclerosis. Some of the eicosanoids that include leukotrienes B4 and C4 are known to be pro- inflammatory. EETs on the other hand have anti-inflammatory properties in the vessels and kidneys and are cardio-protective. Some eicosanoids, or oxygenated 20-carbon polyunsaturated fatty acids (PUFAs), are thought to play key roles in the progression of atherosclerosis. The epoxyeicosatrienoic acids (EETs) derived from arachidonic acid (AA) have anti-inflammatory and cardio-protective actions, and polymorphisms in the genes regulating their formation and metabolism have been associated with atherosclerosis and cardiovascular health risks (Katherine 2012).The vasoactive and inflammatory effect on the vascular endothelium is specifically thought to be facilitated by lipolysis of the anti- inflammatory esterified oxylipins (EETs).

5.1.2 Arachidonic acid derivatives and Pre- Eclampsia

The imbalance between coagulation and fibrinolysis at the placental level is implicated as one of the pathophysiological causes of PE. It is thought that this disparity is caused by an imbalance between two cyclooxygenase metabolites of arachidonic acid, prostacyclin and thromboxane. Prostacyclin causes vasodilatation and is anti- platelet aggragator whereas thromboxane is a vasoconstrictor and favours platelet aggregation (Moncada 2004). What tips this balance in favour of thromboxane is speculated to be oxidative stress in pre-eclampsia.

In mild pre -eclampsia this imbalance is mainly due to a fall in the synthesis of prostacyclin in the endothelium. Severe pre – eclampsia is characterised by increase in the production of platelet thromboxane. Whereas, in the placenta and leukocytes the production of thromboxane is increased and prostacyclin production is reduced in both mild and severe pre-eclampsia. This is most likely manifested by the oxidative stress in pre-eclampsia by enhanced lipid peroxidation and reduced antioxidant production. The lipid peroxides thus produced activates the cyclooxygenase enzyme tipping the balance in favour of thromboxane (Scott 2007).

5.1.3 Oxylipins and PCOS

Although PCOS had been associated with cardiovascular disease and PE, and prostaglandin derived products have been associated both with PE and cardiovascular disease, I was unaware of any published studies measuring prostaglandin-derived products in women with PCOS, especially the oxylipins. This was particularly

important given that the strategic enzyme integral to the regulation of the androgen synthesisis in PCOS, P450, also played a role in arachidonic acid metabolism as previously outlined. Specifically, studies have shown that epoxy- and dihydroxy-eicosatrienoic acids (EETs and DHETs) are vasoactive cytochrome P450 metabolites of arachidonic acid, with EETs primarily recognized for eliciting vasodilatory, anti- inflammatory, and thrombolytic effects (Campbell 2010),(Nithipatikom 2010),(Elmarakby 2012).

The aim of the study in this chapter was to measure oxylipins in plasma of women with PCOS compared with controls.The long-term strategic objective was to aid a planned subsequent overall computational analysis to potentially reveal a single unifying pathway, which would form the basis for future prospective studies aiming to improve the diagnosis of PCOS and predict which women with PCOS would develop, pre-eclampsia.

5.1.4 Methods

As outlined in chapter 3 (methods), this was a cross sectional study involving 59 women with PCOS and 61 controls. Their fasting blood samples were collected and plasma was extracted after centrifugation for 10 minutes. The samples were stored at -80 degrees centigrade. After Solid Phase Extraction, (SPE), Liquid Chromatograph Mass Spectrometry (LCMS) was used for analyzing the samples. The samples were tested for 19 oxylipins in a targeted manner. Regardless of their source or the pathways and receptors on which they act, oxylipins will ultimately exert their effects (complementary or opposing; inflammatory or pro inflammatory) at the cell and tissue level.The strategy I considered was to "phenotype" the action oxylipin profiles on biomarkers of interest rather than measure the effects of individual species. Although an almost unlimited mixture of

oxylipins is possible, only a limited number will occur in biology. This "oxylipin- omics" offers the potential to be more informative than focusing on one metabolite, one pathway at a time, a situation that does not occur in vivo. Such approaches have been shown to prove worthwhile using FAs in similar contexts (Gregory 2009) to the point of outperforming not only individual FA prediction–based models but also combined metrics using traditional risk markers (e.g, Framingham scores)(Gregory 2009).

5.1.5 Results

Patient Demographics (Data on age, ethnicity, clinical history, anthropometric measurements were collected)

Variable (mean)	PCOS (n=59)	Controls (n=61)	p values
Age (years)	32.06	33.14	0.23
Waist (cm)	92.97	80.09	0.00
Hip (cm)	114.14	102.98	0.00
WHR	0.81	0.78	0.01
Height (m)	1.66	1.64	0.24
Weight (kg)	81.00	67.90	0.00
BMI	29.48	25.34	0.00
Systolic(mm Hg)	118.60	111.20	0.07
Diastolic(mmHg)	71.55	66.35	0.18
Parity	0.36	0.38	0.83
LH(U/L)	13.96	5.21	0.00
FSH(U/L)	5.99	7.75	0.00
LH/FSH ratio	2.32	0.74	0.00
Testosterone(nmol/L)	2.09	1.43	0.00
SHBG(nmol/L)	46.32	60.77	0.01
FAI	6.00	2.60	0.00
PRL(mu/L)	224.45	191.82	0.09
TSH(mu/L)	1.89	2.12	0.34
FT4(pmol/L)	15.13	14.97	0.71
17-OHP(nmol/L)	5.47	4.32	0.14
Fasting insulin(mu/L)	8.39	5.82	0.07
Fasting Glucose(mmol/L)	4.73	4.76	0.77
I/G	1.54	1.19	0.16
Cholesterol(mmol/L)	4.57	4.42	0.33
TG(mmol/L)	1.09	0.78	0.01
HDL(nmol/L)	1.44	1.59	0.02

Table 6: Patients and controls demographics and p values

OXYLIPIN	PCOS Mean(SD)	CONTROL Mean(SD)	Mean Difference	Mean(CI) Lower	Upper	P value
c_5.6DHET	0.0463(0.027)	0.0415(0.016)	0.0048	-0.0035	-0.013	0.254
c_8.9DHET	0.065(0.0311)	0.0586(0.0219)	0.00684	-0.0028	0.0165	0.165
c_11.12DHET	0.163(0.0516)	0.1602(0.0494)	0.00315	-0.0151	0.0214	0.733
c_14.15DHET	0.234(0.077)	0.2061(0.0644)	0.0279	0.00212	0.0536	0.034
c_5HETE	0.370(0.347)	0.350(0.287)	0.0195	-0.0955	0.1346	0.737
c_8HETE	0.037(0.0161)	0.0350(0.0136)	0.00266	-0.0027	0.0080	0.331
c_11HETE	0.0401(0.0261)	0.0347(0.0172)	0.00535	-0.0026	0.0133	0.187
c_12HETE	1.380(2.478)	1.166(0.976)	0.2139	-0.462	0.8909	0.533
c_15HETE	0.1579(0.0805)	0.154(0.063)	0.00346	-0.0227	0.0296	0.794
c_16HETE	0.056(0.0185)	0.059(0.0206)	-0.0031	-0.0102	0.0039	0.377
c_19HETE	0.231(0.261)	0.159(0.0979)	0.0721	0.00108	0.1432	0.047
c_20HETE	0.149(0.086)	0.132(0.0608)	0.0173	-0.0096	0.0443	0.205
c_TXB2	0.214(0.397)	0.1319(0.229)	0.0821	-0.0348	0.1990	0.167
c_9oxoODE	0.243(0.120)	0.2284(0.136)	0.01519	-0.0314	0.0618	0.520
c_13oxoODE	0.757(0.329)	0.720(0.486)	0.0374	-0.1133	0.1881	0.62
c_9HODE	2.253(1.141)	2.505(1.785)	-0.2997	-0.8236	0.2242	0.260
c_13HODE	2.253(1.141)	2.538(1.466)	-0.2850	-0.7611	0.1910	0.238
c_14.15EET	0.0267(0.0312)	0.0268(0.0364)	-0.0001	-0.0123	0.0121	0.986
c_17HDoHE	4.94(2.857)	4.900(2.518)	0.0413	-0.9313	1.014	0.933

Table 7: Oxylipins in PCOS compared with controls

OXYLIPIN	WAIST: HIP Mean difference	95%CI Lower	p – value	Upper	BMI Mean difference	95% CI Lower	Upper	p-value
c_5.6DHET	0.005	-0.004	0.291	0.013	0.003	-0.006	0.012	0.499
c_8.9DHET	0.011	0.001	0.033	.020	0.009	-0.001	0.020	0.086
c_11.12DHET	0.005	-0.014	0.584	0.024	0.010	-0.009	0.030	0.288
c_14.15DHET	0.028	0.002	0.036	0.055	0.034	0.006	0.062	0.016
c_5HETE	0.015	-0.104	0.799	0.135	-0.002	-0.126	0.121	0.968
c_8HETE	0.002	-0.003	0.383	0.008	-0.0003	-0.006	0.005	0.929
c_11HETE	0.004	-0.004	0.310	0.012	-0.00002	-0.008	0.008	0.997
c_12HETE	0.219	-0.482	0.538	0.920	-0.050	-0.768	0.667	0.890
c_15HETE	0.001	-0.026	0.940	0.028	-0.017	-0.043	0.010	0.209
c_16HETE	-0.004	-0.011	0.296	0.003	-0.004	-0.012	0.003	0.273
c_19HETE	0.085	0.012	0.023	0.158	0.072	-0.004	0.149	0.064
c_20HETE	0.014	-0.014	0.311	0.042	-0.001	-0.029	0.026	0.919
c_TXB2	0.060	-0.060	0.326	0.180	0.031	-0.093	0.154	0.623
c_9oxoODE	0.003	-0.045	0.916	0.050	0.001	-0.049	0.051	0.971
c_13oxoODE	-0.004	-0.157	0.956	0.149	-0.015	-0.176	0.145	0.850
c_9HODE	-0.372	-0.912	0.175	0.167	-0.352	-0.916	0.212	0.219
c_13HODE	-0.347	-0.838	0.164	0.144	-0.383	-0.894	0.128	0.140
c_14.15EET	-0.001	-0.013	0.928	0.012	-0.002	-0.015	0.011	0.758
c_17 HDoHE	0.238	-0.758	0.637	1.235	0.275	-0.767	1.317	0.602

Table 8: Oxylipins in PCOS compared with controls, adjusted for waist: hip and BMI

Of the 19 Oxylipins analysed in the plasma samples, 14, 15 DHET and 19 HETE were found to be significantly raised (table 7) in PCOS women compared to the controls (p=<. 05). When adjusted for BMI and waist: hip ratio and using multivariate analysis using IBM SPSS 22, 14,15 DHET and 19 HETE, remained significant, though 19 HETE was marginally significant when adjusted for BMI (Table No.8).

5.1.6 Discussion

As expected the weight, BMI and waist: hip ratio were all higher in PCOS cohort compared with the controls. A study by Gateva et al looked at waist stature ratio (WSR) and waist circumference (WC) and waist: hip ratio (WHR) and concluded that WSR and WC were better markers of cardiovascular morbidity in PCOS women (Gateva 2012). The cut offs for waist circumference are different but most commonly it is the WHO definition that is followed which describes the cut off to be 80 cms in women and this is taken as high risk for developing metabolic syndrome (WHO 2008). In our study we did not calculate the WSR but WHR and WC was statistically significant ($p=<0.05$) in PCOS women.

Matching does not confer any benefit over independent control selection with regard to study validity i.e confounding bias. When proper data analysis methods are applied, those confounding factors, which are different in cases and control but are risk factors of interest, analysis would contribute to effect estimates. Most investigators use logistic regression to overcome the bias. Similarly in matched controls appropriate data analysis used for appropriate variables is the key. Pubmed search by Bloom M et al, revealed only one study out of 23 that had used appropriate analytical methodology for matched variables (Bloom 2007).

Selecting controls is often a difficult task in studies. The controls can be matched or unmatched. In my study, the controls were not matched. The reason being, that it is challenging to control for all the known and un-known confounding factors and therefore difficult to recruit enough numbers. If the controls are

very closely matched, it might not represent the general population (Mann 2003).

The targeted approach of metabolomics was adapted as analysis of pre-determined metabolomics reduced the likelihood of analytical errors and inaccuracies in data analysis. This can also reduce interference by great quantity metabolites.

My colleague also used a new specialised extraction method and used it for her thesis. LC -MS was used because of local expertise and availability.

This study found, that of the 19 Oxylipins analysed in the plasma samples, 14,15 DHET AND 19 HETE were found to be significantly raised in PCOS women compared to the controls (p=<. 05). When adjusted for BMI and waist: hip ratio and using multivariate analysis using IBM SPSS 22, 14, 15 DHET and 19 HETE, remained significant, though 19 HETE was marginally significant when adjusted for BMI.

This is the first time that oxylipins have been analyzed in women with and without PCOS.

14, 15 EET'S are known to be cardio-protective and they are very rapidly hydrolyzed to stable compounds called DHET'S. The 14, 15 DHET are physiologically less active and might depict low levels of 14, 15 EET. The high level of 14, 15 DHET in women with PCOS may therefore be indicative that these women are more prone to develop cardiovascular disease in later life and could be used as a biomarker if validated. The confounding effect of obesity can however not be excluded given that this difference did not disappear when the results were corrected for BMI.

19 HETE is an analogue of 20 HETE. CYP metabolizes AA to several biologically active eicosanoids by two distinct enzymatic reactions. CYP epoxygenases convert AA to 4 already described regioisomeric epoxyeicosatrienoic acids (EETs) and CYP hydroxylases convert AA to hydroxyeicosatetraenoic acids (HETEs) including 19- and 20-HETE.

20 HETE is produced in response to angiotension II and is a potent vasoconstrictor. 19 HETE blocks the vasoconstrictor effect of 20 HETE in renal and cerebral arteries. In this context, the raised level of 19 HETE in women with PCOS needs to be further investigated to ascertain whether its high level might be conferring some kind of cardio-protective action. This is consistent with previous research on mortality from cardiovascular disease in PCOS, which has not shown an increased risk despite women with PCOS having increased cardiovascular morbidity (Wild 2000). 20 HETE is an arachidonic acid derivative that is produced not only in vascular smooth muscle cells but also by myloid cells in the peripheral blood and bone marrow. It is also known to be produced in neutrophils and platelets (Samantha 2015). Androgens (raised in PCOS) are also known to induce 20 HETE production (Wu 2011).

20 HETE (hydroxyeicosatetraeonic acid) also plays a vital role in renal function, vascular tone and arterial pressure. In the vessels, 20-HETE is a potent vasoconstrictor, it’s up -regulation promotes inflammation, oxidative stress, endothelial dysfunction, elevation in peripheral resistance. This effect is found in various conditions like obesity, diabetes, ischemia/reperfusion and vascular oxidative stress (Waldman 2016). 20 HETE also mediates its pro-coagulatory effect via platelet adhesions in endothelial cells. This action of enhanced platelet aggregation is thought to be through

increased secretion of von Willibrand fraction. Inhibiting the actions of 20 HETE might be beneficial in thrombosis and cardiovascular disease. This was a conclusion in an experiment by Jiaxiang W using cultured human umbilical vein and rat thrombosis model (Wang 2015).

As outlined above, the enzyme P450 also metabolises arachidonic acid to many other biologically active compounds which include 5.6,8,9,11,12 and 14, 15 epoxyeicosatrienoinc acids (EET's) and their corresponding dihydroxyeicosatetraneoic acid (DHET's) and 19,20-hydroxyeicosatetraenoic acids (HETE's). All these compounds are strong regulators of vascular tone. The EET's exert this effect via intracellular pathways. EET's act via Ca 2+ sensitive K+ channels (BKCa) in vascular smooth muscle cells which causes hyperpolarization of the resting membrane potential and results in vasodilatation of coronary circulation (Campbell 1996),(Fisslthaler 1999), (Fleming 2004).

This could be an important biomarker discovery for long-term prognosis of cardiovascular disease, and possibly pre-eclampsia but needs validation. It is a vital discovery as our cohort of controls and PCOS were not matched for weight and BMI but doing multivariate analysis adjusted the group for BMI. Studies have shown that EET's demonstrate anti- inflammatory, thrombolytic and angiogenic properties in the vasculature (Node 1999).

Women with PCOS are at increased risk of developing type 2 diabetes in later life and there is a role of 20 HETE in diabetes as well. Diabetes makes humans prone to cardiac ischemia resulting in endothelial damage and inflammation in the vessels and 20 HETE is known to be at an increased level in these conditions

(Issan 2013). It has also been reported that 20 HETE impairs the insulin signalling in the endothelium by phosphorylation of insulin receptor substrate-1 (IRS-1) (Li 2014). This may therefore play a role in the insulin resistance in PCOS, but more mechanistic studies are required to test this hypothesis. On the other hand, Ward et al in 2006 reported that there was no association between insulin levels, insulin resistance and 20-HETE and this was observed even after adjusting for age, gender, BMI and blood pressure. Whereas, previously the same group had described a positive correlation between BMI and 20-HETE (Ward 2006). In contrast, Laffer et al observed that there was no correlation between BMI and 20-HETE in a population of obese hypertensive subjects and they also found that there was a negative correlation with insulin levels and 20-HETE (Laffer 2004).

In my study 19 HETE was marginally raised when adjusted for BMI. As discussed above, 19 HETE is analogue of 20 HETE. Laffer et al in their study on rats found a very strong negative correlation of 20 HETE with obesity, which is in part expalined by increased insulin levels and the effect of raised insulin on cytochrome P450(Laffer 2004). Similarly, when I did multivariate analysis between fasting insulin levels and 20 –HETE, the level of 20-HETE was not significantly expressed in PCOS cohort ($p<0.41$) as also reported by Wang et al. The only difference is that in both these studies, the cohorts were entirely different. In the study by Ward et al (Coronary Artery Risk Development in Young Adults) CARDIA, the cohort of women were followed for 20 years and the diagnosis of PCOS was based on a questionnaire. Among 984 participants only 42(4.3%) women were diagnosed with PCOS (Ward 2012) and urinary 20-HETE was tested whereas we tested fasting plasma and we based our diagnosis of PCOS according to Rotterdam criteria.

In another study by Shen T et al it was demonstrated that there was a role of 15 Lipoxygenase (LO)/15 HETE involvement in the formation of thrombus in pulmonary artery hypertension (PAH) (Shen 2015). The 15- lipoxygenase converts arachidonic acid to 15-HETE which then in turn promotes atherogenesis (Harats 2000). Both 15 HETE and 20 HETE have similar actions and are known to encourage thrombus formation. A connection between these two oxylipins has been proven by Lui et al as well an association with shortening in bleeding time and increasing platelet aggregation in rats (Lui 2010). This finding is similar to the result in a retrospective case control study which analysed plasma samples from acute coronary syndrome patients. Levels of Leukotrien B4, 8-HETE, 11-HETE, 12-HETE and 15-HETE were significantly increased in Major Adverse Cardiovascular Event (MACE) and non- MACE groups (Lingyun 2016).

This was not the case in my study as I did not see any statistical significance difference in the level of 15 HETE.

It is known that p450 metabolites of arachidonic acid play a role in the pathophysiology of pregnancy-induced hypertension and pre-eclampsia as was demonstrated by Catella et al in 1990 (Catella 1990). A recent case control study which looked at women with pre- eclampsia, normotensive pregnant women and non- pregnant women concluded that the levels of EET's, HETE's LT's and TXB2 in the pre -eclampsia group were significantly increased compared to the other two groups. The serum levels of 8,9 HETE, 11, 12 HETE,5 HETE,12 HETE,15 HETE and LTB4 were significantly raised in the pre-eclampsia group. These metabolites were increased both before and after the onset of pre-eclampsia but the levels of 8, 9 EET was only increased after the onset of pre-eclampsia (Long 2016). Other studies have also found that

the levels of 5, 12 and 15 HETE were considerably higher in the placentas of women with pre-eclampsia (Pearson 2010). Of these oxylipins associated with PE, in my study, I found that differential expression of 11 HETE, 15 HETE was not consistent with previous research on oxylipins and PE and may form the basis for future studies into oxylipin biomarkers of PE risk in PCOS. All these findings reveal that these arachidonic acid metabolites have considerable involvement in the pathogenesis of pre-eclampsia and may therefore play a part in the mechanisms leading to a higher risk of pre-eclampsia in PCOS.

.

5.1.7 Conclusion

In conclusion, this study found that of the 19 Oxylipins analysed in the plasma samples, 14, 15 DHET AND 19 HETE were found to be significantly raised in PCOS women compared to the controls. When adjusted for BMI and WHR, 14,15DHET and 19 HETE were still significant, however 19 HETE was only marginally significant when adjusted for BMI. More studies need to be done to ensure these results were not false positives and determine whether EET's and HETE's have any role to play as markers in PCOS and in understanding and predicting the risks of PE and cardiovascular disease in PCOS. These data also form a useful basis to inform sample size calculations for a definitive study.

Arachidonic acid derivatives in the form of oxylipins may prove to be the answer to discovering the biomarkers for the early detection of pregnancy related complications like pre-eclampsia and prognosis of long term health complications of PCOS like cardiovascular disease. The next chapter presents the results of computer based network analysis of lipids differentially expressed in Pre-Eclampsia and Polycystic Ovary Syndrome from Metabolomic Studies. As highlighted in the introduction, this was

to aid a planned subsequent overall computational analysis integrating all the data derived from the individual studies of this thesis. The aim was to potentially reveal a single unifying pathway which would form the basis for future prospective studies aiming to improve the diagnosis of PCOS and predict which women with PCOS would develop pre-eclampsia and cardiovascular disease.

Chapter 6

Network analysis of lipid metabolites differentially expressed in Pre-Eclampsia and Polycystic Ovary Syndrome from Metabolomic Studies.

6.1.1 Introduction

This chapter presents the results of computer based network analysis of lipids differentially expressed in Pre-Eclampsia and Polycystic Ovary Syndrome from Metabolomic Studies. As discussed in the introduction, it has been shown in various meta-analysis that the risk of developing pregnancy induced hypertension and pre -eclampsia in women with PCOS is 3-4 times more than controls (Boomsma 2006), (Kjerulff 2011),(Qin 2013). Early prediction of PE and understanding the pathophysiology of the disease and its link to PCOS might help in the prevention of the PE and its potential life threatening consequences. However,there have been no studies to date ,that would either identify factors that would predict which women with PCOS will go on to develop PE or to predict which factors increase cardiovascular mortality in women with PCOS.The study in this chapter reviewed the literature on lipid biomarkers identified as being differentially expressed in women with PE compared with controls using metabolomics approaches and I carried out the same exercise in the only published study on lipid biomarkers expressed differently in women with PCOS compared with

controls using metabolomics technology. Data was then inputted into a network containing all lipid reactions that were found in public databases to identify smaller sub-regional networks and key genes which potentially played a pivotal role in the mechanisms of PCOS and PE.

6.1.2 Systematic Review on lipid biomarkers of PE and PCOS using metabolomics approaches:

i. METABOLOMIC STUDIES IN PRE-ECLAMPSIA

All eligible published studies on metabolomic biomarkers for PE and PCOS which were identified through MEDLINE (1996-December 2013), EMBASE (1980- December 2013) and Cochrane (1993- December 2013) ISI web of knowledge (v4.2) databases, using the search terms "metabolomics", "metabolomic", "preeclampsia", "preeclamptic toxemia, "metabolomic biomarker", "metabolomic biomarker" and "PE" without any restrictions were evaluated.

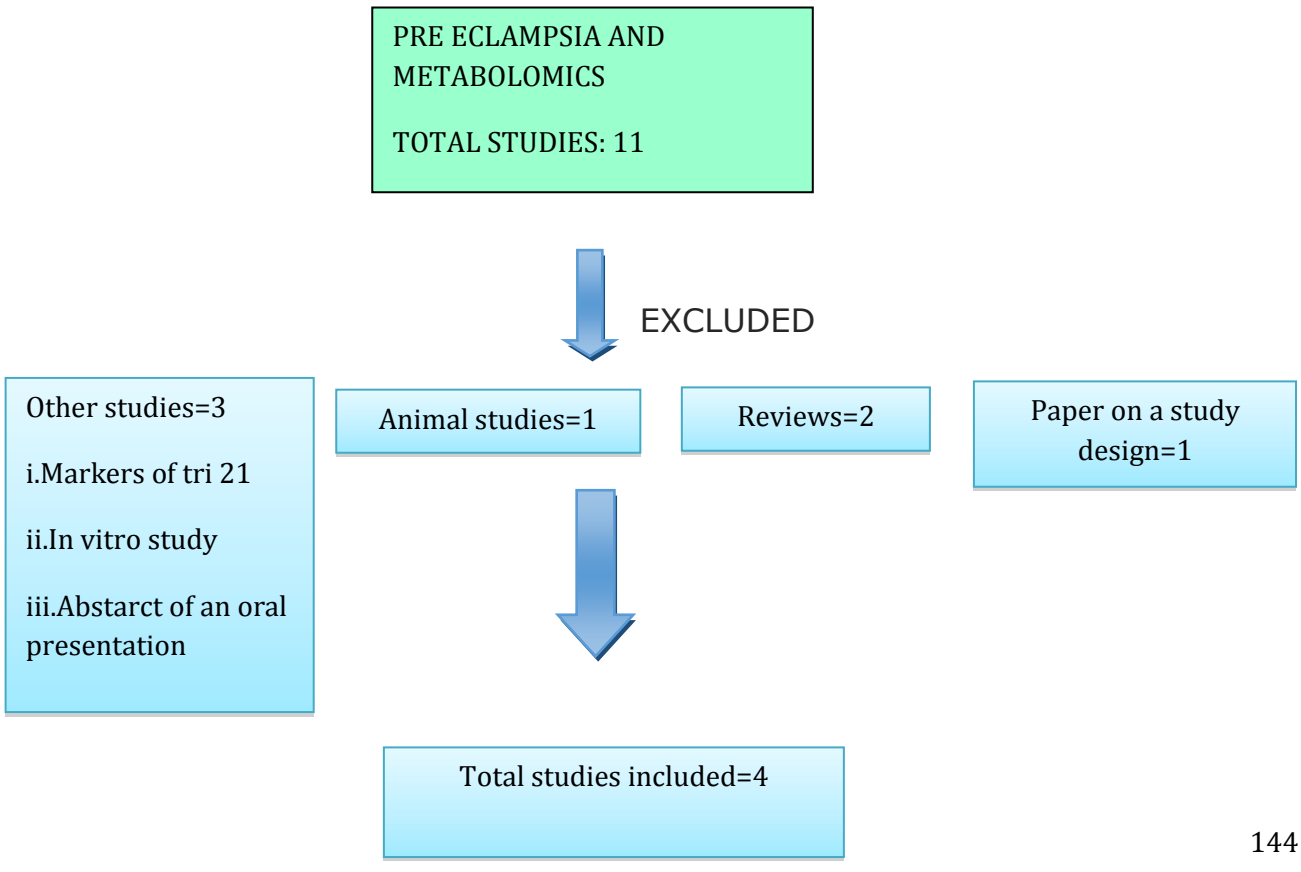


Figure11: Flow chart of the literature search (Metabolomics and Pre eclampsia)

My literature search (Figure 11) identified four relevant studies (W.B.Dunn et al in 2009, Bhado Singh et. al., 2013, Odibo et al and Kenny et. al., 2010).

W.B.Dunn et al in 2009 studied placental villous extracts from PE and normal pregnancies cultured under normtoxic and hypoxic conditions. Ultra-Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS) was used for analysis. The study found that the metabolites in conditioned media from PE cultured under normotoxic conditions were similar to uncomplicated pregnancies cultured under hypoxic conditions. Glutamate (glutamic acid) was at a lower concentration in PE and in hypoxic uncomplicated pregnancy whereas oxypurinol was in higher concentration in both. Another group of metabolomics biomarkers differentially expressed, however, were prostaglandins. COX -1 and COX-2 activity was decreased in hypoxic and PE group. This could be related to oxidative stress, consistent with the study in chapter 4 looking at proteomic biomarkers in PE and PCOS.

A discovery phase study from USA by Odibo et al looked at maternal serum samples at 11-14 weeks of gestation to detect any markers for the prediction of PE in the first trimester. The samples were analyzed using LC-MS/MS. The data showed that there were seven metabolites that were up regulated in PE compared to controls. This included 5 amino acids and two hydroxylated carnitine esters. Subsequent analysis taking into consideration cofounding factors found four metabolites remained

significantly differentially expressed including C6OH (hydroxyhexanoylcarnitine), Phenylalanine, Glutamate and Alanine. The detection of PE at a 10 and 20% false positive rate using the combination of these 4 was 50 and 60 % respectively.

A study by Bhado Singh et al in 2013 looked at first trimester metabolomic markers to detect late onset PE. Maternal serum was taken at 11-13 weeks and analysed using NMR. 17 metabolites were found to be significantly different in late onset PE compared to normal controls (Table 8). In the study using variable importance in projection (VIP) analysis four metabolites were found to be different in concentration between late onset PE and normal controls. These include glycerol, carnitine, methylhistidine and acetone. Of these four two (glycerol and carnitine) were found to be clearly raised in late onset PE compared to normal controls. Glycerol is a 3-carbon alcohol, which is a basic component of glycolipids and thus plays an important role in lipid metabolism. The link between obesity and late onset PE is well known which was also confirmed in this study (Poon 2010). Carnitine is a quaternary ammonium compound that is vital in lipid transport from cytoplasm to mitochondria. It is made in liver and kidneys and both these organs are affected in PE. Cartinines prevent lipid oxidation thus helps in preventing oxidative stress. Increased cartinine could be in response to tackle excessive oxidative lipid metabolism. This study shows that there is some evidence that lipid metabolism disturbance may be central in the pathogenesis of late onset PE.

Kenny et al did a two-phase discovery/validation study on maternal plasma at 15 weeks gestation in 2010. Samples were analysed using UPLC-MS. In the discovery phase 11 clear classes emerged (Based on Human Metabolome Database). These

included amino acids, carbohydrates, carnitines, eicosanoids, fatty acids, keto or hydroxy acids, lipids, phospholipids, porphyrins, phosphatidylserine and steroids (table 8). Out of 45 metabolites found in the discovery phase 34 were also detected in the validation phase. They showed similar peak response. It was shown in the study after robust mining and modelling techniques that a combination of 14 metabolites produced a robust predictive model with AUC of >0.9. The authors found that the sensitivity of using the 14 metabolites for the possible screening for detection of PE in the discovery and validation phase at 10% false positive rate would be 77% and 73%.

ii.Metabolomic (Lipodomic) studies in PCOS:

I identified one previously published study on lipidomic metabolomics biomarkers in PCOS. Liquid chromatography in conjunction with mass high-resolution mass-spectrometry (LCHRMS) was used to explore whether there were any differences in plasma lipidomic profiles between PCOS and control women (Haoula 2014). The list is outlined in table 9. From the PCOS list, it is possible to see that they were mainly composed of phosphatidylcholine with only 1 FA chain on them, those lipids could have been generated directly from the MG (Monoacylglyceride) or by the elimination of one of the FA chains.

Study	**Study design**	**Population**		**Selection Criteria**		**Metabolite Identified**
		N	Mean Age ± SD & Age Range	**Inclusion**	**Exclusion**	
W.B.DUNN ET AL	Cross sectional	Control n= 6 PE n= 6	NA	Uncomplicated term pregnant patients. New – onset hypertension >140/90mm Hg And proteinuria 0.3gm/24h after 20 weeks gestation.	Pregnancies complicated by other maternal or fetal factors,like essential hypertension and intra uterine growth	Glutamic acid/Glutamine Prostaglandins /Leukotienes Kynurenine metabolism

					retardation.	
Odibo et al	Case control prospective study(11-14 wks)	PE=41 Controls=41	30.5 32.9	Cases who developed PE at any gestational age according to the ACOG,2002 definition. Controls were without any adverse pregnancy outcome.	NA	 C5OH C6OH Phenylalanine Glutamate Arginine Alanine Hydroxyproline
Kenny et al	Prospective ,multicentre cohort Independent case-control (15±1 wk)	*Discovery phase* PE=60 Controls=60 *Validation Phase*	30.2 30.4 22.0	PE was defined as blood pressure ≥140/90mm Hg after 20 weeks gestation(before the onset of labour) or in the postnatal	NA	***Amino acid*** 5-Hydroxytryptopha Urea ***Dicarboxylic acid*** Methylglutaric acid ***Lipid*** Di-(heptadecadienoy

Kenny et al Cont….	SCOPE STUDY	PE=39 Controls=40	23.2	period,with either proteinuria(24-hour urinary protein ≥300mg,spot urine protein:creatinine ratio≥30mg/mmol,or urine dipstick ≥ ++)and /or evidence of multiorgan complications. Controls were women with uncomplicated pregnancies.		-eicosanyl-sn-glycer ***Phosphatidylserin*** Octadecenoyl-sn-glycero-3-phosphosernie ***Bile acid*** 12-Ketodeoxycholic acid. ***Carbohydrates*** Monosaccharide ***Carnitines*** Palmitoylcarnitine Stearoylcarnitine Decanoylcarnitine Octaoylcarnitine

						Acetylcarnitine Dodecanoylcarnitine ***Eicosanoids*** 8,11,14-Eicosatrienc acid 20-Carboxyleukotien B4 Eicosapentaenoic aci ***Fatty acids*** Isovaleric acid Oleic acid Linoleic acid Docosahexaenoic aci Hydroxy-octadecano acid Hexadecanoic acid γ-Butyrolactone

Kenny et al.						2-Oxovaleric acid ***Keto or hydroxy F*** 3-hydroxybutanoic acid Acetoacetic acid ***Lipids*** Hexadecenoyl-eicosatetraenoyl-sn-glycerol Di-sn-glycerol ***Phosphatidylserin*** Octadecenoyl-sn-glycero-3-phosphoserine ***Phospholipids*** Dioctanoyl-sn-glycero-3-phosphocholine

						Sphingosine phosphate Sphinganine1-phosphate ***Steroids*** Vitamin D derivatives Etiocholan-3-α-o17-one
Bahado-Singh et al	Case control (11+0-13+6)	PE=30 Controls=60	31.2 30.8	Definition of PE used was that proposed by the International Society For the study of	HELLP Gestational hypertension.	Glycerol 1-methylhistidine Valine Carnitine Acetone Isopropanol

				hypertension in pregnancy as systolic blood pressure ≥140mmHg or diastolic pressure ≥90mm Hg on ≥ 2 occasions 4 hours apart >20 weeks gestation,in women who were previously normotensive. Proteinuria was defined as a total of 300mg in 24-hour urine collection or 2 readings of at least 2+ protein on a		Pyruvate Hydroxyisovalerate_ Glucose Hydroxybutyrate_2 Creatinine Creatine Citrate Hydroxybutyrate_3 Trimethylamine Acetamide, Dimethylamin

				midstream or catherized urine specimen in the absence of a 24-hour urine collection.		

Table 9: List of metabolomics in PE

Phosphatidylcholine (once given the trivial name 'lecithin') is usually the most abundant phospholipid in animals and plants, often amounting to almost 50% of the total, and as such it is obviously a key building block of membrane bilayers (Lipidlibrary). In animal tissues, phosphatidylcholine tends to exist in mainly in the diacyl form. The fact that it was found in the study in the monoacyl form may indicate that the gene regulating the transformation from phosphatidylcholine to lysophosphatidilcholine has been under-expressed (see figure 12 below). There are at least fifteen different groups of enzymes in the phospholipase A2 super-family, which differ in calcium dependence, cellular location, and structure.

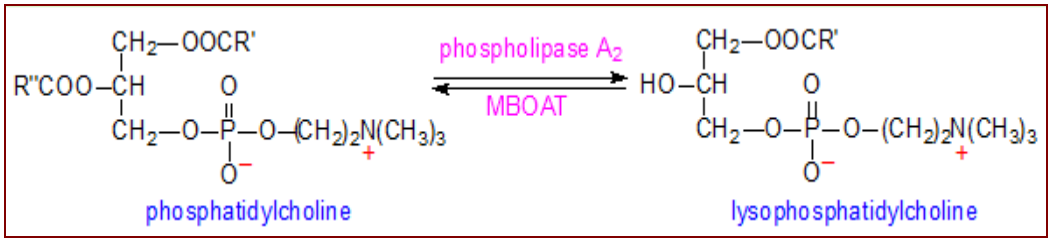


Figure 12: Diagramatic transformation of phosphatidylcholine to lysophosphotidylcholine

Lysophosphatidylcholine was also differentially expressed (Lyso PC). LysoPC has one mole of fatty acid per mole of lipid in position sn-1, and is found in trace amounts in most tissues. Lysophosphatidylcholine has pro-inflammatory properties and it is known to be a pathological component of oxidized lipoproteins (LDL) in plasma and of atherosclerotic lesions; it has been shown to promote demyelination in the nervous system. Recently, it has been found to have some functions in cell signalling, and specific receptors (coupled to G proteins) have been identified. It activates the specific phospholipase C that releases diacylglycerols and inositol triphosphate with resultant increases

in intracellular Ca2+ and activation of protein kinase C (Arouri 2013).

Name on the paper	Name on network	Regulated	Availability
SM (d18:0/20:2)	SM (20:2[8,11])	Up	Done
SM (d18:0/18:0)	SM (18:0)	Up	Done
LysoPC (16:0)	PC (16:0/0)	Down	Done
LysoPC (18:2)	PC (18:2[9,12]/0)	Down	Done
LysoPC (18:1)	PC (18:1/0)	Down	Done
LysoPC (18:0)	PC (18:0/0)	Down	Done
PE (42:1)	PE (42:1)	Down	Multiple
Plasmalogen (40:7)	PC (P-40: 7)	Up	Multiple
Plasmalogen (30:0)	PC (P-30:0)	Down	Multiple
DG (36:2)	DG (36:2)	Up	Multiple
PC (32:4)	PC (32:4)	Down	Multiple
PE (34:0)	PE (34:0)	Down	Multiple
PC (30:0)	PC (30:0)	Down	Multiple
TG (54:7)	TG (54:7)	Up	Multiple
TG (58:5)	TG (58:5)	Up	Multiple
TG (56:4)	TG (56:4)	Up	Multiple
DG (36:3)	DG (36:3)	Up	Multiple
TG (52:4)	TG (52:4)	Up	Multiple

TG (50:0)	TG (50:0)	Up	Multiple

Table 10: List of lipids expressed significantly different in controls and patients for PCOS.

(Multiple was used to input all the different combinations of lipids that were put in the network, where more than one lipid was found in the database with the same weight), almost means that metabolites that were not found in the database but a close precursor was found were, thus not included in the network. Done means where the metabolite was found in the database and were included in the network analysis.

6.1.3 Network Analysis

We did the network analysis with these metabolites excluding the TG because they didn't provide too much information.

The first step in this approach was the creation of a network containing all lipid reactions that are found in public databases. The main skeleton of the network was generated using the information in LIPIDMAPS, due its extensive coverage of lipids for humans. Thanks to literature research, the cover of the network has been expanding with more reactions. Using python scripts and prior knowledge the generation of the 12000 node network was created. A picture of the actual network is presented in the image below (Figure 13).

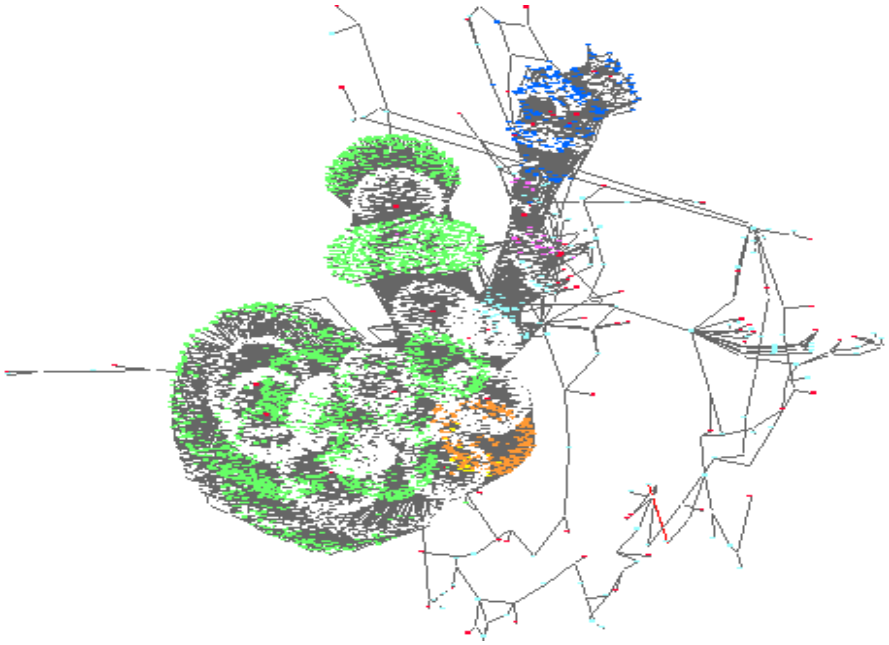


Figure 13: Lipidomic network.

The colouring indicates the different families of lipids (green Glycerolipids and Phosphoglycerolipids, blue Sphingolipids, orange Diglycerides, yellow Monoglycerides, light blue other lipids, red genes). These nodes are linked to the relevant Reaction nodes, coloured white. The organic layout within Cytoscape was used to produce the network view.

When doing a pathway analysis for a global lipidomic dataset, the first step is the identification of the metabolites that are expressed in significantly different concentrations in the disease conditions being investigated. Once the list of lipids is identified, we match the lipid names to those in the lipidomic network. Generally the lipid list also provides a score for each molecule, which reflects its importance in the multivariate analysis. These scores are also inputted into the network as node attributes, and used later.

The first neighbours from each of these nodes are selected to create a smaller network. This is what we call the "network of

study", for example Figure 14, and reveals the main reactions and pathways associated with the change of state in the lipid metabolism. This network can be used to generate scores for the different enzymes, which provide an idea of how important it is for generating the observed difference in lipid profile, see Table 9. The positive and negative values indicate that the reaction is faster or slower. In this example, the FAAH/FAAH2 reaction seems to be the most important point. If we take a closer look, it is possible to see that the other changes in the lipids are generated by initial demand of fatty acids.

Enzyme	Score
FAAH	-23.739
Ppap2a	-9.956
Sphk1/Sphk2	-7.377
Ptdss1	-3.034
Pld1	-3.034
Dgkz	9.956
MOGAT3	11.862
Chpt1	16.445
FAAH2	23.739

Table 11: Node-contribution scores

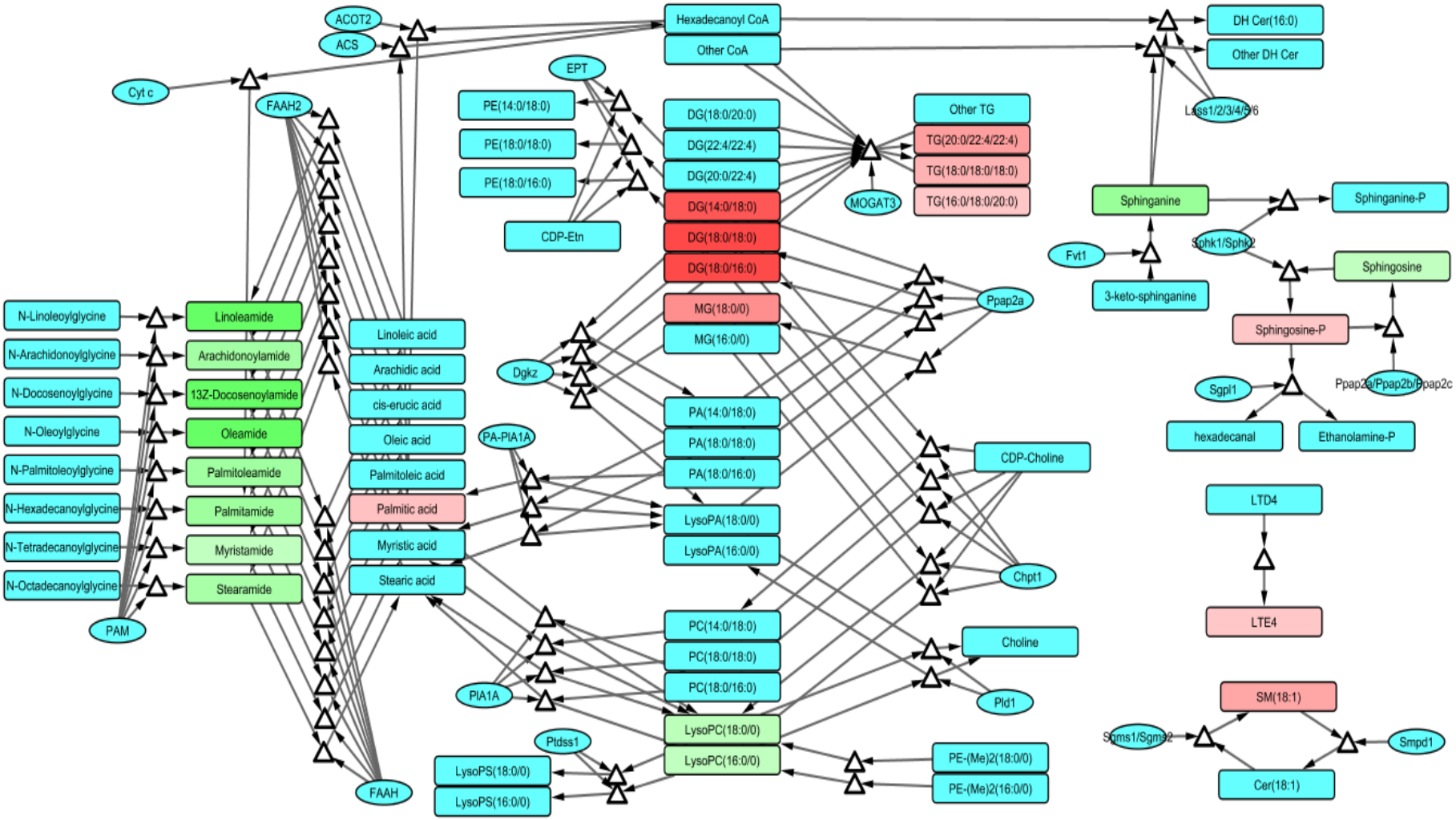

ACOT2
ACS
Hexadecanoyl CoA
Other CoA
DH Cer(16:0)
Other DH Cer
Lass1/2/3/4/5/6
Cyt c
FAAH2
EPT
PE(14:0/18:0)
PE(18:0/18:0)
PE(18:0/16:0)
CDP-Etn
DG(18:0/20:0)
DG(22:4/22:4)
DG(20:0/22:4)
DG(14:0/18:0)
DG(18:0/18:0)
DG(18:0/16:0)
MOGAT3
Other TG
TG(20:0/22:4/22:4)
TG(18:0/18:0/18:0)
TG(16:0/18:0/20:0)
Sphinganine
Sphinganine-P
Sphk1/Sphk2
Fvt1
3-keto-sphinganine
Sphingosine
Sphingosine-P
Ppap2a/Ppap2b/Ppap2c
Sgpl1
hexadecanal
Ethanolamine-P
N-Linoleoylglycine
N-Arachidonoylglycine
N-Docosenoylglycine
N-Oleoylglycine
N-Palmitoleoylglycine
N-Hexadecanoylglycine
N-Tetradecanoylglycine
N-Octadecanoylglycine
PAM
Linoleamide
Arachidonoylamide
13Z-Docosenoylamide
Oleamide
Palmitoleamide
Palmitamide
Myristamide
Stearamide
Linoleic acid
Arachidic acid
cis-erucic acid
Oleic acid
Palmitoleic acid
Palmitic acid
Myristic acid
Stearic acid
FAAH
Dgkz
MG(18:0/0)
MG(16:0/0)
Ppap2a
PA-PlA1A
PA(14:0/18:0)
PA(18:0/18:0)
PA(18:0/16:0)
LysoPA(18:0/0)
LysoPA(16:0/0)
CDP-Choline
Chpt1
LTD4
LTE4
PC(14:0/18:0)
PC(18:0/18:0)
PC(18:0/16:0)
Choline
Pld1
PlA1A
LysoPC(18:0/0)
LysoPC(16:0/0)
Ptdss1
LysoPS(18:0/0)
LysoPS(16:0/0)
PE-(Me)2(18:0/0)
PE-(Me)2(16:0/0)
SM(18:1)
Sgms1/Sgms2
Smpd1
Cer(18:1)

Figure14: A network of study.

The input data had significant changes in 23 lipids in a disease. The shape of a node indicates its type: circles, rectangles and triangles are respectively genes, metabolites and reaction nodes. The node colour indicates its status: red, green and blue respectively for lipids that are at higher concentration in the disease, lipids that are at lower concentration, and metabolites not in the input data but retrieved by this analysis. The intensity of the Red and Green indicate how big the contribution was to the model.

6.1.4 PCOS network analysis

From the network analysis of PCOS lipids, I found 3 key genes played a pivotal role in the network. The genes were Choline Phosphotransferase (CHPT1), Phosphatidylserine Synthetase (PTDSS1) and Phospholipase D (PLD1). Taking in consideration the fact that some of the metabolites of interest were present in a higher concentration or in lower concentration in the disease than in the controls, following my network analysis, I inferred that the stress genes would be acting as outlined in table 12 below in PCOS.

Gene	Stress	State
PLD1	655844	Overexpressed
CHPT1	340802	Under expressed
PTDSS1	5412	Overexpressed

Table 12: Genes expression in PCOS

6.1.5 PE network analysis

For PE the list of lipids (table 9 was bigger but it was not clear, how sure we could trust this identification). For example in the list it can be found the entry "PC (8:0/8:0)" and this phosphatidylcholine is really unusual, as the majority of them have fatty acids (FA) with a length between 16 and 22. But we did not doubt the identification of those lipids.

The most abundant lipids in that analysis were the acylcarnitines (table 9). Acylcarnitines are generated in order to move lipids from the cytosol to the mitochondrial matrix (http://lipidlibrary.aocs.org/Lipids/carnitin/index.htm).

Finding a high amount of acylcarnitines could indicate that once the fatty acids enter the membrane that it is not being used for any other biological function (figure 15 below). Another option would be that there is an excess of carnitine and that's why they generate acylcarnitine. However, as carnitine was not found on the list so we assumed that it was the less probable option.

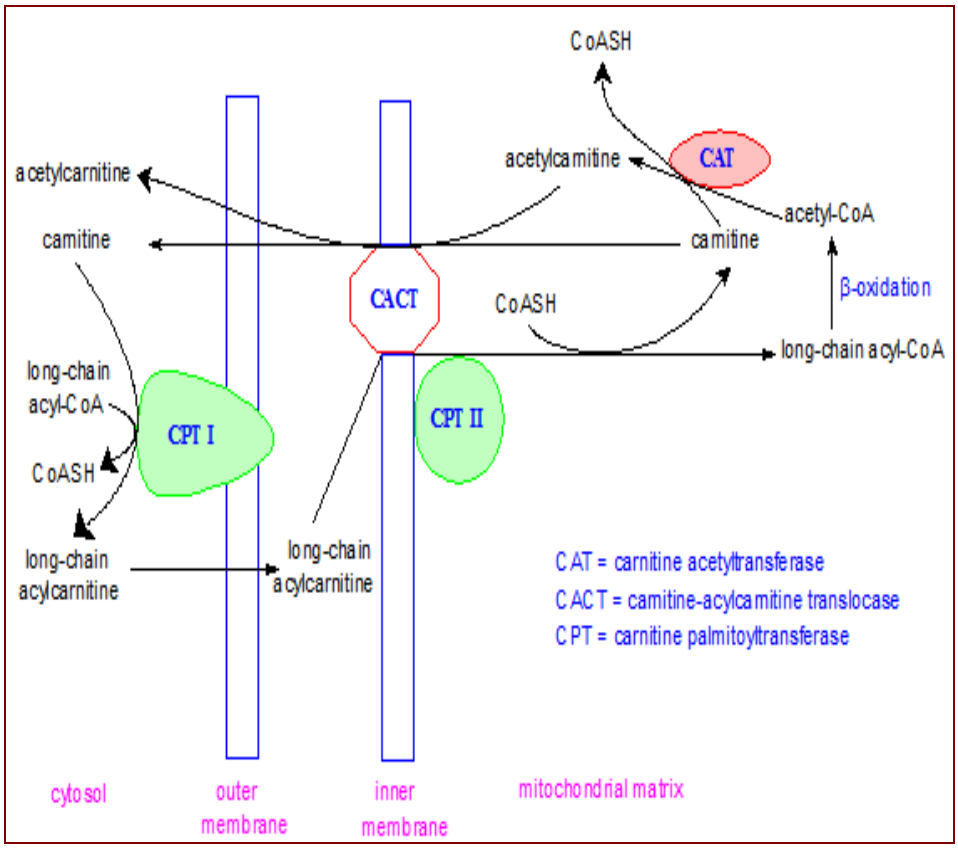


Figure15: Metabolic pathway for the pair CPT1 and CPT2 in acyl carnitines

Name on the paper	Name on network	Regulated	Availability
Palmitoylcarnitine	L-Hexadecanoylcarnitine	Up	Done
Stearoylcarnitine	L-Octadecanoylcarnitine	Up	Done
Decanoylcarnitine	L-Decanoylcarnitine	Up	Done
Octanoylcarnitine	L-Octanoylcarnitine	Up	Done
Acetylcarnitine	L- Acetylcarnitine	Up	Done
Dodecanoylcarnitine	L-Dodecanoylcarnitine	Up	Done
Adipic acid	(Lysine precursor)	Up	No
8,11,14-Eicosatrienoic acid	8,11,14-Eicosatrienoic acid	Up	Done
20-Carboxyleukotriene	(20-OH LTB4)	Up	Almost
Eicosapentaenoic acid (5,8,11,14,17)	(6Z,9Z,12Z,15Z,18Z)-Tetracosapentaenoic acid	Up	Almost
Valeric acid	Odd FA	Up	No
Oleic acid	Oleic acid	Up	Done

Linoleic acid	Linoleic acid	Up	Done
Docosahexaenoic acid	Docosahexaenoic acid	Up	Done
Hydroxy-octadecanoic acid	Intermediate on the synthesis for Octanoyl	Up	Almost
Hexadecanoic acid	Palmitic acid	Up	Done
Eicosatetraenoic acid	AA	Up	Done
Octadecanoic acid	Stearic acid	Up	Done
Butirolactone	(Small molecule)	Up	No
oxo-methylbutanoic acid	(Valine is precursor)	Up	No
3-hydroxybutanoic acid	3-hydroxybutanoic acid	Up	Done
Acetoacetic acid	Acetoacetate	Up	Done
Oxoheptanoic acid	Odd FA	Up	No
Hexadecenoy-eicosatetraenoyl-sn glycerol	DG(C20:4/C16:0)	Up	Done
Di-(octadecadienoyl)-sn glycerol	DG (C18:0/C18:0)	Up	Done
Octadecenoyl hexadecanoyl-sn-glycero-3-	PS (C18:0/C16:0)	Up	Done

phosphoserine			
Octadecenoyl-sn-glycero-3-phosphoserine	PS (C18:0/C0)	Up	Done
Dioctanoyl-sn-glycero-3-Phosphocholine	PC (C8:0/C8:0)	Up	Done
Sphingosine 1-phosphate	Sphingosine-P	Up	Done

Table13: List of lipids expressed significantly different in controls and PE.
The ones that indicate almost is the nearest metabolite found in the network to that lipid, they have not been used in the analysis

Following the network analysis (figure 14) we identified a list of 5 genes of high stress suggesting a possible involvement in influencing the different concentration of the lipids found in PE, Carnitine palmitoyltransferase 1&2 (CPT1, CPT2), Acyl-CoA Thioesterase 2(ACOT2), Phosphatidylserine Synthetase 1(PTDSS1), and Choline Phosphotransferase I (CHPT1).

Taking into account the fact that some of the metabolites of interest were present in a higher concentration in disease (PE) than in control samples, I inferred what behaviour the stress genes were having in women with PE.

Gene	**Stress**	**State**
ACOT2	43010	Overexpressed
PTDSS1	13262	Overexpressed
CPT1	12524	Overexpressed

CPT2	12524	Under expressed
CHPT1	1130	Overexpressed

Table14: Genes expression in PE

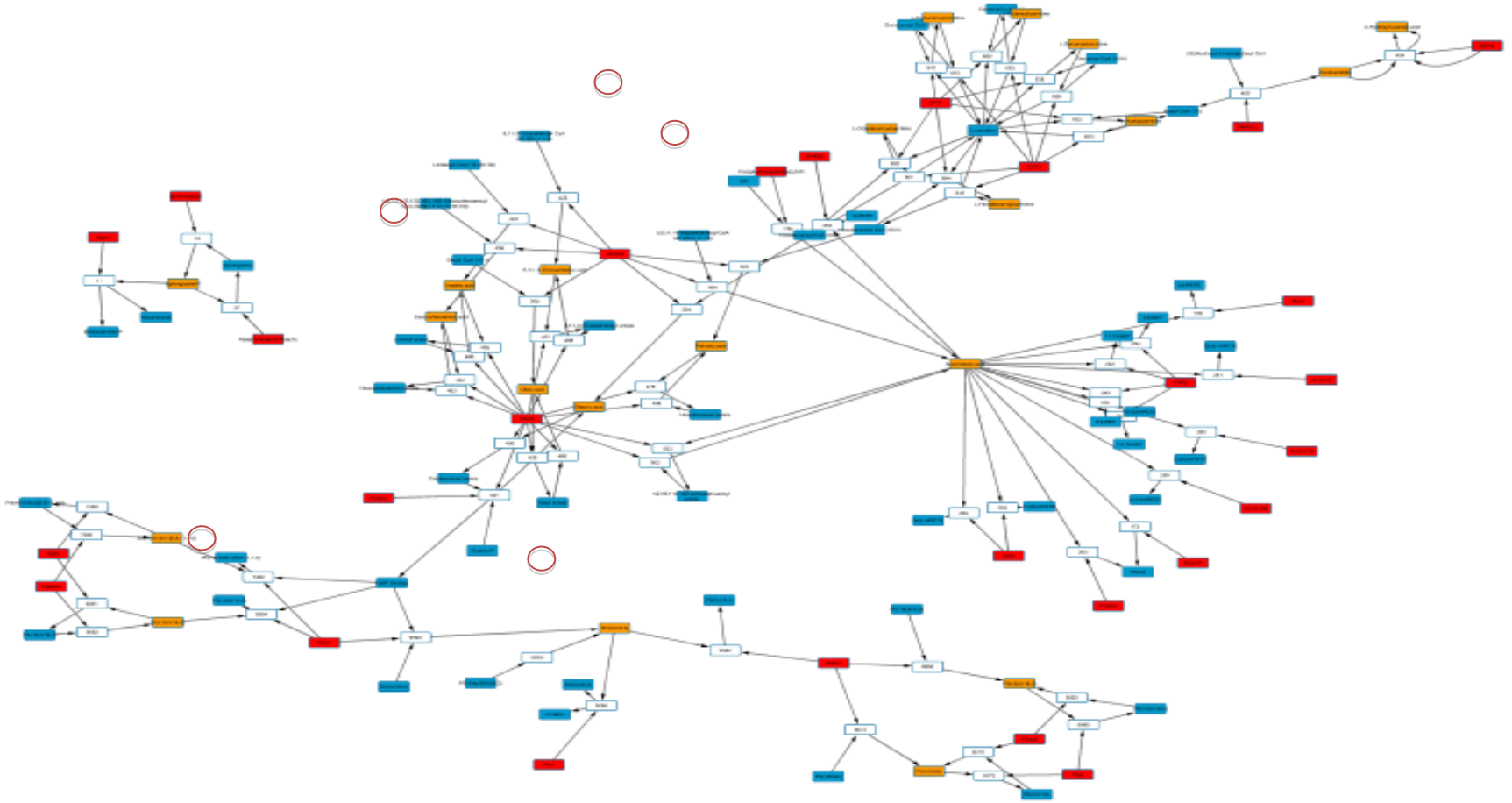


Figure16: Sub network generated from the PE metabolites of interest.

The nodes in white are reaction nodes, the nodes in red are genes the nodes in blue are metabolites, and the nodes in orange are the metabolites of interest. In a circle are shown the 5 genes with high stress.

6.1.6 Discussion

The study in this chapter reviewed the literature on lipid biomarkers identified as being differentially expressed in women with PE compared with controls using metabolomics approaches. I also carried out the same exercise in the only published study on lipid biomarkers expressed differently in women with PCOS compared with controls using metabolomics technology. Data were then inputted into a network containing all lipid reactions that are found in public databases to identify smaller networks and key genes, which potentially played a pivotal role in the mechanisms of PCOS and PE.

I found 3 key genes (CHPT1, PTDSS1 and PLD1) potentially played a pivotal role in the mechanisms of PCOS following the PCOS lipid network analysis and 5 genes in the PE lipid network analysis (CPT1, CPT2, ACOT2, PTDSS1 and CHPT1). Two genes (PTDSS1, CHPT1) were identified in both the PE and PCOS lipid network analysis. PTDSS1 was however the only gene similarly over expressed in both PCOS and PE. To my knowledge there have been no studies that reported any association of these genes with PCOS or PE. These findings suggest that these genes play a role in the aetiology of PCOS and PE and PTDSS1 and CHPT1 might underpin the mechanisms linking PCOS and PE. More mechanistic studies are however to investigate the precise mechanisms these genes play in the aetiology of PCOS and PE.

Phosphatidylserine Synthetase 1 (PTDSS1) is a protein-coding gene. Phosphatidylserine is synthesized by two enzymes PS synthtase1 and PS synthetase2 that converts phosphatidylcholine and phosphotidylthanolamine. A defect in this gene causes Lenz-Majewski hyperostotic dwarfism. Lenz-Majewski syndrome (LMS) is a rare condition characterised by complex craniofascial, dental, cutaneous and limb abnormalities as well as intellectual disability (Sohn 2016).

Choline Phosphotransferase I (CHPT1) is a protein coding gene and is involved in glycerophosphoipid and cholesterol metabolism. The down regulation of CHPT1 has been implicated in kidney ischemia-reperfusion injury (IR) (Feng 2016). It also plays a central role in the formation and maintenance of vesicular membranes.

Phospholipase D (PLD) is known to be a significant factor in tranduction mechanisms in various cells including cardiomyocytes. Poorly controlled diabetes causing hyperglycemia and ketosis causes oxidative stress and as a result could have an impact on the PLD activity during diabetes (Paramjit 2006). There has been also an interest in PLD1 and PLD2 in the setting of cancer, cardiovascular and neurodegenerative and infectious diseases. The PLD1 AND PLD2 genes are extensively expressed in different tissues and cells and their activation occurs via G-protein coupled receptors (GPCR's) and receptor tyrosine kinases (RTK's). In mice studies, it has been shown that PLD inhibition causes blockage of thrombus formation without affecting the haemostasis (Frohman 2015). PLD also plays a possible in cancer. Tumours under hypoxic stress release vascular endothelial growth factor (VEGF) and this stimulates, tumour vascularization leading to the growth of the tumour and PLD1 has been implicated to play a vital role in this process (Zhang 2014).

Phospholipase D is also a catalyst in the hydrolysis of Phosphotidylcholine to produce phosphatidic acid (PA). The enzymes responsible are PLD1 and PLD2. PLD1 as previously discussed, plays a vital role in oxidative stress conditions like diabetes, hypertension and cardio- vascular disease. Essential hypertension can be associated with enhanced activity of PLD dependant pathways as a result of oxidative stress and exaggerated response of angiotension II (ANG II) (Touyz 2001).

High content of adipose tissue influences the development of hyperlipidemia and insulin resistance. A study by Trujillo Viera J et al in

mice suggests that deficiency of PLD1 /PLD2 promotes obesity and diabetes (Trujillo 2016). It is interesting to speculate that this gene plays a role in obesity in PCOS but more studies are required to test this hypothesis. However, Funai K et al tested the hypothesis that skeletal muscle phospholipid metabolism regulates systemic glucose metabolism. For the experiment choline/ethanolamine phosphotransferase 1 (CEPT1), the terminal enzyme in the Kennedy pathway of phospholipid synthesis was agitated. It was concluded that in obese humans, muscle CEPT1 mRNA was inversely linked with insulin sensitivity (Funai 2016).

The approach in my study had several advantages. The first was that it did not require perfect identification from the molecular weights (masses) of the lipids identified in the literature review, which would have been labour intensive to achieve.Simply knowing the family of the compound and the mass was enough. Also most of the procedure was scripted so it was easy to repeat for each datasets and will be for future datasets.

6.1.7Conclusion

Lipidomics has obtained great advantages from recent developments in mass spectrometry. The improvements in resolution and mass accuracy have allowed detection and identification of more and more lipids from animal samples. Thus it is being used to identify possible biomarkers for various human disorders. The challenge is that some disorders present a more complex pathology than just the change in concentration of some particular lipids as highlighted by the studies on PCOS and PE in this chapter.

My aims in this chapter were to combine the list of biomarkers and enzyme reaction information, identified from my literature review of lipid biomarkers of PCOS and PE, and to detect metabolic-pathway changes common to PCOS and PE in a network analysis.

To this end, two resources were developed. The first was a metabolic network, which contained all the metabolic reactions for the different family of lipids. The second was a system for converting generic lipid assignments into lists of specific metabolites as defined in the network. By querying this network with the set of lipids whose levels were statistically significantly different in patients suffering a certain disease, it was possible to extract scored sub-networks that corresponded to disease pathways. This approach was tested with a published lipidomic dataset from patients with Polycystic Ovary Syndrome and Pre- eclampsia. I found 3 key genes (CHPT1 PTDSS1 and PLD1) which potentially played a pivotal role in the mechanisms of PCOS following the PCOS lipid network analysis and 5 genes following the PE lipid network analysis (CPT1, CPT2, ACOT2, PTDSS1 and CHPT1). Two genes (PTDSS1, CHPT1) were identified in both the PE and PCOS lipid network analysis. PTDSS1 was however the only gene similarly over expressed in both PCOS and PE.

The next chapter presents an overall discussion and conclusion to my thesis.

Chapter 7

7.1Summary and Conclusion

The aims and objective of this study were:

1. Identify proteomics biomarkers similarly expressed in women with pre-eclampsia and PCOS to aid the subsequent synthesize of these results with primary metabolomics data and obtain a comprehensive overview of novel pathways potentially linking PCOS with PE.
2. Identify oxylipin biomarkers (metabolomics markers of cardiovascular disease and possible pre-eclampsia) unique to Polycystic Ovary Syndrome.
3. Perform a network analysis of lipids differentially expressed in Pre-Eclampsia and Polycystic Ovary Syndrome from Metabolomic Studies to identify novel pathways implicated in the link between PCOS and PE.

The **null hypothesis** was that women with PCOS did not have a proteomic or metabolomics profile unique to PCOS and PE and that computational analysis would not reveal a single unifying pathway which would form the basis for future prospective studies aiming to improve the diagnosis of PCOS and predict which women with PCOS would develop pre-eclampsia.

The systematic review performed to identify proteomic biomarkers common to both PCOS and PE was undertaken as results from various meta-analysis of pregnancy complications in women with PCOS, implied that there was a higher risk of pre- eclampsia developing in pregnancy in women with PCOS. A database of proteomic markers for PCOS is publicly

available. The list of biomarkers identified from my systematic review was merged with the PCOS proteomic biomarker database to identify whether there were any biomarkers that were similarly expressed in both conditions and to establish any possible link between the two conditions.

One hundred and ninety two proteomic biomarkers for PE were cross-referenced with the PCOS database to determine if any were also differentially expressed in PCOS. Five biomarkers were found to be differentially expressed in women with PE and with PCOS compared to controls. These included Transferrin, fibrinogen alpha, beta and gamma chain variants and kininogen-1 which were raised and annexin 2 and peroxiredoxin 2 were decreased in both women with PCOS and with PE. In PE these biomarkers were found in serum, plasma and placenta respectively where as in PCOS the biomarkers identified were in serum, follicular fluid, ovarian and omental biopsy respectively.

The oxylipins are lipophilic signaling molecules derived from the oxidation of polyunsaturated fatty acids. In the study conducted for oxylipin biomarkers unique to Polycystic Ovary Syndrome, 14, 15 DHET and 19 HETE were significantly raised in women with PCOS compared to controls. When adjusted for BMI and waist: hip ratio, 14, 15 DHET and 19 HETE remained significantly raised in PCOS group,though 19 HETE was only marginally significantaly raised when adjusted for BMI.

Finally, in the network analysis of lipids differentially expressed in Pre-Eclampsia and Polycystic Ovary Syndrome from Metabolomic Studies, I found 3 key genes (CHPT1 PTDSS1 and PLD1) which potentially play a pivotal role in the mechanisms of PCOS ,after analyzing the the PCOS lipid network and 5 genes following the PE lipid network analysis (CPT1, CPT2, ACOT2, PTDSS1 and CHPT1). Two genes (PTDSS1, CHPT1) were identified in both the PE and PCOS lipid network analysis. PTDSS1 was however the only gene similarly over expressed in both PCOS and PE.

As far as I know, this is the first study that has investigated the links between PCOS and PE using this approach. I was not able to identify a single unifying pathway from the list of biomarkers and genes identified in my study. My null hypothesis was therefore NOT rejected.

The proteomic biomarkers common to PCOS and PE were involved in a range of diverse molecular pathways including immunoregulation/inflammation, fibrinolysis, iron transport and blood coagulation. The oxylipins differentially expressed in PCOS were involved in cardio-protection, vasoconstriction, renal function, vascular tone and arterial pressure, inflammation, oxidative stress, diabetes, impaired insulin signaling, platelet aggregation and generation of thrombin. Finally, the two genes (PTDSS1, CHPT1) identified in both the PE and PCOS lipid network analysis was associated with Lenz-Majewski hyperostotic dwarfism, glycerophosphoipid and cholesterol metabolism, kidney ischemia-reperfusion injury and the formation and maintenance of vesicular membranes.

More mechanistic studies are therefore required to investigate the inter-relationships between the proteomic biomarkers, oxylipins and genes identified from my lipidomic network analysis to improve our understanding of the mechanisms linking PCOS and PE. The diverse mechanistic pathways identified so far may however reflect the complexity of both PCOS and PE, and it may well be that a range of factors (molecular, lifestyle, environmental and intra-uterine factors) all interact to result in both phenotypes. Our findings may therefore have identified the effect rather than cause of the diseases.

The strengths of my study were in its originality, as the proteomic database integration of PCOS and PE biomarkers have not been previously done. Oxylipins have also never been previously tested in PCOS women, prior to the onset of my study. This unique combination of omic

approaches complemented by computational analysis of a novel lipid network to identify novel genes and pathways linking PCOS and PE, are the strengths of my study. Some novel potential leads for research were also identified, for example, of the oxylipins associated with PE, in my study, I found the differential expression of 14,15 DHET and 19 HETE and this can form the basis for future studies into oxylipin biomarkers of PE risk in PCOS.

Lipodomics is a subdivision of metabolomics that define the role of lipids at molecular level in the tissues and biofluids. I focused on lipids due to the available local expertise (my supervisors); however, they are biomolecules that have an important activity of sustaining homeostasis. They are a vital component of cell membranes and also store and transport energy. They are involved in communication and signaling. Alteration in the enzymatic metabolism of lipids is related to the pathogenesis of a number of diseases like Atherosclerosis, Insulin-resistant diabetes, cancer, Alzheimer's and schizophrenia. A database of lipids is widely available online and it was used in creating the lipid network used for my study.

It may well be that looking at all metabolites (amino acids, glycoproteins, e.t.c) may have produced a more comprehensive insight into the common pathways involved in both PCOS and PE. On the other hand, by focusing on lipids which are known risk factors for PCOS and its associated health complications, we hoped to reduce the chances of spurious findings, which formed our strategy in the chapter where we measured oxylipins in PCOS.

7.2 Conclusion

The interest in the discovery of biomarkers has generated interest because the wider application of these biomarkers in clinical practice. It potentially has a huge impact on new drug discovery and confronting existing public health challenges. Clinical bioinformatics is a useful tool in translating research into clinical application. In my thesis, I have made an

attempt to address a key health issues related with PCOS (pre-eclampsia) by using these contemporary methods of research. More research is however required to take my findings forward.

Chapter 8

Suggestions for future research

The "omic" methods have the potential for research in discovering novel biomarkers. It's a facsinating and ever evolving field which can use proteomics, metabolomics and genomic approaches as shown in my work.

The aim would be to collaborate and identify areas of interest and validate the already discovered oxylipin biomarkers and also reveal new oxylipins that might lead to the identification of unmapped biochemical pathways.

Incorporation of the "omic" techniques will allow a better understanding of the role of this system in health and disease including PCOS and Pre eclampsia.

It would have been ideal to carry out a prospective study measuring proteomic and oxylipin biomarkers in women with PCOS, prior to pregnancy to identify which biomarkers predicted which women with PCOS developed PE, but this was not feasible in the available timescale for my PhD. Women with PCOS also suffer from infertility and it would have not been feasible to await conception and pregnancy follow-up.

Future research should aim to

1. Validate the five proteomic biomarkers common to PCOS and PE identified in our study in a study of pregnant women with PE.
2. Validate the findings from our study of oxylipins in PCOS in an independent cohort.

3. Investigate oxylipin biomarkers in women with PE to find out if they are similar to those found in our study.
4. Investigate gene expression of the two genes (PTDSS1, CHPT1) identified in both the PE and PCOS lipid network analysis in women with PCOS, PE and women affected with both conditions compared with controls.

If our findings are validated/confirmed in independent study, the ideal long term study would be a prospective follow up study of women with PCOS about to embark on a pregnancy (or in early pregnancy) to identify whether the 5 proteomic biomarkers, oxylipins or the 2 genes identified from my thesis could form the basis for useful biomarkers of PE in women with PCOS.

References

American Diabetes Association. (1998) *Consensus Development Conference on Insulin Resistance*. 5–6 November 1997. Diabetes Care *1998; 21:310–314.*

Arouri, A. and Mouritsen, O. (2013) Membrane-perturbing effect of fatty acids and lysolipids. *Progress in Lipid Research,* 52(1**),** pp.130-140 *Available at:*http://lipidlibrary.aocs.org/Lipids/carnitin/index.htm. Accessed 19th June 2017.

Atger, M., Misrahi, M., Sar, S., Le Flem, L., Dessen, P., and Milgrom, E. (1995). Structure of the human luteinizing hormone-choriogonadotropin receptor gene: unusual promoter and 5' non-coding regions. *Mol Cell Endocrinol,* 111,113–12

Atiomo, W.and Daykin, C.A. (2012) Metabolomic biomarkers in women with polycystic ovary syndrome: a pilot study. *Molecular Human Reprodroduction*: *Basic Science of Rproductive Medicine,* 18(11), 546-53.

Atiomo WU, Khalid S, Ziauddin A, Tooth D, Layfield R. (2009). Framework for a systems approach to proteomic biomarker profiling in polycystic ovary syndrome. Special Report. *Expert Rev Proteomics,* 6,469–499.

Azziz, R et al.(2006) Criteria for Defining Polycystic Ovary Syndrome as a Predominantly Hyperandrogenic Syndrome: An Androgen Excess Society Guideline. *The Journal of Clinical Endocrinology & Metabolism,* 91(11), 4237–4245.

Azziz R, NIH 1990 *vs.* Rotterdam 2003 Criteria for PCOS. *J Clin Endocrinol Metab, March 2006, 91(3): 781–785*

Baek, K.H., Kim, Y.S., Gu, B.H., Kim, M.S., Chung, H.Y.and Choi, B.C. (2010).' Apolipoprotein as a novel gene associated with polycystic ovary syndrome, *Fertility and Sterility'. Conference: Annual Meeting of the American Society for Reproductive Medicine, ASRM 2010 Denver, CO United States. 2010; 94; 4 (suppl 1): S197-S198*

Bahado-Singh, R.O.et al. (2013). First-trimester etabolomic detection of late-onset preeclampsia.*American Journal of Obstetrics and Gynecology.* 208(1):*58.e1-7.*

Balen, A.H., Laven, J.S., Tan,S. L and Dewailly D.(2003) Ultrasound assessment of the polycystic ovary: international consensus definitions. *Human Reprodroduction Update, 9,505–514*

Baranzini, S.E. (2006) Systems-based medicine approaches to understand and treat complex diseases. The example of multiple sclerosis. *Autoimmunity,* 39, 651–662.

Barry, J.A., Azizia, M.M and Hardiman, P.H (2014) Risk of endometrial, ovarian and breast cancer in women with polycystic ovary syndrome: a systematic review and meta-analysis. *Human Reproduction Update,* 20(5) 748–758.

Barth, J.H., Field,H.P., Yasmin,E and Balen,A.H.(2010) Defining hyperandrogenism in polycystic ovary syndrome: measurement of testosterone and androstenedione by liquid chromatography–tandem mass spectrometry and analysis by receiver operator characteristic plots. *European Journal of Endocrinology,* 162 (3), 611–615.

Baumgartner, C., Osl, M., Netzer, M. and Baumgartner,D.(2011). Bioinformatic-driven search for metabolic biomarkers in disease. *Journal of Clinical Bioinformatics,* 1(2).

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

Bergh, C., Carlsson, B., Olsson, J.H., Selleskog, U. and Hillensjö,T.(1993) Regulation of androgen production in cultured human thecal cells by insulin-like growth factor I and insulin. *Fertiity and Sterility*.**59**(2):323-31.

Bergman, R.N., Hope, I.D., Yang, Y.J., Watanabe, R.M., Meador, M.A., Youn, J.H.and Ader, M .(1989). Assessment of insulin sensitivity *in vivo*: a critical review. *Diabetes Metabolism Review* **5**:411–429

Biomarkers Definitions Working Group: Biomarkers and surrogate endpoints: preferred definitions and conceptual framework.(2001). *Clinical Pharmacology and Therapeutics* **69**:89-95.

Blankley, R.T., Gaskell, S.J., Whetton, A.D., Dive, C., Baker, P.N and Myers, J.E.(2009).A proof-of-principle gel-free proteomics strategy for the identification of predictive biomarkers for the onset of pre-eclampsia. *BJOG,* 116(11), 1473-80.

Bloom, S .M.,Schisterman,E.F and Hediger M.L.(2007) The use and misuse of matching in case-control studies: the example of polycystic ovary syndrome. *Fertility and Sterility,* 88(3), 707-710.

Blumenstein, M., McMaster, M.T., Black,M.A., Wu,S., Prakash,R., Cooney, J .et al.(2009). A proteomic approach identifies early pregnancy biomarkers for preeclampsia: novel linkages between a predisposition to preeclampsia and cardiovascular disease. *Proteomics,* 9(11), 2929-45.

Blumenstein, M., Prakash, R., Cooper, G.J and North, R. A. (2009). SCOPE Consotrium. Aberrant processing of plasma vitronectin and high-molecular-weight kininogen precedes the onset of preeclampsia.*Reproductive Science,* 16(12),1144-*52.*

Bodmer, M et al(2011) Use of metformin and the risk of ovarian cancer: A case–control analysis. *Gynecologic Oncology,* 123 (2011) , 200–204.

Boomsa,C.M.et al.(2006). A meta-analysis of pregnancy outcomes in women with polycystic ovary syndrome .*Human Reproduction Update,* 12 (6), 673-683.

Buhimschi, I.A., Zhao, G., Funai, E., Harris, N., Sasson, I., Berstein, I .et al.(2008). Proteomic profiling of urine identifies specific fragments of SERPINA1 and albumin as biomarkers of preeclampsia. *American Journal of Obstetrics and Gynecology,* 199(5), 551.e1-16.

Burton,G.J.,& Jauniaux, E.(2004).Placental oxidative stress from miscarriage to preeclampsia.*Journal of the Society of Gynecological Investigation,* 11(6),342-5.

Campbell, W.B.and Fleming, I. (2010). Epoxyeicosatrienoic acids and endothelium dependent responses. *Pflugers Archive,* 459,881–95.

Campbell, W.B.et al. (1996). Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circulation Research,* 78:415–23.

Catella, F., Lawson, J.A., Fitzgerald, D.J and Fitzgerald, G.A. (1990) Endogenous biosynthesis of arachidonic acid epoxides in humans: Increased formation in pregnancy-induced hypertension. *Proceedings of the National Academy of Sciences of the United States of America*, 87(15), 5893-5897.

Centlow, M., Hansson, S.R, and Welinder, C. (2010). Differential proteome

analysis of the preeclamptic placenta using optimized protein extraction.*Journal of Biomedicine and Biotechnology,* 458748,48-9.

Centre for Maternal and Child Enquiries (CMACE). Saving Mothers' Lives: reviewing maternal deaths to make motherhood safer: 2006–08. The Eighth Report on Confidential Enquiries into Maternal Deaths in the United Kingdom. *BJOG 2011;118 (Suppl. 1):1–203*

Chang, R.J.et al.(1983).Insulin resistance in nonobese patients with polycystic ovarian disease. *Journal of Clinical Endocrinology and Metabolism,* 57,356–359.

Chang, A.Y.et al. (2011).Polycystic ovarian syndrome and subclinical atherosclerosis among women of reproductive age in the Dallas heart study. *Clinical Endocrinology (Oxf),* 74(1),89-96.

Chen, Z.J. et al. (2011).Genome-wide association study identifies susceptibility loci for polycystic ovary syndrome on chromosome 2p16.3, 2p21 and 9q33.3. *Nature Geneicst*,43(1):55-9.

Chittenden, B.G.et al.(2009). Polycysticovary syndrome and the risk of gynaecological cancer: a systematic review. *Reproductive Biomedicine Online,* 19,398–405.

Clifford, K.et al. (1996).Does suppressing luteinising hormone secretion reduce the miscarriage rate? Results of a randomised controlled trial.*British Medical Journal* **312**:1508–11.

ChereauA1844Me´moires pour servir a` l'e´tude des maladies des ovaires. Paris:Fortin, Masson, Cie Rokitansky C 1844. *A manual of pathological anatomy. Vol 2. Philadelphia: Blanchard, Lea, 1855:246 (tr. by Edward Sieveking from original German edition of 1844*)

Conway,G. et al.(2014) European survey of diagnosis and management of the polycystic ovary syndrome: results of the ESE PCOS Special Interest group's questionnaire. *European Journal of Endocrinology*,17(14), 489-498.

Cubedo. J.et al. (2013).High-molecular-weight kininogen and the intrinsic coagulation pathway in patients with de novo acute myocardial infarction.*Thrombosis and Haemostasis* **110 (6):**1121-34.

Cussons, A.J.et al. (2005). Polycystic ovarian syndrome: marked differences between endocrinologists and gynaecologists in diagnosis and management. *Clinical Endocrinology* **62:** 289–295.

DeFronzo, R.A., Tobin, J.D, and Andres, R. (1979). Glucose clamp technique: a method for quantifying insulin secretion and resistance.*The American Journal of Physiology,* 237**,** E214–E223.

De Melo, A.S et al.2015. Pathogenesis of polycystic ovary syndrome: multifactorial assessment from the foetal stage to menopause. *Reproduction,* 150(1), R11–R24.

Diamanti-Kandarakisa, E. et al.(2008).Defects in insulin signalling pathways in ovarian steroidogenesis and other tissues in polycystic ovary syndrome (PCOS). *Journal of Steroid Biochemistry & Molecular Biology,* 109 (3-5), 242–246.

Doia, S.A.,Towers,P.A., Scott,C.J and Al-Shoumera,K.A.(2005) PCOS: an ovarian disorder that leads to dysregulation in the hypothalamic–pituitary–adrenal axis? *European Journal of Obstetrics & Gynecology and Reproductive Biology, 118 (1), 4–16*

Dong, F.et al. (2015). Serum metabolomics study of polycystic ovary syndrome based on UPLC-QTOF-MS coupled with a pattern recognition approach. *Analytical Bioanalytical Chemistry,* 407 (16),4683-95.

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

Dumas, M.E. et al. (2006). Assessment of analytical reproducibilty of 1H NMR spectroscopy based metabonomics for large-scale epidemiological research: the INTERMAP study. *Analytical Chemistry,* 78, 2199–2208.

Dunaif, A.et al (1995). Excessive insulin, receptor serine phosphorylation in cultured fibroblasts and in skeletal muscle: a potential mechanism for insulin resistance in the polycystic ovary syndrome. *Journal of Clinical Investigation,* **96**:801–810.

Dunaif, A. (1992). Polycystic ovary syndrome and obesity. *In: Bjorntorp P, Brodoff BN (eds) Obesity. J.B. Lippincott Co., Philadelphia, pp 594–605*

Dunaif, A and Hoffman, A.R.(1988). Insulin resistance and hyperandrogenism: clinical syndromes and possible mechanisms. *In: Pancheri P, Zichella L (eds) Biorhythms and Stress in the Physiopathologyof Reproduction. Hemisphere Publishing Co, Washington, DC, pp293-317*

Dunaif, A.and Book C-B (1996). Selective intrinsic defects in insulin and IGF-1 action in polycystic ovary syndrome (PCOS) in skin fibroblasts. *Program of the 56th Annual Meeting of The American Diabetes Association, San Francisco, CA, (Abstract 317)*

Dunaif, A. (1997) Insulin Resistance and the Polycystic Ovary Syndrome: Mechanism and Implications for Pathogenesis. *Endocrine Reviews 18(6): 774–800 Copyright © 1997 by The Endocrine Society.*

Dunn, W.B et al (2009). Changes in the metabolic footprint of placental explant-conditioned culture medium identifies metabolic disturbances related to hypoxia and pre-eclampsia.*Placenta, 30(11) 974-80*

Elmarakby, A.A. (2012) Reno-protective mechanisms of epoxyeicosatrienoic acids in cardiovascular disease. *American Journal of Physiology. Regualatory, Integrative and Comparitive Physiology ,* 30*2(3)* R321–30.

Epiney, M.et al. (2012) Comparative analysis of secreted proteins from normal and preeclamptic trophoblastic cells using proteomic approaches. *Journal of Proteomics,* 75(6), 1771-7.

Eriksen, M.B.et al. (2012) Association of polycystic ovary syndrome susceptibility single nucleotide polymorphism rs2479106 and PCOS in Caucasian patients with PCOS or hirsutism as referral diagnosis. *European Journal of Obstetrics, Gynecology and Reproductive Biology,* 163(1*), 39–42.*

Fang, X.et al. (2006) 14, 15-Dihydroxyeicosatrienoic acid activates peroxisome proliferator-activated receptor-alpha. *American Journal of Physiology. Heart and Circulatory Physiology,* 290(1), H55–H63.

Feng,W et al (2016) Identification of Genes and Pathways Associated with Kidney Ischemia-Reperfusion Injury by Bioinformatics Analyses. *Kidney and Blood Pressure Research,* 41(1):48-54.

Ferriman, D and Purdie, A.W. (1979) The inheritance of PCO and possible relationship to premature balding. *Clinical Endocrinology (Oxf),* 11(3), 291–300.

Fisslthaler, B.et al. (199) Cytochrome p450 2c is an edhf synthase in coronary arteries. *Nature,* 401, 493–7.

Fleming, I. (2004) Cytochrome p450 epoxygenases as edhf synthase(s). *Pharmacological Research,* 49(6), 525–33.

Frohman, M.A.(2015) The phospholipase D superfamily as therapeutic targets. *Trends in Pharmacological Science,* 36(3), 137–144.

Funai, K.et al. (2016) Skeletal Muscle Phospholipid Metabolism Regulates Insulin Sensitivity and Contractile Function. *Diabetes,* 65(2), 358-70.

Gateva, A.T.and Kamenov, Z. A. (2012). Markers of visceral obesity and cardiovascular risk in patients with polycystic ovarian syndrome. *European Journal of Obstetrics & Gynecology and Reproductive Biology* 16:61–166 .

Group's Questionnaire. *European Journal of Endocrinology,* 171(4), 489–498.

Gerszten, R.E and Wang, T.J.(2008) The search for new cardiovascular biomarkers. *Nature,* 451(7181), 949-952.

Galazis, N. et al.(2013) Proteomic biomarkers of endometrial cancer risk in women with polycystic ovary syndrome: a systematic review and biomarker database integration. *Gynecological Endocrinology,* 29(7), 638-44.

Galazis,N.,Docheva,N.,Nicolaides,K.H and Atiomo,W.(2013) Proteomic Biomarkers of Preterm Birth Risk in Women with Polycystic Ovary Syndrome (PCOS): A Systematic Review and Biomarker Database Integration. *PLoS One,8(1):* [on line] Available from https://doi.org/10.1371/journal.pone.0053801.[Accessed:16th May 2016]

Gatter,K .C et al.(1983) Transferrin receptors in Human tissues: their distribution and possible clinical relevance. *Journal Clinical of Pathology,* 36(5), 539 – 545.

Gharesi-Fard,B.,Zolghadri,J and Kamali-Sarvestani, E.(2010) Proteome differences of placenta between pre-eclampsia and normal pregnancy *Placenta,* 31(2), 121-5.

Goodarzi, M.O.et al (2012) Replication of association of DENND1A and THADA variants with polycystic ovary syndrome in European cohorts. *Journal of Medical Genetics,* 49(2), 90–95.

Gottschau, M. , Kjaer,S.K.,Jensen,A and Munk,C.(2015) Risk of cancer among women with polycystic ovary syndrome:A Danish cohort study. *Gynecologic Oncology,* 136 (2015), 99–103.

Green., A et al.(2006)Transferrin and iron induce insulin resistance of glucose transport in adipocytes. *Metabolism Clinical and Experimental* ,55, 1042– 1045.

Green top guideline, Royal College of Obstetricians and Gynaecologists (2006). Management of severe pre-eclampsia and eclampsia,

Griffin, J.L. (2003) "Metabonomics: NMR spectroscopy and pattern recognition analysis of body fluids and tissues for characterisation of xenobiotic toxicity and disease diagnosis". *Current Opinion in Chemical Biology,* 7 (5), 648– 54.

Grundy, S.M et al.(2004) Definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. *Circulation 2004;109:433–438.*

Gugliucci, A and Ghitescu, L.(2002). Is diabetic hypercoagulability an acquired annexinopathy? Glycation of annexin II as a putative mechanism for impaired fibrinolysis in diabetic patients. *Medical Hypotheses*, 59 (3),247-51.

Hague, W.H., Adams, J., Reeders,S.T., Peto, T.E.A and Jacobs,H.S. (1988) Familial polycystic ovaries: a genetic disease? *Clinical Endocrinology (Oxf),* 29,593–605.

Harats, D et al.(2000) Overexpression of 15-lipoxygenase in vascular endothelium accelerates early atherosclerosis in LDL receptor-deficient mice. *Arteriosclerosis, Thrombosis and Vascular Biology,* 20(9), 2100–2105.

Hardiman, P., Pillay, O.S and Atiomo, W.(2003) Polycystic ovary syndrome and endometrial carcinoma. *Lancet ,* 361,1810–2.

Haoula, Z., Salman, M and Atiomo, W.(2012) Evaluating the association between endometrial cancer and polycystic ovary syndrome. *Human Reproduction,* 27,1327–31.

Haoula, Z et al. (2015) Lipidomic analysis of plasma samples from women with polycystic ovary syndrome. *Metabolomics,* 11(3), 657–666.

Himelein, M.J and Thatcher,S.S.(2006) Polycystic ovary syndrome and mental health: a review. *Obstetric and Gynecological Survey ,* 61,723–732.

Hojlund, K., Mogensen, M and Sahlin,K. (2008) Mitochondrial Dysfunction in Type 2 Diabetes and Obesity. *Endocrinology and Metabolism Clinics in North America,* 37(3), 713-31.

Hoopes, S. L et al. (2015) Vascular Actions of 20-HETE. *Prostaglandins and Other Lipid Mediators,* 120, 9–16.

Huang, J et al.(2004) Annexin II is a thiazolidinedione responsive gene involved in insulin-induced GLUT4 translocation in 3T3-L1 adipocytes. *Endocrinology,* 145, 1579-86.

Insenser, M., Montes-Nieto, R., Murri, M and Escobar-Morreale, H.F. (2013) Proteomic and metabolomic approaches to the study of polycystic ovary syndrome. *Molecular Cell Endocrinology,* 370:65–77.

Issan, Y et al. (2013) Elevated level of pro-inflammatory eicosanoids and EPCdysfunction in diabetic patients with cardiac ischemia. *Prostaglandins other Lipid Mediators,* 100–101, 15–21.

Jarvela, I.Y et al. (2002) Characterization of normal and polycystic ovaries using three-dimensional power Doppler ultrasonography. *Journal of Assisted Reproduction and Genetics,* 19(12), 582–590

Jensen, R.B., Chellakooty, M., Vielwerth S, et al.(2003) Intrauterine growth retardation and consequences for endocrine and cardiovascular disease in adult life: does insulin-like growth factor-1 play a role? *Hormone Research,* 60 (3l), 136-48.

Jin, H et al. (2008) Analysis of expression and comparative profile of normal placental tissue proteins and those in preeclampsia patients using proteomic approaches. *Analytica Chimica Acta,* 629(1-2) 158-64.

Johnstone, E.D et al (2011) Differential proteomic analysis of highly purified placental cytotrophoblasts in pre-eclampsia demonstrates a state of increased oxidative stress and reduced cytotrophoblast antioxidant defense. *Proteomics,* 11 (20), 4077-84.

Kang, H.M et al. (1999) Role of annexin II tetramer in plasminogen activation. *Trends in Cardiovascular Medicine,*9 (4),92-102

Kathleen Hoeger, MD Obstetrics and Gynecology. *Clinics of North America. Volume 28, Issue 1, 1 March 2001, Pages 85–97*

Kawano, Y et al. (1995) Inhibitory effect of transferrin on progesterone production in the granulosa cell of humans in vivo and porcine granulosa cell in vitro.*Gynecology and Obstetric Investigation,* 40(1), 1-4.

Kedia, K et al. (2016). Global "omics" evaluation of human placental responses to preeclamptic conditions.*American Journal of Obstetrics and Gynecology*. [on line] 215(2):238.e1-238.e20. Available from: doi: 10.1016/j.ajog.2016.03.004. [Accessed 30th July 2017]

Kenny, L.C et al. (2010) Robust early pregnancy prediction of later preeclampsia using metabolomic biomarkers. Screening for Pregnancy Endpoints Consortium.*Hypertension,* 56(4), 741-9.

Kjerulff, L.E., Sanchez-Ramos, L and Duffy, D. (2011) Pregnancy outcomes in women with polycystic ovary syndrome: a meta-analysis. *American Journal Obstetrics and Gynecology, 204(6),* 558.e1–558.e–6.

Kolla,V et al.(2012) Quantitative Proteomic (iTRAQ) Analysis of 1st Trimester Maternal Plasma Samples in Pregnancies at Risk for Preeclampsia. *Journal of Biomedicine and Biotechnology [online] 2012* (2012), 305964, Available from: http://dx.doi.org/10.1155/2012/305964 [Accessed 20th June 2016].

Kosova, G and Urbanek, M. (2013) Genetics of the polycystic ovary syndrome, *Molecular and Cellular Endocrinology* 373 (1-2) 29–38.

Kralova, A et al. (2008) Differential transferrin expression in placentae from normal and abnormal pregnancies: a pilot study. *Reproductive Biology and Endocrinology,* 2(6), 27.

Laffer, C. L.et al. (2004).20-HETE and circulating insulin in essential hypertension with obesity. *Hypertension,* 43(2), 388–392.

Lee C.R et al. (2006) Genetic variation insoluble epoxide hydrolase (EPHX2) and risk of coronary heart disease: The Atherosclerosis Risk in Communities (ARIC) study. *Human Molecular Genetics*, 15(10), 1640–1649.

Legro, R.S et al.(2013) Diagnosis and treatment of polycystic ovary syndrome: an Endocrine Society clinical practice guideline. *Journal of Clinical Endocrinology and Metabolism,* 2013 (98), 4565–4592.

Legro, R.S et al. (2014).Letrozole versus clomiphene for infertility in the polycystic ovary syndrome. *New England Journal of Medicine, 371:119–29*

Legro, R .18th February 2015. Diagnosis and treatment of polycystic ovary syndrome (PCOS): An interview with Richard Legro. *Legro BMC Medicine,* 13:64.

Lemley, K.V. (2007) An introduction to biomarkers: applications to chronic kidney disease. *Pediatric Nephrology*, 22 (11), 1849-59.

Lennon, N.J et al. (2003) Dysferlin interacts with annexins A1 and A2 and mediates sarcolemmal woundhealing. *Journal of Biological Chemistry*,278 (50), 50466–50473.

Lerchbaum, E et al. (2011) Susceptibility loci for polycystic ovary syndrome on chromosome 2p16.3, 2p21, and 9q33.3 in a cohort of Caucasian women. *Hormone and Metabolic Research,* 43(11):743–747

Li, X et al.(2014) 20-Hydroxyeicosatetraenoic acid impairs endothelial insulinsignaling by inducing phosphorylation of the insulin receptor substrate-1 atSer616, *PLoS One[on line] 9 (4),*Available from: https://doi.org/10.1371/journal.pone.0095841. [Accessed: 23rd March 2017].

Liu, B., Xu, Y.ans Voss,C.(2012). Altered Protein Expression in Gestational Diabetes Mellitus Placentas Provides Insight into Insulin Resistance and Coagulation/Fibrinolysis Pathways. *PLoS ONE 7(9)* .Available on line:https://doi.org/10.1371/journal.pone.0044701.Accessed 26th July 2017.

Liu, C et al. (2011) Proteomic analysis of human serum for finding pathogenic factors and potential biomarkers in preeclampsia. *Placenta,* 32 (2), 168-74

Ling, J et al. (2011) Heat shock protein 10 regulated apoptosis of mouse ovarian granulosa cells. *Gynecological Endocrinology,* 27, (1) 63-71

Liu, J.Y et al. (2010) Metabolic profiling of murine plasma reveals an unexpected biomarker in rofecoxib-mediated cardiovascular events. *Proceedings of the National Academy of Sciences USA,* 107(39), 17017–17022.

Long, A., et al Li (2016). Association between the maternal serum levels of 19 eicosanoids and pre-eclampsia. *International Journal of Gynecology and Obstetrics* 133: 291–296.

Long-term consequences of polycystic ovarian syndrome. *RCOG Green Top guideline No.33, 2014.*

Lumbreras, B., Porta, M., Márquez,S et al.(2008). QUADOMICS: An adaptation of the Quality Assessment of Diagnostic Accuracy Assessment (QUADAS) for the evaluation of the methodological quality of studies on the diagnostic accuracy of '-omics'-based technologies. *Clinical Biochemistry* **41**:1316–1325.

Lunde, O., Magnus, P., Sandvik, L and Hoglo, S. (1989) Familial clustering in the polycystic ovarian syndrome. *Gynecological and Obstetric Investigation,* 28(1), 23–30.

Mann, C.J. (2003). Observational research methods. Research design II: cohort, cross sectional, and case-control studies. *Emergency Medical Journal,* 20, 54–60.

Martin, K.A et al. (2008) Evaluation and treatment of hirsutism in premenopausal women: an Endocrine Society clinical practice guideline. *Journal of Clinical Endocrinology and Metabolism,* 93, 1105–1120.

Ma, X et al. (2007) Proteomic analysis of human ovaries from normal and polycystic ovarian syndrome. *Molecular Human Reproduction,* 13 (8), 527-35.

Mihalik, S.J. (2012) Metabolomic profiling of fatty acid and amino acid metabolism in youth with obesity and type 2 diabetes: evidence for enhanced mitochondrial oxidation. *Diabetes Care,* 35,605–611.

Moncada, S. (2004) Adventures in pharmacology, aspirin, prostacyclin and nitric oxide.*Acta Cardiology*,59(1),47-50.

Moran, L.J., Misso, M.L., Wild, R.A and Norman, R.J. (2010) Impaired glucose tolerance, type 2 diabetes and metabolic syndrome in polycystic ovary syndrome: a systematic review and meta-analysis. *Human Reproduction Update,* 16(4), 347-63I.

Mustafa, S et al. (2009) Insulin resistance and endothelial dysfunction: Are epoxyeicosatrienoic acids the link? *Experimental clinical cardiology, 14(2),e41-e50.*

Mutharasan, P. et al. (2013) Evidence for chromosome 2p16.3 polycystic ovary syndrome susceptibility locus in affected women of European ancestry.*Journal of Clinical Endocrinology and Metabolism, 98, E185–E190*

Myers, J.E et al. (2013) Integrated Proteomics pipeline yields novel biomarkers for predicting preeclampsia. *Hypertension,* 61 (6), 1281-8.

Nithipatikom, K and Gross G.J. (2010) Review article: epoxyeicosatrienoic acids: novel mediators of cardioprotection. *Journal of Cardiovascular Pharmacology Ther, 15: 112–19.*

Node, K et al. (1999) Anti-inflammatory properties of cytochrome p450 epoxygenase-derived eicosanoids. *Science,* 285(431), 1276–9.

Odibo, A.O et al. (2011) First-trimester prediction of preeclampsia using metabolomic biomarkers: a discovery phase study. *Prenatal Diagnosis,* 31(10), 990-4.

Ohtoshi, K et al. (2005) Association of soluble epoxide hydrolase gene polymorphism with insulin resistance in type 2 diabetic patients. *Biochemical and Biophysical Research Communications,* 331(1), 347-50

Olsen, C.M et al. (2008) Epithelial ovarian cancer: testing the 'androgens hypothesis'. *Endocrine Related Cancer,* 15, 1061–1068.

Pan, Z and Daniel Raftery. (2007) Comparing and combining NMR spectroscopy and mass spectrometry in metabolomics. *Analytical and Bioanalytical Chemistry,* 387(2), 525-527.

Park, J. S et al. (2008) Identification of proteomic biomarkers of preeclampsia in amniotic fluid using SELDI-TOF mass spectrometry. *Reproduction Science,* 15 (5), 457-68.

Pasquali, R et al. (1982) Insulin and C-peptide levels in obese patients with polycystic ovaries. *Hormone and Metabolic Research,* 14(6), 284–287.

Pearson, T et al. (2010) Measurement of vasoactive metabolites (hydroxyeicosatetraenoic and epoxyeicosatrienoic acids) in uterine tissues of normal and compromised human pregnancy. *Journal of Hypertension,* 28(12), 2429–37.

Peral,B., Camafeita,E and Fernandez-Real J.M.(2009) Tackling the human adipose tissue proteome to gain insight into obesity and related pathologies. *Expert Review of Proteomics,* 6 (4), 353-361.

Poon, L.C et al. (2010) Maternal risk factors for hypertensive disorders in pregnancy: a multivariate approach.*Journal of Human Hypertension,* 24(2), 104-10

Qin, J.Z et al. (2013) Obstetric complications in women with polycystic ovary syndrome: a systematic review and meta-analysis. *Reproductive Biology and Endocrinology* [on line] 11(56).Available from:doi: 10.1186/1477-7827-11-56. [Accessed 26th January 2017]

Qin, K .N and Rosenfield, R.L (1998) Role of cytochrome P450c17 in polycystic ovary syndrome. *Molecular and Cellular Endocrinology,* 145(1-2), 111–121.

Randeva, H.S et al. (2012) Cardiometabolic aspects of the polycystic ovary syndrome. *Endocrine Reviews,*33 (5),812-41

Rasanen, J et al. (2010). Comprehensive maternal serum proteomic profiles of preclinical and clinical preeclampsia. *Journal of Proteome Research,* 9(8), 4274-81.

Redman, C.W. (2011) Preeclampsia: a multi-stress disorder.*Rev Medicine Interne* [on line] 32(1):S41-4 .Available from https://doi.org/10.1016/j.revmed.2011.03.331 [Accessed 10th February 2017].

Report of a WHO Expert Consultation Geneva, *8–11 December 2008* .Waist Circumference and Waist–Hip Ratio.

Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004 Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. *Fertil Steril 81:19–25*

Rumberger, J.M et al. (2004) Transferrin and ironcontribute to the lipolytic effect of serum in isolated adipocytes*.Diabetes,* 53, 2535- 41.

Ryan E. (2003) Hormones and insulin resistance in pregnancy. *Lancet,* 362(9398), 1777-1778.

Saxena, R and Georgopoulos Han, N.A. (2015) Chinese polycystic ovary syndrome risk variants in women of European ancestry: relationship to FSH levels and glucose tolerance. *Human Reproduction, .*30(6), 1454–1459.

Schildkraut, J.M et al. (1996) Epithelial ovarian cancer risk among women with polycystic ovary syndrome. *Obstetrics and Gynecology,* 88(4 Pt 1),554-9.

Schmidt, J., Landin-Wilhelmsen, K et al.(2011) Cardiovascular Disease and Risk Factors in PCOS Women of Postmenopausal Age: A 21-Year Controlled Follow-Up Study. *Journal of Clinical Endocrinology and Metabolism,* 96(12), 3794–3803.

Seli, E., Robert, C and Sirard, M.A (2010) OMICS in assisted reproduction: possibilities and pitfalls.*Molecular Human Reproduction,* 16(8), 513-30.

Shaw, L.J et al.(2008) Postmenopausal Women with a History of Irregular Menses and Elevated Androgen Measurements at High Risk for Worsening Cardiovascular Event-Free Survival: Results from the National Institutes of Health—National Heart, Lung, and Blood Institute Sponsored Women's Ischemia Syndrome Evaluation. *Journal of Clinical Endocrinology and Metabolism,* 93(4), 1276–1284.

Shen, T et al. (2015) 15-Lipoxygenase and 15hydroxyeicosatetraenoic acid regulate intravascular Thrombosis in pulmonary hypertension. *American Journal of Physiology, Lung, Cellular and Molecular Physiology,* 309(5), L449–462.

Shearer, G.C and Newman, J.W. (2009) Impact of Circulating Esterified Eicosanoids and Other Oxylipins on Endothelial Function. *Current Atherosclerosis Reports,* 11(6), 403–410

Shearer, G.C., Pottala, J.V., Spertus, J.A and Harris W.S.(2009) Red blood cell fatty acid patterns and acute coronary syndrome. *PLoS ONE, 4,* e5444.

Shi Y1, Zhao H et al. (2012) Genome-wide association study identifies eight new risk loci for polycystic ovary syndrome.*Nature Genetics*,*44(9),* 1020-5.

Shoupe, D., Kumar, D.D and Lobo, R.A. (1983) Insulin resistance in polycystic ovary syndrome. *American Journal of Obstetrics and Gynecology, 147:588–592.*

Sibai, B., Dekker, G and Kupferminc, M. (2005) Pre-eclampsia. *Lancet. 365,785–799.*

Sohn, M et al.(2016) Lenz-Majewski mutations in PTDSS1 affect phosphatidylinositol 4-phosphate metabolism at ER-PM and ER-Golgi junctions .*Proceedings of National Academy of Sciences U S A* ,113(16),4314-9.

Solá, E et al. (2007) Fibrinogen, plasma viscosity and blood viscosity in obesity. Relationship with insulin resistance. *Clinical Hemorheology and Microcirculation,* 37(4), 309-318.

Solomon, C. and Seely, E.W. (2006) Hypertension in pregnancy. *Endocrinology and Metabolism Clinics in North.America, 35, 157–71.*

Solomon, C .G.et al. (2002).Menstrual Cycle Irregularity and Risk for Future Cardiovascular Disease. *The Journal of Clinical Endocrinology & Metabolism,* 87(5), 2013–2017.

Spector AA, Norris AW: Action of epoxyeicosatrienoic acids on cellular function. *Am J Physiol 2007, 292:C996– C1012.*

Speert H. (1949) Carcinoma of the endometrium in young women. *Surgical Gynaecology and Obstetrics, 88, 332–36.*

Stedman's Medical Dictionary. *27th ed. Baltimore, MD, Lippincott, Williams and Wilkins, 2000, p. 1746*

Stefano Palomba et al, Complications and challenges associated with polycystic ovary syndrome: current perspectives. *International Journal of Women's Health, 31 July 2015*

Stein IF, Leventhal ML 1935 Amenorrhea associated with bilateral polycystic ovaries. *Am J Obstet Gynecol 29:181–191.*

Stepto, N.K et al (2013) Women with polycystic ovary syndrome have intrinsic insulin resistance on euglycaemic–hyperinsulaemic clamp. *Human Reproduction,* 28, (3), 777–784.

Sun L1, Hu W, Liu Q, et al, Metabonomics reveals plasma metabolic changes and inflammatory marker in polycystic ovary syndrome patients. *Proteome Res. 2012 May 4;11 (5):2937-46*.

Szarka, A et al.(2010) Circulating cytokines,
chemokines and adhesion molecules in normal pregnancand preeclampsia determined by multiplex suspension array.*BMC Immunology,*11,59.

Tappia, P.S., Dent, M.R and Dhalla, N.S. (2006) Oxidative stress and redox regulation of phospholipase D in myocardial disease. *Free Radical Biology & Medicine,* 41(3), 349–361.

Teede, H .J et al. (2011) Assessment and management of polycystic ovary syndrome: summary of an evidence-based guideline. *The Medical Journal of Australia,* 195 (6), 65-112.

Tekin, A et al. (2009) Left ventricular function in patients with polycystic ovary syndrome: a Doppler echocardiographic study. *Experimental and Clinical Endocrinology and Diabetes,* 117(4), 165–169.

Thekena, K.N et al. (2012) Evaluation Of Cytochrome P450-Derived Eicosanoids In Humans With Stable Atherosclerotic Cardiovascular Disease. *Atherosclerosis,* 222(2), 530–536.

Toulis, K.A et al. (2011) Meta-analysis of cardiovascular disease risk markers in women with polycystic ovary syndrome.*Human Reproduction Update,* 17(6),741-60.

Tousoulis, D., Andreou, I., Antoniades, C., Tentolouris, C and Stefanadis, C.(2008) Role of inflammation and oxidative stress in endothelial progenitor cell function and mobilization:therapeutic implications for cardiovascular diseases. *Atherosclerosis* ,201(2),236-47.

Touyz, R. M and Schiffrin, E. L (2001) Increased generation of superoxide by angiotensin II in smooth muscle cells from resistance arteries of hypertensive patients: role of phospholipase D-dependent NAD(P)H oxidase-sensitive pathways. *Journal of Hypertension,* 19(7), 1245–1254.

Troisi, R et al. (2003) Estrogen and androgen concentrations are not lower in the umbilical cord serum of pre-eclamptic pregnancies. *Cancer Epidemiological Biomarkers & Prevention,* 12(11pt 1), 1268-70.

Tumani, H et al. (2009) Cerebrospinal fluid biomarkers in multiple sclerosis. *Neurobioogy of Disease,* 35(2), 117–127.

Ulu, A et al. (2008) Soluble epoxide hydrolase inhibitors reduce the development of atherosclerosis in apolipoprotein e-knockout mouse model. *Journal of Cardiovascular Pharmacology, 52(4), 314–323.*

Vascotto, C et al. (2007) Oxidized transthyretin in amniotic fluid as an early marker of preeclampsia. *Journal of Proteome Research,* 6(1)160-70.

Viera, J.T et al. (2016) Phospholipases D1 and D2 Suppress Appetite and Protect against Overweight. *PLoS One*, 11(6), e0157607.

Villoslada, P. (2010) Biomarkers for multiple sclerosis. *Drug News Perspect. 23, 585–595.*

Vinaixa, M et al. (2011) Metabolomics reveals reduction of metabolic oxidation in women with polycystic ovary syndrome after pioglitazone-flutamide-metformin polytherapy. PLoS One, 6(12) e29052.

Waldmana, M., Petersonc, S.J., Aradb, M and Hochhauser,E.(2016) The role of 20-HETE in cardiovascular diseases and its risk factors. *Prostaglandins & other Lipid Mediators*, 125, 108-117.

Walsh, S.W (2004). Eicosanoids in preeclampsia. *Prostaglandins, Leukotrienes and Essential Fatty Acids,* 70 (2), 223–232.

Wang, E.T. et al (2012) Polycystic Ovary Syndrome Is Associated with HigherLeft Ventricular Mass Index: The CARDIA Women'sStudy. *The Journal of Clinical Endocrinology and Metabolism,* 97(12), 4656–4662.

Wang, J et al. (2015) 20-Hydroxyeicosatetraenoic acid involved in endothelial activation and thrombosis. *American Journal of Physiology. Heart and Circulatory Physiology, 308(11),* H1359–H1367.

Wang, W et al. (2015) Detection of urine metabolites in polycystic ovary syndrome by UPLC triple-TOF-MS. *Clinica Chimica Acta,* 448, 39–47.

Ward, N.C et al. (2006) 20-Hydroxyeicosatetraenoic acid is not associated with circulating insulin in lean to overweight humans. *Diabetes Research and Clinical Practice,* 74 (2006), 197–200.

Watanabe H et al. (2004). Proteome analysis reveals elevated serum levels of clusterin in patients with preeclampsia. *Proteomics,* 4(2), 537-43.

Welt, C.K et al. (2012) Variants in DENND1A are associated with polycystic ovary syndrome in women of European ancestry. *Journal of Clinical Endocrinology and Metabolism,* 97(7), E1342-7.

Whigham, L. D et al. (2014) Metabolic Evidence of Diminished Lipid Oxidation in Women With Polycystic Ovary Syndrome. *Current Metabolomics,* 2 (4), 269–278.

Whiting, P et al. (2003) The development of QUADAS: a tool for the quality assessment of studies of diagnostic accuracy included in systematic reviews. *BMC Medical Research Methodology, 10(3), 25.*

Wild, S., Pierpoint, T., McKeigue, P and Jacobs H. (2000) Cardiovascular disease in women with polycystic ovary syndrome at long-term follow-up: a retrospective cohort study. *Clinical Endocrinology (Oxf),* 52(5), 595-600.

Wong, M.K and Takei, Y.(2013) Lack of plasma kallikrein-kinin system cascade in teleosts. *PLoS One* ,8(11),e81057.

Wu, C.C and Schwartzman, M.L. (2011) The role of 20-HETE in androgen-mediated hypertension. *Prostaglandins Other Lipid Mediators, 96(1–4), 45–53.*

Yang, H et al. (2017) A mass spectrometric insight into the origins of benign gynecological disorders. *Mass Spectrometry Reviews*, 36 (3), 450-470.

Yildiz, B.O & Azziz, R. (2007). The adrenal and polycystic ovary syndrome. *Review in Endocrine and Metabolic Disorder,* 8,331–342.

Yun, S.H., Moon, Y.S., Sohn, S.H and Jang, I.S.(2012) Effects of cyclic heat stress or vitamin C supplementation during cyclic heat stress on HSP70, inflammatory cytokines, and the antioxidant defense system in Sprague Dawley rats. *Experimental Animals*, *61(5), 543-53.*

Zawadzki, J.K and Dunaif, A (1992) Diagnostic criteria for polycystic ovary syndrome: towards a rational approach. In: Dunaif A, Givens JR, Haseltine FP, Merriam GR. *Polycystic ovary syndrome. Boston:* Blackwell Scientific; 377–384.

Zeggini, E et al. (2008) Meta-analysis of genome-wide association data and large-scale replication identifies additional susceptibility loci for type 2 diabetes. *Nature Genetics,* 40(5), 638–645.

Zhang. L., Rodriquez, H., Ohno, S and Miller, W.L (1995) Serine phosphorylation of human P450c17 increases 17, 20 lyase activity: implications for adrenarche and the polycystic ovary syndrome. *Proceedings of the National Academy of Science USA,* 92(23), 10619–10623.

Zhao, X et al.(2014) Serum Metabolomics Study of Polycystic Ovary Syndrome Based onLiquid Chromatography−Mass Spectrometry. *Journal of Proteome Research,* 13(12), 1101−1111.

Zhao, Y et al. (2012) Metabolic profiles characterizing different phenotypes of polycystic ovary syndrome: plasma metabolomics analysis. *BMC Medicine*, *30 (10), 153.*

Zhang, A et al. (2012) Modern analytical techniques in metabolomics analysis. *The Analyst,* 137(2), 293-300.

Zhang, Y and Frohman, M.A. (2014) Cellular and physiological roles for phospholipase D1 in cancer. *The Journal of Biological Chemistry,* 289 (330),22567–22574.

Zu, L., Guo, G., Zhou, B and Gao, W. (2016) Relationship between metabolites of arachidonic acid and prognosis in patients with acute coronary syndrome. *Thrombosis Research,* 144 (2016), 192–201.

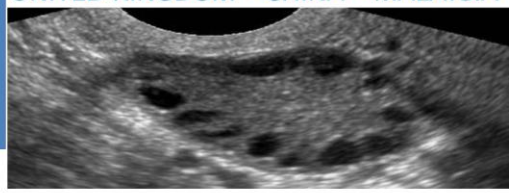
Appendices

Poster presentation

Study of metabolomics and oxylipins to improve the diagnosis and to predict long term health implications in Polycystic Ovarian Syndrome.

Dr Gulafshana Khan*, Mr William Atiomo,*
Prof David Barratt.†
*School of Clinical Sciences.
Division of Human Development.
†*Centre for Analytical Bioscience, School of Pharmacy, University of Nottingham, Nottingham, United Kingdom*

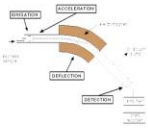
The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA



INTRODUCTION:
Polycystic ovarian syndrome (PCOS) Polycystic ovarian syndrome (PCOS) is the most common endocrine disorder of women, with the a prevalence between 6% and 10% based on the U.S.National Institutes of Health (NIH) criteria and as high as 15% when the broader Rotterdam criteria are applied (Norman R J 2005). It has a major long-term health implications such as diabetes mellitus, cardiovascular disease. The aetiology of PCOS remains obscure and presents major challenges.
Advances in the field of 'OMICS' such as genomics, proteomics and metabolomics provides an insight to unravel the mystery surrounding many medical conditions and may apply to PCOS.This is done by using a single experiment involving genes, proteins and metabolites through the application of novel molecular biology techniques.

Aims: To see if there is a potential role of oxylipins in the diagnosis of PCOS and also whether there is a role as a prognostic indicator for cardiovascular disease in later life in PCOS.

Method: This is a cross-sectional single-centre prospective study in the University hospital department of Obstetrics and Gynaecology at Queens Medical Centre Hospital, Nottingham. Fasting blood samples and 3D US scanning has been obtained from 60 women with PCOS and 60age and weight matched controls in the follicular phase of the menstrual cycle. Women with PCOS were identified from the joint gynaecology/endocrine clinic and the fertility clinics. Controls were identified from women who volunteered to participate. Women who fit the inclusion criteria were recruited.



Due to its high sensitivity and wide range of covered metabolites, MS has become the technique of choice in many metabolomics studies. Its utility lies in its wide dynamic range, reproducible quantitative analysis, and the ability to analyze bio fluids with extreme molecular complexity (Y. Lee do 2010)

CONSENT FORM

PCOS

(Version 2.0 / Aug 2010)

Title of Study: Biochemical and Ultrasound studies to improve diagnosis and management of Polycystic Ovarian Syndrome (PCOS)

REC ref: ***(to be added after approval given)***

Name of Researcher:

Name of Participant: **Please initial box**

1. I confirm that I have read and understand the information sheet version number 1 dated May 2010 for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected. I understand that should I withdraw then the information collected so far cannot be erased and that this information may still be used in the project analysis. ☐

3. I understand that relevant sections of my medical notes and data collected in the study may be looked at by authorised individuals from the University of Nottingham, the research group and regulatory authorities where it is relevant to my taking part in this study. I give permission for these individuals to have access to these records and to collect, store, analyse and publish information obtained from my participation in this study. I understand that my personal details will be kept confidential. ☐

4. I understand and agree that 2 blood samples will be taken for analysis of Hormonal levels and blood Biomarkers. ☐

5. I agree to my GP being informed of my participation in this study and if any incidental or significant findings are found on my blood test and/or scans. ☐

6. I agree to take part in the above study. ☐

7. I consent to my blood sample and US (ultrasound) results to be stored and used for future research. ☐

_____	_____	_____
Name of Participant	**Date**	**Signature**
_____	_____	_____
Name of Person taking consent (if different from Principal Investigator)	Date	Signature
_____	_____	_____
Name of Principal Investigator	Date	Signature

3 copies: 1 for participant, 1 for the project notes and 1 for the medical notes

CONSENT FORM

Controls

(Version 2.0 / Aug 2010)

Title of Study: Biochemical and Ultrasound studies to improve diagnosis and management of PolyCystic Ovarian Syndrome (PCOS)

REC ref: ***(to be added after approval given)***

Name of Researcher:

Name of Participant: **Please initial box**

1. I confirm that I have read and understand the information sheet version number 1 dated May 2010 for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at anytime, without giving any reason, and without my medical care or legal rights being affected. I understand that should I withdraw then the information collected so far cannot be erased and that this information may still be used in the project analysis. ☐

3. I understand and agree that 2 blood samples will be taken for analysis of Hormonal levels and blood Biomarkers. ☐

4. I agree to my GP being informed of my participation in this study and if any incidental or significant findings are found on my blood test and/or scans. ☐

5. I agree to take part in the above study. ☐

6. I consent to my blood sample and US (ultrasound) results to be stored and used for future research. ☐

_____	_____	_____
Name of Participant	**Date**	**Signature**
_____	_____	_____
Name of Person taking consent (if different from Principal Investigator)	Date	Signature
_____	_____	_____
Name of Principal Investigator		
Signature		

Queen's Medical Centre Nottingham NHS
University Hospital NHS Trust

3 copies: 1 for participant, 1 for the project notes and 1 for the medical notes

Patient Information Sheet for Women with PCOS

Biochemical and Ultrasound studies to improve diagnosis and management of Polycystic Ovarian Syndrome

Researchers: Mr W Atiomo, Dr C Daykin, Mr N Raine-Fenning, Dr Gulafshana H Khan.

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like further information. Take time to decide whether or not you wish to take part.

WHAT IS THE PURPOSE OF THE STUDY? The aim of our project is to identify specific markers in the blood (Biomarkers) in addition to check for the thickness of endometriun (lining of the womb), number and size of follicles and the blood flow to the ovaries which may be altered in women with PolyCystic Ovarian Syndrome (PCOS) (using new scientific techniques which now make this possible) compared with women without it to determine what is different. In this project, 100 women with PCOS and 100 women without PCOS will be approached. The purpose of the study will be explained, and following informed consent, a fasting blood test and a pelvic 3D ultrasound test will be obtained. This project may improve our understanding of PCOS and may help us identify new factors which could be targeted in the future to improve management of women with PCOS. On the other hand, we may not find any significant changes between women with and without PCOS in this study.

WHY HAVE I BEEN CHOSEN? You have been approached because your doctor has diagnosed you with PCOS. We do not intend to approach women who do not know if they have been diagnosed with PCOS for inclusion in the study group. We intend to recruit 200 women in total for this study (100 women with PCOS and 100 without).

DO I HAVE TO TAKE PART? It is up to you to decide whether to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

WHAT WILL HAPPEN TO ME IF I TAKE PART?

This study may involve an extra visit to the hospital in addition to visits required as part of your clinical care. As external funding has not been secured for this study, there will unfortunately be no financial compensation /travel expenses for this visit.

Clinical Interviews:

A mutually convenient date when you will be invited to come up to the hospital will be arranged. At the visit, details will be obtained from you about your medical history. Your height, weight and blood pressure will be checked. Measurements of your hip and waist circumference will also be performed.

Blood Tests:

You will be asked to come in first thing in the morning before you have had breakfast for the blood tests, as the results of some of the hormones can be affected by the time of the day when they are obtained. One sample of blood will be obtained from you and collected into two vacutainers (containers for small amounts of blood) for the tests of metabolites expressed.

Storage of Blood Samples

On completion of the analysis of the Biomarkers in the blood tests obtained from you, with your consent, we would like to retain some of the remaining blood samples for storage to allow further future studies, if newer technology for carrying out these studies become available. However, if you do not wish to have your blood sample retained for this part of the study, the sample will be dispensed with according to the established protocols in the laboratory immediately after the experiments. Permission will be sought form our local research ethics committee before any further studies are carried out using any stored blood samples.

Ultrasound scans:

A pelvic ultrasound scan using 3-Dimensional imaging (gives 3- dimensional view of the organs) will be performed to check in detail for the thickness of the lining of the womb, the number and size of follicles and the blood flow to your ovaries. We would recommend that these scans are internal (vaginal) scans as they provide a better picture of the ovary. However, the scans can also be done through the lower abdomen. You will be given the choice of either abdominal or vaginal scan.

WHAT DO I HAVE TO DO? You will be advised not to take any medication, refrain from smoking and avoid any alcohol from 12 midnight prior to the blood tests for metabolite

experiments as these may alter the levels found in the blood. And when you have your 3D scan you need to have an empty bladder.

WHAT ARE THE SIDE EFFECTS OF TAKING PART? We do not envisage any major problems with side effects, however following the blood tests, there may be some slight bruising of the area in the area where blood was obtained from. However all the blood tests will be taken be an experienced Doctor which should minimize this risk.

WHAT ARE THE POSSIBLE DISADVANTAGES AND RISKS OF TAKING PART? We may identify incidental factors in your blood tests, which are associated with an increased risk of adverse conditions like heart disease, diabetes and cancer. Should this occur, the results will be made available to you and you will be referred to your General Practitioner for appropriate counselling. On the other hand, we may not find any significant differences between women with PCOS and controls. We may find that we need to do a further study on more women to obtain any meaningful results.

WHAT ARE THE POSSIBLE BENEFITS OF TAKING PART?

The information we get from this study may help us understand more about PCOS.

WHAT IF NEW INFORMATION BECOMES AVAILABLE?

Sometimes during the course of a research project, new information becomes available. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw, your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form. Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue.'

WHAT HAPPENS WHEN THE RESEARCH STUDY STOPS?

Your clinical care will continue as deemed appropriate. Your personal results will also be made available to you if you so wish.

WHAT IF SOMETHING GOES WRONG?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms may be available to you." Concerns about this study can be reported to the public advice and information centre on B Floor, at the Queens Medical Centre in Nottingham- Patient Advice and Liaison Service on free phone: 0800 183 0204.

WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?

All information, which is collected, about you during the course of the research will be kept strictly confidential. Any information used will be anonymised. With your permission and if you wish your general practitioner will be informed about your participation in this study.

WHAT HAPPENS TO THE RESULTS OF THE RESEARCH STUDY?

The results of the study will be presented at scientific meetings and published in medical journals. To get a copy of the results, you can contact the study co-ordinator whose names and addresses are outlined below. You will not be identified in any report/publication.

WHO IS ORGANISING AND FUNDING THE RESEARCH? **The research is organised by the University of Nottingham.**

WHO HAS REVIEWED THIS STUDY? The study will be reviewed by Nottingham Local Research Ethics Committee 2.

CONTACT FOR FURTHER INFORMATION. Mr William Atiomo. Senior Lecturer and

Consultant in Gynaecology, D Floor, East Block, Queens Medical Centre, Clifton Boulevard, Nottingham, NG7 2UH. 0115 9249924. Email: william.atiomo@nottingham.ac.uk and Dr Zeina Haoula, email: zeina.haoula@nottingham.ac.uk.

Thank you for reading this information sheet! You will be given a copy of the information sheet and a signed consent form to keep if you choose to participate.

Information Sheet for Women without PolyCystic Ovarian Syndrome (PCOS)

Biochemical and Ultrasound studies to improve diagnosis and management of Polycystic Ovarian Syndrome

Researchers: Mr W Atiomo, Dr C Daykin, Mr N Raine-Fenning, Dr Gulafshana Hafeez Khan

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like further information. Take time to decide whether or not you wish to take part.

WHAT IS THE PURPOSE OF THE STUDY? The aim of our project is to identify specific markers in the blood (Biomarkers) in addition to check for the thickness of endometriun (lining of the womb), number and size of follicles and the blood flow to the ovaries which may be altered in women with PolyCystic Ovarian Syndrome (PCOS) (using new scientific techniques which now make this possible) compared with women without it to determine what is different. In this project, 100 women with PCOS and 100 women without PCOS will be approached. The purpose of the study will be explained, and following informed consent, a fasting blood test and a pelvic 3D ultrasound test will be obtained. This project may improve our understanding of PCOS and may help us identify new factors which could be targeted in the future to improve management of women with PCOS. On the other hand, we may not find any significant changes between women with and without PCOS in this study.

WHY HAVE I BEEN CHOSEN? Thank you for considering taking part to act as a control for women with PCOS. We intend to recruit 200 women in total for this study (100 women with PCOS and 100 without).

DO I HAVE TO TAKE PART? It is up to you to decide whether to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

WHAT WILL HAPPEN TO ME IF I TAKE PART?

This study may involve 3 extra visits to the hospital. As external funding has not been secured for this study, there will unfortunately be no financial compensation /travel expenses for these 3 visits.

Following the initial interview, scan and blood tests, if it is deemed that you are suitable to participate in the study. (Confirm that you ***do not have PCOS*** (if not already done), the rest of the steps will be followed.

Clinical Interviews:

A mutually convenient date when you will be invited to come up to the hospital will be arranged. At the visit, details will be obtained from you about your medical history. Your height, weight and blood pressure will be checked. Measurements of your hip and waist circumference will also be performed.

Blood Tests:

You will be asked to come in first thing in the morning before you have had breakfast for the blood tests, as the results of some of the hormones can be affected by the time of the day when they are obtained. One sample of blood will be obtained from you and collected into two vacutainers (containers for small amounts of blood) for the tests of metabolites expressed.

This will occur three times in your cycle, early, midcycle and end of your cycle.

Storage of Blood Samples

On completion of the analysis of the Biomarkers in the blood tests obtained from you, with your consent, we would like to retain some of the remaining blood samples for storage to allow further future studies, if newer technology for carrying out these studies becomes available. However, if you do not wish to have your blood sample retained for this part of the study, the sample will be dispensed with according to the established protocols in the laboratory immediately after the experiments. Permission will be sought form our local research ethics committee before any further studies are carried out using any stored blood samples.

Ultrasound scans:

A pelvic ultrasound scan using 3-Dimensional imaging (gives 3- dimensional view of the organs) will be performed to check in detail for the thickness of the lining of the womb, the number and size of follicles and the blood flow to your ovaries. We would recommend that these scans are internal (vaginal) scans as they provide a better picture of the ovary. However, the scans can also be done through the lower abdomen. You will be given the choice of either abdominal or vaginal scan.

In total, this will be performed 3 times; early in your cycle, midcycle and late in your cycle.

WHAT DO I HAVE TO DO? You will be advised not to take any medication, refrain from smoking and avoid any alcohol from 12 midnight prior to the blood tests as these may alter the levels found in the blood. And when you have your 3D scan you need to have an empty bladder.

WHAT ARE THE SIDE EFFECTS OF TAKING PART? We do not envisage any major problems with side effects, however following the blood tests, there may be some slight bruising of the area in the area where blood was obtained from. However all the blood tests will be taken be an experienced Doctor which should minimize this risk.

WHAT ARE THE POSSIBLE DISADVANTAGES AND RISKS OF TAKING PART? We may identify incidental factors in your blood tests, which are associated with an increased risk of adverse conditions like heart disease, diabetes and cancer. Should this occur, the results will be made available to you and you will be referred to your General Practitioner for appropriate counselling. On the other hand, we may not find any significant differences between women with PCOS and controls. We may find that we need to do a further study on more women to obtain any meaningful results.

WHAT ARE THE POSSIBLE BENEFITS OF TAKING PART?

The information we get from this study may help us understand more about PCOS.

WHAT IF NEW INFORMATION BECOMES AVAILABLE?

Sometimes during the course of a research project, new information becomes available. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw, your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form. Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue.'

WHAT IF SOMETHING GOES WRONG?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms may be available to you." Concerns about this study can be reported to the public advice and information centre on B Floor, at the Queens Medical Centre in Nottingham- Patient Advice and Liaison Service on free phone: 0800 183 0204.

WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?

All information, which is collected, about you during the course of the research will be kept strictly confidential. Any information used will be anonymised. With your permission and if you wish your general practitioner will be informed about your participation in this study.

WHAT HAPPENS TO THE RESULTS OF THE RESEARCH STUDY?

The results of the study will be presented at scientific meetings and published in medical journals. To get a copy of the results, you can contact the study co-ordinator whose names and addresses are outlined below. You will not be identified in any report/publication.

WHO IS ORGANISING AND FUNDING THE RESEARCH? **The research is organised by the University of Nottingham.**

WHO HAS REVIEWED THIS STUDY? The study will be reviewed by Nottingham Local Research Ethics Committee 2.

CONTACT FOR FURTHER INFORMATION. Mr William Atiomo. Senior Lecturer and

Consultant in Gynaecology, D Floor, East Block, Queens Medical Centre, Clifton Boulevard, Nottingham, NG7 2UH. 0115 9249924. Email: william.atiomo@nottingham.ac.uk and Dr Zeina Haoula, email: zeina.haoula@nottingham.ac.uk.

Thank you for reading this information sheet! You will be given a copy of the information sheet and a signed consent form to keep if you choose to participate.



Biochemical and Ultrasound studies to improve diagnosis and management of Polycystic Ovarian Syndrome (PCOS)

Version 1.0 2.0

June 2010 April 2011

Short title: Biochemical and Ultrasound studies of Polycystic Ovarian Syndrome

Trial Registration: www.clinicaltrials.gov

REC reference: 10/H0408/69

Trial Sponsor: University of Nottingham

Funding Source: University of Nottingham

TRIAL / STUDY PERSONNEL AND CONTACT DETAILS

Sponsor: University of Nottingham

Mr Paul Cartledge

Head of Research Grants and Contracts

Research Innovation Services

King's Meadow Campus

Lenton Lane

Nottingham

NG7 2NR

Chief investigator: Mr William Atiomo

Clinical Associate Professor and Consultant Gynaecologist

Division of Human Development

MABBS MA DM FHEA FRCOG

Queens Medical Centre

Nottingham University Hospitals

NG7 2UH

Phone: 0115 8230712

Email: William.atiomo@nottingham.ac.uk

Co-investigators: Dr C Daykin, Mr N Raine-Fenning*,

Dr Z Haoula^, Mr D Hanwar**,Dr Gulafshana H Khan

*Lecturer in school of Pharmacy, University of Nottingham, *Consultant Gynaecologist and Senior Lecturer in Reproductive Medicine, ^Clinical research Fellow in O&G, **PhD student in School of Pharmacy*

Trial / Study Statistician: Dr Clare Daykin

School of Pharmacy

Room C10 Boots Science Building

University of Nottingham

University Park

NG7 2RD

Phone: 0115 951 5100

Email: clare.daykin@nottingham.ac.uk

Trial / Study Coordinating Centre: Department of Obstetrics and Gynaecology

Division of Human Development

Queens Medical Centre

Nottingham University Hospitals

NG7 2UH

SYNOPSIS

Title	Biochemical and Ultrasound studies to improve diagnosis and management of Polycystic Ovarian Syndrome (PCOS)
Short title	Biochemical and Ultrasound studies of Polycystic Ovarian Syndrome
Chief Investigator	Mr William Atiomo
Objectives	*1. To define the role of biomarkers as identified by NMR spectroscopy for diagnosing PCOS and identifying its varied clinical spectrum.* *2. To define the role of 3D US and automated software, sono-AVC in improving the diagnosis of PCOS and distinguishing its wide clinical spectrum.*

	3. To evaluate the correlation between the biomarkers and 3D US markers of PCOS in relation to clinical outcomes.
Trial Configuration	Cross-sectional single-centre observational study
Setting	Secondary care, Queens Medical Centre Nottingham University Hospitals
Sample size estimate	2 arms of study: estimated sample size for each arm 100 women
Number of participants	Total of 200 women (PCOS and Control)
Eligibility criteria	<u>PCOS</u> -Women between ages of 18-40 -Definition of PCOS: PCOS will be defined as in women with two or more of the following in the absence of other endocrine causes of oligo-and/or anovulation (17). 1. Oligo-and/or anovulation 2. Clinical and/or biochemical signs of hyperandrogenism 3. Polycystic ovaries morphology on ultrasound scan, defined as the presence of 12 or more follicles in each ovary (with one ovary is sufficient for diagnosis) measuring 2-9 mm in diameter, and/or increased ovarian volume (> 10 ml). <u>Controls</u> Consenting age and weight matched women without PCOS who have regular 21-35 day menstrual cycles will be used as controls. Specific inclusion criteria will include the following 1. Ages between 18-40. 2. Regular 21-35 day menstrual cycles. 3. No Ultrasound evidence of PCO 4. No evidence of hyperandrogenism. Not using hormonal contraception
Description of interventions	Venepunctures for endocrine assays and metabolomic assays. 5. Pelvic 2D/3D US to measure endometrial thickness, follicular count, size and blood flow.
Duration of study	Planned start date as soon as ethics are available and for 3 years from start date

Outcome measures	Primary outcome measure: Metabonomic expression profile in women with PCOS compared to controls. Secondary outcome measure: Women with PCOS express specific 3D US parameter that is different from women without Polycystic Ovarian Syndrome
Statistical methods	Statistical analysis will be performed using SPSS version 17.0 (Chicago, IL). Data distribution will be tested to assess the degree of normality using a normal probability plot and appropriate parametric or non-parametric tests depending on the data distribution will be applied to assess the differences between means of the study and control population. Any association between the primary outcome, biomarkers and known metabolic and endocrine features of PCOS will be evaluated using linear or multiple logistic regressions as deemed appropriate. For the metabonomic biomarkers, study of inherent groupings within the data will be carried out using multivariate analysis tools such as principal components analysis (PCA), hierarchical cluster analysis (HCA) and by application of the COSA (clustering objects on sets of attributes) algorithm.

ABBREVIATIONS

3D US	3-dimensional ultrasound scan
COSA	Clustering Objects on Sets of Attributes
CI	Chief Investigator overall
CRF	Case Report Form
DAP	Data Analysis Plan
DMC	Data Monitoring Committee
EOT	End of Trial
GCP	Good Clinical Practice
HCA	Hierarchical Cluster Analysis
ICF	Informed Consent Form
NHS	National Health Service
NMR	Nuclear Magnatic Resonance
PCA	Principal Components Analysis
PCOS	Polycystic Ovarian Syndrome
PI	Principal Investigator at a local centre
PIS	Participant Information Sheet
REC	Research Ethics Committee
R&D	Research and Development department
TMG	Trial Management Group
TSC	Trial Steering Committee

TABLE OF CONTENTS

TRIAL / STUDY PERSONNEL AND CONTACT DETAILS.....2

1.2.2 Evolution of and challenges with the diagnosis of PCOS:.....28

1.4.1 HYPERANDROGENISM in PCOS:.....36

Figure: 3.....50

The insulin resistance-hyperinsulinemia-hyperandrogenemia cycle in Polycystic Ovary Syndrome. PCOS is dominated by three major endocrine disruptions: insulin resistance, hyperinsulinemia, and hyperandrogenemia. The cycle leads to the manifestations of PCOS and infertility, complications during pregnancy, and chronic cardio metabolic co morbidities. Joselyn Rojas, 2014,The Role of Metformin in Metabolic Disturbances during Pregnancy: Polycystic Ovary Syndrome and Gestational Diabetes Mellitus, Int J Reprod Med. 2014; 2014: 797681.....50

***a.INCLUSION CRITERIA*.....86**

***b.EXCLUSION CRITERIA*.....87**

Michael Bodmer, Claudia Becker , Christian Meier , Susan S. Jick , Christoph R. Meier, Use of metformin and the risk of ovarian cancer: A case–control analysis. *Gynecologic Oncology 123 (2011) 200–204*.....**Error! Bookmark not defined.**

Xinjie Zhao, Fang Xu, Bing Qi, et al, Serum Metabolomics Study of Polycystic Ovary Syndrome Based onLiquid Chromatography−Mass Spectrometry. *J. Proteome Res. 2014, 13, 1101−1111*.....200

Name of Participant Date Signature.....204

Name of Participant Date Signature.....206

SYNOPSIS.....218

ABBREVIATIONS.....221

TRIAL / STUDY BACKGROUND INFORMATION AND RATIONALE.....225

STUDY PURPOSE AND OBJECTIVES.....228

PRIMARY OBJECTIVE:.....228

SECONDARY OBJECTIVES:.....228

TRIAL / STUDY DESIGN.....228

TRIAL / STUDY CONFIGURATION.....228

Primary endpoint.....229

Secondary endpoint.....229

Safety endpoints.....230

Stopping rules and discontinuation.....230

TRIAL MANAGEMENT.....230

DURATION OF THE TRIAL / STUDY AND PARTICIPANT INVOLVEMENT 230
End of the Trial 230
SELECTION AND WITHDRAWAL OF PARTICIPANTS 230
Recruitment 230
Inclusion criteria 231
Exclusion criteria 232
Expected duration of participant participation 232
Removal of participants from assessments 232
Informed consent 232
STUDY REGIMEN 233
Compliance 233
Criteria for terminating trial 233
TRANSPORT AND STORAGE OF THE TISSUES 233
LABORATORY ANALYSES 234
STATISTICS 234
Methods 234
Multiple linear and logistic regression analysis will be used to assess the relationship between the biomarkers and the ultrasound characteristics and receiver operative characteristic (ROC) curve analysis will be performed to assess the predictive ability of the ultrasound variables for identifying sub groups of PCOS women who experience adverse outcomes and metabolic syndromes in later life. 234
Sample size and justification 234
Assessment of efficacy 234
Procedures for missing, unused and spurious data 235
Definition of populations analysed 235
ADVERSE EVENTS 235
ETHICAL AND REGULATORY ASPECTS 235
ETHICS COMMITTEE AND REGULATORY APPROVALS 236
INFORMED CONSENT AND PARTICIPANT INFORMATION 236
RECORDS 237
Case Report Forms 237
Source documents 237
Direct access to source data / documents 237
DATA PROTECTION 237
QUALITY ASSURANCE & AUDIT 238
INSURANCE AND INDEMNITY 238

TRIAL CONDUCT **238**
TRIAL DATA **238**
RECORD RETENTION AND ARCHIVING **238**
DISCONTINUATION OF THE TRIAL BY THE SPONSOR **238**
STATEMENT OF CONFIDENTIALITY **239**
PUBLICATION AND DISSEMINATION POLICY 239
USER AND PUBLIC INVOLVEMENT 239
STUDY FINANCES 239
Funding source 239
Participant stipends and payments 239

TRIAL / STUDY BACKGROUND INFORMATION AND RATIONALE

Polycystic ovary syndrome is a common, complex, endocrine condition which affects 5-10% of women of reproductive age (1). Women with PCOS usually have problems with infertility, infrequent ovulation, infrequent menstrual cycles, hirsutism, acne and obesity. Endocrine features include raised testosterone and free androgens and a raised Luteinising Hormone/Follicle Stimulating Hormone ratio. An ultrasound scan of the ovaries usually reveals that they are enlarged and contain more than 10 2-9 mm follicles arranged around the periphery of an enlarged ovary with a very bright stroma (2). PCOS is also associated with insulin resistance and an increased risk of non-insulin dependent diabetes on the longer term (3). There is also some evidence suggesting a potential increased risk of cardiovascular mortality, endometrial cancer and possibly breast cancer in these women (4, 5). Despite the complexity of PCOS as hitherto described, most research approaches have primarily focused on the search for a single molecule or factor as the single underlying cause, but no such factor has been consistently validated in all research projects. There is therefore a need for an approach that is able to analyse all the complex potential molecules involves in the aetiology of PCOS in an integrated way so that simple algorithms can be derived to better characterise the pathophysiology of PCOS.

Aetiology (Causes)

Traditional concepts of PCOS as a primarily endocrine condition secondary to aberrations in the hypothalamo-pituitary ovarian axis manifesting as high luteinising hormone/follicle stimulating hormone ratios, increased androgen production and high oestrone levels from peripheral conversion in adipose tissue of androgens are increasingly being challenged. New developments in research into the pathophysiology of PCOS have focused on the role of genetics, insulin resistance and the interrelationships between obesity and ghrelin (a gastric peptide with adipogenic activity) (2).

The familial pattern and identification of a possible male phenotype in PCOS makes it a condition that hypothetically is predominantly genetic in origin. Overall, studies on the genetics of PCOS do suggest that a gene or several genes may be associated with PCOS, based on the clustering of PCOS in families and studies of theca muscle and adipocytes

from women with PCOS which have highlighted a unique molecular phenotype (6). Results from some family studies suggest a possible autosomal dominant phenotype (7). However, the largest twin study (8) on women with PCOS found a high degree of discordance among twins with polycystic ovaries, which suggests a more complex pattern of inheritance than an autosomal dominant pattern of inheritance. Similarly, although some cytogenetic studies suggested that PCOS may be associated with a large deletion of chromosome 11 (6) and X chromosome aneuploidies, larger cytogenetic studies have failed to reveal any karyotypic abnormalities. Studies on candidate genes have focused on those involved in steroid hormone synthesis, genes involved in carbohydrate metabolism, genes involved in gonadotrophin action and the major histocompatibility region. These candidate genes include CYP11A, CYP 17, CYP 21, Androgen receptor, SHBG gene, Insulin receptor gene, Insulin Gene, Insulin receptor substrate gene, Capain 10, FSH beta subunit gene, Dopamine receptor gene and follistatin (7). Although there is no overwhelming evidence supporting linkage from many of these studies, the strongest evidence can be found in support of the region near the insulin receptor gene as it has been found in two different studies. In these two studies an association was found between a marker (D19S884 at 19p13.3) that is located 2 megabases centromeric from the insulin receptor (9). A unique molecular phenotype of skeletal muscle and ovarian theca cells from women with PCOS also provides some evidence in support of a genetic aetiology. In one study, increased insulin receptor serine phosphorylation of skeletal muscle was found in 50% of a sample of women with PCOS. Although it could be argued that epigenetic alterations could have occurred during the in-vitro cultures of these cells, the results from microarray analysis of ovarian theca cells and skeletal muscle cultures from women with PCOS support the concept of a unique tissue molecular phenotype (10, 11). Larger and confirmatory studies are however awaited. An overall appraisal of the evidence in support of a genetic basis for PCOS would therefore suggest that although there is some evidence that genetics underpins PCOS, the evidence is not overwhelming, and several genes or an interaction between several genes and environmental factors may be involved.

It is now clearly established that insulin resistance is present in obese and non-obese women with PCOS, however the exact mechanisms of insulin resistance in PCOS remain elusive. Recent studies on mechanisms of insulin resistance in PCOS have focused on polymorphisms in genes regulating carbohydrate homeostasis as discussed above. However none of these genes have been consistently shown to be related with PCOS. One central paradox regarding the insulin resistance in PCOS is the high responsiveness to insulin by the ovary, as opposed to the resistance of the whole body, and this model has been used to explain ovarian hyperandrogenaemia as it is thought to arise from a direct stimulatory effect of insulin on ovarian stromal cells. A recent study from China (12) appears to however contradict this hypothesis. In a study of 44 women undergoing IVF treatment, of whom 11 had polycystic ovaries and 33 had normal ovulation, significant decreases in insulin-stimulated glucose incorporation into glycogen in cultured ovarian granulosa cells treated with troglitazone from women with PCOS were found compared with controls suggesting that ovarian cells are also insulin resistant. Although admittedly, these were granulosa and not theca cells and they had been exposed to in-vitro cultures, it does raise the question of whether selective ovarian sensitivity does truly occur in PCOS.

Approximately 10-65% of women with PCOS are obese, and obesity in PCOS tends to be distributed around the abdomen (visceral fat). Generally, a significant correlation exists between obesity and infertility and obesity and miscarriage but the mechanisms have not been completely elucidated. There have however recently been studies looking at the role of a complex interaction between obesity and reproductive function in the aetiology of PCOS mediated by Ghrelin. Ghrelin is a novel gastric peptide which has orexigenic and adipogenic properties. Circulating ghrelin concentrations are influenced by nutritional status and, probably, regulate food intake and body weight. Ghrelin has been shown to modulate the secretory pattern of pituitary hormones, and it may exert direct effects on peripheral organs such as the gonads and endocrine pancreas. Four recent studies (13, 14, 15, 16) have examined the role of ghrelin in women with PCOS and produced conflicting results. In one study ghrelin levels were no different from controls, in another, ghrelin levels were lower in obese women with PCOS compared with controls, and in the third paper, women with PCOS had lower ghrelin levels compared with lean and obese controls. In the most recent study however, circulating Ghrelin levels were found to rise following antiandrogen treatment in women with PCOS, suggesting that androgens were an independent modulator of Ghrelin levels. The complexity of the interrelationships between PCOS, androgens, Ghrelin, obesity and infertility however remain to be un-ravelled. A new complexity/holistic approach to PCOS research is required.

Current Treatment

Treatment of PCOS is currently symptom driven and not directed at the underlying cause. Thus women with infertility are offered Clomiphene, gonadotrophins, ovarian diathermy or assisted conception; Hirsutism is treated with anti-androgens or the combined oral contraceptive pill which increases sex hormone binding globulin thus reducing free testosterone. Acne is treated with the combined pill. More recently, in an attempt to direct treatment at the underlying pathophysiology by encouraging weight loss through lifestyle modification and Metformin use have been advocated as these improve insulin resistance. Improved insulin resistance is associated with improved fertility and a resumption of ovulation. Androgen levels also drop. Unfortunately, not all women respond to Metformin and lifestyle modification is not always effective in the clinical setting. Screening for diabetes is also advocated because of the increased risks. However, as there is also currently no clear evidence on the associated risks of cancer or cardiovascular disease, clinical interventions to reduce the risks of these problems in PCOS are less clear.

Given the outlined clinical and research dilemmas in PCOS, a novel research approach is required to improve the diagnosis, treatment and enable better characterisation of the potential long term health risks. In a pilot study, titled: A study of The Gene and Protein Expression in Women with Polycystic Ovary Syndrome, an NMR spectroscopy based study of metabolomic biomarkers in PCOS was carried out using deproteinised plasma from 5 patients with PCOS and 5 age and BMI-matched controls (unpublished data). This study revealed a number of potential biomarkers at the metabolite level which were consistent between all matched pairs of subjects. However, these findings need to be validated in a definitive study.

Hence, this project aims to do this by investigating PCOS using metabolomics and 3-dimensional scanning. There have been no previous publications using these techniques in PCOS.

STUDY PURPOSE AND OBJECTIVES

Aims/Purpose:

1. To investigate the molecular basis for Polycystic Ovary Syndrome (PCOS) using metabolomic technology.
2. To define the role of 3D US and automated software, sono-AVC in improving the diagnosis of PCOS and distinguishing its wide clinical spectrum.
3. To evaluate the correlation between the biomarkers and 3D US markers of PCOS.
4. To identify potential novel targets for treatment of PCOS

PRIMARY OBJECTIVE:

To find out whether women with PCOS have a specific biomarker profile that is different from women without Polycystic Ovarian Syndrome.

SECONDARY OBJECTIVES:

1. To find out whether women with PCOS express specific 3D US parameter that is different from women without Polycystic Ovarian Syndrome.
2. To evaluate the correlation between the biomarkers and 3D US markers of PCOS.
3. To utilise the defined biomarkers and 3D US markers for identifying sub groups of PCOS women who experience adverse outcomes and metabolic syndromes in later life.

TRIAL / STUDY DESIGN

TRIAL / STUDY CONFIGURATION

This will be a cross-sectional single-centre observational study in the University hospital department of Obstetrics and Gynaecology at Queens Medical Centre Hospital, Nottingham. Women already attending the gynaecology /endocrine clinic and the fertility clinics who are known to suffer with PCOS will be recruited over an 18 month period. Controls will be identified from consenting age and weight matched women without PCOS who have regular 21-35 day menstrual cycles, no signs of PCOS on scan and no evidence of hyperandrogenism, who volunteer to participate. Women will be approached verbally or in writing asking if they would like to participate in this study. An explanation of the study will be provided, following which, a written information sheet will be provided. Patients will be given the opportunity to ask any questions they want, following which a consent form will be provided for signature.
The following observations and investigations will be carried out:
1. Clinical Interview

2. Baseline measurement of Blood Pressure, Height, Weight, Waist and Hip Circumference
3. Venepunctures for endocrine assays and metabolomic assays- Fasting blood samples.
4. Pelvic 2D/3D US to measure endometrial thickness, follicular count, size and blood flow.
Women with PCOS will be invited once in their cycle for fasting blood venepuncture and 3D US scanning.
Women without PCOS will be invited three times in their cycle in the follicular, mid cycle and secretory phase for fasting blood venepuncture and 3D US scanning. Women will be asked about their cycles and confirm what day are they in their cycle.
All measurements will be taken by the clinical research fellow following standard operating procedures. This all will take place in a private room in at Nottingham Queens Medical Centre Hospital.

Plan of research
5-10% of women suffer with PCOS. As Queens Medical Centre is a tertiary centre and provides care for a large population, we have special clinics designed for this purpose and hence we see a large number of women with this condition. The aim is to recruit 100 women with PCOS and 100 age and weight matched controls. Power calculations are not possible as there is no existing data on the outcome measures in such women. Data distribution will be tested to assess the degree of normality and appropriate statistical tests then applied to assess the differences between means of any given population. Any association between biomarkers and known metabolic and endocrine features of PCOS will be evaluated using linear or multiple logistic regressions as deemed appropriate. For the metabonomic biomarkers, study of inherent groupings within the data will be carried out using 'unsupervised' multivariate analysis tools such as principal components analysis (PCA), hierarchical cluster analysis (HCA) and by application of the COSA (clustering objects on sets of attributes) algorithm, in order to allow unbiased study of inherent variation between the sample profiles. Samples will then be unblinded, allowing more detailed 'supervised' data analysis to be carried out using methodologies such as partial least squares-discriminant analysis (PLS-DA), which will allow samples to be classified into specific groups based on their metabolite. Validation of any models produced will be carried out by building models on a sub-set of spectra (training set) and checking whether the remaining spectra are classified into the correct group using the remaining spectra (test set), thus allowing the accuracy of prediction to be established. Study of the 'loadings' plots from these models will allow identification of spectral regions which differ between controls and PCOS women. These spectral regions will be assigned to specific metabolites by the use of 2D NMR spectroscopy.

The aim is to commence the project as soon as approved. Data will be inserted into a database as the study continues and statistical analysis will take place throughout the project. It is estimated that the analysis and final report will be completed by July 2013.

Primary endpoint

Metabonomic expression profile in women with PCOS compared to controls

Secondary endpoint

1- Women with PCOS express specific 3D US parameter that is different from women without Polycystic Ovarian Syndrome

2- Correlation between the biomarkers and 3D US markers of PCOS.

3- Define biomarkers and 3D US markers for identifying sub groups of PCOS women who experience adverse outcomes and metabolic syndromes in later life.

Safety endpoints

1. To define the role of biomarkers as identified by NMR spectroscopy for diagnosing PCOS and identifying its varied clinical spectrum.
2. To define the role of 3D US and automated software, sono-AVC in improving the diagnosis of PCOS and distinguishing its wide clinical spectrum.
3. To evaluate the correlation between the biomarkers and 3D US markers of PCOS in relation to clinical outcomes.

Stopping rules and discontinuation

Patients who do not fit the inclusion criteria will be excluded. We do not anticipate any adverse effects to participants as the main intervention is investigations.

TRIAL MANAGEMENT

Trial will be managed by the chief investigator (Mr Atiomo) and the research team. Monthly meetings will be held to ensure continuous communication, data collection and progress review.

DURATION OF THE TRIAL / STUDY AND PARTICIPANT INVOLVEMENT

Women with PCOS will be invited once in their cycle for blood venepuncture and 3D US scanning.
Women without PCOS will be invited three times in their cycle in the follicular, mid cycle and secretory phase for blood venepuncture and 3D US scanning.
All measurements will be taken by the clinical research fellow following standard operating procedures. This all will take place in a private room in at Nottingham Queens Medical Centre Hospital.

Each patient will be enrolled after informed consent obtained and their enrolment will cease after they complete their last visit to the hospital (as above). The aim is to recruit for 18 months.

End of the Trial

The trial will be ended after the last visit of the last recruited participant, not including any follow-up period.

SELECTION AND WITHDRAWAL OF PARTICIPANTS

Recruitment

The study is set at the University hospital department of Obstetrics and Gynaecology at Queens Medical Centre Hospital, Nottingham.

Women with PCOS will be identified from the gynaecology/endocrine clinic and the fertility clinics at Queens Medical Centre Nottingham. Women with PCOS who fit the inclusion

criteria will be approached verbally or in writing asking if they would like to participate in this study. The initial approach will be from a member of the patient's usual care team (which may include the investigator), and information about the trial will be on display in the relevant clinical areas. A patient information sheet will be given to the women, and following informed consent, they will be recruited to the study by the clinical research fellow.

Controls will be identified from women who volunteer to participate. Women without PCOS who fit the inclusion criteria will be approached verbally or in writing asking if they would like to participate in this study. The initial approach will be from a member of the patient's usual care team if they were attending Gynaecology clinic (which may include the investigator), and information about the trial will be on display in the relevant clinical areas or staff of Nottingham University and Queens Medical Centre who will volunteer or any member of the public who wishes to volunteer. A participant information sheet will be given to the women, and following informed consent, they will be recruited to the study by the clinical research fellow.

Posters will be put up in the gynaecology wards, gynaecology clinics, obesity clinic, hospital corridors and on University of Nottingham boards to advertise the study to patients and staff. In addition, we will advertise on websites.

Due to lack of funding for interpreting services, women who are non-English speakers will not be included in the study. It will be explained to the potential participant that that entry into the trial is entirely voluntary and that their treatment and care will not be affected by their decision. It will also be explained that they can withdraw at any time but attempts will be made to avoid this occurrence. In the event of their withdrawal it will be explained that their data collected so far cannot be erased and we will seek consent to use the data in the final analyses where appropriate.

Inclusion criteria

PCOS

-Women between ages of 18-40

-Definition of PCOS:

PCOS will be defined as in women with two or more of the following in the absence of other endocrine causes of oligo-and/or anovulation (17).

1. Oligo-and/or anovulation
2. Clinical and/or biochemical signs of hyperandrogenism
3. Polycystic ovaries morphology on ultrasound scan, defined as the presence of 12 or more follicles in each ovary (with one ovary is sufficient for diagnosis) measuring 2-9 mm in diameter, and/or increased ovarian volume ($>$ 10 ml).

Controls

Consenting age and weight matched women without PCOS who have regular 21-35 day menstrual cycles will be used as controls.

Specific inclusion criteria will include the following

6. Ages between 18-40.
7. Regular 21-35 day menstrual cycles.
8. No Ultrasound evidence of PCO
9. No evidence of hyperandrogenism.

10. Not using hormonal contraception.

Exclusion criteria

Exclusion Criteria for both groups:

- Thyroid disease
- Current pregnancy
- Delivery or miscarriage occurring within the preceding 3 months
- Recent surgery (within 3 months)
- History of myocardial infarction
- Use of aspirin or heparin
- Sex steroid therapy
- A history of haematological disease, malignancy or liver disease
- Hyperprolactinaemia
- A positive synacthen test following raised 17-hydroxyprogesterone levels
- A history of thrombosis
- Recent Viral Illness
- Women that cannot speak the English language (no funding available for interpreters)

Expected duration of participant participation

Study participants will be participating in the study until they complete their last visit to the hospital.

Removal of participants from assessments

Participants may be withdrawn from the trial either at their own request or at the discretion of the Investigator. The participants will be made aware that this will not affect their future care. Participants will be made aware (via the information sheet and consent form) that should they withdraw the data collected to date cannot be erased and may still be used in the final analysis.

Informed consent

All participants will provide written informed consent. The Informed Consent Form will be signed and dated by the participant before they enter the trial. The Investigator will explain the details of the trial and provide a Participant Information Sheet, ensuring that the participant has sufficient time to consider participating or not (minimum of 24 hours). The Investigator will answer any questions that the participant has concerning study participation.

Informed consent will be collected from each participant before they undergo any interventions (including physical examination and history taking) related to the study. One copy of this will be kept by the participant, one will be kept by the Investigator, and a third will be retained in the patient's hospital records.

Should there be any subsequent amendment to the final protocol, which might affect a participant's participation in the trial, continuing consent will be obtained using an amended Consent form which will be signed by the participant.

STUDY REGIMEN

Women with PCOS will be identified from the gynaecology/endocrine clinic and the fertility clinics at Queens Medical Centre Nottingham. Women with PCOS who fit the inclusion criteria will be approached verbally or in writing asking if they would like to participate in this study. The initial approach will be from a member of the patient's usual care team (which may include the investigator), and information about the trial will be on display in the relevant clinical areas. A patient information sheet will be given to the women, and following informed consent, they will be recruited to the study by the clinical research fellow. Women with PCOS will be invited once in their cycle for fasting blood venepuncture and 3D US scanning arranged at mutual convenient time.

Controls will be identified from women who volunteer to participate. Women without PCOS who fit the inclusion criteria will be approached verbally or in writing asking if they would like to participate in this study. The initial approach will be from a member of the patient's usual care team if they were attending Gynaecology clinic (which may include the investigator), and information about the trial will be on display in the relevant clinical areas or staff of Nottingham University and Queens Medical Centre who will volunteer or any member of the public who wishes to volunteer. A participant information sheet will be given to the women, and following informed consent, they will be recruited to the study by the clinical research fellow. Women without PCOS will be invited three times in their cycle in the follicular, mid cycle and secretory phase for fasting blood venepuncture and 3D US scanning. Women will be asked about their cycles and confirm what day are they in their cycle arranged at mutual convenient time.

All measurements will be taken by the clinical research fellow following standard operating procedures. This all will take place in a private room in at Nottingham Queens Medical Centre Hospital.

Compliance

Women who decide to join the study do so voluntarily and so we expect that they will comply with the study regimen.

Criteria for terminating trial

We have no intention to terminate the study before its completion day.

TRANSPORT AND STORAGE OF THE TISSUES

Samples will be stored in the University Department of Pharmacy. They will be stored in a freezer in a laboratory. The access to the department is controlled via swipe card entry. Samples will be labelled with the study number only. The paper record of the patient's details and study number will be stored in a separate part of the department.

The Clinical Research Fellow will be responsible for the samples. Only the clinical research fellow, her supervisors and the laboratory assistant and manager will have access to the samples.

Samples will be stored under the UoN research licence for those participant's who consented for their samples to be stored for future research. And for those, where consent has not been given samples will be disposed of in accordance with the HTA code of practice.

LABORATORY ANALYSES

Analysis of plasma samples are carried out by 1H NMR spectroscopy at the centre for Biomolecular sciences at the University of Nottingham and the data are analyzed by multivariate statistics. All multivariate analysis is carried out using SIMCA-P version 11.5 (Umetrics, Umeå, Sweden).

STATISTICS

Methods

Statistical analysis will be performed using SPSS version 17.0 (Chicago, IL). Data distribution will be tested to assess the degree of normality using a normal probability plot and appropriate parametric or non-parametric tests depending on the data distribution will be applied to assess the differences between means of the study and control population.

Any association between the primary outcome, biomarkers and known metabolic and endocrine features of PCOS will be evaluated using linear or multiple logistic regressions as deemed appropriate. For the metabonomic biomarkers, study of inherent groupings within the data will be carried out using 'unsupervised' multivariate analysis tools such as principal components analysis (PCA), hierarchical cluster analysis (HCA) and by application of the COSA (clustering objects on sets of attributes) algorithm, in order to allow unbiased study of inherent variation between the sample profiles. Samples will then be unblinded, allowing more detailed 'supervised' data analysis to be carried out using methodologies such as partial least squares-discriminant analysis (PLS-DA), which will allow samples to be classified into specific groups based on their metabolite. Validation of any models produced will be carried out by building models on a sub-set of spectra (training set) and checking whether the remaining spectra are classified into the correct group using the remaining spectra (test set), thus allowing the accuracy of prediction to be established. Study of the 'loadings' plots from these models will allow identification of spectral regions which differ between controls and PCOS women. These spectral regions will be assigned to specific metabolites by the use of 2D NMR spectroscopy.

Multiple linear and logistic regression analysis will be used to assess the relationship between the biomarkers and the ultrasound characteristics and receiver operative characteristic (ROC) curve analysis will be performed to assess the predictive ability of the ultrasound variables for identifying sub groups of PCOS women who experience adverse outcomes and metabolic syndromes in later life.

Sample size and justification

With studies like this, there is a challenge with traditional sample size calculations because of the numbers of potential biomarkers that maybe identified. Therefore, our decision to recruit 200 patients has been pragmatic. We believe this would provide enough data on the parameters of interest and the variability to enable a post hoc sample size calculation.

Assessment of efficacy

Primary efficacy endpoint:

1-Metabonomic expression profile – difference between women with PCOS compared with controls.
Secondary efficacy endpoints:
3D Ultrasound power Doppler ovarian vascularity indices, ovarian volume and antral follicule count-

-difference between women with PCOS compared with controls.

-difference in these variables between sub groups of PCOS women who experience adverse outcomes and metabolic syndromes in later life.

Procedures for missing, unused and spurious data

We will exclude those data from women that don't attend as requested from the analysis. The study will be continued until we reach our target sample size.

Definition of populations analysed

Per protocol set: All participants in the Full Analysis set who are deemed to have no major protocol violations that could interfere with the objectives of the study.

ADVERSE EVENTS

No adverse events are expected. Patients will only be actively involved when visiting the hospital. Approximate duration in hospital 45 minutes.
All women will be seen in a private room at Queens Medical Centre, Nottingham University Hospital. Study group women will be asked to attend on one extra occasion and control group women on three extra occasions (mutual convenient time).

1. Blood tests will be done, a maximum of 20 mls of blood (approximate estimate of an egg cup) will be required and this will have no physical effect on the patient. Possible adverse event will be bruising at the needle site and hence mild discomfort. This will be discussed prior to obtaining blood samples and if it occurs will be dealt with as a normal procedure and will not need reporting.

2. 3D US scanning will be performed which is no difference to a routine internal 2D pelvic scan and this as well has no physical effect on the patient.

ETHICAL AND REGULATORY ASPECTS

ETHICS COMMITTEE AND REGULATORY APPROVALS

The study will not be initiated before the protocol, informed consent forms and participant information sheets have received approval from a Research Ethics Committee (REC) and the respective National Health Service (NHS) Research & Development (R&D) department.

Should a protocol amendment be made that requires REC approval, the changes in the protocol will not be instituted until the amendment and revised informed consent forms and participant and GP information sheets (if appropriate) have been reviewed and received approval / favourable opinion from the REC and R&D departments. A protocol amendment intended to eliminate an apparent immediate hazard to participants may be implemented immediately providing that the REC are notified as soon as possible and an approval is requested. Minor protocol amendments only for logistical or administrative changes may be implemented immediately; and the REC will be informed.

The study will be conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki, 1996; the principles of Good Clinical Practice and Good Laboratory Practice, the DH Research Governance Framework, 2nd ed, 2005 and in accordance with the Human Tissue Act, 2004.

INFORMED CONSENT AND PARTICIPANT INFORMATION

The process for obtaining participant informed consent will be in accordance with the REC guidance, and Good Clinical Practice (GCP) and any other regulatory requirements that might be introduced. The investigator and the participant shall both sign and date the Consent Form before the person can participate in the study.

The participant will receive a copy of the signed and dated forms and the original will be retained in the Trial Master File. A second copy will be filed in the participant's medical notes and a signed and dated note made in the notes that informed consent was obtained for the trial.

The decision regarding participation in the study is entirely voluntary. The investigator shall emphasize to them that consent regarding study participation may be withdrawn at any time without penalty or affecting the quality or quantity of their future medical care, or loss of benefits to which the participant is otherwise entitled. No study-specific interventions will be done before informed consent has been obtained.

If the Informed Consent Form is amended during the study, the investigator shall follow all applicable regulatory requirements pertaining to approval of the amended Informed Consent Form by the REC and use of the amended form (including for ongoing participants).

RECORDS

Case Report Forms

Data will be stored on University Computers but all identifying information will be removed and the patient will be identified using a study number only. The trial identity code number will use their initials (of first and last names) and date of birth (dd/mm/yy). The investigator will make a separate confidential record of the participant's name, date of birth, local hospital number or NHS number, and Participant Trial Number (the Trial Recruitment Log), to permit identification of all participants enrolled in the trial. The study number consist of ZDNPorC (n=numerical number starting with 001 as first recruited and so on and P for patients with PCOS and C for controls).
All paper forms shall be filled in using black ballpoint pen. Errors shall be lined out but not obliterated by using correction fluid and the correction inserted, initialled and dated.
The Chief or local Principal Investigator shall sign a declaration ensuring accuracy of data recorded. CRFs will be treated as confidential documents and held securely in accordance with regulations.
Direct access will be permitted when required to all source documents and other study documentation e.g. signed consent forms, for the purpose of study monitoring and audit and other lawful regulatory inspection by the Chief Investigator, Sponsor's designee and inspection by relevant regulatory authorities.

Source documents

Source documents shall be filed at the investigator's site and may include but are not limited to, consent forms and laboratory results.

Only study staff shall have access to study documentation other than the regulatory requirements listed below including Human Tissue Act and Human Tissue Authority.

Direct access to source data / documents

The CRF and all source documents, including progress notes and copies of laboratory and medical test results shall made be available at all times for review by the Chief Investigator, Sponsor's designee and inspection by relevant regulatory authorities.

DATA PROTECTION

All study staff and investigators will endeavour to protect the study participants' rights to privacy and informed consent, and will adhere to the Data Protection Act, 1998. Only the minimum required information for the purposes of the study shall be collected. Documents will be held securely, in a locked cabinet. Access to the information will be limited to the study staff and investigators and relevant regulatory authorities (see above). Computer held data including the study database will be held securely and password protected. All data will be stored on a secure dedicated web server. Access will be restricted by user identifiers and passwords (encrypted using a one way encryption method).
Information about the study in the participant's medical records / hospital notes will be treated confidentially in the same way as all other confidential medical information.
Electronic data will be backed up every 24 hours to both local and remote media in encrypted format.

QUALITY ASSURANCE & AUDIT

INSURANCE AND INDEMNITY

Insurance and indemnity for trial participants and trial staff is covered within the NHS Indemnity Arrangements for clinical negligence claims in the NHS, issued under cover of HSG (96)48. There are no special compensation arrangements, but trial participants may have recourse through the NHS complaints procedures.

The University of Nottingham has taken out an insurance policy to provide indemnity in the event of a successful litigious claim for proven non-negligent harm.

TRIAL CONDUCT

Trial conduct will be subject to systems audit of the Trial Master File for inclusion of essential documents; permissions to conduct the trial; Trial Delegation Log; CVs of trial staff and training received; local document control procedures; consent procedures and recruitment logs; adherence to procedures defined in the protocol (e.g. inclusion / exclusion criteria, timeliness of visits); adverse event recording and reporting; accountability of trial materials and equipment calibration logs.
The Trial Coordinator shall carry out a site systems audit at least yearly and an audit report shall be made to the Trial Steering Committee.

TRIAL DATA

Monitoring of study data shall include confirmation of informed consent; source data verification; data storage and data transfer procedures; local quality control checks, back-up and disaster recovery of any local databases and validation of data manipulation. The Study Coordinator, or where required shall carry out monitoring of study data as an ongoing activity.
Study data and evidence of monitoring will be made available for inspection by REC as required.

.

RECORD RETENTION AND ARCHIVING

In compliance with the Good Clinical Practice guidelines, regulations and in accordance with the University Of Nottingham Research Code Of Conduct, the Chief Investigator will maintain all records and documents regarding the conduct of the study. These will be retained for at least 7 years or for longer if required
The Study Master File and study documents held by the Chief Investigator on behalf of the Sponsor shall be finally archived at secure archive facilities at the University of Nottingham. This archive shall include all trial databases and associated meta-data encryption codes.

DISCONTINUATION OF THE TRIAL BY THE SPONSOR

The Sponsor reserves the right to discontinue this trial at any time for failure to meet expected enrolment goals, for safety or any other administrative reasons. The Sponsor shall take advice from the Trial Steering Committee and Data Monitoring Committee as appropriate in making this decision.

STATEMENT OF CONFIDENTIALITY

Individual participant medical information obtained as a result of this study are considered confidential and disclosure to third parties is prohibited with the exceptions noted above.
Participant confidentiality will be further ensured by utilising identification code numbers to correspond to treatment data in the computer files.
Such medical information may be given to the participant's medical team and all appropriate medical personnel responsible for the participant's welfare.
Data generated as a result of this trial will be available for inspection on request by the participating physicians, the University of Nottingham representatives, the REC, local R&D Departments and the regulatory authorities.

PUBLICATION AND DISSEMINATION POLICY

The research will be registered on a public database following ethical approval. The intention is to report and disseminate the research results through Peer reviewed scientific journals, internal reports and conference presentations. This study will be written up as part of educational qualification (MD). Identifiable personal data will not be used. Patients will be offered the option to register an interest in receiving information about the outcome of the study after its completion, whether the findings of clinical significance are for their own health or for their relatives. A lay summary will be prepared for circulation to those who have expressed such an interest.

USER AND PUBLIC INVOLVEMENT

The planned research was verbally discussed with women who suffer with PCOS and they have showed interest and have agreed to the additional time required to undertake further tests.

STUDY FINANCES

Funding source

This study is funded by the Academic unit of Obstetrics and Gynaecology at the University of Nottingham.

Participant stipends and payments

Participants will not be paid to participate in the study. As external funding has not been secured for this study, there will unfortunately be no financial compensation /travel expenses.

SIGNATURE PAGES

Signatories to Protocol:

Chief Investigator: (name)_____

Signature:_____

Date: _____

Co- investigator: (name) _____

Signature:_____

Date: _____

Trial Statistician: (name)_____

Signature:_____

Date: _____

REFERENCES:

1. Polson DW, Adams J, Wadsworth J, Franks S (1998). Polycystic ovaries – a common finding in normal women. Lancet ;i:870–2.
2. Sharma A, Atiomo W. Recent Developments in Polycystic Ovary Syndrome. Current Obstetrics and Gynaecology 2003*;* 13, 281-286

3. Peppard HR, Marfori J, Iuorno MJ, Nestler JE. (2001). Prevalence of polycystic ovary syndrome among premenopausal women with type 2 diabetes. Diabetes Care.; 24(6):1050-2.
4. Atiomo W, El Mahdi E, Hardiman P (2003). Familial Associations in PCOS. Fertil Steril.; 80(1):143-5.
5. Hardiman, Pillay, Atiomo (2003). Polycystic Ovary Syndrome and Endometrial cancer. The Lancet . 361,1810-12.
6. M.F. Meyer, F. Gerresheim, A. Pfeiffer, J.T. Epplen and H. Schatz (2000), Association of polycystic ovary syndrome with an interstitial deletion of the long arm of chromosome 11. Exp Clin Endocrinol Diabetes 108, 519–523
7. R. Legro and J. Strauss,(2003), Molecular progress in infertility: polycystic ovary syndrome Fertil Steril, 78, (3) 569-576
8. S. Jahanfar, J.A. Eden, P. Warren, M. Seppala and T.V. Nguyen (1995), A twin study of polycystic ovary syndrome. Fertil Steril 63, 478–486
9. S. Tucci, W. Futterweit, E.S. Conception, D.A. Greenberg, R. Villanueva, T.F. Davies et al (2001), Evidence for association of polycystic ovary syndrome in Caucasian women with a marker at the insulin receptor gene locus. J Clin Endocrinol Metab 86, 446–449
10. A. Dunaif, J. Xia, C.B. Book, E. Schenker and Z. Tang 1995. Excessive insulin receptor serine phosphorylation in cultured fibroblasts and in skeletal muscle. A potential mechanism for insulin resistance in the polycystic ovary syndrome. J Clin Invest 96. 801–810
11. Wood, J.R., Nelson, V.L., Ho, C., Jansen, E., et al. (2003) The molecular phenotype of polycystic ovary syndrome (PCOS) theca cells and new candidate PCOS genes defined by microarray analysis. J Biol Chem, 278, 26380-90.
12. Wu XK, Zhou SY, Liu JX, Pollanen P, Sallinen K, Makinen M, Erkkola R (2003). Selective ovary resistance to insulin signalling in women with polycystic ovary syndrome. Fertil Steril.80(4):954-65.
13. Gambineri A, Pagotto U, Tschop M, Vicennati V, Manicardi E, Carcello A, Cacciari M, De Iasio R, Pasquali R (2003). Anti-androgen treatment increases circulating ghrelin levels in obese women with polycystic ovary syndrome. J Endocrinol Invest. 26(7):629-34
14. Orio F Jr, Lucidi P, Palomba S, Tauchmanova L, Cascella T, Russo T, Zullo F, Colao A, Lombardi G, De Feo P (2003).Circulating ghrelin concentrations in the polycystic ovary syndrome.J Clin Endocrinol Metab. Feb;88(2):942-5.
15. Pagotto U, Gambineri A, Vicennati V, Heiman ML, Tschop M, Pasquali R (2002).Plasma ghrelin, obesity, and the polycystic ovary syndrome: correlation with insulin resistance and androgen levels. J Clin Endocrinol Metab;87(12):5625-9.
16. Schofl C, Horn R, Schill T, Schlosser HW, Muller MJ, Brabant G (2002)..Circulating ghrelin levels in patients with polycystic ovary syndrome.
J Clin Endocrinol Metab.;87(10):4607-10
17. Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group (2003). Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. Fertil Steril.81(1):19-25.

NHS
National Research Ethics Service
NRES Committee East Midlands - Nottingham 2
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Tel: 0115 8839368
Fax: 0115 9123300

27 April 2011

Mr William Atiomo
Clinical Associate Professor and Consultant Gynaecologist Division of Human Development
Queens Medical Centre Campus Nottingham University Hospitals
University of Nottingham D Floor
East Block, Queens Medical Centre C
Nottingham University Hospitals
NG7 2UH

Dear Mr Atiomo

Study title: **Biochemical and Ultrasound studies to improve diagnosis and management of Polycystic Ovarian Syndrome**
REC reference: **10/H0408/69**
Amendment number: **1**
Amendment date: **06 April 2011**

The above amendment was reviewed at the meeting of the Sub-Committee held on 18 April 2011.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Poster	1.0	30 June 2010
Protocol	2.0	30 April 2011
Notice of Substantial Amendment (non-CTIMPs)		06 April 2011

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval