Novel interventional therapies to improve cardiovascular risk in women with polycystic ovary syndrome

Zeeshan Javed

M.B.B.S., MD-USA

Dip. Endocrinology & Diabetes

A thesis submitted for the degree of Doctor of Philosophy

The University of Hull and The University of York

Hull York Medical School

April 2018

Abstract:

Introduction:

Polycystic ovary syndrome (PCOS) is associated with a diverse range of endocrine, metabolic and cardiovascular risk factors. Low vitamin D in PCOS is associated with multiple cardiovascular risk factors and vitamin D replacement therapy has been suggested as a promising alternative for the prevention and treatment of PCOS. Empagliflozin; a sodium-glucose cotransporter 2 (SGLT2) inhibitor, has been shown to have several favourable effects on inflammatory and cardiovascular risk factors in both type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) patients and could potentially be a treatment option in women with PCOS.

Methods:

The first study was a randomized, double-blind, placebo-controlled trial with an objective to determine the effect of vitamin D supplementation on liver fibrosis markers. Forty overweight and obese women with PCOS were randomization to either vitamin D 3200 IU daily or placebo for 3 months. The second trial was a randomised open-label parallel study to look at the effects of empagliflozin versus metformin on hormonal, metabolic and cardiovascular risk factors in women with PCOS. Forty overweight and obese women with PCOS were randomization to either empagliflozin 25mg or metformin 1500mg daily for 3 months.

Results:

For vitamin D treatment, there were significant reductions in individual liver fibrosis markers [hyaluronic acid (HA), N-terminal pro-peptide type-III pro-collagen (PIIINP), tissue inhibitor of metalloproteinase-1 (TIMP-1)] and their cumulative enhanced liver fibrosis (ELF) score was associated with a significant improvement in alanine aminotransferase (ALT) levels in patients randomized to vitamin D, whereas there were no changes in any of these parameters in the placebo group. There were no changes in free androgen index (FAI), insulin resistance

(Homeostasis model assessment-insulin resistance; HOMA-IR), other anthropometric, inflammatory and body composition parameters in either group. There were no significant changes in endothelial microparticles (EMPs) in the vitamin D group as compared to the placebo group. In the second study, there were significant reductions in anthropometric and body composition parameters in patients randomized to empagliflozin while patients on metformin had significant increases in these parameters as compared to baseline. Between groups comparisons at the end of the study showed that the percentage reductions in anthropometric and body composition parameters were statistically significant in the empagliflozin group as compared to the metformin group. There was significant reduction in total testosterone levels in the metformin group only but not in the empagliflozin group. There were no significant changes in FAI, HOMA-IR, reactive hyperaemic index (RHI), fasting lipids and other hormonal and metabolic markers in both the groups. However, there were significant increases in cluster of differentiation 54 (CD54) and cluster of differentiation 62 (CD62) microparticles in the empagliflozin group and cluster of differentiation (CD106) microparticles in both the empagliflozin and metformin groups.

Conclusions:

Direct beneficial effects of vitamin D supplementation on markers of hepatic fibrosis were seen in overweight and obese women with PCOS shown by a reduction in the ELF score and its constituent liver fibrosis markers (HA, PIIINP, TIMP-1). However, vitamin D supplementation did not improve endothelial function as suggested by no significant changes in EMPs in the vitamin D group as compared to the placebo group. In the second trial, empagliflozin improved anthropometric and body composition indices after three months of treatment. However, there was significant increase in CD54 and CD62 microparticles after empagliflozin and CD106 microparticles after both empagliflozin and metformin treatments suggesting that the effects of empagliflozin and metformin could be partly mediated through modulation of endothelial function. This research work suggests both vitamin D and SGLT2

inhibition (empagliflozin) as potential treatment options in women with PCOS for improving future cardiovascular risk.

List of publications and posters from this research work:

Publications:

Javed Z, Kilpatrick ES, Mann V, Corless L, Abouda G, Rigby AS, Atkin SL, Sathyapalan T. *A randomised controlled trial of vitamin D treatment on markers of liver fibrosis in women with polycystic ovary syndrome.* Submitted to Scientific Reports (Under review), 2018

Conference presentation:

1. Javed Z, Kilpatrick ES, Mann V, Corless L, Abouda G, Rigby AS, Atkin SL, Sathyapalan T; *A randomised controlled trial of vitamin D treatment on markers of liver fibrosis in women with polycystic ovary syndrome*
   Presented at Society for Endocrinology BES 2017, Birmingham, UK
   Endocrine Abstracts **(P323)**

2. Javed Z, Kilpatrick ES, Mann V, Corless L, Abouda G, Rigby AS, Atkin SL, Sathyapalan T; A randomised controlled trial of vitamin D treatment on markers of liver fibrosis in women with polycystic ovary syndrome
   Oral presentation – ENDO 2018 – Chicago USA

Abbreviations:

25OHD	25 hydroxyvitamin D
AGEs	Advanced glycation end-products
AI	Augmentation index
AIRg	Acute insulin response to glucose
Akt/PKB	Protein kinase B
ALT	Alanine aminotransferase
AMH	Anti-mullerian hormone
AST	Aspartate aminotransferase
ATGL	Adipocyte triglyceride lipase
BMI	Body mass index
BMR	basal metabolic rate
cAMP	Cyclic adenosine 3', 5'- monophosphates
CD32	Cluster of differentiation 32
CD54	Cluster of differentiation 54
CD62	Cluster of differentiation 62
CD105	Cluster of differentiation 105
CD106	Cluster of differentiation 106
CD142	Cluster of differentiation 142
CD144	Cluster of differentiation 144
CETP	Cholesterylester transfer protein
cIMT	Carotid intima-media thickness
CRP	C-reactive protein
CTIMP	Clinical trial of an investigational medicinal product

CT-scan	Computed tomography
CV	Cardiovascular
DBP	Diastolic blood pressure
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DHT	Dihydrotestosterone
DKA	Diabetic ketoacidosis
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
ELF	Enhanced liver fibrosis
EMPs	Endothelial microparticles
ENG	Endoglin
FAI	Free androgen index
FCS-H	Forward scatter-Height
FDA	Food and Drug Administration
FFA	Free fatty acids
FFM	Fat free mass
FIB-4	Fibrosis-4 index
FLI	Fatty liver index
FMD	Flow mediated dilatation
FSC	Forward scattered light
FSH	Follicle stimulating hormone
GDM	Gestational diabetes mellitus

GLP-1 analogue	Glucagon-like peptide-1 analogue
GLUT4	Glucose transporter 4
GnRH	Gonadotrophin releasing hormone
GS	Glycogen synthase
GSK3	Glycogen synthase kinase3
GWAS	Genome-wide association studies
HA	Hyaluronic acid
HAn	Hyperandrogenism
HC	Hip circumference
HDL	High density lipoprotein
HEC	Hyperinsulinaemic euglycaemic clamp
HOMA-IR	Homeostasis model assessment-insulin resistance
HSCs	Hepatic stellate cells
HSL	Hormone sensitive lipase
HUVECs	Human umbilical vein endothelial cells
ICAM-1	Inter cellular adhesion molecules 1
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IRS-1	Insulin-receptor substrate-1
ISI	Insulin sensitivity index
IVF	In Vitro fertilisation
IVGTT	Intravenous glucose tolerance test
LDL	Low density lipoprotein
LFA-1	Leukocyte function associated antigen-1

LH	Luteinizing hormone
Lp(a)	Lipoprotein(a)
LPL	Lipoprotein lipase
MCAM	Melanoma Cell Adhesion Molecule
MDA	Malondialdehyde
MHRA	Medicines and healthcare products regulatory authority
MI	Myocardial infarction
MPs	Microparticles
MRI-scan	Magnetic resonance imaging
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NHANES	National Health and Nutrition Examination Study
NIH	National Institutes of Health
NO	Nitric oxide
OD	Ovarian dysfunction
OGTT	Oral glucose tolerance test
OSA	Obstructive sleep apnoea
P13K	Phosphatidylinositol 3-kinase
PAI-1	Plasminogen activator inhibitor-1
PAT	Peripheral Arterial Tone
PBS	Phosphate buffered saline
PCOM	Polycystic ovarian morphology
PCOS	Polycystic ovary syndrome
PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1

PET	Positron emission tomography
PFP	Platelet free plasma
PIIINP	N-terminal pro-peptide type-III pro-collagen (PIIINP)
PTH	Parathyroid hormone
QUICKI	Quick insulin sensitivity check index
RCT	Randomized controlled trial
RCTs	Randomized controlled trials
REC	Research ethics committee
RHI	Reactive hyperaemia index
SBP	Systolic blood pressure
SGLT2	Sodium-glucose cotransporter 2
SHBG	Sex hormone binding globulin
SI	Separation index
SSC	Side scattered light
SSC-H	Side scatter-Height
T2DM	Type 2 diabetes mellitus
TBW	total body water
TC	Total cholesterol
TF	Tissue factor
TG	Triglycerides
TIMP-1	Tissue inhibitor of metalloproteinase-1
uPAR	Urokinase receptor
US	Ultrasound
VCAM-1	Vascular cell adhesion molecule 1

VDD	Vitamin D deficiency
VDR	Vitamin D receptor
VDRs	Vitamin D receptors
VE-cadherin	Vascular Endothelial Cadherin
VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoprotein
WC	Waist circumference
WHO	World Health Organization

Acknowledgements:

First and foremost, I would like to express my most sincere gratitude to my supervisor, Professor Thozhukat Sathyapalan. This work would have not been completed without his excellent leadership, guidance, understanding, trust and encouragement. I sincerely thank Professor Stephen L. Atkin and Professor Eric S. Kilpatrick for their intellectual and insightful input into every part of this work and kind support throughout the journey.

I am grateful to my co-supervisor Dr Leigh Madden for inspiration and guidance acquired regarding flow cytometry and evaluating endothelial microparticles.

I should also like to extend my thanks and gratitude to my colleagues; Maria Papageorgiou and Dr Ahmed Al-Qaissi for their patience during the challenging patient recruitment and especially for their skills, knowledge and support.

I thank Professor Alan S Rigby for the statistical support.

My thanks also go to the staff at the centre for Diabetes, Endocrinology and Metabolism Research. In particular, I would like to thank, Gill Byne, Lucy Newton, Anneli James, Carrie Willby, Dawn Lowsley, Dana Schofield, Donna Jacklin, Zoe Sugdon, Lee Rollins, Nalumon Parnwell and finally Lisa Baldwin for their assistance and guidance all the way.

I am deeply indebted to all of the participants whose trust and willingness made this work possible.

Finally, and most especially, I would like to thank my wife, Dr Unaiza Qamar, for her continuous support and care, also essentially my father, mother and rest of my family for their prayers and encouragement, without which, this work would not have been completed.

Author's Declaration:

I confirm that this work is original and that any diagram(s) or passage(s) have been copied from academic books, papers, the internet or any other sources are clearly identified by the use of references cited and quotation marks. I certify that other than where indicated this is my work and does not breach the regulation of HYMS, the university of Hull or University of York regarding plagiarism or academic conduct in examinations. I have read the HYMS Code of Practice on Academic Misconduct, and state that this piece of work is my own and does not contain any unacknowledged work from other resources. I can confirm that any information obtained regarding participants in both studies has been appropriately anonymised.

Table of Contents:

List of Figures:

List of Tables:

1 Chapter 1: Introduction:

1.1 Polycystic ovary syndrome:

1.1.1 Background:

Stein and Leventhal, in 1935, published a case series of seven women with varying degrees of cystic ovaries, amenorrhea and hirsutism and were the first to provide the description of polycystic ovary syndrome (PCOS) (1), though the findings of polycystic ovaries (sclerocystic ovaries or cystic oophoritis) had already been suggested at least a century before their interpretation (2-5).

PCOS is now acknowledged as the most common endocrine disorder of women in their reproductive age (4). PCOS is composed of several key features, as characterized by physiologic studies (6), such as enhanced gonadotrophin releasing hormone (GnRH) pulse frequency and quantity (7), high androgen levels (8), high LH:FSH (Luteinizing hormone : Follicle stimulating hormone) ratio (8, 9), insulin resistance and elevated insulin levels (10, 11), elevated anti-mullerian hormone (AMH) levels (12) and polycystic ovarian morphology (1, 13). Additionally, PCOS women who had polycystic ovarian morphology were consistently found to have elevated androgen levels and oligomenorrhea. Interestingly, insulin and androgen levels were high in these women with polycystic ovarian morphology even when their menstrual cycles were regular (14-16). However, the definition of PCOS has been subjected to several revisions due to the advancement in ability to measure hormone concentrations, development of ultrasonography and awareness of the role of insulin resistance and metabolic abnormalities associated with it (4, 13, 17). Further clinical, biochemical and endocrinological studies have revealed a range of underlying abnormalities in patients having PCOS, and ovarian morphology is no longer an essential component for its diagnosis (4).

1.1.2 Defining PCOS:

In 1990, an international meeting was held at United States National Institutes of Health (NIH) (18), where a group of investigators recommended that diagnostic criteria of PCOS should include oligo-anovulation associated with hyperandrogenism (clinical and/or biochemical); excluding other endocrinopathies which include hyperprolactinaemia, thyroid dysfunction, Cushing's syndrome, non-classical adrenal hyperplasia, drug-induced excess androgen and androgen-producing tumours as well as pregnancy (19). However, in 2003, European and American investigators attended a meeting at Rotterdam (Rotterdam Consensus) and suggested that PCOS diagnosis should include at least two of the following features: 1) oligo-anovulation, 2) hyperandrogenism (clinical and/or biochemical), 3) Polycystic ovaries excluding other endocrinopathies as mentioned above (19, 20). In 2006, androgen-excess society for PCOS (AE-PCOS) proposed a compromise approach and argued that hyperandrogenism should be an integral part of PCOS diagnosis as it is an androgen excess disorder. The other essential criterion could be either oligo-anovulation or polycystic ovary morphology (4). Nevertheless, there was a general consensus that PCOS diagnosis must be established on the presence of at least two of the following:

1) Chronic anovulation
2) Hyperandrogenism (clinical and/or biochemical)
3) Polycystic ovarian morphology

Table 1.1 explains and compares the diagnostic criteria for PCOS.

Table 1.1: Diagnostic criteria for PCOS

NIH/NICHD	**Rotterdam Criteria 2003 (ASRM/ESHRE)**	**Androgen Excess PCOS Society 2006**
Includes all of the following: • Clinical or/and biochemical hyperandrogenism • Menstrual dysfunction	Includes 2 of the following: • Anovulation or oligo-ovulation • Clinical or/and biochemical hyperandrogenism • Polycystic ovaries	Includes all of the following: • Clinical or/and biochemical hyperandrogenism • Polycystic ovaries and/or ovarian dysfunction
Abbreviations: NIH/NICHD (National Institutes of Health/National Institute of Child health and Human disease); ASRM/ESHRE (American Society for Reproductive Medicine/European Society for Human Reproduction and Embryology Adapted from Endocrine Practice (21)		

In a recent meeting, NIH research experts have revised the criteria and have suggested use of broader ASRM/ESHRE 2003 diagnostic criteria but, in addition, included the detailed description of PCOS phenotype (22). They have classified PCOS patients into four phenotypes as summarized below in table 2.

Table 1.2: PCOS phenotypes

Phenotype A	HAn + OD + PCOM
Phenotype B	HAn + OD
Phenotype C	HAn + PCOM
Phenotype D	OD + PCOM
Abbreviations: HAn (Hyperandrogenism), OD (Ovarian dysfunction), PCOM (Polycystic ovarian morphology) Adapted from Lizneva et. al. (23)	

It has been suggested that dividing PCOS patients phenotypically has several practical applications, for instance helping to distinguish PCOS patients who are at high risk of metabolic dysfunction (phenotypes A and B). Secondly, it will be helpful for researchers to categorize their study outcomes to specific PCOS phenotypes while conducting clinical trials, which would be helpful in better understanding outcome variability in trials in the same population (24).

Several PCOS suggestive features like multi-follicular ovaries and menstrual irregularities are very common with normal pubertal transition to adulthood, and there is still no general consensus about defining PCOS in adolescents (25-27). Therefore, clinicians and researchers should be very careful about making a diagnosis of PCOS in this group and categorising them into different phenotypes. Recently, the Endocrine society (28) and ESHRE/ASRM (29) have published two sets of guidelines to define PCOS in adolescents. They suggested that PCOS should be diagnosed on the basis of hyperandrogenism (clinical or biochemical) associated with primary amenorrhea by age 16 years and/or persistent oligo-amenorrhea for at least 2 years after menarche and/or polycystic ovarian morphology (ovarian volume >10 cm^3) after exclusion of secondary causes. Similarly, the diagnosis of PCOS could be challenging in peri-menopausal and postmenopausal women due to age related changes as most of them

regain menstrual regularity; display normal serum androgen levels along with decrease in ovarian volume and number of follicles which could ameliorate PCOS clinical presentation (30-33). The Endocrine society guidelines (28) have suggested that these patients could be diagnosed on the basis of previous medical history of menstrual irregularities plus high androgen levels during the reproductive period as polycystic ovarian morphology is less likely to be found in peri/postmenopausal women owing to age related changes, however, it can be considered as a supportive sign.

Although obesity and insulin resistance are very common in patients having PCOS (34) and play a vital role in the pathogenesis of excessive androgen production and enhancing susceptibility to development of glucose intolerance and type 2 diabetes (T2DM), they are not part of the diagnostic criteria (35).

1.1.3 Pathophysiology and aetiology of PCOS:

The fundamental underlying pathophysiological defect in PCOS is still not fully understood, and these patients have various interrelated features such as hyperandrogenism, insulin resistance and altered gonadotrophin dynamics (36). The most consistent feature of PCOS is persistently high frequency GnRH pulsatile release and disordered gonadotrophin secretion associated with raised LH and low or normal FSH levels (19, 37). The current understanding of PCOS involves multiple factors including genetic, environmental, foetal and metabolic have been implicated in its pathophysiology (38, 39) and several theories have been suggested to explain it. Figure1.1. (Adapted from Dumesic et al. (19))

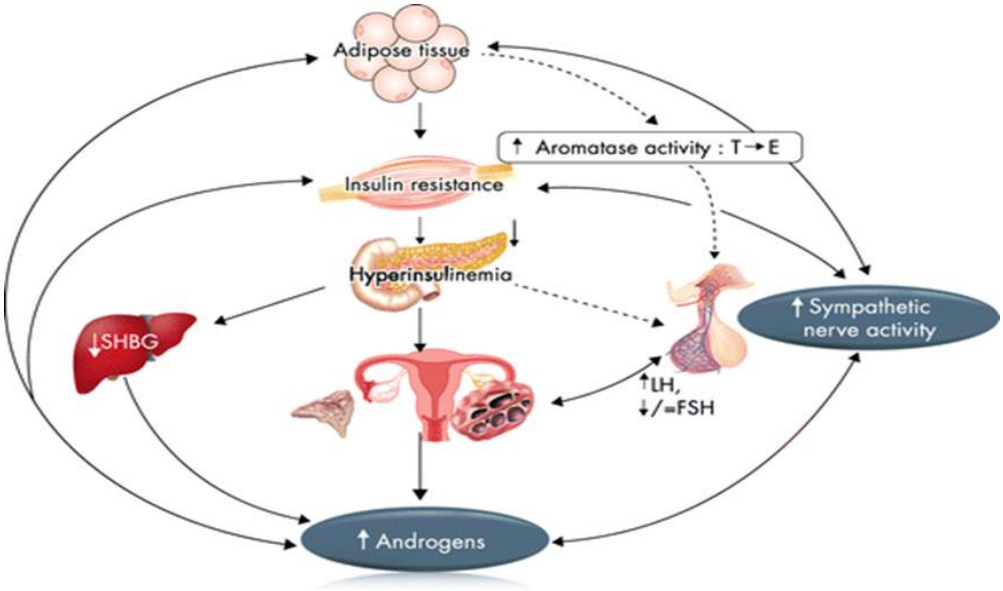

Figure 1.1: PCOS pathophysiology

There is an intrinsic defect in ovarian androgen production due to neuroendocrine defects resulting in enhanced amplitude and pulse frequency of LH with relatively low FSH and insulin resistance associated with compensatory hyperinsulinaemia. There is also excessive adrenal androgen secretion due to altered cortisol metabolism.

Abbreviations: SHBG, Sex hormone binding globulin; LH, Luteinizing hormone; FSH, Follicle stimulating hormone; ↑, increase; ↓, decrease; T, Testosterone; E, Estradiol.

In order to establish the genetic basis of PCOS, the initial evidence came from the familial aggregation study done by Cooper et al. in 1968 (40, 41). The studies on the cultured cells from PCOS patients have revealed that the defects in insulin sensitivity in their skin fibroblasts and augmented androgen secretion from their theca cells persevered even after over several passages (42). The genes in PCOS related to metabolism, obesity, insulin resistance, beta cell dysfunction, ovarian androgen production and folliculogenesis have been studied using a candidate approach technique (42, 43). Family based studies suggested an autosomal dominant inheritance pattern with a high prevalence of PCOS related traits in siblings (44). The siblings of PCOS-probands were suggested to more likely be affected with

symptoms and signs of PCOS (menstrual irregularities and hirsutism in PCOS females and male pattern baldness in male family members), with higher rates of metabolic abnormalities in first-degree relatives especially insulin resistance, beta cell dysfunction, dyslipidaemia and metabolic syndrome (19). Later, it was suggested that a single gene might be responsible for the high prevalence of signs and symptoms in first-degree relatives of PCOS patients (45). In addition, family members (sisters and mothers) of women with PCOS were found to have a higher prevalence of insulin resistance, hyperandrogenism and metabolic syndrome as compared to the family members of women who did not have PCOS (age and BMI matched), suggesting genetic susceptibility to these defects (46-49). The evidence was further supported by twin studies, where PCOS heritability was assessed in mono and dizygotic twin pairs and it was proposed that 72% of the variance in PCOS risk has a genetic basis (50, 51). In order to establish PCOS risk genes, researchers initially used a candidate gene analysis technique, which although it pointed out several promising genes related to PCOS susceptibility, not one gene has been successfully identified as causative for PCOS across all studies (19, 52). Some of the genes identified and replicated by this technique in family based and case control studies were fibrillin-3 gene (53-55), insulin-receptor gene, insulin-receptor substrate-1 (IRS-1) (56), the fat mass and obesity-associated gene (FTO) (57-59), calpain-10, FSH receptor and SHBG gene (52, 60). The application of this technique has been found to be unsuccessful in complex diseases, with reasons cited for lack of replication of these genes being incomplete candidate gene coverage and small sample size, which could result in false negative or false positive findings. It has also been suggested that the technique lacks the capability of finding novel genes or rare variant genes, and can only be useful if there is an a priori hypothesis about a gene's involvement in the disease aetiology (19, 52).

Genome-wide association studies (GWAS) have been very successful in establishing strong associations between PCOS and several genes. The first GWAS reported for PCOS was

published by Chinese researchers who identified candidates within three loci that were significantly associated with PCOS: LH receptor, FSH receptor and DENND1A genes on chromosome 2 and thyroid adenoma associated (THADA) gene on chromosome 9 (61). Only a few of them, notably DENND1A and THADA, have been replicated in European studies (62, 63). Further, GWAS has led to the discovery of almost 16 loci having significant association with PCOS pathophysiology. The loci identified have genes with distinct roles either in metabolic or reproductive dysfunction, as is found in PCOS patients (40). These findings promise better understanding of PCOS pathophysiology with future incorporation of genetic testing in early disease prediction, diagnosis and treatment.

1.1.4 Prevalence of PCOS:

PCOS has been recognized as one of the most prevalent endocrine/metabolic disorders. The first evidence related to PCOS prevalence ranging from 4% - 6.6% came from two studies (64, 65) done in USA using NIH 1990 criteria. Subsequently, several epidemiological studies reported PCOS prevalence in various populations using multiple diagnostic criteria and worldwide prevalence of PCOS has been reported between 4% and 21% (66-78). The prevalence of PCOS reported in these studies partly depends on the diagnostic criteria used. For example, the prevalence ranges between 5-10% according to NIH 1990 criterion, 10-15% according to AE-PCOS 2006 criterion and 6-21% according to ASRM/ESHRE 2003 criterion (23). The higher prevalence of PCOS by using either Rotterdam 2003 or AE-PCOS 2006 criteria as compared to NIH 1990 criterion is mainly attributed to their wider definition and recruitment of additional phenotypes of PCOS. But overall the use of NIH 1990 criterion is associated with diminished variability in PCOS prevalence across countries (23).

1.2 Cardiovascular risk factors in PCOS:

There is increasing evidence to suggest that PCOS is associated with high cardiovascular (CV) risk as compared to age matched controls. Studies have shown more extensive coronary

artery disease and seven times higher risk of myocardial infarction (MI) in these patients as compared to healthy controls (79, 80). There are several underlying cardiovascular risk factors associated with PCOS patients as is discussed below.

1.2.1 Clinical risk factors:

1.2.1.1 Obesity and metabolic syndrome:

The features of metabolic syndrome, which is a constellation of several cardiovascular risk factors especially central obesity, dyslipidaemia, impaired fasting glucose and elevated blood pressure, are highly prevalent in women with PCOS (65, 81). The relationship between obesity and PCOS is still not well understood, but obesity is a common finding in patients with PCOS and it is suggested to worsen many of its metabolic and reproductive features (82). Between 40-85% of PCOS patients are either overweight or obese compared to healthy age-matched controls with a greater waist-hip ratio (83). Furthermore, studies have shown that 50-70% of PCOS patients are associated with an android fat distribution, regardless of BMI, which in turn contributes to the increased prevalence of metabolic abnormalities, especially impaired glucose tolerance, insulin resistance, type 2 diabetes, hypertension, dyslipidaemia and abnormal platelet activity (83, 84). Interestingly, insulin has been acknowledged as a central link among these coexisting abnormalities which, along with visceral obesity, affects both obese and lean women with PCOS, putting them at high risk of developing cardiometabolic abnormalities independent of obesity (81, 85). Weight loss is crucial for overweight/obese PCOS women given the fact that obesity substantially increases the severity of clinical features related to PCOS (Table 3). Studies have shown that even 5-10% weight loss improves symptoms and endocrine profiles in PCOS (86).

Table 1.3: Deleterious effects of obesity in women with PCOS

Androgenic features	Metabolic features	Reproductive features & outcomes
Increased severity of: • Androgen secretion • Acne • Hirsutism Reduced SHBG levels	• ↑ Insulin resistance & hyperinsulinaemia • ↑ Type 2 diabetes • ↑ Dyslipidaemia	• ↑ Menstrual irregularities • ↑ Subfertility • ↑ Gestational diabetes & pre-eclampsia • ↑ Preterm birth & birth-weight • ↑ chances of caesarean sections
Adapted from: (87, 88)		

1.2.1.2 Hypertension:

Systemic arterial hypertension, generally, has been shown to be very common in patients with PCOS. Several studies have suggested up to 40% prevalence of hypertension in PCOS and have proposed insulin resistance as the main underlying potential determinant of such a high prevalence (89). Studies have also demonstrated an inverse relationship between systolic arterial hypertension and insulin sensitivity in PCOS patients. Though, it remains controversial whether hypertension is associated with PCOS independent of obesity; evidence suggests that early detection and subsequent treatment of hypertension in women with PCOS decreases future risk of having cardiovascular disease (90).

One of the mechanisms suggested is hyperinsulinaemia associated with insulin resistance stimulates ovaries to produce excessive androgens, which may directly regulate the renin-angiotensin system and thus increase extracellular volume and blood pressure (89, 91, 92). However, obesity is a significant risk factor for hypertension, common in women with PCOS, which has not been accounted for in many studies and whether the BMI has been adjusted for in the data is not always transparent (90, 93). Similarly, it is very important to analyse the data critically, as in several studies that have demonstrated a high prevalence of hypertension in women with PCOS, these women were not matched in terms of race and ethnicity, or the

studies were done in post-menopausal women who had undergone ovarian wedge resection that may also have altered the results (90).

1.2.1.3 Obstructive sleep apnoea/Sleep-disordered breathing:

Obstructive sleep apnoea (OSA) has been suggested to have a significantly higher prevalence in adult women with PCOS compared to women without the disorder and therefore screening for OSA has been recommended in PCOS patients (94). Although, the underlying pathophysiological mechanism for such a high prevalence of OSA in PCOS has not been well established, three features of PCOS, including obesity, insulin resistance and hyperandrogenism, have been suggested to contribute to this effect (28). There is no causal relationship between insulin resistance and OSA; however, several trials have established an association between insulin resistance and OSA (95). In a cohort study, OSA, apnoea/hypopnea index, oxygen desaturation index and minimal oxygen saturations were independently associated with insulin resistance over an 11-year follow-up period even after adjustment for age, baseline BMI and hypertension (96). Further, studies in healthy lean men established OSA to be associated with insulin resistance even in the absence of obesity (97).

However, the high prevalence of OSA in PCOS could not be explained fully on the basis of above mentioned three factors only. As, in some studies sleep apnoea did not correlate with BMI and women with PCOS were found to have 9 times higher occurrence of day time sleepiness and 30 times higher prevalence of sleep-disordered breathing as compared to controls even after adjusting for BMI (28). Similarly, several other studies in women with PCOS had found to have a significantly higher prevalence of sleep related disorders compared to their BMI-matched controls (98, 99). Lower estradiol levels have been reported to be associated with poor sleep quality among post-menopausal women and progesterone is thought to promote direct stimulation of respiratory drive to both hypercapnea and hypoxia (100) and therefore, reduce airway resistance by increasing upper airway dilator

muscle activity (101). As women with PCOS have low circulating progesterone concentrations which may contribute to the high prevalence of OSA. Furthermore, women with PCOS taking oral contraceptives had been shown to have less sleep-disordered breathing (102). Likewise, hormone replacement therapy was associated with a lower likelihood of sleep disordered breathing among postmenopausal women (103). Lastly, the mean apnoea-hypopnea index was significantly higher in PCOS women (22.5 ± 6.0 vs. 6.7 ± 1.7; $P<0.01$) as compared to weight-matched controls that is not completely explained by presence of obesity only suggesting the interplay of multiple other factors in addition to obesity (103).

1.2.2 Biochemical risk factors:

1.2.2.1 Hyperandrogenism:

It has been shown that women with PCOS have elevated cardiovascular risk despite their young age and one of the underlying factors might be the excessive production of androgens in these patients (104). Recent studies have demonstrated that hyperandrogenism in PCOS women is associated with atherosclerosis and increased cardiovascular risk as compared to controls (105). Biochemical hyperandrogenism in women is determined when total testosterone or free androgen levels are greater than the upper limit of the normal range. Clinical hyperandrogenism (in the absence of biochemical hyperandrogenaemia) is determined with the combination of hirsutism, excessive male-type hair growth, acne or androgenic alopecia. Hirsutism is assessed by using the Ferriman and Gallwey scoring system, where a score of ≥ 8 indicates hirsutism (106). Almost 60% of women diagnosed with PCOS have been found to have hirsutism.

Hyperandrogenism in women with PCOS results from both the ovaries and adrenal glands. Furthermore, low circulating levels of sex hormone binding globulin (SHBG) in these patients increase, and thereby worsen, hyperandrogenaemia, which is exacerbated in overweight and obese women due to obesity-induced hyperinsulinaemia, which further inhibits SHBG production (87). The chief circulating androgens in women are

dehydroepiandrosterone sulphate (DHEAS), dehydroepiandrosterone (DHEA), androstenedione, testosterone and dihydrotestosterone (DHT) in descending order of their serum concentration. Most of the androgenic effects are induced by testosterone and its biologically active metabolite DHT, as the first three androgens require conversion to testosterone and DHT to induce their androgenic effects (pro-androgens). Only 1-2% of testosterone in the circulation is unbound and thus biologically active as the majority of androgens are bound to carrier proteins predominantly SHBG and albumin (107). Therefore, measurement of the free androgen index (FAI) (calculated by Total Testosterone/SHBG × 100) is of importance when determining androgenic potential and effects. The FAI calculation is considered to be a more reliable measurement for androgen excess due to significant technical difficulties in measuring free testosterone levels (108).

1.2.2.2 Insulin resistance and impaired glucose metabolism in PCOS:

Insulin is secreted by pancreatic β-cells after a meal in response to increased serum levels of glucose and amino acids. Insulin helps the body in maintaining glucose homeostasis and in promoting effective glucose utilisation (109). Insulin is known to be a potent anabolic hormone that stimulates glucose oxidation, inhibits lipid-oxidation, and augments lipogenesis and glycogenesis. Insulin exerts its action after binding to its specific insulin receptor, which are present in almost every cell of the body, but their concentration varies in different cells, with the highest density being on adipocytes and hepatocytes (110). The insulin receptor is a transmembrane receptor that belongs to the class of tyrosine kinase receptors. It is comprised of two extracellular α-subunits that are each connected to transmembrane β-subunits by disulphide bonds. The alpha subunits have the insulin binding sites whereas beta subunits possess the tyrosine kinase activity (Figure 1.2 Adapted from (111, 112)). The gene for the insulin receptor is located on the short arm of chromosome 19 in humans (113).

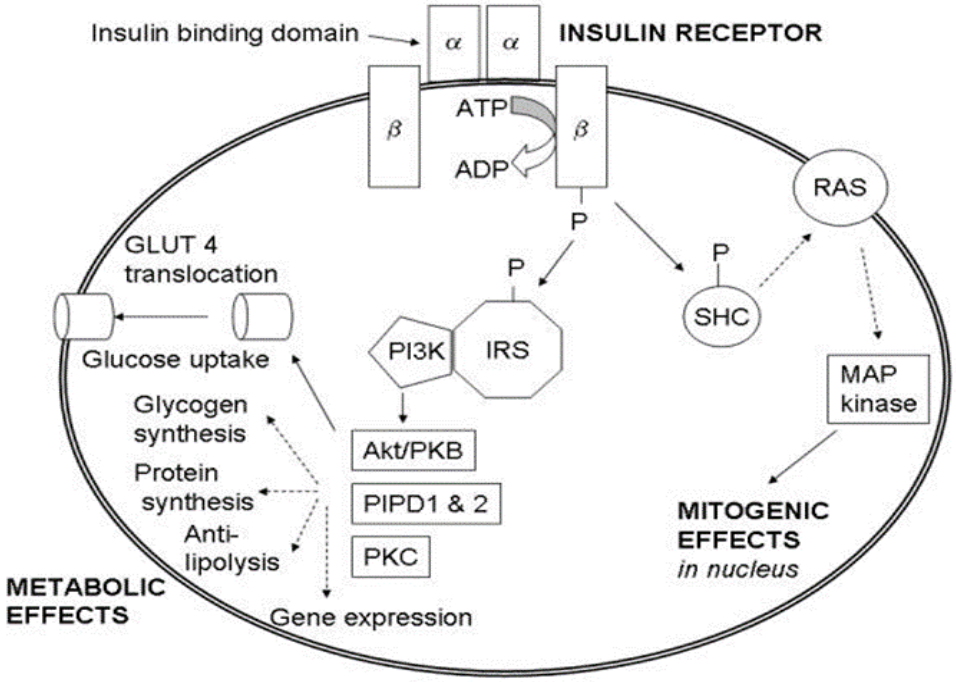

Figure 1.2: Insulin signalling pathways

Legend: Abbreviations: Akt/PKB, protein kinase B; ATP, adenosine triphosphate; IR, insulin receptor substrate; ADP, adenosine diphosphate; MAP kinase, mitogen activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PIPD1 & 2, phosphatidylinositol dependent protein kinases 1 & 2 glucose-dependent insulinotropic polypeptide; PKC, protein kinase C; RAS, rat sarcoma protein; GLUT 4, glucose transport protein 4; SHC, adaptor protein with src-homology.

After the insulin is released from pancreatic beta cells, it binds to cell surface insulin receptors and initiates a complex series of intracellular events that lead to enhanced glucose uptake, glycogen production, and fat storage (114). Tyrosine kinase mediates the insulin response through phosphorylation of tyrosine residues on intracellular endogenous target proteins such as insulin receptor substrates (IRS) 1 and 2. This leads to activation of targeted internal proteins, including PI3K (phosphatidylinositol 3-kinase). PI3K then stimulates downstream protein kinases, particularly protein kinase B (Akt/PKB), which ultimately leads

to increased translocation of glucose transporter 4 (GLUT4) molecules to the cell membrane and subsequently enhanced insulin-mediated glucose uptake into cells. The same cascade also leads to reduced lipolysis of fat cells by insulin mediated inhibition of hormone sensitive lipase (HSL) via intracellular reduction in cyclic adenosine 3', 5'- monophosphates (cAMP). It also results in increased glycogen synthesis by deactivating glycogen synthase kinase3 (GSK3), which in turn fails to deactivate glycogen synthase (GS). In addition, insulin suppresses genes involved in fatty acid synthesis, induces those involved in lipogenesis and exerts an effect on adipocyte differentiation (115).

1.2.2.2.1 Insulin sensitivity & glucose tolerance:

Insulin encourages the uptake, metabolism and storage of glucose by acting on the cells throughout the body. Insulin has variable effects on glucose metabolism that depend upon the target tissue. In the liver, insulin promotes storage of glycogen and decreases glucose production by inhibiting gluconeogenesis and glycogenolysis. In adipose and muscle tissue, insulin enhances the uptake, storage and utilization of glucose. Insulin also decreases the circulating levels of free fatty acids by inhibiting lipolysis in the muscle and adipose tissue and by promoting lipid synthesis in the liver and adipose tissue (34, 116). There are certain factors that have independent effects on insulin sensitivity, such as body fat distribution, obesity and muscle mass. Impaired insulin sensitivity (insulin resistance) is defined as a reduced ability of insulin to mediate its metabolic activities on glucose production, uptake and lipolysis that results in an increased requirement for the amount of insulin to achieve euglycaemia (34). This leads to compensatory hyperinsulinaemia due to increased production and secretion of insulin by the pancreas, characteristic of insulin resistance. Although there is compensatory hyperinsulinaemia, glucose levels rise slightly, though stay within the normal range before reaching the diagnostic criteria for impaired glucose intolerance.

Obesity, lack of physical activity and genetic aspects are important risk factors underlying insulin resistance. Moreover, insulin resistance is closely linked with major public health issues, especially obesity, metabolic syndrome, hypertension, dyslipidaemias and cardiovascular disorders. Impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) are intermediate metabolic states between euglycaemia and diabetes, but are known risk factors for future diabetes and cardiovascular disease development (117).

The development of type 2 diabetes originates mainly from insulin resistance emerging in adipose and muscle tissues, resulting in decreased uptake of glucose from blood associated with an initial compensatory increased production of insulin from pancreatic β-cells. Therefore, type 2 diabetes is considered as a metabolic disorder characterized by chronic hyperglycaemia due to defects in insulin secretion or action, or both (118). The World Health Organization (WHO) criteria for diagnosing IFG; IGT and diabetes are shown below in table 1.4.

Table 1.4: WHO criteria 2006 for diagnosing IFG, IGT and diabetes

Diagnosis	Glucose concentration in plasma (mmol/l)
Diabetes mellitus	
Fasting glucose	≥ 7.0
2-hour glucose	≥ 11.1
Impaired glucose tolerance	
Fasting glucose	< 7.0
2-hour glucose	≥ 7.8 and < 11.1
Impaired fasting glycaemia	
Fasting glucose	6.1 to 6.9
2-hour glucose	< 7.8
(World Health Organization 2006)	

1.2.2.2.2 Insulin resistance/sensitivity assessment:

The best technique of quantifying insulin sensitivity in PCOS is still unclear because insulin resistance is influenced by several factors, especially stress, ageing and obesity, and is related to genetic and ethnic variability. The two methods considered to be the gold standards for assessing insulin sensitivity are the hyperinsulinaemic euglycaemic clamp (HEC) and the frequently sampled intravenous glucose tolerance test (IVGTT) (119). In the HEC, the M-value is used to measure insulin sensitivity that is defined as the insulin stimulated glucose disposal rate. The insulin sensitivity index (ISI) is then measured after a defined period (usually 20-30 minutes) of the HEC by dividing the average M-value by average plasma insulin levels. The alternative way of calculating insulin resistance is by calculating the disposition, which is determined by multiplying the acute insulin response to glucose (AIRg) and insulin sensitivity index (120).

Although, these tests are considered as the gold standards for assessing insulin resistance, they are expensive, time consuming and they are labour intensive, which led to the development of a simplified approach for insulin sensitivity quantification. In contrast to these dynamic approaches, insulin resistance was expressed using steady-state calculations of insulin sensitivity such as determining fasting insulin levels, fasting glucose to insulin ratio, the homeostasis model assessment-insulin resistance (HOMA-IR), that is defined as insulin pmol/L x glucose mmol/L /22.5 (121), and a recently established technique called QUICKI (quick insulin sensitivity check index), calculated as 1/(log [insulin]) + log [glucose]) (122). Whilst, HEC is a gold standard technique for determining insulin resistance, a fasting glucose to insulin ratio >4.5 delivered a sensitivity of 95% and specificity of 84% and was recommended as a screening tool for predicting insulin resistance (123).

The HOMA-IR model has proven to be a robust clinical and epidemiological instrument for the insulin resistance calculation. HOMA-IR is also very a helpful assessment tool in situations where only the fasting insulin and glucose values are available, especially in large epidemiological studies. Similarly, QUICKI has been shown to have a considerably better linear correlation with insulin sensitivity assessments by glucose clamp techniques than other minimal-model evaluations, especially in diabetes and obese subjects (122).

1.2.2.2.3 Insulin resistance an independent risk factor in PCOS:

Almost 60-80% of all women with PCOS have some level of insulin resistance that appears to be important in PCOS development and is thought to be an independent risk factor for cardiovascular disease (124, 125). It has been demonstrated that older and obese PCOS women have more severe insulin resistance that has a greater prevalence in those having biochemical hyperandrogenism associated with a family history of diabetes even after matching for age and BMI. The data regarding the role of insulin resistance in non-obese PCOS patients is still conflicting and remains unclear (126).

The underlying pathophysiology of insulin resistance in PCOS is complex and multifactorial and has been suggested to be related to insulin secretion, its action or clearance. The vital feature of PCOS is selective resistance to insulin effects upon glucose metabolism, especially in adipose and muscle tissues. Several studies have reported a post receptor insulin signalling defect between glucose transport and a receptor kinase in the skeletal muscle as the main underlying aetiology for insulin resistance in PCOS, which is independent of BMI, metabolic derangement and hyperandrogenism (127, 128). Furthermore, reduced suppression of hepatic glucose production has been observed in obese PCOS women, and the fact that insulin resistance is clustered in PCOS families suggests the role of both obesity and genetics in influencing insulin resistance in these patients (129).

Studies have also suggested an intrinsic pancreatic beta cell function defect in PCOS women as an independent predictor of insulin resistance and androgen levels. The NHANES III (The National Health and Nutrition Examination Study) demonstrated a considerably stronger relationship between insulin resistance and an intrinsic beta cell defect in PCOS women compared to controls (130). But the data is conflicting, as other studies have shown intact beta cell function and adaptation in all women with PCOS (131, 132). Similarly, the data related to abnormal metabolic insulin clearance in PCOS is controversial, as in one study insulin diminished in hyperinsulinaemic PCOS patients independent of their BMI, but no such change was observed in other studies (133, 134).

1.2.2.2.4 Impaired glucose tolerance and cardiovascular risk in PCOS:

60-80% of PCOS women suffer from insulin resistance associated with compensatory hyperinsulinaemia that plays a vital role in its pathogenesis, independent of obesity (135). Insulin resistance has been acknowledged as a key risk factor for the development of type 2 diabetes and this concept is supported by several clinical trials showing PCOS is linked to a higher prevalence of impaired glucose tolerance, type 2 diabetes and gestational diabetes mellitus (GDM) (35, 136, 137). Moreover, in PCOS women, a positive correlation has been shown between insulin resistance and FAI, C-reactive protein (CRP) and triglyceride levels with a negative correlation to SHBG levels (138).

A recent meta-analysis has shown that in women with PCOS the odd ratios for IGT and type 2 diabetes are considerably enhanced as compared to their BMI matched controls and, therefore, this has suggested that these patients should be screened for underlying glucose intolerance using the oral glucose tolerance test (OGTT) as a screening tool (28). These conditions have been shown to be more severe in overweight or obese PCOS women compared to their BMI matched controls. However, even though the risk of IGT is further augmented by obesity, lean PCOS women have also been shown to have increased rates of

insulin resistance and IGT when compared with their BMI matched healthy controls in several clinical trials (135, 139, 140).

Furthermore, the risk of impaired glucose tolerance and subsequent development of type 2 diabetes and cardiovascular risk have been demonstrated to vary among the different PCOS phenotypes; lowest among the patients who have polycystic ovarian morphology with anovulation but without hyperandrogenaemia, and highest among the patients who have hyperandrogenaemia in addition to ovulatory dysfunction (141, 142). In addition, to having a high risk of glucose intolerance and the subsequent development of type 2 diabetes; PCOS women also have a high prevalence of developing GDM, revealed in a recent meta-analysis that showed an increased odds ratio of 2.94 for PCOS and the development of GDM (143). Similarly, large population-based studies have shown that PCOS women, especially those with a previous history of GDM, have a twofold increased risk of developing GDM as compared to controls (144, 145).

1.2.2.3 Impaired lipid metabolism:

Approximately 70% of women with PCOS have dyslipidaemia (146). The abnormal serum lipid profile is similar to that observed in diabetic patients with elevated LDL and triglyceride levels, low HDL levels and associated altered quality of LDL cholesterol (146). It has been suggested that both hyperinsulinaemia and hyperandrogenism increase catecholamine-induced lipolysis in adipocytes with subsequent release of free fatty acids into the circulation. The increased free fatty acids then stimulate production of very low-density lipoprotein (VLDL) in the liver, which finally leads to increased triglyceride levels (147). The severity of the lipid abnormalities appears to vary according to the phenotype of PCOS, with the most severe dyslipidaemia affecting the traditional variant, and less severe with other phenotypes such as the ovulatory variant (148). Furthermore, it was found that lipoprotein(a) (also called Lp(a)), an independent risk factor for atherosclerotic diseases, and small dense LDL-

cholesterol were higher in the anovulatory PCOS subjects as compared to the ovulatory variant. Insulin resistance was independently correlated with increased small dense LDL-cholesterol and low HDL levels (148). The Androgen Excess–PCOS Society suggests a complete lipid profile in all women with PCOS, including LDL-cholesterol, triglycerides, HDL-cholesterol and non-HDL-cholesterol (149). It has been recommended further that women with PCOS should be screened for dyslipidaemia for effective cardiovascular risk prevention, as these lipid abnormalities are associated with PCOS regardless of BMI (150).

Insulin resistance, hyperandrogenism and anovulation can affect numerous steps in lipid metabolism (151). The insulin resistance with subsequent hyperandrogenism in PCOS enhances lipolysis in visceral adipocytes due to increased hormone sensitive lipase (HSL) activity, while adipocyte triglyceride lipase (ATGL) activity is normal, which results in an enhanced delivery of free fatty acids (FFA) to the liver, that then serve as a substrate for triglyceride synthesis and results in greater VLDL-1 production (151). Additionally, lipoprotein lipase (LPL) activity can be reduced by insulin resistance. Increased amounts of triglyceride-rich lipoproteins result in enhanced formation of small dense LDL particles. In addition, there is augmented cholesterylester transfer protein (CETP) mediated exchange of cholesterol esters and triglycerides between apolipoprotein-B containing lipoproteins and HDL-cholesterol. This leads to an escalation in triglyceride-enriched HDL that then leads to additional modification by hepatic lipase, resulting in the formation of small HDL-3 particles. In addition, there may be a delay in LDL-cholesterol particle clearance by means of diminished expression of the LDL-receptors (151). These changes have been summarised in figure 1-3 below:

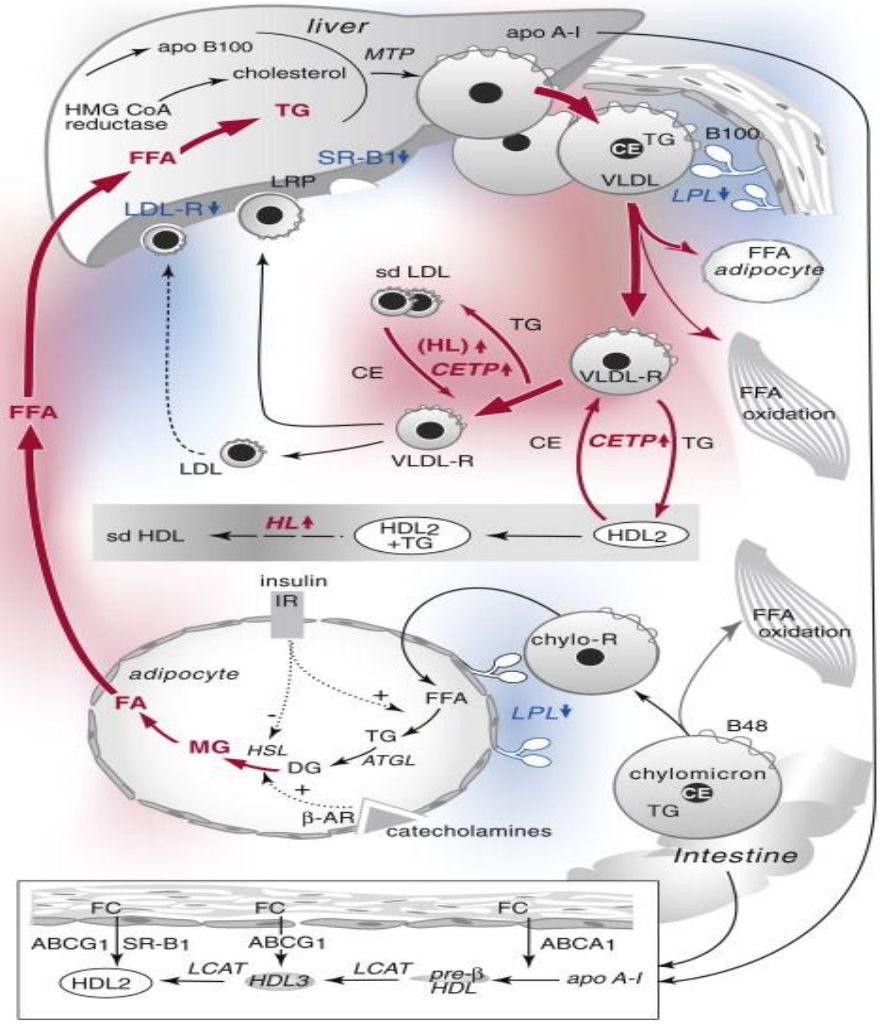

Figure 1.3: Lipoprotein metabolism in PCOS

Abbreviations. IR: insulin resistance; HAn: hyperandrogenism; AO: anovulation; TG: triglycerides; DG: diglycerides; MG: monoglycerides; FFA: free fatty acids; VLDL: very low density lipoprotein; VLDL-R: very low density lipoprotein-remnant; LDL: low density lipoprotein, HDL: high density lipoprotein; apo A-I: apolipoprotein A-I, apo B: apolipoprotein B, MTP: microsomal transferprotein;, sdHDL: small dense high density lipoprotein; sdLDL: small dense low density lipoprotein; FC: free cholesterol; SR-B1: scavenger receptor B1; CETP: cholesterylester transfer protein; PLTP: phospholipids transfer protein; LCAT: lecithin:cholesteryl acyl transferase; LPL: lipoprotein lipase; HL: hepatic lipase. ABCA1: ATP binding cassette A-I, ABCG1: ATP binding cassette G1; FC: free cholesterol; ATGL: adipocyte triglyceride lipase; HSL: hormone sensitive lipase; beta AR: beta adreno receptor. (cited from (151))

Lifestyle intervention is the treatment of choice for overweight and obese women with PCOS. Even a 5% reduction in weight associated with a more active lifestyle modification can improve many problems related to PCOS. Although the effects on plasma lipids are limited, it is a safe intervention for young PCOS women who are planning to conceive (151, 152). However, in women who do not plan to become pregnant, supportive treatment with anti-obesity medicines could be an option.

Based on cardiovascular disease data and related guidelines, lipid lowering treatments will rarely be indicated in PCOS women. Nevertheless, other therapies related to PCOS can affect lipid metabolism. The effects of several PCOS related drugs have been summarised below in Table 1.5.

Drug therapy	Effects on plasma lipids
Lifestyle intervention	HDL-C ↑
Metformin	LDL-C ↓, HDL-C ↑ or =
Rosiglitazone	HDL-C ↑ or = or ↓, LDL-C = or ↓, TG=
Pioglitazone	HDL-C ↑, chol =, TG =
Spironolactone	(HDL ↑)
Finasteride	(=)
Flutamide	TG ↓ or =, LDL-C ↓ or =
Oral contraceptives	TG ↑ or =, HDL-C ↑or =, LDL-C = or ↓ or ↑
Clomiphene	(TG ↑)
Orlistat	=
Sibutramine	TG ↓, apo B ↓
Simvastatin	TG ↓, LDL-C ↓
Acipimox	TG ↓, LDL-C ↓, HDL-C =

Table 1.5: Effects of drug therapy on plasma lipids in PCOS

Abbreviations: apo B: apolipoprotein B; LDL-C: low density lipoprotein-Cholesterol, HDL-C: high density lipoprotein-Cholesterol; TG: Triglyceride; ↓: decreased; ↑: increased; =: no change (adapted from (151))

1.2.2.4 Adipokines and pro-inflammatory factors (chronic inflammation):

Atherosclerosis is exacerbated by an underlying chronic inflammatory process. CRP (C-reactive protein) is a marker of inflammation that is commonly quantified by a highly sensitive technique, though its use in routine clinical practice is still not common. CRP contributes to the vascular inflammation in addition to acting as a marker of inflammation, and is produced by the liver in response to interleukin-6 (153). A chronic low-grade inflammatory process is associated with persistent moderately elevated CRP concentrations, though still within the normal range. As compared to other inflammatory markers including LDL, CRP has been revealed as an independent risk factor for future cardiovascular events, including myocardial infarction, coronary revascularization procedures, stroke and cardiovascular death in asymptomatic subjects (154-156). Moreover, there is increasing evidence that atherosclerotic lesions are a consequence of a series of extremely specific responses at the cellular and molecular levels that result in inflammatory disease. Additionally, inflammation plays a major role in predisposing individuals to plaque rupture by weakening of the fibrous cap in coronary plaques, and thereby increasing the risk of coronary thrombosis (157). In an *in vitro* study, it was suggested that CRP may a have direct role in enhancing the inflammatory component of the atherosclerotic processes (158). Increased levels of CRP have been shown to be associated with the metabolic syndrome, obesity and cigarette smoking in several studies. Similarly, numerous trials have demonstrated increased levels of CRP in women with PCOS as compared to their controls, even after adjustment of their age and BMI (159, 160). The reports have also revealed positive relationships between CRP levels and body weight, adipose tissue mass and insulin resistance (159, 161, 162). Likewise, women with PCOS have been shown to have high levels of other low grade chronic inflammatory markers such as cellular adhesion molecules, including E-selectin, inter cellular adhesion molecules 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) (159). Furthermore, levels of pro-inflammatory

cytokines, especially interleukin-18, are elevated in women with PCOS, which stimulates the synthesis of interleukin-6 (163).

1.2.2.5 Subclinical atherosclerosis:

The first measurable stage of atherosclerotic change is thickening of the arterial wall. The measurement of common carotid intima-media thickness (carotid-IMT) by ultrasound is a morphological marker used to assess early atherosclerotic changes (164). Carotid-IMT measures the thickness of the two layers (intima and media) of the carotid arterial walls, the largest conduits of blood supply to the brain (165). It has been shown in a recent meta-analysis that increased carotid-IMT is an independent predictor for future cardiovascular events, such as myocardial infarction and stroke (164). Reports indicate that increased carotid-IMT is linked with obesity, metabolic syndrome and increasing insulin resistance in young women with PCOS when compared with their matched control women (166, 167), in addition to women with PCOS who are older than 40 years (168). Moreover, severe carotid atherosclerotic changes, assessed by carotid plaque index of three or more, have been observed in PCOS (7.2% of women with PCOS and 0.7% of their controls) (168, 169). The extent of atherosclerotic changes in the cardiac arteries may be assessed by measuring coronary artery calcification. The presence of coronary calcium is related to the presence of coronary atherosclerosis, without the presence of any symptoms (165). Studies have shown that women with PCOS have a considerably greater prevalence and extent of coronary and aortic artery calcification as compared to age matched ovulatory control women (169, 170). However, in one study, adjustment for BMI in PCOS predicted coronary calcification (169), but not in another (170). Similarly, diastolic dysfunction is considered to be an early marker of coronary artery disease and, in women with PCOS, several unfavourable changes in diastolic function have been observed when compared with BMI matched control women, suggesting women with PCOS may be likely to develop diastolic dysfunction (171).

1.3 PCOS and non-alcoholic fatty liver disease:

1.3.1 Introduction:

Non-alcoholic fatty liver disease (NAFLD) comprises a spectrum of diseases that range from simple steatosis to non-alcoholic steatohepatitis (NASH) and finally to cirrhosis (172, 173). NAFLD is acknowledged as a principal cause of cryptogenic cirrhosis and is recognized as the most common form of liver disease in the western world (174, 175). Hepatic steatosis ensues as a result of anomalous lipid handling by the liver that sensitizes liver to injury and then to inflammation, while NASH is categorized by increased injury and apoptosis of hepatocytes (176). The changes seen in simple hepatic steatosis are usually benign and reversible and which seldom progress to NASH, and carry only 1-2% risk of progressing to hepatic cirrhosis (177). In contrast, NASH has high risk (up to 30%) of rapidly progressing to fibrosis and subsequently to liver cirrhosis or hepatocellular carcinoma (177, 178). The estimated median prevalence of NAFLD is 20% worldwide (approximately between 6.3-33%) in the general population (179). However, the prevalence of NAFLD could increase up to 75% in the presence of metabolic syndrome, obesity and type 2 diabetes mellitus. In addition, several other risk factors could enhance the development of NAFLD such as impaired lipid metabolism, male gender, increasing age, sleep apnoea syndrome, hypogonadism, hypothyroidism and hypopituitarism (180, 181). It has been revealed in several longer follow up studies that NAFLD patients have a 2.6 fold increased mortality risk as compared to the general population (178, 182, 183). The diagnosis of NAFLD could be made by either imaging or histology showing evidence of hepatic steatosis with the absence of other possible causes of hepatic fat deposition, including hepatitis C infection, nutritional disorders, excess alcohol consumption, teratogenic medication use and hereditary disorders (181).

PCOS has emerged as a substantial risk factor for NAFLD development in young women of reproductive age (65). The metabolic and biochemical abnormalities in PCOS, such as

obesity, insulin resistance, type 2 diabetes mellitus and hyperandrogenism further increase that risk and subsequently increased future cardiovascular risk and liver related morbidity (181).

1.3.2 Prevalence of NAFLD in PCOS:

The evidence linking PCOS and NAFLD was first published in 2005 and subsequent retrospective studies confirmed this relationship (184). The prevalence of NAFLD in women with PCOS depends on the diagnostic criteria used both for PCOS and NAFLD and is estimated between 15-55%, with more in metabolic PCOS identified using NIH criteria (184, 185). A chart review in a retrospective study on adolescent women with PCOS showed a prevalence rate of 15.4% of elevated aminotransferases (ALT). Similarly, another study revealed that increased liver fat in obese and overweight adolescent girls with PCOS is associated with worsening dyslipidaemia, growing abdominal adiposity, increasing age and insulin sensitivity (186). It was also found in this study that the highest contribution to liver fat development was age and total testosterone levels. However, the data related to prevalence of NAFLD in lean women with PCOS is still controversial. Notably, NAFLD has been found to affect only 5% of lean women with PCOS as compared to 28% of obese patients (72). The available evidence is less clear on whether or not NAFLD is also more prevalent in lean women with PCOS. In one study, the authors included both lean and obese PCOS patients and found that 39% of lean women with PCOS had NAFLD, suggesting that PCOS itself is a risk for the development of NAFLD independent of obesity (72). However, two other trials (187) that included 17 young women with PCOS, as well as an additional study that recruited 34 lean women with PCOS, have not demonstrated the same results when compared to their appropriately matched lean controls. Some data propose that the prevalence of NASH and advanced fibrosis do not vary significantly between lean and obese NAFLD patients; however, the lean patients tend to have less severe disease at presentation (188).

Further, it has been suggested that the underlying pathophysiology of lean NAFLD patients may be quite different such as genetic predispositions, visceral obesity, fructose and cholesterol-rich diets and dyslipidaemia (188).

Schwimmer *et al* reported a 30% risk of ALT elevation amongst a group of PCOS women attending a fertility clinic. Another trial found only 15% of PCOS patients had elevated aminotransferases, but they used a higher cut-off value for abnormal ALT (> 60 U/L) as compared to the previous trial that used >35 U/L. The prevalence, however, increased to 28% by applying the same ALT elevation criteria as used by Schwimmer et al (>35 U/L) (189). Furthermore, one retrospective study revealed that 55% of PCOS women had hepatic steatosis using ultrasound as the diagnostic modality, but there was no control group to compare. The first prospective study that included a control group was done in Chile and demonstrated a prevalence of hepatic steatosis in women with PCOS up to 41.5% using ultrasound and only 19.4% in their matched controls (184, 190). ALT was elevated in 39% of women with PCOS as compared to 3.2% of their controls.

Conversely, it has been shown that the presence of PCOS is also very common among NAFLD patients attending liver clinics. It was found that reproductive aged women attending these clinics had a PCOS prevalence of 71% that was confirmed using liver biopsy. The trials have also shown (191) that biopsies in PCOS women revealed advanced fibrosis despite their young age, suggesting that women with PCOS are at increased risk of developing more advanced disease at an earlier age (185).

1.3.3 Evidence associating NAFLD to PCOS:

The evidence suggests that the main factors related to underlying NAFLD in women with PCOS are obesity, especially central adiposity, and insulin resistance. There are several clinical trials that have demonstrated significant elevation of ALT levels in PCOS women and have revealed its substantial association with recognised risk factors such as obesity,

waist circumference, age, SHBG levels, LDL-cholesterol, HDL-cholesterol, triglycerides and the degree of insulin resistance, even though the techniques used to measure insulin resistance differed (using QUICKI, HOMA-IR and by the gold standard euglycaemic clamp technique) (192). Similarly, significant associations have been seen between indices indicating hepatic steatosis, such as fatty liver index (FLI) and age, waist circumference, obesity, LDL-cholesterol, HDL-cholesterol and insulin resistance. Furthermore, the studies using imaging modalities to detect hepatic steatosis have also revealed substantial relationships between age, waist circumference, obesity, SHBG, levels, LDL-cholesterol, HDL-cholesterol, triglycerides and the magnitude of insulin resistance, as evaluated using various techniques including HOMA-IR, QUICKI and euglycaemic insulin clamps (192, 193). These findings have been further supported by the evidence suggesting that dietary and lifestyle intervention in the form of exercise and weight loss have beneficial effects on NAFLD in PCOS, either alone or in combination with pharmacological intervention, especially metformin (193).

However, a question arises whether the high prevalence of NAFLD in PCOS is merely a consequence of having shared risk factors or due to PCOS itself, as many of the features of metabolic syndrome are also associated with the development of NAFLD in PCOS women, with the prevalence reaching up to 100% in some studies (174, 193). The diagnosis of PCOS has a substantial association with NAFLD as suggested by two studies that demonstrated this independent relationship after adjustment for age, obesity, waist circumference and dyslipidaemia (194). Since hyperandrogenism is the predominant feature of PCOS, further evidence comes from the studies evaluating the putative role of androgens in NAFLD development. These studies have shown that high levels of androgens in PCOS are significantly associated with NAFLD development. One of the studies revealed significant association of clinical hyperandrogenaemia, mainly hirsutism, with raised levels of ALT in

seventy women with PCOS, though serum androgen levels were not measured in these patients (189). A positive correlation has been shown between testosterone levels and FAI and ALT levels in a case control study in overweight and obese women with PCOS when compared to their matched controls. Similarly, another trial (195) has revealed that obese PCOS women with high fatty liver index (FLI) values, suggesting NAFLD, were found to have high levels of free testosterone levels as compared to those patients who had lower FLI values. The same findings have been explained in a large-scale case control study in PCOS women (196) even after adjustment of associated confounding factors such as impaired lipid metabolism, insulin resistance and obesity. Furthermore, the trials that used imaging modalities for NAFLD diagnosis in obese PCOS women have also shown a substantial positive relationship between high testosterone levels and NAFLD prevalence. In one of the trials, high FAI levels were significantly related to the diagnosis of NAFLD in both PCOS women and their appropriate controls evaluated using ultrasound. Likewise another trial (197) that used abdominal ultrasound for detecting NAFLD in obese PCOS women found a positive correlation with increasing FAI values and an inverse relationship with SHBG levels when compared to their age matched controls. Likewise, PCOS patients in a case control study with high testosterone and FAI levels showed substantially high levels of liver fat as compared to their PCOS controls with normal androgen levels. Interestingly, this finding remained significant even after correction for insulin resistance, visceral and total adipose tissues. In a recent study that used CT scan to detect hepatic fat content in obese and overweight women with PCOS, the results revealed that high androgens and age are independent risk factors for the development of NAFLD (186). In contrast, there are a few small studies that have not reported any significant differences in androgen levels between PCOS patients, with or without NAFLD (193). There are also findings revealing significantly enhanced levels of apoptotic markers in the serum of PCOS women. These findings were

confirmed with gold standard liver biopsy that revealed PCOS patients with NAFLD had high levels of an apoptotic marker when compared to their BMI and age matched controls, which suggest that PCOS women may have higher cell turnover (198). There are suggestions that high androgen levels may stimulate the development of NAFLD in PCOS women, either by direct effects on hepatic stellate cells or by indirectly through insulin sensitivity, and by enhancing visceral adipose tissue deposition (198).

There are numerous susceptible genetic loci have been identified in women with PCOS suggested to be associated with the development of NAFLD in these patients, but the evidence is limited due to small sample size and use of diverse diagnostic criteria for PCOS (199).

1.3.4 NAFLD associated cardiovascular risk:

The metabolic syndrome is very common in PCOS being seven times more frequent as compared to the general population of the same age and sex, with prevalence around 50%. PCOS is considered as an ovarian manifestation of metabolic syndrome, while NAFLD has been proposed as the hepatic manifestation of metabolic syndrome (200). The cardiovascular risk factors that encompass the metabolic syndrome, such as obesity, insulin resistance, type 2 diabetes mellitus, endothelial function abnormalities, impaired lipid metabolism and vascular atherosclerosis, are associated with both NAFLD and PCOS (35, 200). There is an ample amount of data available suggesting that NAFLD is linked with long-term risk of adverse cardiovascular outcomes [36] and should be considered as a substantial independent risk factor for both sub-clinical and clinical cardiovascular diseases (201). The long-term cardiovascular follow up studies have demonstrated that the most common cause of death in NAFLD patients is cardiovascular disease. Furthermore, much evidence links NAFLD with well-recognized markers for cardiovascular disease, such as vascular endothelial dysfunction, augmented coronary artery calcification and enhanced carotid intima media thickness (202).

However, the data in PCOS related to long term cardiovascular outcomes is still controversial. A recent trial compared cardiovascular risk in PCOS with associated NAFLD to those with PCOS alone and has suggested that NAFLD presence does place PCOS patients at high risk of developing cardiovascular disease. There were no significant differences in cardiovascular risk factors in both groups in, for instance, fasting lipid profile, systolic and diastolic blood pressure and endothelial function, though BMI was higher in the NAFLD group (203). Nevertheless, PCOS patients have all the risk factors that may increase long-term cardiovascular outcomes later in life, which warrants further studies in this regard in order to apply earlier interventions.

1.3.5 Screening for NAFLD and its implications in PCOS:

The first diagnosis of NAFLD in PCOS was reported in 2005 when a young, obese, non-diabetic PCOS patient, with no history of alcohol but with raised aminotransferase levels, underwent liver biopsy which revealed NASH (204). It was then proposed that, since insulin resistance and metabolic syndrome are common features in both PCOS and NAFLD patients, NAFLD might be prevalent in certain women having PCOS. Therefore, the importance and frequency of screening PCOS patients for NAFLD was realised, especially by looking at their liver function. The symptoms of NAFLD are usually non-specific, such as malaise, fatigue and right upper quadrant discomfort, but most of the time patients are asymptomatic. The signs of chronic liver disease and hepatomegaly are very rare in these patients (181). The diagnosis of NAFLD is usually made by assessing liver function and with the help of imaging techniques, after excluding all the secondary causes of fatty liver disease (genetic causes, excessive alcohol consumption, toxins, drugs, bariatric surgery, metabolic and nutritional factors, autoimmune liver disease, viral infections etc.). The gold standard method for diagnosis and staging of NAFLD is liver biopsy; however, this could not be routinely performed because it is an invasive method with associated complications (181, 205).

1.3.5.1 Use of laboratory markers in NAFLD:

There are several studies that investigated the presence of surrogate markers of NAFLD in PCOS patients as raised levels of serum aminotransferase levels. In one of the retrospective studies that involved 70 obese PCOS patients having numerous ethnicities, including Hispanic, White, Blacks and Asians with no controls, raised alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were described in only 30% and 12%, respectively. The authors used ≥35 U/L and ≥40 U/L cut-off levels for raised ALT and AST respectively (189).

Several interventional studies have also shown elevated levels of serum aminotransferase levels in obese and overweight PCOS patients. In one of the studies, reviewing the effects of metformin, 57.8% of patients out of 140 participants were found to have elevated serum levels of ALT, while another study performed in Chile found a substantial difference with raised serum levels of ALT in obese PCOS (39%) patients as compared to BMI and age compared controls (3.1%) (193). In agreement, two studies done in Greece revealed similar statistically significant differences in serum aminotransferase levels between overweight and obese PCOS patients as compared to age and weight matched controls, although the cut-off values used and the number of patients used in both of these studies were different (187, 206). Caspase-3 has been established as a serum marker of increased hepatic apoptotic activity, suggesting the early development of NASH in PCOS patients. In one of the case control studies, in which 186 women with PCOS and 73 age-matched controls were recruited, caspase 3, which is a cleaved fragment of cytokeratin-18, was significantly raised even after correction for BMI (207). Another case control study evaluated the presence of hepatic steatosis in PCOS patients by using an algorithm called fatty liver index (FLI) and two fibrosis indices. FLI is calculated using BMI, waist circumference, triglycerides and ɣ-GT of the patients and the 2 fibrosis indices used were AST-to-platelet ratio index and Fibrosis-4 (FIB-4) index (208). FLI is easy to employ as its each discrete component is a routine

measurement in clinical practice and it has been validated in several large epidemiology studies as a practical, reliable, and economic technique to diagnose NAFLD (209, 210). The study showed significantly greater FLI levels in 611 women with PCOS as compared to 139 age and BMI matched controls (195). However, this significant increased prevalence of raised FLI was found only in obese and overweight PCOS patients as compared to obese and overweight controls, but FLI levels were not significantly raised in lean PCOS patients as compared to their lean controls. In addition, no difference was detected in fibrosis indices in either PCOS patients or their matched controls (193, 195).

Enhanced liver fibrosis (ELF) panel is comprised of three markers: hyaluronic acid (HA), the N-terminal pro-peptide type-III pro-collagen (PIIINP) and tissue inhibitor of metalloproteinase- 1 (TIMP-1), that reflect the ongoing fibrogenesis in liver cells (211). It was first developed after a major multicentre study in which the ELF panel was established for its cross-sectional capability to identify the stage of hepatic fibrosis. The ELF score was further validated in both adult and paediatric NAFLD patients (212, 213). Several large trials have tested the ability of the ELF score in predicting liver related clinical outcomes and have reported that ELF was at least as accurate as liver biopsy in predicting early fibrotic changes (211).

1.3.5.2 Using imaging techniques:

Due to the invasive nature and limitations associated with liver biopsy, several non-invasive imaging techniques have been used to diagnose NAFLD in patients. The most commonly used imaging techniques in detecting NAFLD include ultrasound (US), computed tomography (CT-scan) and magnetic resonance imaging (MRI-scan), and have been shown to have comparable sensitivity and specificity for diagnosing hepatic fatty changes (between 94% and 80% respectively) (214, 215). Another technique called Proton H1 magnetic resonance spectroscopy has been found to have similar sensitivity to US, CT scan and MRI scan;

however, it is more costly and is not widely available (215). Further, none of these techniques offer any reliable information regarding the presence and detection of early development of hepatic fibrosis (216).

Ultrasound has been suggested as the imaging technique of first choice for early detection of fatty deposition in liver, because it is considered safe with less exposure of patients to radiation as compared to other imaging modalities and, secondly, it is less expensive than CT-scan and MRI-scan (216). However, the sensitivity of ultrasound scan is reduced to between 60 to 90% if fatty changes in liver are present in less than 30% of hepatic cells (180, 217). Similarly, MRI scan has reduced sensitivity in detecting fatty changes in liver in patients with advanced fibrosis (218). Additionally, none of these imaging modalities could distinguish between hepatic steatosis and NASH and NAFLD (180, 217). Fibroscan (transient elastrography) is a newly established alternative technique for assessing liver stiffness that correlates directly with the degree of fibrotic changes in liver (219-221). However, it also has its limitations, which preclude its use in certain groups of patients, such as the morbidly obese and patients who have enormous amounts of fat over the chest wall that is common in women with PCOS (219, 222). Some novel techniques that have been used have a similar sensitivity as transient elastrography and are based on the same principle. Such imaging techniques include Acoustic radiation force impulse (ARFI) (a new ultrasound wave based elastrography method that is incorporated in the conventional ultrasound machine), real-time shear wave elastography (SWE) and magnetic resonance elastography (MRE) (223, 224). All of these novel techniques have demonstrated similar levels of sensitivity and specificity in detecting early liver fibrotic changes as compared to Fibroscan and, additionally, could also provide useful information in obese patients. The drawback to these techniques is their limited availability, the chief hindrance in implementing their use in diagnosing NAFLD and early hepatic fibrosis (223, 225).

1.4 Endothelial dysfunction:

Vascular endothelium plays a fundamental role in regulating vascular tone and blood flow. Endothelial cells line the entire circulatory system, from the heart to the tiniest capillaries (226). Healthy endothelial cells are able to react to both chemical and physical stimuli and help in regulating the vascular environment by maintaining vascular tone, monitoring cellular adhesion, vascular inflammation and proliferation of vascular smooth muscle cells through the production of various vasoactive factors (227). The vital role of the vascular endothelium was first recognized due to its effects on the vascular tone that is attained by the production and release of various vasoactive peptides and molecules that help the vessels in relaxation or constriction. The vascular endothelium also maintains vascular tone by responding to other vasoactive peptides in the circulation, like thrombin and bradykinin (228). This spontaneous oscillation of the blood vessels plays a pivotal part in balancing oxygen supply and the metabolic requirements of the tissues, mainly by regulation of vascular diameter and tone. These vasomotor changes also help in maintaining organ perfusion through the remodelling of vascular assembly (229-231). Nitric oxide (NO) was the first recognized factor produced by the endothelium in several pioneering experiments (5). The main enzyme required for the production of nitric oxide from L-arginine is NO-synthase that requires the presence of cofactor tetrahydrobiopterin (231). The crucial activator of endothelial NO-synthase is shear stress in normal physiology that helps in adjusting body organ perfusion to changes in cardiac output (232, 233). Furthermore, certain other molecules activate NO-synthase, such as serotonin, bradykinin, vascular endothelial growth factor (VEGF) and adenosine (234). The endothelium also uses nitric oxide independent pathways to maintain vascular tone through hyperpolarization and consequent propagation of the depolarization wave across the vascular smooth muscle cells by increasing potassium conductance (234). Nitric oxide plays a vital part in maintaining the vascular wall in a dormant state through cellular proliferation,

inhibition of excessive inflammation, and by pacing thrombosis (235). The underlying mechanism by which nitric oxide achieves such targets is through a form of post-translational modification (S-nitrosylation) of cysteine residues of target proteins (nuclear factor NF-KB, cell-cycle monitoring proteins, and those related to the generation of tissue factor) that helps in moderating their biological activities (236, 237). The above mechanism (endothelial cell derived hyperpolarizing factors) could be helpful in maintaining vascular tone in situations where nitric oxide production is reduced or its bioavailability is diminished. However, these hyperpolarizing factors, derived from endothelial cells, are only understood partially, and differences between various vascular beds have been observed (235). There is another factor derived from endothelium that helps in maintaining vascular tone independent of nitric oxide, called prostacyclin that is produced through the cyclooxygenase pathway. However, prostacyclin has a more limited role in humans in maintaining vascular tone but does support other regulatory mechanisms (238, 239). Vascular endothelium is not only recognized as an endocrine organ that releases vasoactive substances and thereby controls vascular tone but it also controls the vascular tone by stimulating the release of vasoconstrictor factors, such as angiotensin II, endothelin and protanoids, that have systemic effects in addition to local actions in order to control vascular remodelling and structure (240). PCOS is linked to endothelial dysfunction in addition to unbalanced gonadotropins secretion, along with aberrations in release of androgens, insulin, prolactin and hormones secreted by adipose cells (241). It is still unclear whether endothelial dysfunction in women with PCOS is related to abnormal endocrine changes, markers of inflammation or associated cardiovascular risk factors. Evidence suggests that each one of these could add independently to the endothelial abnormality found in PCOS patients (242). Moreover, recent studies have shown inconsistent evidence regarding the traditional assumptions that endothelial dysfunction in PCOS is linked to high androgens and insulin resistance entirely. Numerous other hormonal imbalances have

been implicated as an underlying mechanism for endothelial dysfunction in these patients (243, 244). Further studies are looking for other factors to unravel the underlying mysteries related to endothelial dysfunction in PCOS, as currently known factors only partially explain such changes in these patients, and further knowledge would be helpful in improving future cardiovascular risk in women with PCOS (245).

1.4.1 Methods to evaluate endothelial function:

Various methods have been used for endothelial dysfunction assessment in the past but, in 1986, Ludmer and his co-workers performed the first experiment to demonstrate endothelial dysfunction in diseased coronary vessels using a combined technique involving intracoronary infusion of acetylcholine with quantitative angiography (246). Their innovative work changed the understanding provided insight into human vascular atherosclerosis, as previously it was considered only a structural disease. The functional manifestations of vascular atherosclerosis were further explored with regard to its association with poor blood supply to organs due to excessive vascular tone and constriction. Over time, less invasive procedures were established, in which the main surrogate for the assessment of coronary arteries was forearm circulation (247-249). Different techniques that have been developed have their own pros and cons and, most importantly, each method uses its own distinct vascular bed for assessment (250).

The normal response of healthy arteries is (1) dilatation in response to pharmacological stimuli such as vasodilator drugs (endothelium dependent) including serotonin, acetylcholine and bradykinin via release of nitric oxide from endothelial cells and/or (2) other endothelium derived vasoactive factors or (3) they normally dilate through a phenomenon called flow mediated vasodilatation (reactive hyperaemia) (251). However, such response (dilatation) is usually reduced or absent in disease states. Whatever technique is used and whatever area is used to determine the endothelial status, the vascular responses are revealed by assessing both

the structural condition and functional status of the vasculature. Further, in order to distinguish the endothelium independent responses from the endothelium dependent responses, exogenous factors are used such as glycerol trinitrate, which is nitric oxide donor, or adenosine, which acts though pathway independent of nitric oxide. The endothelial dysfunction that is endothelium independent is usually associated with alterations in smooth muscles cells or structural changes in vasculature (252).

1.4.1.1 Coronary microvascular function assessment:

The main advantage of this method, used to assess endothelial function of coronary arteries, is that it measures endothelial function directly to give accurate result at this area of the vasculature, but it is invasive. The intravascular ultrasonography or quantitative angiography is used to look at the epicardial coronary vessels in order to assess their responses, via analysing changes in their diameters and cross-sectional areas after applying endothelial dependent interventions (253). The normal response from the healthy endothelium is vasodilatation while vasoconstriction is caused if the endothelium is disrupted or damaged (246). In order to induce endothelium-dependent responses, physical stimuli usually used are exercise (254) or tachycardia caused by pacing (substitute for exercise) (255, 256). In diseased endothelium, vasoconstriction will be more prominent due to smooth muscle cells constriction, mimicking effects of acetylcholine test (257, 258). Coronary vessels flow reserve, is measured by dividing peak coronary blood flow by the blood flow in the vessels during resting stage. The flow reserve of ≤ 2.0 is usually considered abnormal (259). As the above tests are invasive in nature and not possible to perform routinely, various non-invasive tests have been developed to demonstrate functional assessment of the microvasculature such as echocardiography, myocardial perfusion imaging technique, PET (positron emission tomography) scan and blood oxygen level dependent MRI (260-262).

1.4.1.2 Methods to evaluate endothelial function in peripheral circulation:

Several less invasive surrogate methods have been developed in order to evaluate endothelial function in both macro and microvasculature as routine performance of coronary angiogram is not possible due to its invasive nature. While these non-invasive techniques are not able to evaluate vascular endothelial function directly in the coronary arteries, they have been shown to correlate with those invasive methods (263, 264). It is important to remember while performing and analysing these non-invasive methods that they assess the generalized function of the vasculature and certain phenomena could not be explained by just evaluating systemic endothelial dysfunction. Certain local factors also need consideration such as flow patterns and local disturbed shear stresses especially at branch points, which might also contribute to cardiovascular disease (265, 266).

1.4.1.2.1 Assessment of the forearm vasculature and response to vasoactive factors:

In this technique blood flow is measured in both forearms by venous plethysmography before and after infusion of vasoactive factors directly into the brachial artery. The results are reproducible that are measured and stated as the ratio of the blood flow changes in both arms. While this test is still limited due to its small invasive nature (248, 267), the main advantage is that endothelial function and dysfunction can be observed directly in a dose dependent manner during the infusion of vasoactive factors, hormones and drugs such as acetylcholine, nitroglycerin etc. Additionally, the contralateral arm could serve as a control because the drug doses required have minimal systemic effects (267-269). The vital role of nitric oxide is explained by substantially reduced response by the endothelium to acetylcholine when monomethyl L-arginine is infused, while there is no change in response after acetylsalicylic acid (270). The main disadvantage of this method is that whilst drug induced vasodilation is caused during this technique that provides remarkable information regarding micro-vascular pathophysiology; it does not essentially mimic vasodilation of microvasculature seen in exercise or transient ischemia. Furthermore, in patients who have multiple risk factors,

further consideration is required while estimating the endothelial dysfunction as these hyperpolarizing substances also play pivotal role in maintain resting micro-vascular tone and drug stimulated dilatation (271-273). Additionally, this method is well equipped to assess blood flow variations in response to various stimuli only in individuals as comparisons between different groups and serial measurements in the same subject have limited value due to factors such as differences in initial mean blood pressures, different forearm sizes and variations in blood flow in the forearm (274).

1.4.1.2.2 Flow-mediated dilatation:

Flow mediated dilatation (FMD) is a non-invasive technique that measures the ability of the arteries to dilate with nitric oxide released from endothelium after a 5 minutes period of occlusion called reactive hyperaemia. It is now widely used and the most common artery used is the brachial artery that is occluded with a blood pressure cuff for 5 minutes. The endothelial function evaluated by FMD on the peripheral circulation gives a very close estimate of the coronary vasculature endothelial function (264). It was first demonstrated by Celermajer et al. that this vasodilatation is mainly dependent on nitric oxide release from the endothelial cells with much less contribution from the other pathways. They used ultrasound to assess the respective changes in the diameter of radial and brachial arteries (275-277). The only disadvantage of this technique is that its application is not technically easy and it requires substantial training time for reproducibility that includes, preparation for the acquisition of the image, proper selection of the site, probe positioning sphygmomanometer, occlusion time of cuff, correct use of machine software, and accurate description of the FMD assessment (278-280).

Reactive hyperaemia with increased blood flow and the induced shear stress on the endothelial cells (flow mediated dilatation) evaluates channel artery vessels allowing functional evaluation of peripheral micro-vasculature (281, 282). Though, FMD has been

demonstrated to have strong correlations with risk factors for coronary heart disease; however, the measurement of stimulus for FMD itself (both velocity changes and subsequent hyperaemia induced shear stress) have shown to have even stronger correlations with cardiovascular risk factors than FMD, and are suggested as better predictors for assessing future cardiovascular outcomes (283, 284). Moreover, current evidence from several multi-centre trials have revealed that simple baseline diameter measurements from the brachial vessels can be correlated with cardiovascular clinical outcomes to almost the same level as FMD (285). The potential explanation for this observation is that as the diameter of the blood vessel increases the shear stress induced is diminished with less stimulation of endothelium and the percentage changes in the FMD are very small and less sensitive (286, 287). However, the data is contradictory regarding an effort for the normalization of the dilatational responses to shear stress. Therefore, it is suggested that other physiological and methodology related factors should also be considered because they could also influence the results (278). It has been suggested that FMD is a measure of blood flow following a stimulus like shear stress, but it does not give any useful information regarding the changes in the flow in the vasculature while in the resting state, and no assessment could be made about the release of resting vasoactive factors (288). Therefore, another technique was introduced that assessed the vasoconstriction of the brachial artery after the application of supra-systolic pressure over the wrist with a cuff, changes that are mediated via the release of endothelin. This concept led to the concept of low flow mediated vasoconstriction (289, 290). This new technique measures the changes in the diameter of the brachial artery in reaction to a reduction in the flow of blood in them and due to shear tension created by putting the pressure cuff distally with occlusion of the artery. Recent studies have revealed a strong correlation between impairment of low flow mediated vasoconstriction and cardiovascular risk factors with future development of cardiovascular outcomes especially coronary artery disease (291).

1.4.1.2.3 Fingertip EndoPAT:

This technique uses a new proprietary machine called (EndoPAT 200; Itamar Medical) (292, 293) that was developed to assess pulsatile blood flow changes in the finger. This technique has gained much popularity recently because it is observer independent and gives very accurate estimations of the endothelial function with peripheral arterial tonometry (also called finger plethysmography). The pneumatic probes of the EndoPAT are applied over the index fingers usually that records beat-to-beat plethysmographic readings as pulse wave amplitudes of the small arteries. In order to avoid pooling and subsequent distension of distal venous system in the fingers that could lead to reflex arteriolar responses, a small pressure of 70 mm Hg is applied over the digits with the probes (292). In order to evaluate the endothelial function or dysfunction, a blood pressure cuff is placed on the upper arm, as it is placed in flow mediated dilatation technique that is inflated about 60 mmHg above the systolic blood pressure after recording the baseline blood volume variations. The cuff is then deflated after 5 minutes in order to induce and record the resultant reactive hyperaemia in the same arm that in normal situations augments the amplitude of measured signals in EndoPAT due to the subsequent increased flow of blood in the artery. The other arm usually serves as a control and used to correct the systemic drifts in the vascular tone occurred during the procedure. The final index that is obtained has been validated as a measure of the underlying endothelial function. The increased response measured as augmented amplitude of the pulse due to increased blood flow (reactive hyperaemia) after the ischemia is considered a very complex response after ischemia, which is independent of nitric oxide release and reflects variations in flow and dilatation of micro-vasculature (294). Several clinical trials have shown and validated that endothelial function measured in the small fingers by EndoPAT gives an estimate of the microvascular function of coronary arteries and helpful in predicting future cardiovascular risk in humans (295). In several bigger trials it has been demonstrated that vascular dysfunction measured in the digits is more significantly associated with metabolic

and other cardiovascular risk factors as compared to FMD that is only modestly associated. Two large studies of 1900 patients and a cross-sectional study that included 5000 subjects (Gutenberg Heart study) confirmed these findings on vascular function and dysfunction (293, 296, 297).

1.4.1.2.4 Endothelial microparticles:

Endothelial microparticles (EMPs) are complex tiny vesicular structures, ranging from 0.1 to 1.0 μm in size, shed either from endothelial cells when they are activated or during apoptotic changes. EMPs contain vital cellular information like DNA, RNA, microRNAs (miRNAs), receptors and specific proteins related to cell of origin (298). They have been suggested to play a significant role in endothelial dysfunction, cellular inflammation, coagulation and angiogenesis thus predispose to cardiovascular diseases by interrupting the vascular homeostasis. EMPs carry numerous endothelial proteins but most commonly expressed are CD31, Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1): CD54, Intercellular Adhesion Molecule-1(ICAM-1): CD62, Cell Adhesion Molecule E-selectin: CD105, Endoglin (ENG): CD106, Vascular Cell Adhesion Molecule-1 (VCAM-1): CD142, Tissue factor (TF): CD144, Vascular Endothelial Cadherin (VE-cadherin) and CD146, Melanoma Cell Adhesion Molecule (MCAM) (Figure 1.4 adapted from (299)).

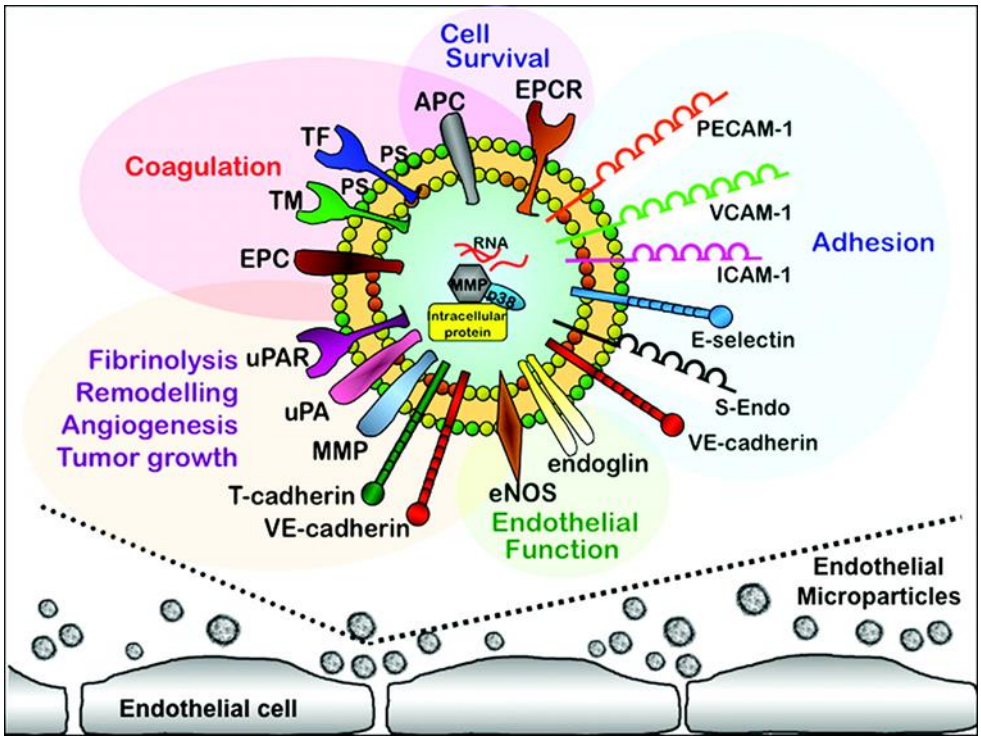

Figure 1.4: Schematic representation of surface markers expressed by EMPs

Figure Legend: EPCR, endothelial protein C receptor; PECAM-1, platelet endothelial cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular cell adhesion molecule-1; E-selectin, endothelial selectin; S-Endo, CD146, melanoma cell adhesion molecule; VE-cadherin (vascular endothelial cadherin); eNOS, endothelial NO synthase; MMP, matrix metalloproteases; uPAR, urokinase plasminogen activator receptor; EPC, endothelial protein C; TM, thrombomodulin.

In patients with vascular pathologies, raised levels of EMPs have been found to correlate with endothelial dysfunction and serve as a surrogate marker of endothelial function. However, recent data have challenged the presumed harmful effects of EMPs and suggested that EMPs could support cellular survival, produce anti-inflammatory effects, counteract coagulation processes and encourage endothelial regeneration. Flow cytometry is largely used to detect and quantify microparticles (MPs) in clinical samples and are defined by their size and by expression of specific antigens related to their parent cell.

1.4.2 Endothelial function in PCOS:

It has been demonstrated in several studies that PCOS is directly correlated with endothelial dysfunction and vascular anomalies and patients are at increased cardiovascular risk (159, 300-302). In one study, flow mediated dilatation has been demonstrated to be substantially reduced in PCOS patients along with a decreased vasodilator response to nitrates in PCOS compared to age and weight matched control women (301). Endothelial dysfunction can also be evaluated by quantifying specific indicators of coagulation such as plasminogen activator inhibitor-1 (PAI-1), markers of inflammation like soluble intracellular cell adhesion molecule-1 (sICAM-1) and soluble VCAM-1 (sVCAM-1), or circulating markers derived from endothelium such as ADMA (asymmetric dimethylarginine). The levels of such markers of endothelial dysfunction (PAI-1, ADMA, sVCAM-1, and sICAM-1) have also shown to be higher in PCOS patients as compared to their appropriate controls in a range of studies (243, 303-306). In one of the studies, 107 participants were recruited and the study revealed that high levels of ADMA have direct positive correlation with increased testosterone levels, fasting insulin, HOMA-IR and fasting lipid levels (307). Similarly, in another study that recruited 81 PCOS patients and revealed that these patients had significantly high levels of ADMA that was independent of their age and weight (243). PCOS patients also found to have increased aggregation tendency of platelets and have substantially poor responses to the inhibitory effects of sodium nitroprusside on platelet aggregation as compared to their appropriately matched controls (308). Conversely, plethysmography method in a small study that involved only 23 patients has shown no significant difference in endothelial dysfunction between PCOS patients and their controls (79). Moreover, in a study on PCOS women that used cardiovascular magnetic resonance to measure endothelial function of vessels established that endothelial dysfunction (significantly reduced $P < 0.01$) was seen only in flow-mediated dilatation, but not with glyceryl trinitrate that is endothelial independent (244).

In a recent meta-analysis that studied and compared the results from several studies revealed that PCOS patients have significantly higher prevalence of endothelial dysfunction (low flow mediated dilatation) with subsequent higher risk of future cardiovascular events as compared to their age and weight matched controls. They also explained that these effects are independent of their (young) age and normal BMI. However, there was significant variation in the results due to difference in the measurements and the techniques (242). Similar findings were revealed in another meta-analysis that involved 2835 PCOS patients and 1930 non-PCOS healthy women and explained that PCOS patients had substantially higher degree of endothelial dysfunction (raised levels of ADMA) and had higher levels of other inflammatory and cardiovascular markers such as PAI-1 and CRP (245). Moreover, it has been suggested that the different methods that are used for evaluating endothelial dysfunction have very weak interrelationship. This was explained when correlation between different techniques of measuring endothelial dysfunction and intima-media thickness was explored in a small study on women with PCOS; there was no correlation between IMT and flow mediated dilatation nor between flow mediated dilatation with EndoPAT, augmentation index (AI), low flow mediated vasoconstriction and pulse wave velocity (309).

1.4.3 Relationship of endothelial dysfunction in PCOS with endocrine anomalies:

1.4.3.1 High free androgen index (androgens):

Clinical and biochemical hyperandrogenism are diagnostic criteria for the diagnosis of PCOS. Studies in men have shown that endothelial dysfunction is significantly correlated with lower total and free testosterone levels independent of other risk factors (310-312) and testosterone replacement has revealed to improve or even reverse these adverse effects (313). Several trials have demonstrated that hyperandrogenaemia in PCOS patients is associated with endothelial dysfunction (159, 301, 304, 307, 314). In one of the studies, it was explained that high ADMA levels in PCOS patients were directly related with the high free androgen index

levels when compared to controls (304). Similar findings were found in another study that recruited 102 participants including both PCOS patients and controls (315). In a cross sectional study that involved obese PCOS patients, it was found that higher levels of endothelial dysfunction markers including ADMA and PAI-1 were significantly correlated with fasting lipids, inflammatory and hormonal markers compared to their age and BMI matched controls. The flow-mediated dilatation was also significantly impaired in PCOS group (307). However, in a small study involving only 12 overweight or obese PCOS patients with high androgen levels compared to age and BMI matched controls, it was found that flow mediated dilatation did not differ (316). The role of androgens has also been explored in several studies suggesting that anti-androgens (such as spironolactone) treatment in addition to improvement in androgen levels resulted in significant improvement in endothelial dysfunction as shown by a substantial increase in FMD (317). The spironolactone induced improvement in endothelial function could also be partly due to inhibition of renin-angiotensin system (RAS) with subsequent reduction in aldosterone and renin levels, common in PCOS patients, as shown in several trials investigation cardiovascular outcomes (317-320). It has been suggested that while assessing the results of different studies in PCOS looking at the endothelial dysfunction; gender variation (321) and the likelihood of estrogenic effects of testosterone (after being transformed by aromatase) should also be taken into consideration (322).

1.4.3.2 Gonadotrophins:

Whilst the LH/FSH ratio is typical elevated in PCOS it does not form part of the diagnostic criteria (159). Several studies in PCOS patients looking at the endothelial dysfunction and cardiovascular diseases have revealed that luteinizing hormone levels were consistently elevated in them. However, there are not any specific studies available that could assess the specific associations in the PCOS patients (323).

1.4.3.3 Endothelial dysfunction and insulin resistance:

It is a known fact the endothelial dysfunction in women with PCOS has direct relationship with insulin resistance, common in PCOS, as evidenced from a range of studies done in the last 2 decades (301, 314, 324). PCOS patients with insulin resistance have also been established to be associated with other metabolic, cardiovascular and inflammatory factors such as abnormal lipids, elevated hs-CRP etc. though the outcomes are not very consistent (138). In one of the prospective studies that recruited 50 young PCOS patients and 50 age and weight matched controls found that insulin resistance measured in these patients was positively correlated with endothelial dysfunction and inversely related to adiponectin levels (301). These findings could be supported by findings in another study that revealed that flow mediated dilatation (marker of endothelial dysfunction) in obese PCOS patients who have normal insulin levels was not significantly different from their appropriate controls (316). In a small study, involving only 8 overweight and obese PCOS patients explained that levels of markers of endothelial dysfunction were directly correlated with the degree of insulin resistance present. As the severity of insulin increased, the levels of ADMA, PAI-1 increased and flow mediated dilatation decreased significantly (307). Further evidence came from the study that revealed that high levels of PAI-1 were directly correlated with the insulin levels in PCOS patients. There was elevated expression of PAI-1 expression in these PCOS when infused with insulin but it was found that effect was delayed and non-sustained in those who had insulin resistance (325). Indirect indication of insulin resistance being related to endothelial dysfunction can be obtained from clinical trials in which PCOS patients taking metformin showed significant improvements in flow mediated dilatation and ADMA levels along with inflammatory markers (306). Likewise, treatment with metformin for 12 weeks in PCOS patients resulted in significantly increased levels of DHEA-S while it decreased the levels of hormones like androstenedione, FSH, along with decrease in levels of glucose and inflammatory factors like tumour necrosis factor, interleukin-6, ICAM-1 and E-selectin (326).

Moreover, the levels of advanced glycation end-products (AGEs) (pro-inflammatory marker) and their receptors (RAGE) were established to be raised in patients with obesity and was determined that ovarian function could be compromised by excessive weight, common in patients PCOS but exact underling mechanism was not clear (327). The levels of AGEs have been established to be raised in PCOS patients as compared to their controls found in a separate study (328) and established that they play a central role in relating metabolic and reproductive disorders in patients having PCOS. In addition, interventions used to block the expression of RAGE have proved to be beneficial in these patients by improving insulin resistance (329). The expression of RAGE was much lower in the theca cells of ovaries in PCOS patients as compared to granulosa cells, which were predominant as compared to healthy controls (330). However, the evidence regarding association between insulin resistance and endothelial dysfunction in PCOS is still controversial as evidence in some of the studies that did not reveal any connection between them (331). In a study, that involved 54 overweight PCOS patients revealed that in spite of having insulin resistance there was no significant difference in endothelial dysfunction from the overweight health controls. The methods used to assess endothelial dysfunction and vascular health were evaluation of levels of ADMA, EndoPAT, PAI-1, and augmentation index and pulse wave velocity (243). Similarly, another study that involved 19 overweight and obese women with PCOS found endothelial dysfunction was not associated with obesity or insulin resistance. The authors used EndoPAT device to measure reactive hyperaemia index (RHI) for measuring endothelial dysfunction (332). Same findings were observed in a study that involved healthy PCOS women showing normal endothelial function despite having insulin resistance (331). Furthermore, substantially high levels of ADMA and increased insulin resistance measured by HOMA-IR were found in a study involving 50 participants as compared to controls while nitric oxide levels were very low in PCOS patients as compared to their controls. Although,

the patients had significantly high levels of insulin resistance but still there no significant correlation was found between insulin resistance and increased levels of markers of endothelial dysfunction (ADMA) and lower levels of nitric oxide (243). In conclusion, the evidence suggests that other cardiovascular risk factors are also involved in PCOS patients in addition to insulin resistance.

1.4.3.4 Endothelial dysfunction and inflammatory mediators:

There are several factors that have been suggested to play a vital role in the development of endothelial dysfunction in overweight and obese women having PCOS such as pro-inflammatory mediators (IL-6, IL-8), retinol, retinol binding protein-4, adipocytokines (adiponectin, leptin), visfatin and resistin (333). In a review, recently published, have suggested a possible role of adipocytokines in the regulation of insulin sensitivity in PCOS patients and explained their diverse role in various metabolic changes commonly found especially in this patient group (334). A recent research presented that high levels of visfatin found in PCOS patients were directly to high androgens and endothelial dysfunction (flow mediated dilatation. They also revealed that high visfatin as a predictor for endothelial dysfunction independent of age, weight, body fat mass, androgens, and insulin resistance (315). In summary, adipocytokines role in PCOS has been suggested in current literature but further large-scale studies are required to explore and validate this vital connection.

1.5 Management of PCOS:

Menstrual irregularity is one of the major presenting features of PCOS. Evidence suggests that the menstrual dysfunction and ovulation in PCOS patients could be improved with even just 5% of weight loss (86). COCPs are cornerstone of treatment in PCOS patients in which weight loss is unable to improve menstrual irregularity, with an additional benefit of contraception. COCPs could also be helpful for PCOS patients who also possess symptoms

of excessive androgens. Metformin decreases insulin stimulated secretion of androgens from ovarian theca cells (335). In a meta-analysis, it was proposed that metformin decreases serum androgen levels in PCOS; however, COCPS are more potent in improving serum androgens than metformin. Metformin can improve menstrual dysfunction in women with PCOS; however, it is not a treatment of choice if fertility is the primary concern (336).

Hirsutism is also a common presenting characteristic of PCOS patients. It is defined as male pattern excessive hair growth. Eflornithine is approved as a topical treatment for mild to moderate form of hair growth over the face only (337). The Endocrine Society recommends the use of either drug treatment or other forms of permanent hair removal such a laser therapy or electrolysis if the initial cosmetic treatment is not helpful (28). Systemic treatments are recommended for patients with severe form of hirsutism +/- acne after the addition of physical forms of hair removal. COCPs alleviate hirsutism in patients through several mechanisms including suppression of endogenous production of gonadotropins via negative feedback, by suppressing ovarian production of androgens, by augmenting SHBG and finally by preventing dihydrotestosterone (DHT) binding to its receptor (338). Cyproterone acetate is most commonly used for its anti-androgen properties. It acts via decreasing 5α-reductase production and by decreasing the binding of androgens to their receptors. It also decreases androgen production in ovaries via inhibiting secretion of gonadotropins (339). Spironolactone is an alternative to cyproterone acetate and is helpful in improving excessive hair growth in PCOS patients. It has anti-androgenic properties, binds to androgen receptor but is also a mineralocorticoid receptor antagonist. In addition, it suppresses the production of androgens in ovaries and adrenal glands (340). Flutamide and finasteride are competitive inhibitors of 5α-reductase enzyme sometimes used to treat clinical hyperandrogenaemia. Finasteride has shown to improve excessive hair growth as effective as spironolactone and is

safe to use (341). The use of flutamide is restricted because it has a small risk of hepatotoxicity (342).

Evidence suggests that approximately 40% of PCOS patients suffer from infertility issues, and the majority of cases are due to either oligo-ovulation (irregular or infrequent ovulation) or anovulation (343). It has been suggested in the guidelines that PCOS patients who are overweight or obese should be initially treated with diet and exercise therapy to reduce weight before starting any pharmacological treatment because evidence suggests that weight reduction could improve infertility significantly in these patients (343). Clomiphene citrate is an estrogen receptor antagonist that has been suggested by ESHRE/ASRM as a first-line of pharmacological treatment for reduced fertility in PCOS patients (344). It has been described that clomiphene usually could induce ovulation in approximately 80% of PCOS patients (344).

Obesity is one of the reasons that amplify insulin resistance in PCOS patients because it could prime the development of low-grade chronic inflammation in their adipose tissue (345). Further, excessive weight and obesity are associated with an augmented risk of infertility, impaired glucose tolerance, diabetes and coronary heart disease. The first step to treat overweight and obese PCOS patients is to offer diet and exercise therapy as the evidence suggests in bariatric surgery PCOS patient that weigh reduction causes significant improvements in androgen levels, insulin resistance and ovarian dysfunction (346). Other treatment options should be considered if lifestyle interventions are not successful after 6 months to one year. Several drug treatments have been suggested in the literature that could be potentially used in these patients**.** Orlistat is one of the drugs that has sound safety profile and has well-known place in treating obesity, including PCOS patients. However, it is not effective always. Orlistat decreases the absorption of fat from the intestine by irreversibly binding to and inhibiting the action of gastric and pancreatic lipases. Several studies have

shown that orlistat is more effective in causing weight loss in obese patients as compared to diet and exercise therapy or placebo. It has also shown to improve metabolic and cardiovascular risks associated with PCOS patients (347). However, the common side effects associated with orlistat that could limit its compliance and overall efficacy are flatulence and loose stools. Liraglutide, a glucagon-like peptide-1 analogue (GLP-1 analogue) has revealed to cause significant weight reductions along with improvements in fasting glucose, and markers of liver cirrhosis after 6 months of treatment in obese PCOS patients having NAFLD. However, they are very notorious in causing nausea and vomiting and long-term safety data is not available especially in females who want to conceive (348). Somatostatin analogues such as octreotide can also be potentially useful for treating obesity in PCOS patients. It has been revealed that weight loss associated with octreotide therapy was very effective in improving insulin levels and ovulation rate in obese PCOS patients as compared to placebo treatment (349). However, further evidence regarding the safety and efficacy of somatostatin analogues is required before they can be used safely in PCOS women during their reproductive age.

1.5.1 Impaired glucose tolerance:

The guidelines suggest that in PCOS patients only fasting plasma glucose measurement is not enough to evaluate their glycaemic status (35). They should have standard 75g oral glucose tolerance test (OGTT) especially who have additional risk factors for development of diabetes mellitus and with BMI $\geq$30 kg/m^2. Diet and exercise therapy is the most effective intervention to prevent diabetes risk in PCOS as shown in Diabetes Prevention Program trial that the risk reduced by 60% while metformin alone reduced it to 31%. Further, 10 years follow up revealed that lifestyle therapy was still better than metformin in retarding the risk of future diabetes development (350). The endocrine society recommends OGTT to screen IGT and T2DM in adolescents and adult women with PCOS, because they are at high risk for

such abnormalities. Rescreening in women with PCOS is suggested every 3–5 years, or more frequently only if clinical factors such as central adiposity, significant weight gain, and/or symptoms of diabetes develop (350).

1.5.2 Impaired lipid metabolism:

PCOS patients are associated with impaired lipid metabolism, have decreased level of HDL-C and elevated levels of total cholesterol, LDL-C and triglycerides (351). Statin therapy in PCOS women helps in reducing androgen levels and improves dyslipidaemia. In vitro, statins have been revealed to hinder the production of steroids within theca cells of ovaries (352). A recent meta-analysis including RCTs in PCOS patients determined that statins substantially decrease serum testosterone and LDL-C levels (353). Further evidence demonstrates that flutamide decreased LDL-C and triglycerides levels in PCOS patients via androgen receptor blockade; however, it can cause hepatotoxicity (353).

1.5.3 Metformin in PCOS:

Metformin is most commonly used drug for controlling blood glucose in diabetes. It decreases liver glucose production and improves insulin sensitivity of cells using insulin for utilising glucose. The evidence suggests that women with PCOS have insulin resistance so metformin has also been used to treat PCOS. Studies suggest that metformin increases ovulation rate in PCOS patients but it is still debatable either this beneficial effect is independent of associated weight loss or not (354). In a meta-analysis of randomized controlled trials it was recommended that ovulation rate was significantly higher in PCOS group (46%) on metformin as compared to those who were on placebo (24%) (355). Moreover, ovulation rate observed in PCOS patients was substantially higher (76%) in PCOS patients who were on both metformin and clomiphene citrate as compared to those who were on clomiphene alone (42%). The efficacy of metformin inducing ovulation in PCOS patients has been questioned. In one of the double blind studies that included 228 PCOS patients

detected that clomiphene alone showed better ovulation rate (72%) as compared to co-administration of clomiphene and metformin (64%). Legro *et al.* observed the same results in another randomized trial of a large group (626) of anovulatory infertile obese PCOS women (356). They revealed that conception rate was twofold higher in clomiphene alone or in combination with metformin group as compared to metformin monotherapy. Furthermore, live birth rate was threefold higher in clomiphene alone or in combination with metformin group as compared to metformin monotherapy. However, a recent meta-analysis revealed that metformin treatment improved the pregnancy rate in PCOS patients. Further, pregnancy rate was better with metformin and clomiphene combined therapy as compared to clomiphene monotherapy. Though, there was no clear evidence that metformin therapy increased live birth rate whether it was used alone or in combination with clomiphene citrate. Two further meta-analyses printed in the recent years looked at whether clomiphene or metformin treatment was better in improving infertility in non-obese PCOS (357). The authors did not find any statistically significant difference in ovulation rates, pregnancy rates or live births between patients who were either in metformin monotherapy group or in clomiphene monotherapy group. In brief, evidence suggests that metformin monotherapy increases clinical pregnancy rates in PCOS women but with no evidence that this effect is independent of weight loss (354). Likewise, it is also not clear whether metformin addition to clomiphene is better than clomiphene citrate monotherapy in improving fertility in this patient group and also metformin role in managing anovulatory infertility remains unproven in those PCOS patients who do not respond to clomiphene monotherapy. The subclinical markers indicative of increased future cardiovascular risk such as increased coronary artery calcification and increased thickness of carotid-artery-intima-media (cIMT) are high in PCOS patients (358). Evidence suggests that insulin sensitizers (metformin, thiazolidinediones) could be helpful as they improve blood flow to tissues by improving endothelial dysfunction in these patients

(358). Increased androgens in PCOS are also an important risk factor for future cardiovascular events; however, the data related to it is also still debateable. There is evidence that androgens modulate body vasculature in addition to modulating lipid and glucose metabolism. However, women with regular menstruation and high androgens (isolated hyperandrogenaemia) have lower occurrence of markers of future cardiovascular events as compared to women who have PCOS (359).

1.6 Role of vitamin D in PCOS:

Vitamin D (colecalciferol) is a fat-soluble vitamin that was recognised as a calciferol in the 20th century there are two main forms of colecalciferol: vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol). Although, human body can synthesise some colecalciferol, the amount may not be sufficient due to several reasons, suggesting additional sources are required to sustain sufficient levels. Vitamin D has been classified as a hormone, rather than just a vitamin as specific vitamin D receptors are present nearly on all cells of human's body [7]. Since vitamin D3 (colecalciferol) is more potent it is recommended over vitamin D2 (ergocalciferol) supplementation. Vitamin D acts through vitamin D receptors (VDRs) to regulate transcription of genes. VDRs are found on various tissues all over the body such as bones, ovaries and parathyroid glands (360). Vitamin D deficiency increases parathyroid hormone (PTH) production, which is regulated through levels of serum calcium and vitamin D, and increased PTH is independently associated with PCOS, anovulatory infertility and increased testosterone (361). It has been suggested that the combination of vitamin D deficiency and dietary calcium insufficiency (because serum calcium regulates PTH release) may be largely responsible for the menstrual irregularities associated with PCOS (362). However, it has been proposed that vitamin D sufficiency is more important than high calcium intake at maintaining desired values of PTH (363). VDRs play a vital role in the production of ovarian hormones especially oestrogen and it has been proposed that vitamin D

maintains the production of oestrogen via regulating extracellular calcium homeostasis through regulating the aromatase enzyme gene (362, 364). It has been shown in several studies that vitamin D stimulates the production of estrogen and progesterone in ovarian tissue through amplification of aromatase activity (364). Moreover, studies have shown that menstrual cycles are affected by sunshine (365). In summer, increased ovarian activity (increased ovarian follicle size, frequency of ovulation and shorter menstrual cycle) has been observed than in winter in women living in a continental climate at temperate latitudes. This could be due to the impact of natural light; the more sunshine 2–3 days before the presumed day of ovulation, the shorter the cycle (365).

Low vitamin D levels have been described in PCOS patients, with the majority of patients having values ≤20ng/ml and with average values between 11-31ng/ml (366, 367). Conversely, in a study to compare PCOS women to age and BMI matched control healthy women, PCOS patients had a considerably higher levels of vitamin D (29·3 ng/ml vs 19·4 ng/ml). Therefore, whether low vitamin D levels are associated with PCOS is unclear. Studies have described an inverse relationship of BMI, body fat and waist measurements with serum vitamin D levels in PCOS patients (368). As vitamin D is fat soluble, it is suggested that low vitamin D deficiency in PCOS patients is related to obesity because a higher percentage of it being sequestered in fat tissue thus decreasing bioavailability (368). However, others suggest that there is reduced biosynthesis of vitamin D in obese subjects because they spend less time exposed to sunlight. There is also a possibility that vitamin D metabolism and dietary habits of both obese and non-obese subjects may vary, but a study of vitamin D across BMI ranges showed no differences (366).

1.6.1 Relationship between vitamin D and insulin resistance in PCOS:

Low vitamin D in PCOS patients has been associated with insulin resistance and metabolic syndrome (369). Vitamin D acts via multiple pathways to enhance insulin action such as

increasing the synthesis and release of insulin, augmented insulin-receptor expression and via decreased production of inflammatory cytokines (370). Vitamin D levels have been negatively correlated with fasting insulin levels and insulin resistance as measured by HOMA-IR; however, this significant correlation was lost after adjustment of BMI in the control group. Similar findings were found in as study that demonstrated that low levels of vitamin D were linked with HOMA-IR and BMI (371). Obesity has been suggested a confounding factor in all those studies looking at the relationship between vitamin D deficiency and insulin resistance in PCOS women. However, one study revealed that PCOS patients who had severe vitamin D deficiency had severe insulin resistance that was independent of their weight and other metabolic features, and others have shown a negative correlation between vitamin D levels, insulin resistance and BMI (372). The RCTs looking at the effects of vitamin D supplementation in vitamin D deficient PCOS patients have shown beneficial effects on insulin secretion and resistance (366). However, one pilot study did not shown any benefit of vitamin D replacement on insulin resistance in lean PCOS patients (373). In summary, there is evidence, suggesting a relationship of vitamin D deficiency with insulin resistance in PCOS patients, but data related to vitamin D replacement is lacking and needs further studies (366).

1.6.2 Vitamin D and fertility in PCOS:

Vitamin D has been suggested to be important in reproduction in PCOS with of vitamin D deficiency being associated with ovarian follicular arrest, menstrual irregularity and infertility due to impaired regulation of calcium metabolism (362). This is supported by animal studies showing impaired follicular growth with vitamin D deficiency. In several observational studies looking at the association of vitamin D with infertility in PCOS women high follicular levels of vitamin D were associated with an increased pregnancy rate during In Vitro fertilisation (IVF) following adjustment for age, weight, and ethnicity (374). In accord with

this others reported that PCOS patient with low vitamin D had decreased rates of follicular development and a lower pregnancy rate after clomiphene stimulation, independent of their age and weight. Vitamin D therapy has shown significant improvements in menstrual irregularity with subsequent pregnancy rates in PCOS patients suggesting the importance of vitamin D replacement if there is vitamin D deficiency (375).

1.6.3 Vitamin D status and hyperandrogenaemia in PCOS:

Observational studies have found relationships between markers of hyperandrogenism (DHEAS, testosterone, SHBG and FAI) and vitamin D status (376). Women with PCOS and excessive hair growth (hirsutism) have been found to have significantly lower levels of vitamin D compared to women with PCOS without hirsutism (367). In women with PCOS, vitamin D levels have been positively correlated with SHBG and negatively correlated with the degree of hirsutism, FAI, total testosterone and DHEAS (367, 371, 372, 377). Additionally, SHBG levels were lower in women with PCOS with severe vitamin D deficiency; however, this was no longer significant after adjusting for BMI and waist hip ratio (372). A similar result was found in two further studies where a relationship between SHBG and vitamin D status was no longer significant after controlling for BMI, suggesting obesity as the common determinant for both vitamin D and SHBG (367, 371). It has been further proposed that the associations between hyperandrogenism and vitamin D status may be due to the reduction in SHBG that results from obesity (371, 372). Very few studies have looked at the effects of vitamin D supplementation on measures of hyperandrogenism and have shown no significant changes in levels of testosterone, FAI and SHBG (373, 378). One small uncontrolled study where women with PCOS were supplemented with vitamin D combined with calcium reported clinical improvement of acne vulgaris (362), however, there were no other clinical improvements in the other signs of hyperandrogenism such as hirsutism, acanthosis nigricans and alopecia (362). The authors have suggested further

randomized controlled trials to investigate the effect of vitamin D supplementation on hirsutism and androgen levels in women with PCOS.

1.6.4 Vitamin D and cardiovascular risk factors in PCOS:

Vitamin D deficiency is associated with a high risk of future cardiovascular disease and increased cardiovascular related mortality (379). Adverse associations of vitamin D deficiency with risk factors such as fasting glucose levels, fasting lipid profile, systolic and diastolic blood pressures, leptin and hs-CRP have been reported (367). A large observational study that included 206 PCOS patients found that patients with metabolic syndrome had low levels of vitamin D as compared to those PCOS women who did not have any features of metabolic syndrome (367). The interventional studies looking at the effects of vitamin D replacement therapy have shown to improve several cardiovascular risk factors in addition to improving insulin resistance in PCOS patients. These trials have reported improvements in dyslipidaemia, decreased fasting plasma glucose and abdominal circumference, though there were no significant improvements in BMI, HDL-C and blood pressure (373). Recently, a trial described the improvement in hs-CRP after atorvastatin therapy was due to significant increase in vitamin D levels as compared to those patients who were on placebo suggesting important role of vitamin D supplementation in PCOS patients for improving future cardiovascular risk (380).

In summary, it remains unclear if vitamin D deficiency is more prevalent in PCOS patients; however, it is related to insulin resistance, reduced fertility, hirsutism and increased cardiovascular risk factors. Evidence suggests that vitamin D has a role in the pathogenesis of PCOS being correlated to insulin resistance, obesity, menstrual irregularities, subfertility and hyperandrogenaemia. Vitamin D replacement therapy has shown small improvements in insulin resistance, improved menstrual regularity and pregnancy rate though these need confirmation.

1.7 Role of sodium glucose cotransporter 2 inhibitors (empagliflozin) in PCOS:

Currently there are no studies of sodium-glucose cotransporter 2 (SGLT2) inhibitors in PCOS but there are theoretical reasons why they may be of value. Empagliflozin, is a direct inhibitor of SGLT2, which is a new treatment option for T2DM, that has recently shown to induce favourable effects on arterial stiffness and vascular resistance in both T1DM and T2DM patients (381, 382), and noted above to be features reported in PCOS. It improved cardiovascular risk significantly in patients with T2DM, reduces oxidative stress, and suppresses markers of inflammation (a feature of PCOS) and fibrosis (383). There was a dose-response effect for 10mg and 25mg empagliflozin (384), but in the EMPA-REG study empagliflozin 10mg and 25mg dose were used separately without titration (381). Moreover, empagliflozin has an insulin-independent mechanism (promote urinary glucose excretion by inhibiting glucose reabsorption in the kidney) and does not cause hypoglycaemia in normoglycaemic individuals (381, 384). Hence, in empagliflozin treatment group, patients were randomised to empagliflozin 25mg daily to get the maximum metabolic response with comparable duration to the metformin treatment group who received metformin SR 1500mg, (385). Given the reported cardioprotective effects of empagliflozin and the associated changes in insulin resistance, plasma volume, weight/fat mass and inflammation that are common pathological features of PCOS, this would suggest a pilot trial of empagliflozin on hormonal, metabolic and cardiovascular risk factors in patients with PCOS would be warranted.

The recommended starting dose for empagliflozin is 10 mg once daily for monotherapy and add-on combination therapy with other medicinal products for the treatment of diabetes. In patients who have an eGFR $\geqslant$60 ml/min/1.73 m^2 the dose can be increased to 25 mg once daily. The maximum daily dose is 25 mg. Empagliflozin is used for insufficiently controlled

type 2 diabetes mellitus as an adjunct to diet and exercise either as monotherapy when metformin is considered inappropriate due to intolerance or in addition to other medicinal products for the treatment of diabetes.

The most common side effects are increase in urination frequency or amount of urine, painful urination, urinary tract infections, genital infections, itching and dyslipidaemia. The other uncommon side effects are symptoms and signs of volume depletion, hypotension, syncope and increased haematocrit. Diabetic ketoacidosis (DKA) is a serious problem that happens to people with diabetes when chemicals called “ketones” build up in their blood. As PCOS, patients recruited did not have diabetes then this was expected rarely.

1.8 Hypothesis and aims of this thesis:

PCOS is the commonest endocrine disorder of young females in their reproductive age that is associated with increased future cardiovascular risk. There are several risk factors found in PCOS that put them at increased cardiovascular risk including obesity, insulin resistance, and hyperandrogenaemia. The studies have revealed that all such factors contribute to the development of endothelial dysfunction in PCOS that could be the underlying mechanism leading to the development cardiovascular events. Recently, low vitamin D in PCOS has been found to have an inverse correlation with obesity, insulin resistance, hyperandrogenaemia, endothelial function and NAFLD and put them at high risk of cardiovascular events. Therefore vitamin D replacement may represent an attractive option for the improvement of cardiovascular risk in women with PCOS; however, clinical data are lacking. Additionally, the effects of vitamin D supplementation on liver fibrosis markers in young overweight and obese women with PCOS are not known. The SGLT2 inhibitor empagliflozin, has been shown to improve multiple risk factors in type 2 diabetes patients in both animal and human studies including insulin resistance, endothelial dysfunction and resulted in weight loss thus suggesting the therapeutic potential of this drug in PCOS also.

1.8.1 Hypothesis:

Hypothesis for vitamin D study:

Three months of vitamin D supplementation will improve hormonal, metabolic and cardiovascular risk markers in vitamin D deficient, overweight and obese women with PCOS.

Hypothesis for empagliflozin vs metformin study:

Three months of empagliflozin treatment will improve hormonal, metabolic and cardiovascular risk markers in overweight and obese women with PCOS.

1.8.2 Aims:

The work I conducted during this PhD was aimed to assess the therapeutic potential of vitamin D and SGLT2 inhibitor (empagliflozin) by answering the following questions:

1) Does vitamin D supplementation improve cardiovascular risk factors especially insulin resistance, hyperandrogenaemia and NAFLD in vitamin D deficient, overweight and obese women with PCOS?

2) Specifically does vitamin D supplementation improve underlying endothelial dysfunction in vitamin D deficient, overweight and obese women with PCOS?

3) Does empagliflozin improve cardiovascular risk factors in overweight and obese women with PCOS better than or equal to treatment with metformin?

4) Specifically, does empagliflozin improve endothelial dysfunction in overweight and obese women with PCOS better than or equal to treatment with metformin?

1.8.3 Primary and secondary endpoints:

1.8.3.1 Primary endpoints:

Vitamin D study:

Cardiovascular risk assessment by hs-CRP, HOMA (fasting glucose & insulin), fasting lipid profile and ELF markers

Empagliflozin versus metformin study

Endothelial function as measured by RHI (reactive hyperaemia index)

1.8.3.2 Secondary endpoints:

1.8.3.3 Vitamin D study:

1. Hormonal parameters including testosterone, SHBG and FAI
2. Inflammatory marker hs-CRP
3. Endothelial function by EndoPAT and microparticles

1.8.3.4 Empagliflozin versus metformin study

1. Inflammatory markers (hs-CRP)
2. Insulin resistance as measured by HOMA-IR (fasting glucose & insulin)
3. Body weight
4. Blood pressure
5. Fasting lipid profile
6. Hormonal parameters including free androgen index
7. Endothelial function measured by microparticles

2 Chapter 2: Methods and materials:

2.1 Study designs and protocols:

2.1.1 Trial approvals:

Both the studies were approved and sponsored by the local Research and Development department of Hull and East Yorkshire Hospitals NHS Trust. The vitamin D study was a double blind randomised placebo-controlled study to look at the effects of colecalciferol (Vitamin D) on hormonal, metabolic and cardiovascular risk factors in patients with PCOS, approved by Health Research Authority, NRES Committee Yorkshire & The Humber - Leeds East (REC reference: 14/YH/1125). The Empagliflozin study was a randomised open-label parallel study to look at the effects of empagliflozin versus metformin on hormonal, metabolic and cardiovascular risk factors in women with PCOS. Empagliflozin study was a CTIMP (clinical trial of an investigational medicinal product) (EudraCT number: 2016-004435-20), therefore, was first approved by MHRA (medicines and healthcare products regulatory authority) (Ref: 21411/0254/001-0001) and then by Health Research Authority, Yorkshire & The Humber - Leeds East Research Ethics Committee (REC reference: 17/YH/0118).

2.1.2 Subjects recruitment:

For both the randomized trials, the PCOS patients were primarily recruited from the outpatient clinic endocrine clinics. The patients were also recruited from the PCOS biobank from those patients who had already consented to be contacted to participate in future. After identifying potentially suitable patients, an invitation letter from the research team was sent to them together with participant information asking them to contact the research centre should they be interested in taking part. After contacting the research team an appointment was booked to discuss the study. They had the opportunity to go home and discuss the study with

members of their family or with the General Practitioner. If they decided to take part, the participants were asked to sign the informed consent form at the research centre before or at the time of the screening visit; written informed consent was taken. No vulnerable subjects or non-English speakers were recruited. Reasonable parking and travel expenses were reimbursed.

2.2 Interventions:

2.2.1 Vitamin D (colecalciferol):

2.2.1.1 Dosage:

In this randomized double blind trial, each participant received either Fultium-D_3 3,200 IU capsule (colecalciferol 3200IU) or placebo daily for three months. Each capsule contained: 3,200 IU Colecalciferol that is equivalent to 80 micrograms vitamin D3. Full details about Fultium D3, 3200IU is available on the underlying website:

http://www.mhra.gov.uk/home/groups/par/documents/websiteresources/con428314.pdf

The study drug was supplied by Internis Pharmaceuticals and was despatched to trial site's pharmacy for storage. The study drug was stored and dispensed by the trials site pharmacy in accordance with Good Clinical Practice and Good Manufacturing Practice.

2.2.2 Bolamyn-SR 500mg prolonged release tablets (metformin hydrochloride):

One prolonged-release tablet contains 500 mg metformin hydrochloride that is equivalent to 390 mg metformin.

2.2.2.1 Dosage and administration:

In the empagliflozin versus metformin trial, half of patients were on metformin (Bolamyn) 1500mg daily for three months. The usual starting dose is one tablet once daily. The

maximum recommended dose in PCOS is three tablets daily (1500 mg). The dose was increased in increments of 500 mg every week, up to a maximum of 1500 mg once daily with the evening meal.

For the treatment of vitamin D deficiency, National Institute for Clinical Excellence (NICE-UK) recommends fixed loading doses of vitamin D (up to a total of about 300,000IU) given either as weekly or daily split doses. https://cks.nice.org.uk/vitamin-d-deficiency-in-adults-treatment-and-prevention. It's also normal clinical practice to supplement 3,200IU of vitamin D daily for 3 months for vitamin deficiency. The vitamin D levels were only measured at baseline (before vitamin D supplementation) and at final visit (post vitamin D supplementation). Since the study was a double blind placebo control study I did not measure their vitamin D levels during the study.

In a recently published meta-analysis several trials have been mentioned that used daily vitamin D regimens (doses ranging from 500-5000IU daily) for 12 weeks and have shown significant beneficial effects in patients with type 2 diabetes (386). In the current study, vitamin D levels normalised after 12 weeks of 3200IU daily of vitamin D supplementation, however, longer duration studies are required to see whether there will be a further improvement in these markers in women with PCOS.

2.2.3 Empagliflozin (jardiance):

In the empagliflozin versus metformin trial, half of patients were on empagliflozin (Jardiance) 25 mg daily for three months.

2.3 Procedures:

2.3.1 EndoPAT 2000:

EndoPAT 2000 (Itamar Medical Ltd) is a medical device that is used to measure endothelial function non-invasively. EndoPAT is based on the Peripheral Arterial Tone (PAT™) signal

technology. It is Food and Drug Administration (FDA) approved and is CE-marked. The FDA approval was obtained by comparing results of PAT Technology with a more invasive catheterization technique that directly evaluated endothelial dysfunction in coronary vasculature. EndoPAT is simple to perform, and is independent of both operator and interpreter. EndoPAT has been used for major epidemiological studies such as the Framingham Heart Study.

2.3.1.1 PAT signal:

EndoPAT uses the PAT signal that is recorded from the fingertip by measuring pulsatile arterial volume changes in the fingers using unique bio-sensors. These pulsatile fluctuations are produced by making a downstream response of reactive hyperaemia prompted by a standard occlusion of the artery for 5 minutes. The occlusion is done using a regular blood pressure cuff. When the blood pressure cuff is released, the resulting increased blood flow causes dilatation of artery, Flow Mediated Dilatation (FMD), which is endothelium dependent. The resulting hyperaemia causes an upsurge in the PAT signal amplitude which is recorded by EndoPAT. The total test duration is 15 minutes and the results of the test are automatically determined and finally an EndoScore is produced. This score represents the current health state of endothelial cells.

Figure 2.1: EndoPAT 2000 apparatus

2.3.1.2 EndoPAT 2000 procedure:

In order to evaluate the effects of vitamin D, empagliflozin and metformin on cardiovascular, hormonal and metabolic risk factors reactive hyperaemic index (RHI) was measured using EndoPAT 2000 at the baseline visit and at the end of the intervention (final visit) in both the colecalciferol & PCOS trial and Empagliflozin versus Metformin in PCOS studies.

PCOS patients attended the research centre fasting. After having the initial demographics done, the patients were sat comfortably for 15 minutes to attain a steady cardiovascular state. In the study room the temperature was maintained between 21-24ºC. The fingers of subjects were examined for any deformities that could affect the study results. The blood pressure was recorded from the patients' control arm (that was not used for occlusion) for at least 5 min before the beginning of the test as recommended in the EndoPAT manual. Then the patient was seated comfortably and supported the hands with arm supports to be kept them at the level of the heart. A blood pressure cuff was then placed on the upper arm of the nominated

test side. The PAT probes were placed inside the holes of the arm support and the patient was advised to insert both index fingers into the probes.

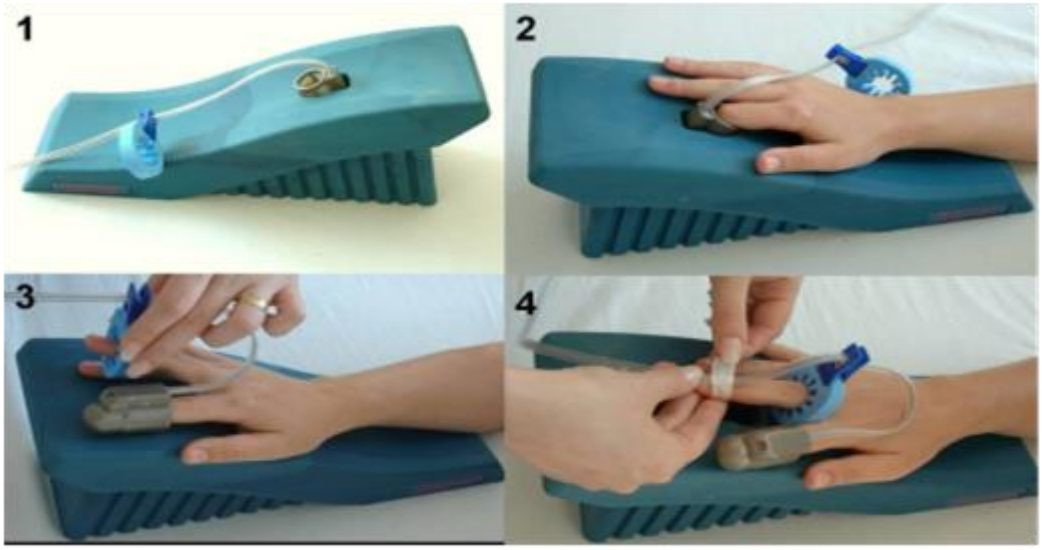

Figure 2.2: Applying and preparing PAT probes

The probes were connected via pneumoelectric tubes to the EndoPAT device and were controlled via its software installed in the computer. The patient was advised to remain relaxed during the test and refrain from talking as it could affect the results. The PAT signals were recorded in three steps as follows:

- Baseline phase for 5 minutes in a relaxed state
- Occluded phase for 5 minutes in which the brachial artery was occluded in the test arm by blood pressure cuff inflation to a supra-systolic level (the suggested cuff pressure is 60 mmHg above systolic blood pressure of patient and not less than 200 mmHg)
- Post-occlusion phase for 5 minutes in which the pressure cuff was deflated quickly.

2.3.1.3 EndoPAT correlation with cardiovascular events:

Endothelial dysfunction has been suggested to correlate with cardiovascular risk and future cardiovascular events in patients at high risk. The large scale population based studies such as

the Framingham Heart study have used the EndoPAT for endothelial function assessments. The authors in a large cross-sectional analysis of data from the Framingham heart study established a significant inverse correlation between RHI and several cardiovascular risk factors especially smoking, diabetes, total cholesterol, HDL-cholesterol and lipid lowering therapy (293). Further in a recent trial, patients with unexplained chest pain (low-risk findings during stress testing) and/or the absence of new obstructive lesions by an invasive coronary angiogram were assessed using EndoPAT. It was revealed that patients with low RHI values were independently associated with higher cardiovascular adverse events (cardiac death, myocardial infarction, revascularization or cardiac hospitalization) during a 7-year follow-up period (295).

2.3.1.4 Reproducibility of EndoPAT results:

The coefficient of variation was 18% when the reproducibility and viability of EndoPAT analysis was evaluated in adult patients (387). The EndoPAT test is both operator and interpreter independent as suggested while comparing the reliability and reproducibility of EndoPAT and flow mediated dilatation results that revealed lower within-day variability for the flow mediated dilatation as compared to EndoPAT measurements (10% versus 18%). The between-day variability was similar (11%) and reactive hyperaemic index measurements done by flow mediated dilatation and EndoPAT were strongly correlated (388).

2.3.2 Flow cytometry:

2.3.2.1 Principles behind flow cytometry:

Flow cytometry has the capability to calculate the optical and fluorescence features of any particle or even a single cell in a fluidic stream when they pass through a light source including nuclei, microorganisms and chromosomal preparations. The different features of cells are analysed and are differentiated from each other on the basis of their size, granularity

and fluorescence (389). The basic working principle behind flow cytometry is scattering of a light source with fluorescence emission, which happens when a light commonly from a laser beam (excitation source) hit the moving particles in the sample as explained in figure 2.3 adapted from (389).

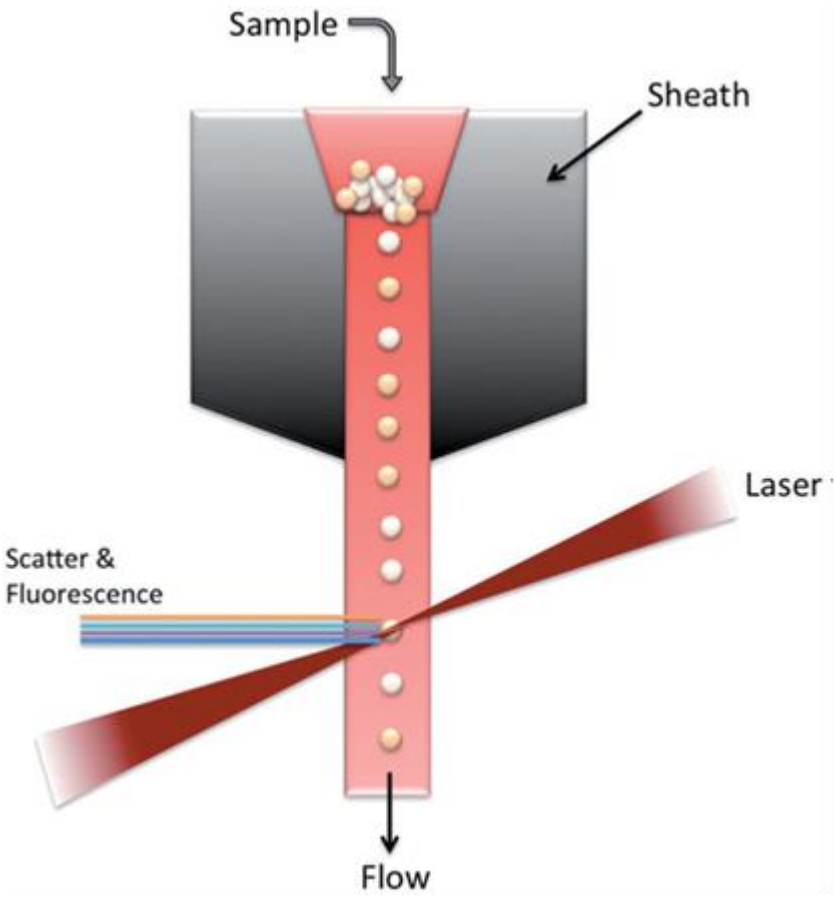

Figure 2.3: Basic working principle behind flow cytometry.

Depending on the detection of nuclear antigens, cytoplasmic features or membrane components flow cytometry can be used in numerous applications. Furthermore, in addition to detecting whole cells, small cellular portions can also be detected using flow cytometry including nuclei, chromosomes, protein components, DNA, RNA, cytokines and hormones. It is also used to quantify the number of microparticles within a cell suspension that pass through a flow cell after being injected in the machine. The cell suspension injected lies

within the centre of the surrounding sheath fluid. The pressure of the sheath fluid creates the flow that creates and transports cells and microparticles in a single file that are then exposed to the laser source.

The laser light scatters in either in a forward direction or to the side direction. The forward scattered (FSC) light is captured by forward scatter detector, which measures cell size or microparticles and distinguishes viable cells and cellular debris. Side scatter detectors collect the side scattered (SSC) light, which provides information about the cellular contents, internal complexity or granularity of the cell (figure 2.4) adapted from (389).

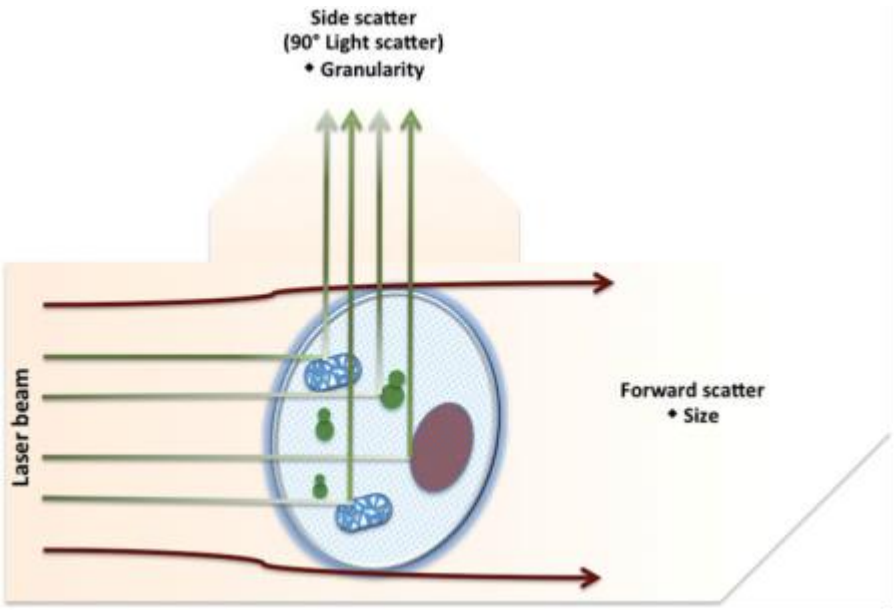

Figure 2.4: Forward light scatter is proportional to size while side scatter is proportional to cellular internal complexity or granularity

While analysing the cells or microparticles fluorescent label antibody is added to the sample that forms a complex with the antigen under investigation. The subsequent complex formed emits a certain wavelength fluorescent light that is detected by the flow cytometer. The detected fluorescent light is transformed to an electric signal that correlates with the number

of antigens on the cellular surface or on microparticles, which are then quantified. The different components of flow cytometry are explained in figure 2.5 adapted from (389, 390).

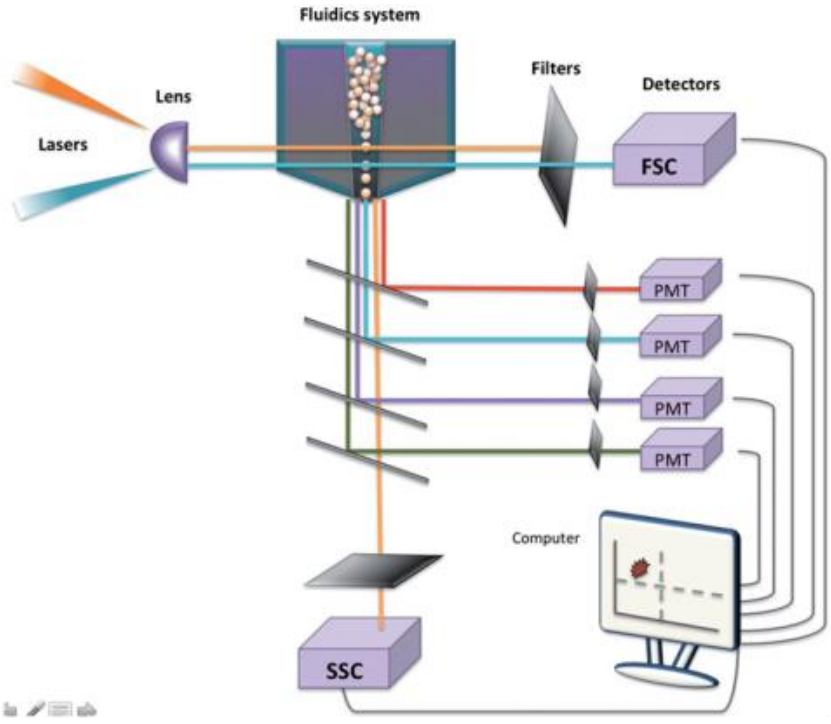

Figure 2.5: Flow cytometer structure and components.
Legend: Abbreviations: PMT, Photomultiplier tube (photodetectors); FSC, Forward scatter; SSC, Side scatter

2.3.2.2 Preparation and separation of endothelial microparticles (EMPs):

Blood samples were taken from an antecubital vein when the patient presented fasting to the department of Academic Diabetes Endocrinology and Metabolism, Hull Royal Infirmary. The sample was collected in EDTA (ethylenediaminetetraacetic acid) containing vacutainer. Platelet free plasma (PFP) samples were prepared using serial centrifugation steps; firstly sodium citrate-anticoagulated whole blood tubes were spun at 180 x g for 10 min (at room temperature to avoid cold-induced platelet activation) and then platelet-rich plasma obtained

was then subsequently spun at 12,000 x g for 10 min to remove platelets. From this, 25µL of PFP was incubated with 5 µL of either antibodies (CD31, CD54, CD62, CD105, CD106, CD144 and CD146 in vitamin D PCOS study and CD31, CD54, CD62, CD105, CD106, CD142 and IgG Negative control in Empagliflozin versus Metformin study), in the dark at room temperature for 30 minutes. Quantification of samples was achieved by adding 150 µL of filtered (0.1 µm) phosphate buffered saline (PBS) (Fisher Scientific, UK) and counting beads (25 µL, UK) immediately prior to analysis by flow cytometry. Table 2.1 explains the different microparticles in detail, adapted from (391).

Origin and types of microparticles	
Origin	**Types**
Endothelial Cells	CD31 (PECAM-1) CD54 (ICAM-1) CD62E (E-selectin) CD105 (Endolgin) CD106 (VCAM-1) CD144 (VE-Cadherin) CD146 (MCAM)
Subendothelial tissue	CD142 (tissue factor)

Table 2.1: Showing different types of microparticles

Legend: CD, Cluster of differentiation; PECAM-1, platelet endothelial cell adhesion molecule-1; ICAM-1, intercellular cell adhesion molecule-1; E-selectin, endothelial selectin; VCAM-1, vascular cell adhesion molecule-1; VE-cadherin (vascular endothelial cadherin); MCAM, melanoma cell adhesion molecule

2.3.2.3 EMPs quantification:

EMPs assessment was done using flow cytometry technique on a Becton Dickinson FACS Calibur instrument (BD Biosciences, Oxford, UK) in Vitamin D study and on Accuri® C6 (BD Biosciences, Oxford, UK) in Empagliflozin versus Metformin Study. Both machines are calibrated, cleaned and serviced on a regular basis. Both are reliable instruments and had been used for multiple publications before (392-394).

2.3.2.3.1 Gating and the threshold setting for microparticles:

The upper and lower thresholds were established in order to detect an event by the flow cytometry. It was necessary to increase the chances of getting the maximum population of interest and to eliminate debris, noise and any other undesired events. The higher threshold

was set to demarcate the population of interest (EMPs) from platelets and other minor cellular debris.

2.3.2.3.2 Establishing lower threshold:

The first step was to create the noise floor as all flow cytometry machines have an intrinsic noise due to signals from tiny particulate matter in numerous channels. This is not avoidable, and it is a balance between losing the population of interest and false positives due to the noise. In order to establish the lower threshold, deionised (DI) 0.2 µm filtered water was first run and counted the number of events at several thresholds. The BD Accuri machine has a better separation index (SI) with a side scatter scale, therefore, side scatter (SSC) was used for setting the gate. At forward scatter-Height (FCS-H) and side scatter-Height (SSC-H) gates only 3 -4 events were recorded for DI water that were labelled as noise events.

2.3.2.3.3 Setting the gates:

Side scatter Megamix beads were used that consists of numerous sizes ranging from 0.16 to 0.8 µm. The predominant populations are 0.16, 0.24, 0.3, 0.5 and 0.8 um bead sizes. Since 0.16 um beads fell in the noise region and being aware of the limitation of the BD Accuri for having a low separation index (SI) for microparticles of less than 0.3um, the lower gate was set at 0.3um fluorescent laser-1 (FL1) peak of SSC megamix. FL1 peaks were used as megamix beads are fluorescein isothiocyanate (FITC) conjugated. The higher gate was set at 0.8 um as beyond this size other cellular fragments interfere with calculation of events.

2.3.2.3.4 Counting of events:

Counting beads ranging from the size 4-6 µm were used for quantifying the number of microparticles per microliter of plasma. A separate gate R5 was formed around these beads population, whereas, the microparticles were counted at R4 gate. The microparticles were calculated for every 5000 counting beads in R5 gate. Since there are 1000 beads per µl of the

counting beads mix, the absolute count for microparticles/µl of plasma was calculated using the formula:

$$\frac{Number\ of\ events\ in\ R4}{R5(5000)} \times 1000$$

2.3.2.3.5 Compensation:

As all the antibodies and beads were FITC conjugated, there was no need for the application of compensation. In order to rule out any non-specific bindings of antibodies, an IgG auto control was run along with all samples to further validate the results.

2.3.3 TANITA-body composition analyser:

TANITA-body composition analyzer, Model: BC 418 MA (TANITA Corporation Itabashi-ku, Tokyo, Japan), was used to calculate basal metabolic rate, body fat percentage, body fat mass, fat free mass, bioelectrical impedance and total body water. It is shown in figure 2.6 below. The patients were asked to stand on the TANITA scale bare footed to measure percentage of body composition. This procedure takes 5 minutes and was done both at baseline and at the final visit after the supplementation of vitamin D, empagliflozin and metformin therapy.

Figure 2.6: TANITA Body Composition Analyser

2.3.4 Anthropometric measures, blood sampling and biochemical analysis:

Height and weight were recorded with participants wearing light clothing and no shoes using Marsden medical weighing scale, Model number: MS-4202L, (Marsden Weighing Machine Group Limited, Rotherham, UK) with attached stadiometer. BMI (Body mass index) was calculated as weight in kilograms divided by the square of height in meters (kg/m2). Blood pressure was measured using an automated device (NPB-3900; Nellcor Puritan Bennett, Pleasanton, CA) during each study visit. Blood pressure measurements were performed after the subjects had been seated quietly for at least 5 minutes and with the right arm supported at heart level. Three readings were taken, each at least 2 minutes apart, and then the average of the readings was obtained. The waist and hip circumference was measured using a specific abdominal circumference tape measure by wrapping it around the patient's waist at the midway point between the top of iliac crest and the bottom of the ribs. The participants were advised to relax their abdominal muscles and breathe naturally during the procedure.

In both trials, following an overnight fast, blood samples were collected and weight and blood pressure were measured both at the baseline and final visit (end of 3-month period). The fasting venous blood was collected into fluoride oxalate and serum gel tubes. Samples were separated by centrifugation at 2000g for 15 minutes at 4°C, and the aliquots were stored at -80°C within 1 hour of collection. Overnight urine samples were collected in vitamin D study and aliquots were stored at -80°C until batch analysis.

The following assays were performed in NHS laboratory (Hull Royal Infirmary) accredited by United Kingdom National External Quality Assessment Scheme (UK NEQAS) and United Kingdom Accreditation Service (UKAS). The serum insulin was assayed using chemiluminescent immunoassay on the Beckman Coulter UniCel DxI 800 analyser (Beckman Coulter UK Ltd, High Wycombe). The plasma glucose was measured using a Beckman AU 5800 analyser (Beckman-Coulter, High Wycombe, United Kingdom) and

according to the manufacturer's recommended protocol. Serum vitamin D levels, testosterone and androstenedione were quantified using isotope-dilution liquid chromatography tandem mass spectrometry (LC-MS/MS). SHBG and DHEAS were measured using a chemiluminescent immunoassay on the Beckman Coulter UniCel DxI 800 analyser, applying the manufacturer's recommended protocol. The free androgen index (FAI) was calculated as: (total testosterone/SHBG) x 100. Total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C), alanine aminotransferase (ALT) and high sensitivity C-reactive protein (hs-CRP) levels were measured enzymatically using a Beckman AU 5800 analyser (Beckman-Coulter, High Wycombe, UK). Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald equation.

2.3.5 ADVIA centaur enhanced liver fibrosis test:

The enhanced liver fibrosis (ELF) score provides a single value using an algorithm combining quantitative serum measurements of three biomarkers including hyaluronic acid (HA), tissue inhibitor of metallo-proteinases-1 (TIMP-1) and amino-terminal propeptide of type III procollagen (PIIINP). We used the ADVIA Centaur XP/XPT immunochemical analyzer (Siemens Healthcare Diagnostics Inc., Erlangen, Germany) according to manufacturer's instructions figure 2.7. The ELF score was calculated directly by the instrument employing the following equation:

$$\text{ELF score} = 2.278 + 0.851 \ln(C_{HA}) + 0.751 \ln(C_{P3NP}) + 0.394 \ln(C_{TIMP1})$$

Concentrations (C) of each assay are in ng/mL

Figure 2.7: ADVIA Centaur® XP System

2.3.6 Homeostatic model assessment of insulin resistance (HOMA-IR):

HOMA-IR was calculated using the following formula that includes the measurement of fasting plasma glucose fasting insulin levels (121):

$$HOMA - IR = \frac{Fasting\ Plasma\ Insulin\ \left(\frac{\mu U}{mL}\right) \times Fasting\ Plasma\ Glucose\ (mmol/L)}{22.5}$$

HOMA-IR was calculated in both Vitamin D in PCOS and Empagliflozin versus Metformin in PCOS studies, at both baseline and final visits.

3 Chapter 3: Effect of vitamin D treatment on markers of liver fibrosis in obese women with polycystic ovary syndrome

3.1 Introduction:

PCOS is associated with increased prevalence of cardiovascular risk factors (395, 396). Low serum concentrations of 25 hydroxyvitamin D (25OHD) are associated with higher cardiovascular risk (397, 398), are independently associated with insulin resistance and predict the development of hyperglycaemia in non-diabetic Caucasians (399, 400). Vitamin D deficiency is very common in women with PCOS, with 67-85% having serum concentrations of 25OHD <20 ng/ml that correlate with obesity and increased insulin resistance, testosterone and DHEAS levels (368, 371, 401). Studies have revealed that vitamin D replacement therapy may have beneficial effects on insulin resistance and improve menstrual frequency and influence steroidogenesis of the sex hormones oestradiol and progesterone in obese women with PCOS (378, 402). Furthermore, studies have shown that increased exposure to sunlight and an increased consumption of oily fish and fish oils, that contain large amounts of vitamin D, protect against coronary heart disease (403-405). We have shown that in patients with PCOS, atorvastatin increases vitamin D levels that correlate with a reduction in inflammatory and oxidative stress markers (380, 406). However, there are studies which have not shown any benefits of vitamin D supplementation in women with PCOS. In one of the trials administering single oral doses of 300,000 units of vitamin D3 did not cause any significant changes in fasting glucose, fasting insulin, testosterone and DHEAS levels (378). Similarly, in another two trials high doses of vitamin D had no significant effects on insulin sensitivity but lipid profile was improved (407, 408). Furthermore, in a recent systematic review inverse relationship between vitamin D and metabolic disturbances has been suggested but controversy in many studies has made it difficult to draw a definite conclusion,

and the authors have suggested that further drug-placebo clinical trials could lead to more accurate results (409).

Women with PCOS are also at increased risk of developing NAFLD as a hepatic manifestation of the metabolic syndrome, which encompasses a spectrum of diseases progressing from simple steatosis to NASH and ultimately to cirrhosis (185, 410). NAFLD is recognized as the most common form of liver disease and the leading cause of cirrhosis in the Western world with a worldwide prevalence of 6.3-33%, increasing to 75% in the presence of obesity and type 2 diabetes (179, 182), and with a 2.6 fold increased mortality. NAFLD's association with type 2 diabetes has recently been suggested as a novel cardiovascular risk marker (411). Hepatic steatosis develops due to anomalous lipid handling by the liver that sensitizes it to injury and inflammation. Subsequent increased hepatocyte injury characterises NASH that is associated with a mixed acute and chronic lobular inflammation, hepatocellular ballooning and perisinusoidal fibrosis (177). As a likely consequence, NASH is associated with a 30% risk of developing progressive fibrosis leading to cirrhosis and hepatocellular carcinoma.

Women with PCOS have a high prevalence of metabolic syndrome and its associated NAFLD (ranges from 15-55% depending on the diagnostic index used) that is linked to increased long-term risk of cardiovascular outcomes (174, 412). Several studies have shown that NAFLD in PCOS is directly related to BMI with a higher prevalence with obesity (410). Emerging evidence has associated NAFLD with vitamin D deficiency and, epidemiologically, both of these conditions share multiple cardiometabolic risk factors (413).

It has been recommended that early recognition of NAFLD in women with PCOS, and its subsequent monitoring and intervention, is important because these women can develop a more severe and progressive liver disease at a much younger age, especially those with insulin resistance, with or without the metabolic syndrome (414). The progression of

NAFLD is potentially reversible, and therefore its identification is important (206). Liver biopsy remains the gold standard diagnostic and staging test but it has a recognised morbidity and mortality and is used sparingly. Consequently, there is a strong consensus that non-invasive markers should be the first choice to exclude significant fibrosis due to their good inter-laboratory reproducibility, high reliability and widespread availability (415), reserving liver biopsy for cases where non-invasive tests are unable to exclude it. The ELF test is one of the first commercially available serum multi-marker fibrosis tests (416). ELF has been validated in a mix of patient groups in a large multicentre study and was subsequently found to be accurate in detecting early fibrosis and at least as good as liver biopsy at predicting liver disease related outcomes (417, 418). Furthermore, it is suggested that ELF may be better than liver biopsy for predicting clinical outcomes, reflecting the on-going disease progression that a biopsy cannot follow (211).

This double blind randomized placebo controlled study was conducted to assess the metabolic, hormonal and cardiovascular effects of vitamin D supplementation in PCOS women with vitamin D deficiency.

3.2 Subjects and methods:

3.2.1 Ethical approval and informed consent:

This study was registered in the ClinicalTrials.gov registry as NCT02513381. Explained in chapter 2 section 2.1.1

3.2.2 Patient selection and recruitment:

Fifty-four reproductive-aged women (18–45 years) diagnosed with PCOS based on all three diagnostic criteria of the Rotterdam consensus were screened for vitamin D deficiency (20). The participants were considered vitamin D-deficient when their serum 25OH-D level was less than 50 nmol/L (20 ng/mL). None had NAFLD on ultrasound.

The value of 50nmol/l was taken from Endocrine Society Clinical Practice Guideline, which recommends using the serum circulating 25-hydroxyvitamin D [25(OH)D] level to evaluate vitamin D status in patients who are at risk for vitamin D deficiency. Vitamin D deficiency is defined as a 25(OH)D below 20 ng/ml (50 nmol/liter), and vitamin D insufficiency as a 25(OH)D of 21–29 ng/ml (52.5–72.5 nmol/liter) (419).

There is an extensive literature suggesting that vitamin D level below 50nmol/liter is associated with increased cardiovascular events. For example, data from National Health and Nutrition Examination Survey has shown that 25-hydroxyvitamin D levels were inversely related to the following cardiovascular risk factors: blood pressure >140/90 mm Hg, blood glucose >125 mg/dL (6.95 mmol per L), and BMI of 30 kg per m^2 or greater (420). Similarly, Framingham Offspring Study cohort showed that there was a 62% higher risk of cardiovascular events in patients whose 25-hydroxyvitamin D level < 15 ng/mL (38 nmol/L), compared with those whose level was 15 ng per mL or greater (421).

3.2.3 Recruitment criteria:

3.2.3.1 Inclusion criteria:

1) Caucasian women, aged 18-45 years, with confirmed diagnosis of PCOS based on all three diagnostic criteria of the Rotterdam consensus (422).
2) Irregular periods with raised FAI
3) Vitamin D < 50 nmol/L.

3.2.3.2 Exclusion criteria:

1) Non-classical 21-hydroxylase deficiency, hyperprolactinaemia, Cushing's disease and androgen-secreting tumours were excluded by appropriate tests if clinically indicated.

2) Any concurrent illness including type 2 diabetes, subjects who were on any medication (including medications that interfere with calceotrophic hormones) for the preceding 6 months.
3) Women planning to conceive.
4) Women who were using any oral or implantable contraceptives or any other treatments likely to affect ovarian function, insulin sensitivity or lipids for at least 3 months before entering the study. Stable dose of metformin for 3 months was allowed. Subjects were advised to use barrier contraception during the study period.
5) Estimated Glomerular Filtration rate (eGFR) <60.
6) Hypersensitivity to vitamin D or any of the excipients in the product.
7) Peanut or soya allergy.
8) Nephrolithiasis.
9) Diseases or conditions resulting in hypercalcaemia and/or hypercalciuria.

All participants were advised to maintain their usual dietary and lifestyle habits during the study. In addition, participants were also advised not to modify lifestyle factors, such as smoking status, sunlight exposure, and physical activity during the study that could affect the levels of vitamin D and metabolic indices.

3.2.4 Study visits:

This study included three visits to the Diabetes & Endocrinology Research Centre, Brocklehurst Building, Hull Royal Infirmary. The details for study visit are given below in the Table 3.1:

	Visit1 (Screening)	Visit 2 (Week 0)	Visit 3 (Week 12)
Consent	x		
Medical history	x		
Inclusion/Exclusion	x		
Full physical examination	x		
BP, Pulse rate	x	x	x
Height, weight, hip & waist circumference, TANITA		x	x
Stiffness of arteries-EndoPAT		x	x
Fasting overnight		x	x
Blood tests	x	x	x
Urine sample		x	x
Adipose tissue biopsy (Optional)		x	x
Study medications dispensed		x	
Adverse event recording			x

Table 3.1: Vitamin D study timetable
Legend: BP, Blood pressure; TANITA, Body composition analyzer; EndoPAT, Endothelial-Peripheral arterial tonometry

3.2.5 Randomization and blinding:

A double blind, randomized, placebo-controlled study was undertaken using vitamin D 3200 IU daily. Forty patients who fulfilled both inclusion and exclusion criteria were randomly assigned to the vitamin D 3200 IU or placebo group based on a computer-generated randomization list by Essential Nutrition Ltd, UK. The National Osteoporosis Society guidelines recommend treating patients who have serum 25OHD levels between 30–50

nmol/L and are at increased risk of developing vitamin D deficiency in the future because of reduced exposure to sunlight, common in PCOS (423).

3.3 Study measurements:

3.3.1 Anthropometric measures:

Already explained in chapter 2 section 2.3.4

3.3.2 Blood sampling and laboratory analysis:

Already explained in chapter 2 section 2.3.4

3.3.3 Enhanced liver fibrosis score:

Already explained in chapter 2 section 2.3.5

3.4 Statistical plan:

3.4.1 Sample size calculation:

In order to look at the inflammation marker hs-CRP, the minimum difference worth detecting/observed difference was 32.7%, estimated within-group SD was 11.1; therefore, for 90% power and a significance level of 5%, a sample size of 16 per group was calculated (411). Specifically for liver biomarkers, the sample size was based on the study by Kahal et al (348), on the change in PIIINP in a group of patients with PCOS treated for their metabolic dysfunction. The minimum difference worth detecting/observed difference was 0.9 with an effect size of 1.12. Adjusting for a possible 20% dropout rate, a total of 40 patients were recruited (nQuery, Statistical Solutions, Saugus, USA).

3.4.2 Statistical analysis:

The two randomized groups were compared statistically at baseline. Comparisons between both the two groups from baseline were carried out using the paired *t*-test. The Wilcoxon

signed rank test was applied to data that violated the assumptions of normality when tested using the Shapiro-Wilk test. The effect of treatment on MPs was first evaluated by calculating the percentage change from baseline and then the percentage change for each variable in each patient group, thus negating the differences in the baseline values of the two groups. The formula used for measuring percent change was:

$$\frac{\boldsymbol{New\ Value(V3) - Baseline\ Value(V2)}}{\boldsymbol{Baseline\ Value\ (V2)}} \times \mathbf{100}$$

Between-group comparison of percent changes was performed using independent-samples *t*-test. The Wilcoxon rank-sum test (Mann–Whitney U test) was applied to data that violated the assumptions of normality when tested using the Shapiro-Wilk test for normality. For all analyses, a two-tailed $P \leq 0.05$ was considered to indicate statistical significance. Statistical analysis was performed using IBM SPSS Statistics for Windows, version 24.0 (Armonk, NY: IBM Corp.).

3.5 Results:

3.5.1 Recruitment and progress of patients in trial:

Thirty-seven patients completed the 3-months study period. Two patients from the vitamin D group and one patient from placebo group dropped out of the study (figure 3.1).

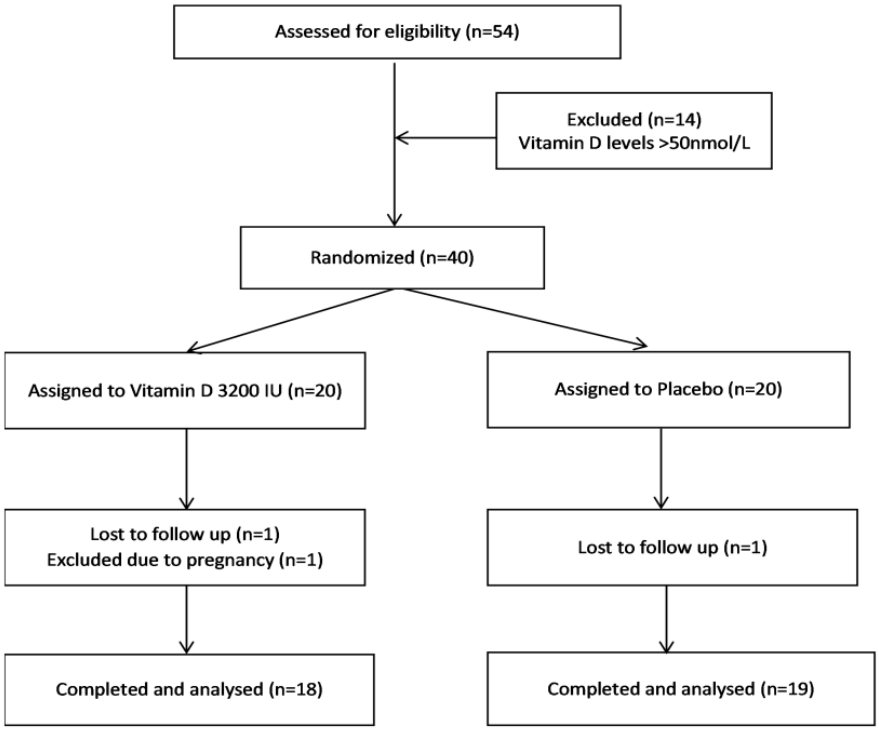

Figure 3.1: Flow chart showing the progress of patients through the trial

3.5.2 Compliance:

The compliance with treatment was calculated by counting the returned medications. After their exclusion, compliance was 99% in both groups by counting returned medication. None of the subjects developed any significant side effects in the course of the study.

3.5.3 Baseline characteristics of patients and changes after vitamin D or placebo supplementation:

Parameters	Vitamin D group	Placebo group	P value
Age (years)	28.6 ± 5.5	29.1 ± 7.5	0.8
BMI (kg/m^2)	35.4 ± 10.6	33.8 ± 7.2	0.6
Waist circumference (cm)	98.9 ± 21.1	98.3 ± 16.1	0.9
Hip circumference (cm)	119.1 ± 19.5	116 ± 15.3	0.6
SBP (mmHg)	116.7 ± 9.0	121.3 ± 15.6	0.3
DBP (mmHg)	72.2 ± 10.3	80.9 ± 10.7	0.01*
RHI	2.0 ± 0.5	1.6 ± 0.4	0.02*
AI	1.3 ± 13.3	4.6 ± 11.8	0.3
FAI	6.3 ± 5.1	5.5 ± 3.1	0.6
Testosterone (nmol/L)	1.40 ± 0.8	1.1 ± 0.4	0.3
SHBG (nmol/L)	29.7 ± 19.6	23.4 ± 10.7	0.5
Androstenedione (nmol/L)	5.7 ± 3.2	4.6 ± 1.9	0.2
DHEAS (µmol/L)	6.4 ± 3.9	6.4 ± 3.5	0.8
PTH (pmol/L)	5.0 ± 1.8	3.0 ± 0.9	<0.01*
ELF	8.2 ± 0.4	7.8 ± 0.6	0.02*
HA (ng/mL)	19.5 ± 9.8	12.0 ± 5.9	0.01*
TIMP-1(ng/mL)	167.7 ± 29.4	164.2 ± 44.7	0.9
PIIINP (ng/mL)	7.8 ± 2.0	6.7 ± 1.6	0.09
ALT (IU/L)	27 ± 12.2	20.1 ± 5.6	0.05*
25OHD (nmol/L)	25.6 ± 11.4	31.3 ± 10.7	0.1
hs-CRP (mg/L)	6.1 ± 8.3	5.6 ± 5.2	0.7
FSH (IU/L)	5.5 ± 1.6	6.4 ± 3.0	0.4
	9.4 ± 6.7	10.5 ± 6.9	0.5

LH (IU/L)			
Estradiol (pmol/L)	283.9 ± 214.9	238.7 ± 193.0	0.3
17OHP (nmol/L)	2.2 ± 1.3	1.9 ± 1.7	0.7
TC (mmol/L)	4.7 ± 0.9	4.9 ± 0.8	0.9
LDL (mmol/L)	3.0 ± 0.7	3.7 ± 0.7	0.9
HDL (mmol/L)	1.4 ± 0.3	1.3 ± 0.3	0.7
Triglycerides (mmol/L)	1.2 ± 0.6	1.2 ± 0.5	0.6
Cholesterol-HDL ratio	3.8 ± 1.0	3.9 ± 0.9	0.8
Non-HDL cholesterol	3.5 ± 0.8	3.6 ± 0.8	0.8
Fasting glucose (nmol/L)	4.7 ± 0.4	4.8 ± 0.4	0.4
Fasting insulin (μIU/L)	14.1 ± 10.7	11.7 ± 6.5	0.6
Basal metabolic rate (kcal)	1710.4 ± 337.6	1628.9 ± 214.2	0.4
Fat %	42.4 ± 9.3	44.0 ± 8.0	0.6
Fat mass (kg)	44.0 ± 22.1	42.9 ± 17.1	0.9
Fat free mass (kg)	53.8 ± 9.5	51.0 ± 5.6	0.3
Total body water (kg)	39.4 ± 6.9	37.4 ± 4.1	0.3
Whole body impedance (Ω)	598.1 ± 94.4	641.2 ± 56.9	0.1
Trunk fat %	39.5 ± 9.7	42.9 ± 8.9	0.3
Trunk fat mass (kg)	21.4 ± 10.3	22.7 ± 9.5	0.7
Trunk fat free mass (kg)	29.8 ± 4.7	28.1 ± 2.8	0.2

Table 3.2: Baseline anthropometric, hormonal and metabolic measurements between the vitamin D group and placebo group

Legend: Abbreviations: 25OHD, 25-hydroxyvitamin D; ELF Score, Enhanced Liver Fibrosis; HA, Hyaluronic acid; PIIINP, Amino-terminal propeptide of type III procollagen; TIMP-1, Tissue inhibitor of metallo-proteinases-1; ALT, Alanine Aminotransferase; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FAI, Free androgen index; SHBG, Sex hormone binding globulin; DHEAS, Dehydroepiandrosterone sulphate; 17OHP, 17-hydroxyprogesterone; PTH, Parathyroid hormone; FSH, Follicle stimulating hormone; LH, Luteinizing hormone; RHI, Reactive hyperaemic index; AI, Augmentation index; TC, total cholesterol; TG, triglycerides; LDL, low density lipoproteins; HDL, high density lipoproteins; hs-CRP, high sensitivity-C-reactive protein. Data are presented as mean ± SD. * = $p<0.05$

The mean age group of patients was 28.6 ± 6.4 years (vitamin D 28.6 ± 5.5 *vs.* placebo 29.1 ± 7.5 years). The baseline comparison between vitamin D levels, anthropometric, hormonal and biochemical parameters of the two groups are given in Table 3.2. There were only few variables including DBP, RHI, PTH, ELF, HA and ALT that were significantly different between groups at baseline.

	Vitamin D group (n = 18)		Placebo group (n = 19)		% Change		
Parameter	**Baseline**	**12 week**	**Baseline**	**12 week**	**Vitamin D**	**Placebo**	**P value**
25OHD (nmol/L)	25.6 ± 11.4	90.4 ± 19.5**	30.9 ± 11.1	47.6 ± 20.5**	319.3 ± 213.9	59.5 ± 56.7	<0.01**
ELF Score	8.2 ± 0.4	7.6 ± 0.8**	7.8 ± 0.6	7.8 ± 0.7	-7.6 ± 9.6	0.4 ± 8.9	0.02*
HA (ng/mL)	19.5 ± 9.8	12.1 ± 6.2**	12.0 ± 5.9	13.0 ± 6.7	-31.2 ± 31.9	16.9 ± 57.1	0.006**
PIIINP (ng/mL)	7.8 ± 2.0	6.2 ± 1.9**	6.7 ± 1.6	7.1 ± 2.2	-18.6 ± 20.4	8.0 ± 27.2	0.003**
TIMP-1 (ng/mL)	167.7 ± 29.4	138.5 ± 44.7*	164.2 ± 44.7	160.4 ± 41.1	-15.3 ± 29.6	0.7 ± 26.8	0.1
ALT (IU/L)	27 ± 12.2	22.1 ± 11.5*	20.1 ± 5.6	24.4 ± 8.7*	-16.7 ± 25.7	21.9 ± 28.3	<0.001**
Weight (kg)	97.9 ± 31.3	98.2 ± 31.9	93.8 ± 22.3	94.3 ± 21.5	0.4 ± 3.8	0.6 ± 2.6	0.8
BMI (kg/m^2)	35.4 ± 10.6	35.5 ± 10.8	33.8 ± 7.2	34.0 ± 7.0	0.4 ± 3.8	0.6 ± 2.6	0.8
Waist (cm)	98.9 ± 21.1	98.8 ± 22.1	98.3 ± 16.1	97.3 ± 16.2	-0.2 ± 3.4	-1.0 ± 2.3	0.4
Hip (cm)	119.1 ± 19.5	118.2 ± 21.4	116 ± 15.3	115.6 ± 15.8	-1.0 ± 3.4	-0.4 ± 2.0	0.5
SBP (mmHg)	116.7 ± 9.0	115.8 ± 14.6	121.3 ± 15.6	122 ± 12.5	-0.9 ± 7.8	1.1 ± 7.6	0.4
DBP (mmHg)	72.2 ± 10.3	73.5 ± 13.5	80.9 ± 10.7	80.6 ± 10.7	1.7 ± 11.8	-0.2 ± 6.9	0.6
FAI	6.3 ± 5.1	5.2 ± 3.0	5.5 ± 3.3	5.9 ± 3.4	5.3 ± 67.7	10.7 ± 31.2	0.8
Testosterone (nmol/L)	1.4 ± 0.8	1.2 ± 0.1	1.1 ± 0.4	1.2 ± 0.4	-0.6 ± 45.3	11.3 ± 27.3	0.3
SHBG (nmol/L)	29.7 ± 19.6	29.7 ± 22.2	23.4 ± 10.7	24 ± 12.5	1.0 ± 16.1	2.8 ± 17.9	0.7
Androstenedione (nmol/L)	5.7 ± 3.3	5.3 ± 2.5	4.6 ± 1.9	4.7 ± 2.0	58.7 ± 271.2	-0.5±34.3	0.4

DHEAS (µmol/L)	6.4 ± 3.9	5.9 ± 3.6	6.4 ± 3.5	6.3 ± 3.1	-7.8 ± 20.6	0.6±21.0	0.2
17OHP (nmol/L)	2.2 ± 1.3	2.7 ± 3.3	1.9 ± 1.7	1.8 ± 1.7	22.8 ± 123.3	25.5 ± 134.8	1.0
PTH (pmol/L)	5.0 ± 1.8	4.0 ± 2.1*	3.0 ± 0.9	3.0 ± 1.3	-18.4 ± 31.1	1.8 ± 34.2	0.1
FSH (IU/L)	5.5 ± 1.6	6.4 ± 2.3	6.4 ± 3.0	6.9 ± 3.3	30.0 ± 73.3	11.8 ±3 6.1	0.4
LH (IU/L)	9.4 ± 6.7	12.1 ± 12.2	10.5 ± 6.9	9.2 ± 5.3	86.3 ± 191.2	18.4 ± 85.8	0.2
Oestradiol (pmol/L)	283.9 ± 214.9	312.6 ± 286.7	238.7 ± 193.0	240.2 ± 170.6	54.3 ± 160.0	28.6 ± 86.3	0.5
Fasting glucose (mmol/L)	4.7 ± 0.4	4.8 ± 0.6	4.8 ± 0.4	4.8 ± 0.5	-2.7 ± 25.8	0.7 ± 7.6	0.6
Fasting insulin (µIU/ml)	14.1 ± 10.7	15.4 ± 12.2	11.7 ± 6.5	12.8 ± 8.0	11.2 ± 43.2	0.7 ± 7.6	0.3
HOMA-IR	3.0 ± 2.3	3.5 ± 3.3	2.6 ± 1.6	2.9 ± 2.0	10.3 ± 57.9	19.3 ± 54.3	0.6
hs-CRP (mg/L)	6.1 ± 8.3	6.5 ± 11.7	5.6 ± 5.2	6.1 ± 5.6	17.7 ± 67.0	15.6 ± 45.5	0.9
RHI	2.0 ± 0.1	1.9 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	-2.3 ± 7.9	-6.6±5.6	0.7
AI	1.3 ± 3.2	1.5 ± 4.2	4.6 ± 2.7	3.9 ± 2.3	-310.9 ±313.0	173.2±152.8	0.2
TC (mmol/L)	4.9 ± 0.7	5.0 ± 0.9	4.9 ± 0.8	4.9 ± 0.8	3.7 ± 10.8	0.9 ± 15.0	0.5
LDL-C (mmol/L)	3.0 ± 0.7	3.1 ± 0.7	3.0 ± 0.7	2.9 ± 0.6	5.7 ± 17.3	0.8 ± 21.9	0.5
HDL-C (mmol/L)	1.4 ± 0.3	1.3 ± 0.4	1.3 ± 0.3	1.3 ± 0.3	-0.4 ± 10.8	-2.5 ± 6.7	0.5
TG (mmol/L)	1.2 ± 0.6	1.3 ± 0.8	1.2 ± 0.5	1.5 ± 0.7	4.4 ± 28.9	22.1 ± 45.9	0.2
Cholesterol : HDL ratio	3.8 ± 1.0	4.0 ± 1.2	3.9 ± 0.9	4.0 ± 0.9	4.6 ± 10.7	3.2 ± 13.7	0.7
Non-HDL Cholesterol	3.5 ± 0.8	3.7 ± 0.9	3.6 ± 0.8	3.6 ± 0.8	6.0 ± 14.3	2.7 ± 20.1	0.6
BMR (kcal)	1710.4 ± 337.6	1722.7 ± 354.2	1628.9 ± 214.2	1648.8 ± 225.4	0.6 ± 2.7	1.2 ± 2.7	0.6

Fat %	42.4 ± 9.3	42.3 ± 9.4	44.0 ± 8.0	43.7 ± 7.4	-0.3 ± 5.2	-0.5 ± 3.8	0.9
Fat Mass (kg)	44.0 ± 22.1	44.1 ± 22.2	42.9 ± 17.1	42.6 ± 16.0	0.4 ± 8.3	0.2 ± 5.1	1.0
FFM (kg)	53.8 ± 9.5	54.2 ± 10.0	51.0 ± 5.6	51.8 ± 6.2	0.7 ± 2.8	1.5 ± 3.2	0.4
TBW (kg)	39.4 ± 6.9	39.7 ± 7.3	37.4 ± 4.1	37.9 ± 4.6	0.7 ± 2.8	1.5 ± 3.2	0.4
WBI (Ω)	598.1 ± 94.4	593.4 ± 99.6	641.2 ± 56.9	625.7 ± 71.9	-0.9 ± 4.2	-2.5 ± 5.2	0.3
Trunk Fat%	39.5 ± 9.7	39.2 ± 9.7	42.9 ± 8.9	42.1 ± 8.0	-0.9 ± 7.3	-1.4 ± 5.8	0.8
Trunk Fat Mass (kg)	21.4 ± 10.3	21.2 ± 9.7	22.7 ± 9.5	22.2 ± 8.7	-0.5 ± 10.0	-1.0 ± 6.8	0.9
Trunk FFM (kg)	29.8 ± 4.7	30.0 ± 5.0	28.1 ± 2.8	28.7 ± 3.2	0.6 ± 2.8	2.2 ± 5.5	0.3
Urine Ca/Cr ratio	0.2 ± 0.1	1.2 ± 0.2	0.3 ± 0.1	1.1 ±0.1	4.0 ± 38.6	0.2 ± 42.5	0.8

Table 3.3: Comparison of anthropometric, metabolic and hormonal parameters before and after Vitamin D or placebo supplementation

Legend: Abbreviations: 25OHD, 25-hydroxyvitamin D; ELF Score, Enhanced Liver Fibrosis; HA, Hyaluronic acid; PIIINP, Amino-terminal propeptide of type III procollagen; TIMP-1, Tissue inhibitor of metallo-proteinases-1; ALT, Alanine Aminotransferase; BMI, body mass index; SBP, systolic blood pressure in; DBP, diastolic blood pressure; FAI, Free androgen index; SHBG, Sex hormone binding globulin; DHEAS, Dehydroepiandrosterone sulphate; 17OHP, 17-hydroxyprogesterone; PTH, Parathyroid hormone; FSH, Follicle stimulating hormone; LH, Leuteinizing hormone; HOMA-IR, Homeostatic Model Assessment of Insulin resistance; RHI, Reactive hyperaemic index; AI, Augmentation index; TC, total cholesterol; TG, triglycerides; LDL, low density lipoproteins; HDL, high density lipoproteins; hs-CRP, high sensitivity-C-reactive protein, BMR, Basal metabolic rate; FFM, Fat free mass; TBW, Total body water; WBI, Whole body bioelectrical impedance; Urine Ca/Cr ratio, Urine calcium creatinine ratio.

Data are presented as mean ± SD. All serum results are obtained from fasting variables. Percent change is the percent difference compared with the baseline. To convert values for glucose to milligrams per deciliter, divide by 0.056. To convert values for insulin to picomoles per liter, multiply by 7. To convert values for cholesterol to milligrams per deciliter, divide by 0.0259. To convert values for triglycerides to milligrams per deciliter, divide by 0.0113.

* = $p<0.05$, **=$p<0.01$, P value = % change of the difference between Groups (95% CI)

3.5.4 Vitamin D supplementation:

The study started in July 2015 and the last patient, last visit was in November 2016. Vitamin D levels increased in both groups but much greater in patients who were randomised to vitamin D, confirming adherence (figure 3.2).

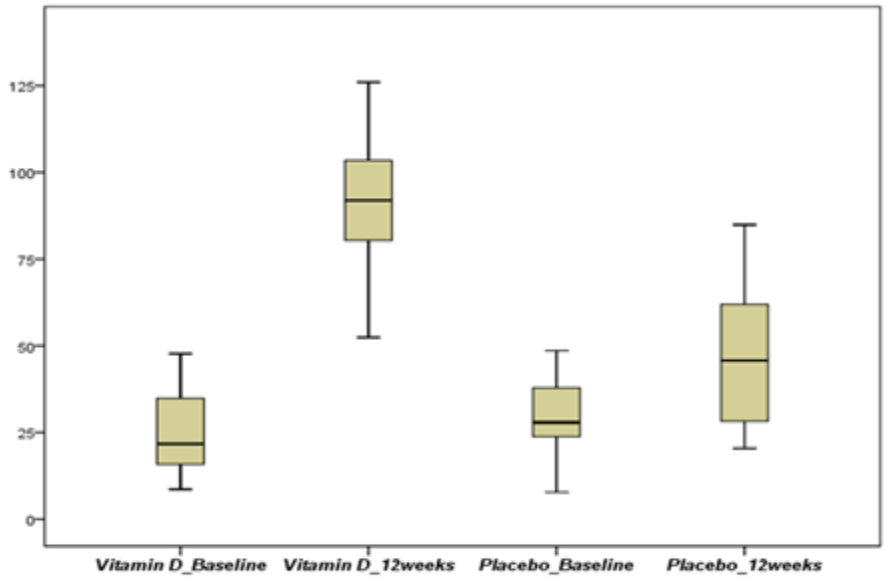

Figure 3.2: Boxplot comparing the change in 25-hydroxyvitamin D after supplementation with either vitamin D 3200IU or placebo daily for 12 weeks

3.5.5 EFL score change after supplementation:

There was a significant reduction in individual liver fibrosis markers (HA, PIIINP, TIMP-1) and their cumulative ELF score in patients randomized to vitamin D (Figure 3.3), whereas there were no changes in any of these parameters in the placebo group (table 3.3).

Figure 3: Boxplot comparing the change in ELF score after Vitamin D 3200 IU or placebo supplementation daily for 12 weeks

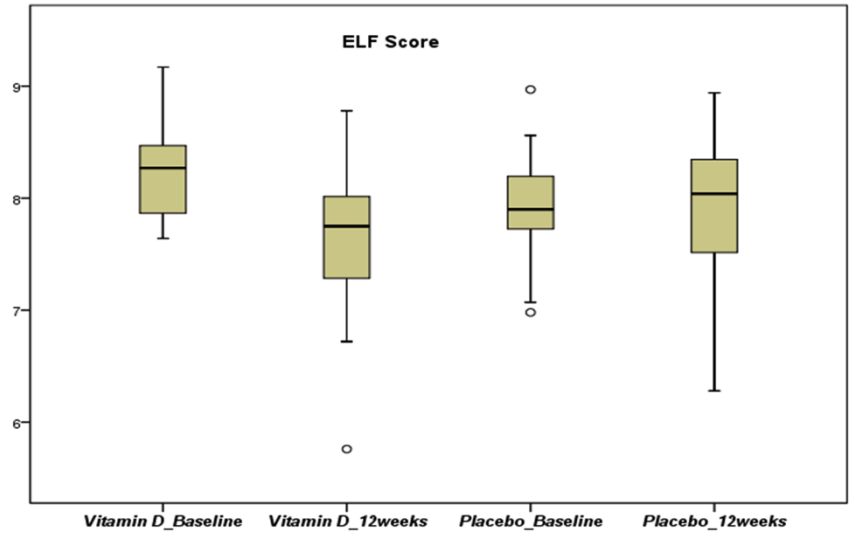

Figure 3.3: Boxplot comparing the change in ELF score after supplementation with either vitamin D 3200IU or placebo daily for 12 weeks

The percent change in liver fibrosis markers (HA, PIIINP) and their cumulative ELF score (vitamin D, -7.6 ± 9.6 vs. placebo, 0.4 ± 8.9; p=0.02) between groups was also significantly higher in the vitamin D group compared to placebo. This reduction in ELF score was also associated with a significant improvement in alanine aminotransferase (ALT) levels in the vitamin D group (-16.7% ± 25.7 vs 21.9 ± 28.3; p<0.001) but unchanged in the placebo group. FAI and testosterone levels did not differ between groups. There were no significant changes in body mass index (BMI), weight, hip circumference, waist circumference, blood pressure, HOMA-IR, hs-CRP, total cholesterol, LDL-C, HDL-C, triglycerides, urine Ca/Cr ratio and body composition parameters (fat mass, fat percent, fat free mass, bioelectrical impedance) in either group. The adipose tissue biopsy was included in the study that was optional and cosmetically unacceptable for the patients. Therefore, there were not sufficient samples available for analysis.

3.6 Discussion:

This is the first study to have shown the direct beneficial effects of vitamin D supplementation on the risk factors of hepatic fibrosis in women with PCOS. Three months treatment with vitamin D 3200 IU in obese women with PCOS resulted in significant improvements in liver fibrosis markers (HA, PIIINP, TIMP-1) and their cumulative ELF score. Although the ALT levels were within the reference range, there was a significant decrease with vitamin D that was associated with the decrease in the ELF score. These changes are in accordance with the recent studies in both animals (424, 425) and humans (426-428) on the effects of vitamin D on hepatic steatosis and NAFLD with or without fibrosis. Significant improvements in hepatic steatosis in vitamin D deficient NAFLD patients, assessed using FibroScan (transient elastrography) were seen after four weeks of vitamin D supplementation (426) that were independent of any change in BMI, but did show improvement in ALT levels in accord with the study here. Others have shown significant improvements in inflammatory and oxidative stress markers, hs-CRP and malondialdehyde (MDA), in NAFLD following four weeks of vitamin D supplementation (428). However, there were no changes in HOMA-IR and hepatic steatosis as measured using ultrasound, despite significant improvements in BMI and ALT levels, a result likely due to ultrasonography having such low sensitivity for detecting hepatic steatosis and early fibrotic changes compared to transient elastrography (426, 429).

There is an emerging data suggesting that ALT has a role as a predictor of mortality independent of liver disease and is an indicator of general health (430). The ALT in this study was within normal range in vitamin D group, but in obese women with PCOS, underlying NAFLD could put them at high risk for development of atherosclerotic cardiovascular disease, and any improvement in ALT levels may reduce that risk.

The gold standard test for diagnosing NAFLD and staging advanced disease is the liver biopsy that is limited due to sampling error, cost and related morbidity and mortality. Endocrine Society clinical practice guidelines (28) and American Association for the Study of Liver Diseases (AASLD) (414) recommend non-invasive serum markers for screening liver dysfunction in women with PCOS, especially those with metabolic risk factors, and liver biopsy should be considered only in those patients with raised serum markers. Several non-invasive markers have been researched but none of them has sufficient sensitivity and specificity in differentiating between NAFLD with or without fibrosis. The National Institute for Health and Care Excellence (NICE) guidelines recommend the ELF score to test for and monitor advanced liver fibrosis in NAFLD patients (431). The ELF score is the first direct marker panel of liver fibrosis, which combines the three serum biomarkers HA, PIIINP and TIMP1, each of which reflect ongoing sinusoidal fibrogenesis in the liver. ELF has been validated to be very accurate in differentiating mild (< 7.7), moderate (≥ 7.7 to < 9.8) and severe fibrosis (≥ 9.8) and is suggested to be a useful prognostic tool, as good as liver biopsy, at predicting clinical outcomes (211, 416). In adjusted models, a unit increase in ELF was associated with a doubling of the risk of liver-related clinical outcomes (211, 418). In our study, the treatment group went from a moderate to a mild fibrosis category indicated by a significant drop in ELF score, suggesting an estimated 60% reduction in liver fibrosis progression in NAFLD patients after vitamin D supplementation. Other non-invasive analyses of liver function are available such as BARD score, APRI score and the FIB4 index that correlate well with each other and the ELF score, though the ELF score may be better at determining liver changes after therapy (432).

Several potential mechanisms have been suggested by which vitamin D reduces the progression of hepatic steatosis to NASH and hepatic fibrosis in NAFLD patients (413). The

key process that leads to the development of liver fibrosis in NAFLD patients is the activation of hepatic stellate cells (HSCs), by fatty infiltration, with subsequent excessive deposition of extracellular matrix (ECM) and destruction of normal liver architecture. The three individual biomarkers in the ELF score reflect the integral ECM components of fibrogenic and fibrolytic processes (413, 433). Recent studies have shown that HSCs express vitamin D receptors (VDR) that when activated can inhibit HSC proliferation by suppressing fibrotic gene expression (433). These data are in accord with in-vitro studies on human cells (427) and an animal model of liver injury (425) that showed vitamin D treatment inhibited fibrogenesis. In addition, vitamin D suppresses hepatic steatosis and fibrosis by enhancing hepatic autophagy in animal studies (424).

No changes in body weight, BMI, insulin resistance, FAI and inflammatory markers were seen in this study, in accord with recent vitamin D trials with or without metformin in women with PCOS (376, 401, 434). This suggests that vitamin D may act on hepatic cells directly reducing hepatic fibrosis markers rather than indirectly via insulin resistance. Similarly, no changes were seen in testosterone levels in accord with a recent meta-analysis on vitamin D effects in PCOS (435). There was no significant improvement in endothelial function, measured by EndoPAT machine and expressed as RHI and AI, after 12 weeks of vitamin D supplementation. These results are in accord with the findings seen in recent trials that revealed improvement in RHI after vitamin D treatment but was not significant as compared to placebo group (436, 437). Similarly, low vitamin D levels have been shown to be associated with dyslipidaemia and abnormal body composition parameters (fat mass, fat percent, fat free mass, bioelectrical impedance) but its supplementation has not shown to improve these parameters (438-440). The most likely possible, reason for no improvement in all these parameters could be either the dose of vitamin D or the duration of treatment was

not enough to make significant changes as shown in the evidence that the effects of vitamin D are dependent on the dose and duration of treatment (441).

A limitation of the study was that a liver biopsy was not undertaken; while this might have added weight to our findings, clinically the biopsy was not warranted. Another limitation was that the vitamin D levels were also raised slightly in placebo group due to seasonal variations; however, the increase was not as significant as in the vitamin D group and also remained within vitamin D deficiency range as shown in figure 2. Whilst a stable dose of metformin was an inclusion criteria in our study that has been shown to improve biochemical and metabolic variables in NAFLD (442, 443). It is unlikely that metformin would have contributed to the vitamin D effects reported since more patients on stable dose of metformin were in the placebo group (8 patients in the placebo group and only 3 in the active group were on metformin).

A major strength of the study was the low dropout rate and the high level of compliance with medication (99%). In addition, the method used to detect the early fibrosis in these patients has been validated to be accurate in differing patient groups.

3.7 Conclusion:

This is the first study that has shown the direct beneficial effects of vitamin D supplementation on markers of hepatic fibrosis in women with PCOS shown by a reduction in the ELF score and its constituent liver fibrosis markers (HA, PIIINP, TIMP-1).

4 Chapter 4: The effect of vitamin D on endothelial microparticles as a measure of endothelial dysfunction in women with PCOS

4.1 Introduction:

In the previous chapter, it was shown that vitamin D supplementation improved metabolic risk factors in PCOS with an improved ELF score. PCOS is also associated with endothelial dysfunction that is one of the earliest signs and a prognostic marker of future atheromatous cardiovascular disease (444). The endothelial cells play an important role in the maintenance of a healthy vascular wall through maintenance of relaxed vascular tone with low levels of oxidative stress by releasing mediators, especially endothelial nitric oxide (NO). In addition, the endothelium actively controls vascular permeability to plasma constituents, leukocytes and platelets adhesion and aggregation, and thrombosis (305). Dysfunctional endothelium results in abnormal activation and adhesion of platelets and leukocytes as well as the release of cytokines resulting in increased permeability of the vessel wall to oxidized lipoproteins and inflammatory mediators, causing in arterial wall structural damage and atherosclerotic plaque formation (445). Multiple metabolic cardiovascular risk factors, many that are common to PCOS, accelerate the process of endothelial dysfunction leading to an increased risk of cardiovascular events (446).

Microparticles (MPs) are a heterogeneous population of extracellular vesicles (0.1–1 μm) released from the cell membrane during cell activation and apoptosis that contribute to the induction of endothelial cell modifications, differentiation, inflammation and angiogenesis (447, 448). EMPs levels have been shown to be increased in a variety of cardiovascular and atherothrombotic diseases such as diabetes, obesity, end-stage renal disease, acute coronary syndromes, cancers, inflammatory disorders and autoimmune diseases (449). In acute

coronary syndrome and diabetic patients, EMPs correlate positively with the extent and severity of coronary stenosis and represent a more robust predictor of cardiovascular events occurrence in diabetic patients as compared to traditional markers of endothelial activation (450). Similar results have been found in patients with end-stage renal disease, obesity and pulmonary hypertension (449). Thus, increased levels of EMPs in plasma not only reflect endothelial cell activation or apoptosis, but also represent endothelial dysfunction. Recent trials have revealed that PCOS patients have higher levels of EMPs that are suggested to promote endothelial dysfunction by suppressing the production of NO and prostacyclin and are an emerging surrogate marker of endothelial dysfunction (447, 448). The microparticles released from endothelial cells express specific surface proteins. The flow cytometry analysis of EMPs relies on the use of these markers for the identification and quantification of endothelial origin of MPs. The most common endothelial proteins expressed are CD31, CD54, CD62, CD105, CD106, CD142, CD144, and CD146 (389).

Recent evidence suggests that vitamin D deficiency (VDD) in the general population is associated with increased future cardiovascular risk, carotid intima-medial thickness and is positively correlated with endothelial dysfunction (451). EMPs were found to be elevated in a diabetes population compared to non-diabetes subjects that were negatively correlated with vitamin D levels (452). Several trials have shown that vitamin D supplementation improved endothelial function in various diseases associated with vitamin D deficiency, and a recent in vitro study has revealed that vitamin D suppressed the oxidative stress induced release of endothelial microparticles in cultured human umbilical vein cells (453, 454). Compared to the general population a relatively higher prevalence of VDD has been observed among women with PCOS (approximately 67%–85%) and studies have shown that VDD in PCOS was associated with hyperandrogenism, insulin resistance, menstrual irregularities, ovulatory dysfunction, obesity and high cardiovascular disease risk factors (366).

Additionally, vitamin D supplementation has been shown to improve various endocrine and metabolic dysfunction in women with PCOS (455, 456) but studies in PCOS looking at the effects of vitamin D supplementation on endothelial microparticles are lacking.

In this study, the aim was to investigate whether vitamin D supplementation could improve endothelial dysfunction as evaluated by decreased EMPs production in obese, vitamin D deficient women with PCOS.

4.2 Material and methods:

4.2.1 Patient selection:

The patient selection is explained in chapter 3 section 3.2.2.

4.2.2 Ethical approval and informed consent:

This study was registered in the ClinicalTrials.gov registry as NCT02513381. Further details are mentioned in chapter 2 sections 2.1.1 and 2.1.2.

4.2.3 Patient recruitment criteria:

The inclusion and exclusion criteria has been discussed in chapter 3 section 3.2.3

4.2.4 Study visits:

The study visits has been explained in chapter 3 section 3.2.4

4.2.5 Randomization and blinding:

Explained in chapter 3 section 3.2.3

4.2.6 Anthropometric measures:

Described in section 2.3.4

4.2.7 Blood sampling and laboratory analyses:

Described in section 2.3.4

4.2.8 Microparticles quantification:

The details have been discussed in chapter 2 section 2.3.2.

In this study, a Becton Dickinson FACS Calibur instrument (BD Biosciences, Oxford, UK) was used to quantify EMPs. Firstly, citrated whole blood tubes were spun at 180 x *g* for 10 minutes and then subsequently spun at 12,000 x *g* for 10 min to remove platelets. The antibodies used in this study were FITC Mouse Anti-Human CD31, FITC Mouse anti Human CD54, FITC Mouse anti Human CD62, FITC Mouse Anti-Human CD 105, FITC Mouse Anti-Human CD 106, FITC Mouse Anti-Human CD 144 and FITC Mouse Anti-Human CD 146.

4.3 Statistical plan:

4.3.1 Sample size calculation:

The sample size calculation has been explained in chapter 3 section 4.4.1

4.3.2 Statistical analysis:

The two randomized groups were compared statistically at baseline. Comparisons between both the two groups from baseline were carried out using the paired *t*-test. The Wilcoxon signed rank test was applied to data that violated the assumptions of normality when tested using the Shapiro-Wilk test. The effect of treatment on MPs was first evaluated by calculating the percentage change from baseline and then the percentage change for each variable in each patient group, thus negating the differences in the baseline values of the two groups. The formula used for measuring percent change was:

$$\frac{\boldsymbol{New\ Value(V3) - Baseline\ Value(V2)}}{\boldsymbol{Baseline\ Value\ (V2)}} \times \mathbf{100}$$

Between-group comparison of percent changes was performed using independent-samples *t*-test. The Wilcoxon rank-sum test (Mann–Whitney U test) was applied to data that violated the assumptions of normality when tested using the Shapiro-Wilk test. For all analyses, a two-tailed $P \leq 0.05$ was considered to indicate statistical significance. Statistical analysis was performed using IBM SPSS Statistics for Windows, version 24.0 (Armonk, NY: IBM Corp.).

4.4 Results:

4.4.1 Baseline characteristics and microparticles of patients:

The baseline comparison of anthropometric, hormonal and cardiovascular markers between vitamin D and placebo groups has been explained in chapter 3, section 3.5.3 and table 3.2. The baseline comparison of microparticles between vitamin D and placebo groups is shown below in table 4.1. There were no significant differences between groups in any of the microparticles at baseline.

Parameters	Vitamin D group	Placebo group	P value
CD31	5693.5 ± 6367.9	5346.8 ± 3872	0.3
CD54	380.9 ± 238.4	284.0 ± 197.2	0.3
CD62	500.1 ± 637.4	361.0 ± 212.5	0.6
CD105	91.2 ± 106.2	71.3 ± 91.3	0.7
CD106	383.6 ± 579.2	339.8 ± 507.1	0.8
CD144	215 ± 358.1	192.7 ± 229.8	0.4
CD146	10.8 ± 20.3	6.7 ± 7.1	0.7

Table 4.1: Baseline microparticles comparison between vitamin D and placebo group

Legend:

Abbreviations: CD31, Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1): CD54, Intercellular Adhesion Molecule-1(ICAM-1): CD62, Cell Adhesion Molecule E-selectin: CD105, Endoglin (ENG): CD106, Vascular Cell Adhesion Molecule-1 (VCAM-1): CD144, Vascular Endothelial Cadherin (VE-cadherin): CD146, Melanoma Cell Adhesion Molecule (MCAM):

Data are presented as mean ± SD. * = $p<0.05$

4.4.2 Recruitment of patients:

Detailed in chapter 3 section 3.2.2

4.4.3 Compliance:

Detailed in chapter 3, section 3.5.2

4.4.4 Seasonal variations and changes in Vitamin D after supplementation:

The vitamin D levels increased significantly in the vitamin D group as compared to the placebo group, as detailed in Chapter 3 section 3.5

4.4.5 Microparticles:

In this study, no significant differences were observed in endothelial microparticles (CD31, CD54, CD62, CD105, CD106, CD144, and CD146) between vitamin D and placebo groups when percentage change was measured (figure 4.1, table 4.2).

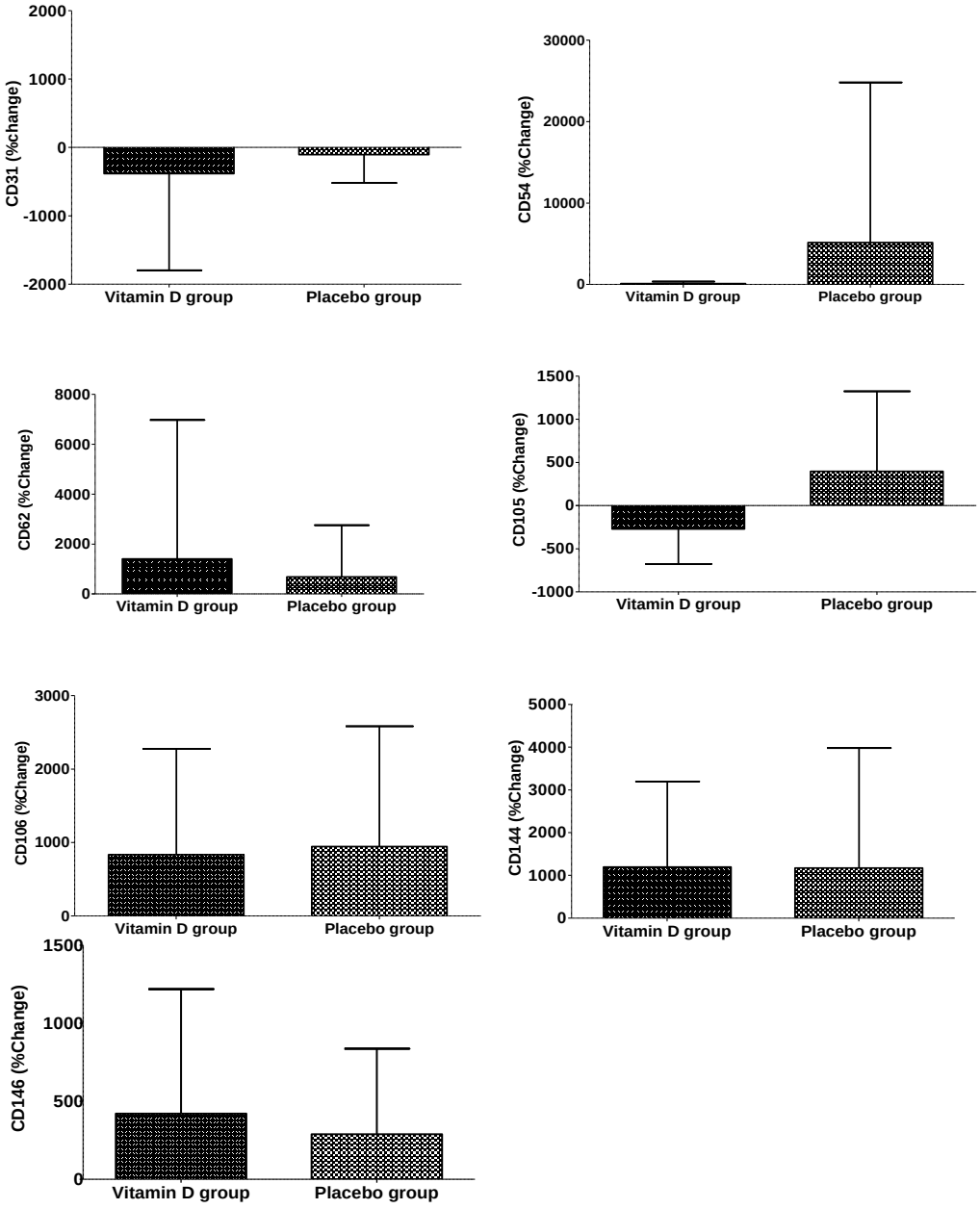

Figure 4.1: Graphical representation of effects of vitamin D and placebo supplementation on endothelial microparticles.

Legend: CD31, Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1): CD54, Intercellular Adhesion Molecule-1(ICAM-1): CD62, Cell Adhesion Molecule E-selectin: CD105, Endoglin (ENG): CD106, Vascular Cell Adhesion Molecule-1 (VCAM-1): CD144, Vascular Endothelial Cadherin (VE-cadherin): CD146, Melanoma Cell Adhesion Molecule (MCAM): % Change (Percent change is the percent difference compared with the baseline).

*States statistically significant ($p<0.05$)

	Vitamin D group (n = 18)			Placebo group (n = 19)			% Change		
Microparticles	**Baseline**	**12 weeks**	**P value**	**Baseline**	**12 weeks**	**P value**	**Vitamin D**	**Placebo**	**P value**
CD31	5693.5 ± 6367.9	3906.8 ± 1545.7	0.9	5346.8 ± 3872	4845.5 ± 2636.8	0.5	-381.7 ± 1413.6	-105.5 ± 414.5	0.6
CD54	380.9 ± 238.4	481.9 ± 271.6	0.2	284.0 ± 197.2	6628.6 ± 22293.2	0.00**	124.4 ± 248.5	5136.3 ± 19680.8	0.09
CD62	500.1 ± 637.4	1049.9 ± 1792.8	0.08	361.0 ± 212.5	3271.8 ± 10386.9	0.003**	1406.6 ± 5566.6	682.6 ± 2076.1	0.6
CD105	91.2 ± 106.2	83.3 ± 77.5	1.0	71.3 ± 91.3	316.7 ± 982.0	0.5	-272.6 ± 404.7	396.0 ± 927.7	1.0
CD106	383.6 ± 579.2	684.6 ± 580.9	0.08	339.8 ± 507.1	844.4 ± 921.5	0.1	836.2 ± 1438.8	945.7 ± 1636.2	0.6
CD144	215 ± 358.1	459.8 ± 267.7	0.005**	192.7 ± 229.8	497.5 ± 383.7	0.003**	1194.3 ± 2004	1174.7 ± 2805.7	0.4
CD146	10.8 ± 20.3	11.7 ± 14.8	0.6	6.7 ± 7.1	13.8 ± 19.3	0.3	420.4 ± 798.8	288.3 ± 549.3	1.0

Table 4.2: Comparison of microparticles evaluation before and after vitamin D or placebo supplementation

Legend:

Abbreviations: CD31, Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1): CD54, Intercellular Adhesion Molecule-1(ICAM-1): CD62, Cell Adhesion Molecule E-selectin: CD105, Endoglin (ENG): CD106, Vascular Cell Adhesion Molecule-1 (VCAM-1): CD144, Vascular Endothelial Cadherin (VE-cadherin): CD146, Melanoma Cell Adhesion Molecule (MCAM):

Data are presented as mean ± SD. Percent change is the percent difference compared with the baseline.

* = $p<0.05$, **=$p<0.01$, P value = % change of the difference between Groups (95% CI)

4.4.5.1 CD31: Platelet endothelial cell adhesion molecule-1 (PECAM-1):

There were no significant changes in CD31 microparticles after both vitamin D and placebo supplementation compared to their baseline values (Figure 4.2). Similarly, no significant differences were observed in the vitamin D group as compared to placebo group when percentage change was performed (-381.7 ± 1413.6 vs -105.5 ± 414.5, p=0.6) as shown in Figure 4.1 and Table 4.2.

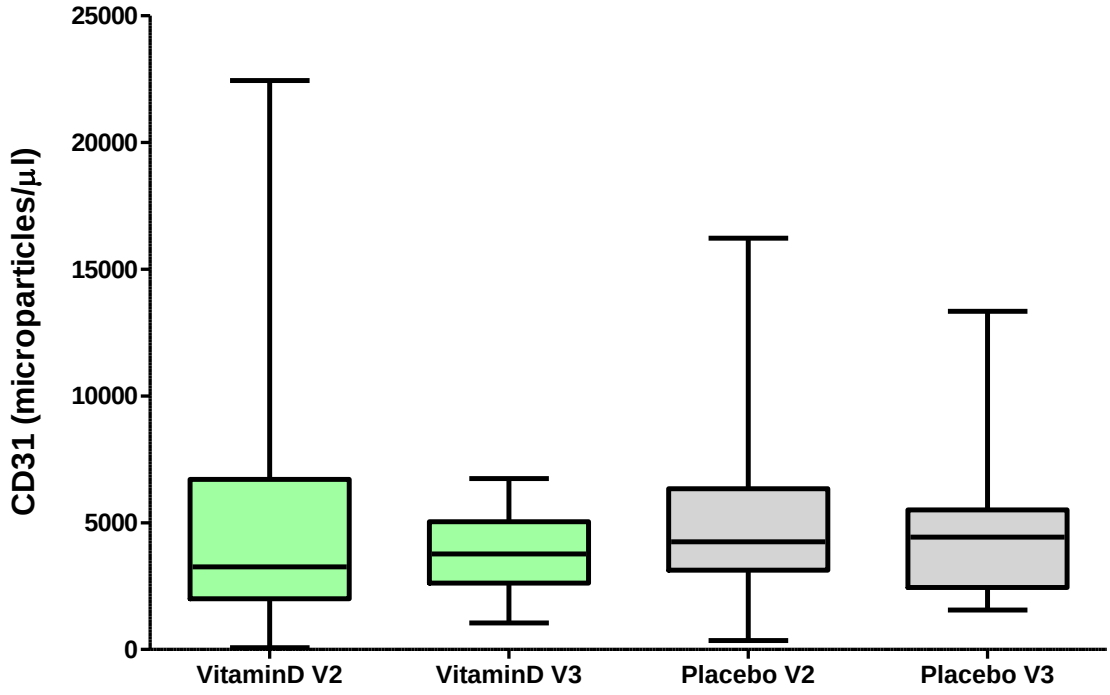

Figure 4.2: Changes in CD31 microparticles before and after vitamin D or placebo supplementation
Legend: Vitamin D V2, Baseline levels of CD31 microparticles in vitamin D group; Vitamin D V3, Levels of CD31 microparticles after vitamin D supplementation; Placebo V2, Baseline levels of CD31 microparticles in placebo group; Placebo V3, Levels of CD31 microparticles after placebo supplementation

4.4.5.2 CD54: Intercellular adhesion molecule-1(ICAM-1):

The CD54 microparticles were increased significantly in the placebo group (284.0 ± 197.2 vs 6628.6 ± 22293.2; p<0.001) but not in vitamin D group when compared to their baseline

values (Figure 4.3). However, no significant differences were observed in the vitamin D group as compared to placebo group when percentage change was performed (124.4 ± 248.5 vs 5136.3 ± 19680.8; p=0.09) as shown in Figure 4.1 and Table 4.2.

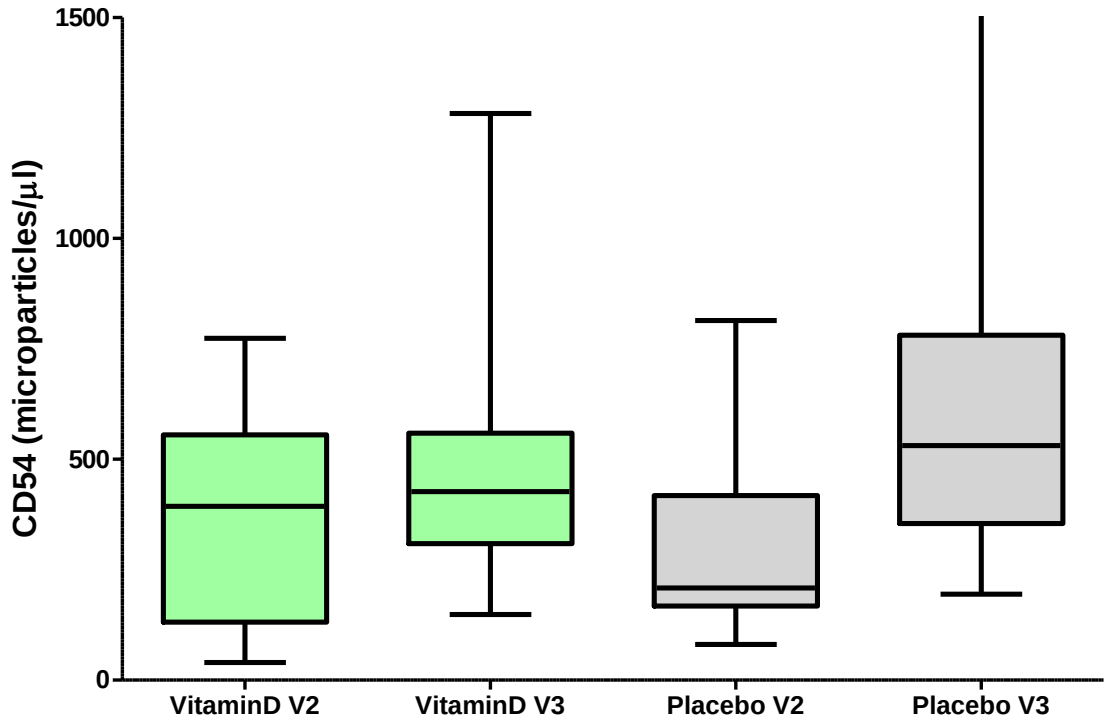

Figure 4.3: Changes in CD54 microparticles before and after vitamin D or placebo supplementation

Legend: Vitamin D V2, Baseline levels of CD54 microparticles in vitamin D group; Vitamin D V3, Levels of CD54 microparticles after vitamin D supplementation; Placebo V2, Baseline levels of CD54 microparticles in placebo group; Placebo V3, Levels of CD54 microparticles after placebo supplementation

4.4.5.3 CD62: Cell adhesion molecule E-selectin:

The CD62 microparticles were increased after both vitamin D and placebo supplementation compared to their baseline values; however, it was significant only in the placebo group

(361.0 ± 212.5 vs 3271.8 ± 10386.9; p=0.003) (Figure 4.4). When percentage change was performed, no significant differences were observed between vitamin D and placebo groups (1406.6 ± 5566.6 vs 682.6 ± 2076.1; p=0.6) as explained in Figure 4.1 and Table 4.2.

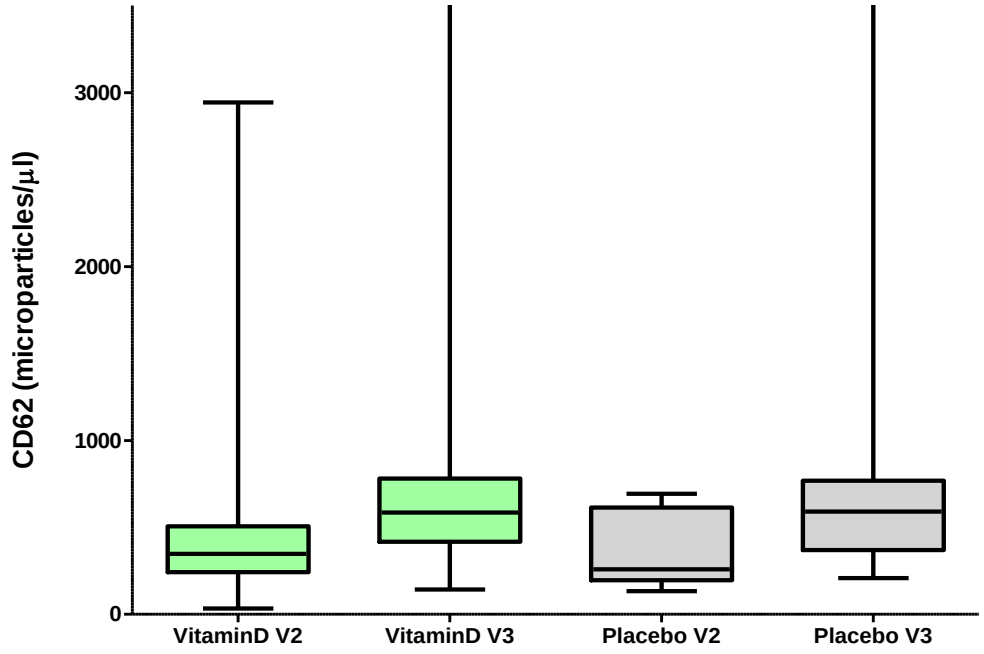

Figure 4.4: Changes in CD62 microparticles before and after vitamin D or placebo supplementation

Legend: Vitamin D V2, Baseline levels of CD62 microparticles in vitamin D group; Vitamin D V3, Levels of CD62 microparticles after vitamin D supplementation; Placebo V2, Baseline levels of CD62 microparticles in placebo group; Placebo V3, Levels of CD62 microparticles after placebo supplementation

4.4.5.4 CD105: Endoglin (ENG):

There were no significant changes in CD105 microparticles compared to the baselines in both vitamin D and placebo groups (Figure 4.5). Similarly, no differences were observed in percentage change in CD105 microparticles between vitamin D and placebo group (-272.6 ± 404.7 vs 396.0 ± 927.7; p=1.0) as revealed in Figure 4.1 and Table 4.2.

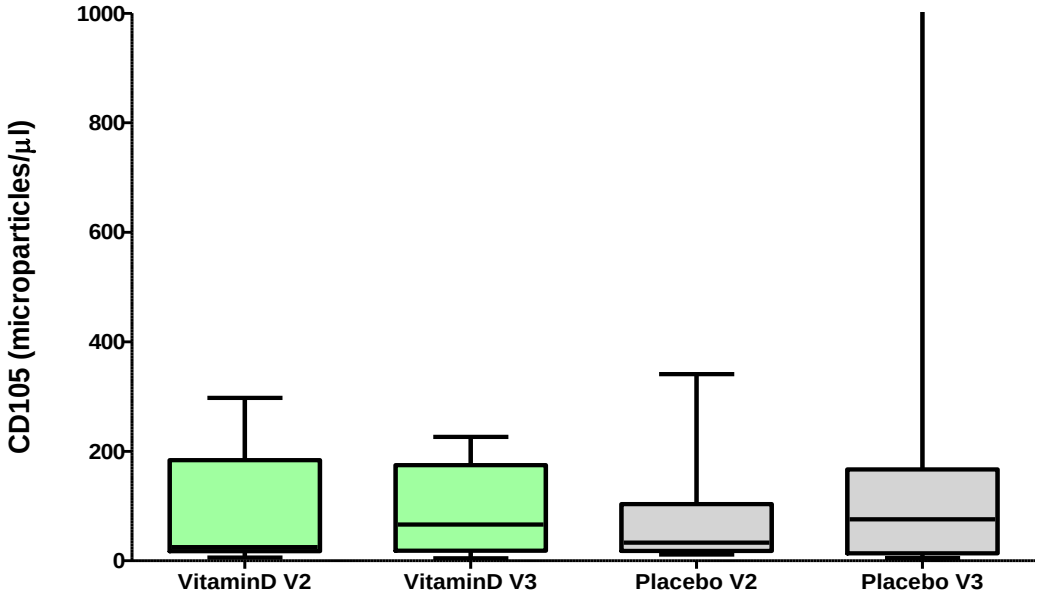

Figure 4.5: Changes in CD105 microparticles before and after vitamin D or placebo supplementation

Legend: Vitamin D V2, Baseline levels of CD105 microparticles in vitamin D group; Vitamin D V3, Levels of CD105 microparticles after vitamin D supplementation; Placebo V2, Baseline levels of CD105 microparticles in placebo group; Placebo V3, Levels of CD105 microparticles after placebo supplementation

4.4.5.5 CD106: Vascular cell adhesion molecule-1 (VCAM-1):

There were no significant changes in CD106 microparticles in both vitamin D and placebo groups when compared to their baseline values (Figure 4.6). Similarly, no significant differences were observed when percentage change was performed (836.2 ± 1438.8 vs 945.7 ± 1636.2; p=0.6) as shown in Figure 4.1 and Table 4.2.

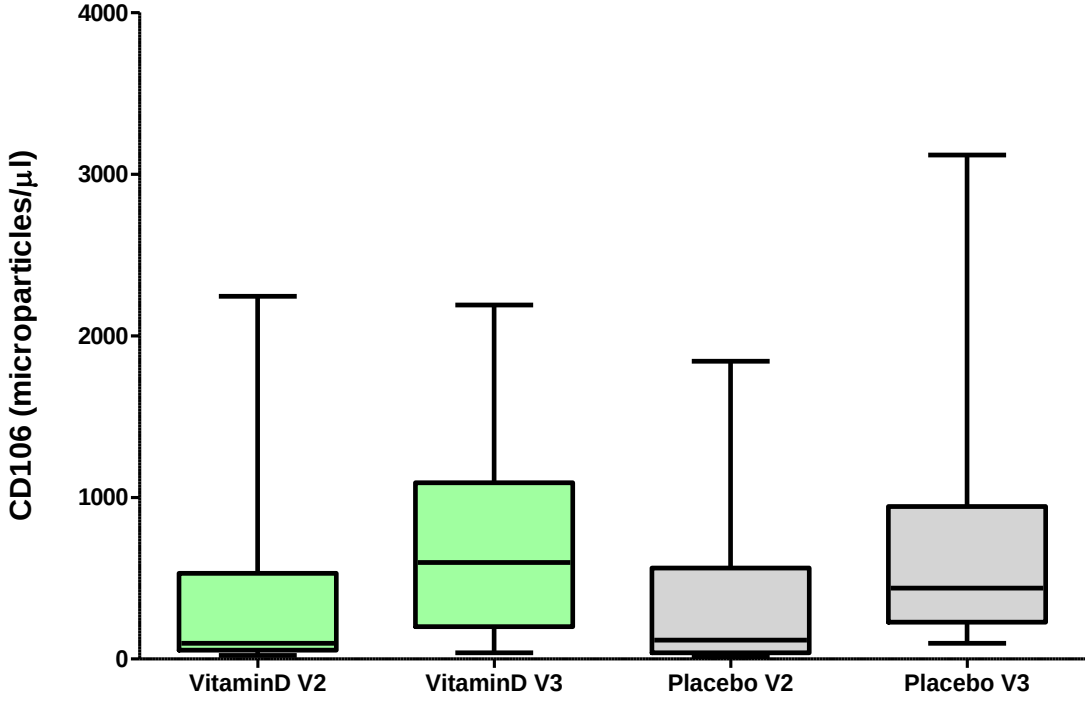

Figure 4.6: Changes in CD106 microparticles before and after vitamin D or placebo supplementation

Legend: Vitamin D V2, Baseline levels of CD106 microparticles in vitamin D group; Vitamin D V3, Levels of CD106 microparticles after vitamin D supplementation; Placebo V2, Baseline levels of CD106 microparticles in placebo group; Placebo V3, Levels of CD106 microparticles after placebo supplementation

4.4.5.6 CD144: Vascular endothelial cadherin (VE-cadherin):

The CD144 microparticles were increased significantly after both vitamin D (215 ± 358.1 vs 459.8 ± 267.7; p=0.005) and placebo supplementation (192.7 ± 229.8 vs 497.5 ± 383.7; p=0.003) as compared to their baseline values (Figure 4.7). However, no difference in percentage change was revealed between vitamin D and placebo groups (1194.3 ± 2004 vs 1174.7 ± 2805.7; p=0.4) as presented in Figure 4.1 and shown in Table 4.2.

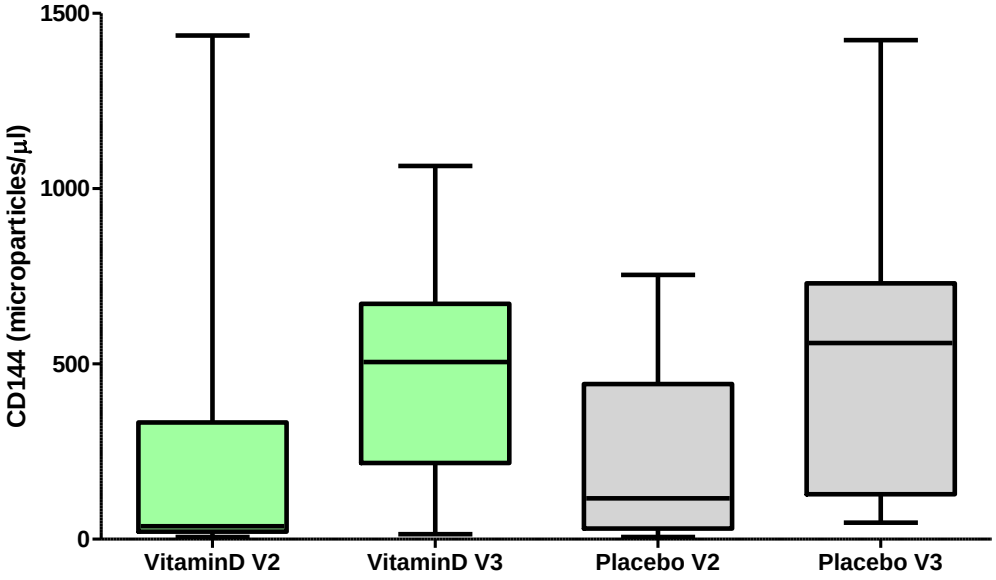

Figure 4.7: Changes in CD144 microparticles before and after vitamin D or placebo supplementation

Legend: Vitamin D V2, Baseline levels of CD144 microparticles in vitamin D group; Vitamin D V3, Levels of CD144 microparticles after vitamin D supplementation; Placebo V2, Baseline levels of CD144 microparticles in placebo group; Placebo V3, Levels of CD144 microparticles after placebo supplementation

4.4.5.7 CD146: Melanoma cell adhesion molecule (MCAM):

There was no change in CD146 microparticles in both vitamin D and placebo groups when compared to their baseline values (Figure 4.8). Similarly, no difference in CD146 microparticles were seen in the vitamin D group as compared to placebo group when percentage change was done (420.4 ± 798.8 vs 288.3 ± 549.3; p=1.0) as shown in Figure 4.1 and Table 4.2.

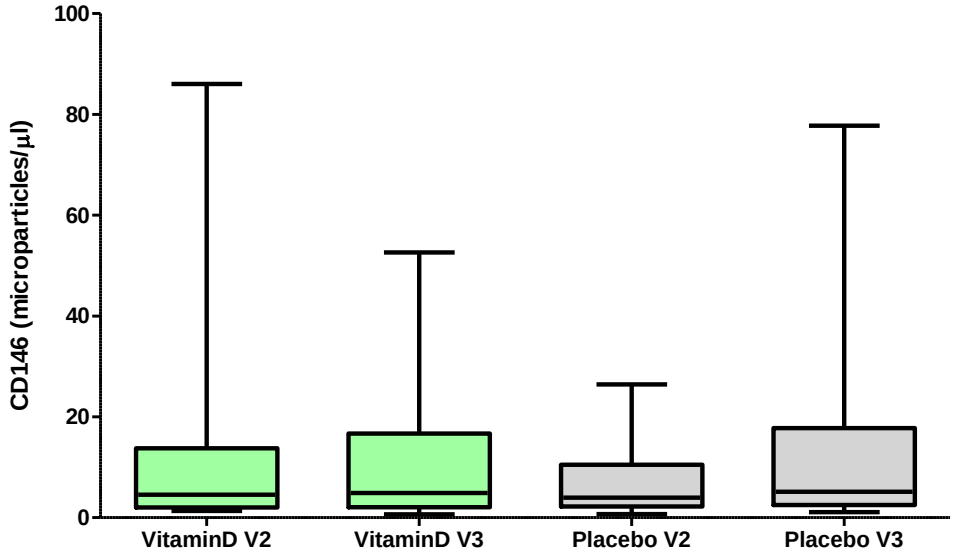

Figure 4.8: Changes in CD146 microparticles before and after vitamin D or placebo supplementation

Legend: Vitamin D V2, Baseline levels of CD146 microparticles in vitamin D group; Vitamin D V3, Levels of CD146 microparticles after vitamin D supplementation; Placebo V2, Baseline levels of CD146 microparticles in placebo group; Placebo V3, Levels of CD146 microparticles after placebo supplementation

4.5 Discussion:

In this study, no statistically significant changes were observed in endothelial microparticles (CD31, CD54, CD62, CD105, CD106, CD144 and CD146) after three months of vitamin D supplementation as compared to the placebo group. However, there was significant increase in CD54 and CD62 microparticles in the placebo group and CD144 microparticles in both vitamin D and placebo groups as compared to their baseline values.

CD54 or ICAM-1 is a cell surface protein that is expressed mainly on the endothelial cells and other cells of immune system such as lymphocytes and macrophages. CD54 is normally expressed in low amounts continuously on the endothelial cells, but upon activation or stimulation, during acute or chronic inflammatory diseases, its expression is increased greatly. CD54 is a ligand for the integrin, which is a leukocyte function associated antigen-1 (LFA-1). LFA-1 attachment with CD54 is a vital step for the transmigration of leukocytes across the endothelial cells into the sites of inflammation, injury, and immune reactions (457). PCOS is

associated with chronic low grade inflammation that may contribute to an increased risk of future cardiovascular events, and may contribute to the underlying pathology of the endocrine and metabolic abnormalities including endothelial dysfunction that is found (324). In this study, vitamin D supplementation had no significant affect on the expression of CD54 microparticles while in the placebo group their expression was significantly increased, suggesting enhanced inflammation and worsening of endothelial function in the placebo group but not in the vitamin D group. Similarly, CD62 or E-selectins play a vital role in the acute and chronic inflammatory processes and are involved in the key steps of leukocytes migration to the sites of inflammation such as capture, rolling and slow-rolling steps with subsequent transmigration across the endothelial cells to the tissues (458). There were no significant changes in CD62 microparticles after vitamin D supplementation in our study however; they were increased significantly in the placebo group suggesting worsening underlying inflammation and endothelial function in the placebo group. In the recent several in-vitro studies, the effects of vitamin D on human umbilical vein endothelial cells (HUVECs) were studied. They revealed that after incubation with vitamin D there was reduced expression of ICAM-1, VCAM-1, the urokinase receptor (uPAR) and membrane type-1 matrix metalloproteinase (MT1-MMP) by the human endothelial cells and CD62P by the platelets (459, 460). The possible underlying mechanism for the reduced production of microparticles after vitamin D supplementation has been explained in a recent study on HUVECs, which describes that vitamin D by acting through its receptors on the endothelial cells, could suppress the production of microparticles by ameliorating the oxidative stress (454).

Both CD144 and CD146 are expressed by activated endothelial cells and are involved in promoting tissue inflammation and maintaining vascular haemostasis (461, 462). Interestingly, there was significant increase in the CD144 microparticles in both the vitamin

D and placebo groups suggesting some worsening of underlying inflammatory process and endothelial function.

When the endothelial cells are activated or stimulated, the microparticles released express certain other (CD31, CD105 and CD106) specific surface proteins in addition to CD54, CD62, CD144 and CD146 (299). CD31 makes up a big portion of endothelial cells intercellular junctions in addition to be present on surface of platelets, neutrophils and monocytes. CD31 plays a major role in tissue inflammation (463). CD105 is a part of transforming growth factor-beta (TGF-β), is expressed in abundant on activated endothelial cells and has important role in angiogenesis and to promote inflammation (464). CD106 mediates the adhesion of leukocytes to vascular endothelium and is involved in the development of chronic inflammation like rheumatoid arthritis and vascular atherosclerosis (465, 466). Similarly, both CD144 and CD146 are expressed by activated endothelial cells and are involved in promoting tissue inflammation and maintaining vascular haemostasis (461, 462).

There was substantial increase in the microparticles levels related to CD31, CD105, CD106, CD144 and CD146 in both the groups but no differences were found between the groups on percentage change analysis. There could be several possible reasons for that. Firstly, the dose and duration of the vitamin D treatment was not enough to suppress the oxidative stress to level where endothelial cells activation and production of all the endothelial microparticles could be reduced. This is supported by evidence suggesting that vitamin D actions on the endothelial cells are dose and time dependent (441). They also found that inhibition of VDR expression on endothelial cells prevented vitamin D induced endothelial activation and proliferation, and concluded that sufficient levels of vitamin D and proper VDR expression are vital for suppressing oxidative stress in endothelial cells (441). Secondly, PCOS patients have chronic low-grade inflammation that is associated with chronic oxidative stress, which

has been demonstrated to downregulates VDRs on the endothelial cells (467). This again possibly suggests either higher doses or longer duration of treatment with vitamin D in PCOS to improve endothelial function was needed.

Several randomized controlled trials (RCTs) have been conducted in a range of patients to evaluate the effects of vitamin D supplementation on endothelial function in the last decade (468) and most of them have shown negative results (469-475). The first possible reason suggested was acute calciotropic effects of vitamin D supplementation, which cause increased influx of calcium into vascular smooth muscle in the short term with subsequent increase in arterial stiffness before its effects at the vascular smooth muscle (476). Secondly, the duration of most of the studies was very small, only for 12 - 16 weeks. Furthermore, it was suggested that if vitamin D concentrations could be maintained for long durations, an improvement in the endothelial function could be seen. Similarly, one recent small RCT has revealed that improved vitamin D status could decrease the expression of certain genes in endothelial cells and improve endothelial function, but higher daily doses of vitamin D are required to maximize these effects on gene expression (476, 477).

4.6 Conclusion:

Vitamin D supplementation for three months had no significant effect on endothelial microparticles in overweight and obese women with PCOS compared to the placebo group.

5 Chapter 5: Effect of empagliflozin on hormonal, metabolic, and cardiovascular risk markers in overweight and obese women with PCOS.

5.1 Introduction:

I have demonstrated above in the previous chapters of my thesis that vitamin D supplementation could be a potential treatment option for women with PCOS who have low vitamin D as it is helpful in improving metabolic dysfunctions related to PCOS and therefore could reduce future cardiovascular risk. In this study, the focus will be placed upon another potential treatment option, empagliflozin, for women with PCOS, which has been shown to exert several anthropometric, metabolic and cardiovascular related benefits in patients with diabetes.

The clinical concerns relating to PCOS range from menstrual and ovulatory dysfunctions to risk of developing diabetes and cardiovascular disease. The treatment is usually symptom based and the search is still ongoing for a single treatment that could address both the reproductive and metabolic abnormalities related to PCOS (478). Hormonal contraceptives have been the cornerstone for treating common symptoms related to PCOS especially menstrual dysfunction and clinical hyperandrogenaemia (hirsutism) (479). Insulin sensitizers including metformin and thiazolidinediones have been very effective in PCOS for the management of metabolic abnormalities (insulin resistance, hyperinsulinaemia, diabetes mellitus) and chronic anovulation; however, their role in the improvement of hirsutism remains unclear (480). Glucagon like peptide-1 receptor agonists `GLP-1RA) and statins comprise novel therapies with diverse metabolic targets that appear to hold promise for PCOS management (28, 481). Advancing evidence have generated interest in a range of complementary and alternative medical treatments including vitamin D for the management

of PCOS (482). Empagliflozin, a direct inhibitor of sodium-glucose cotransporter 2 (SGLT2) that is a new treatment option for T2DM (483), and has recently shown to induce favourable effects on arterial stiffness and vascular resistance in both T1DM and T2DM patients (381, 382). Empagliflozin has an insulin-independent mechanism (promote urinary glucose excretion by inhibiting glucose reabsorption in the kidney) and does not cause hypoglycaemia in normoglycaemic individual (381, 384). Empagliflozin improved cardiovascular risk significantly in patients with T2DM with 38% relative risk reduction in cardiovascular mortality and 32% relative risk reduction in all-cause mortality (383). Furthermore, experimental models of diabetes have shown that SGLT-2 inhibition reduces oxidative stress and suppresses markers of inflammation and fibrosis (263). The suggested underlying cardioprotective effects of empagliflozin, in addition to lowering HbA1c, are multiple interrelated pathways including changes in insulin resistance, plasma volume, weight/fat mass and inflammation which are also very common pathological features of PCOS. A dose-response effect for 10mg and 25mg empagliflozin has been shown in various studies (384) and no titration for the empagliflozin 10mg and 25mg doses were required in the EMPA-REG study (381).

Therefore, the aim of this study was to explore the effects of empagliflozin on hormonal, metabolic and cardiovascular risk factors in patients with PCOS. The comparator to empagliflozin was metformin that is used widely to improve insulin sensitivity, reduce androgen levels, may reduce diastolic blood pressure, dyslipidaemia and BMI in patients with PCOS (484, 485).

5.2 Subjects and methods:

5.2.1 Ethical approval and informed consent:

The ethical approval and informed consent procedure has been explained in chapter 2 sections 2.1.1 and 2.1.2. The trial was registered on clinicaltrials.gov (ID: NCT03008551).

5.2.2 Subject recruitment:

This was an open-label, randomised, therapeutic exploratory study involving 40 women with PCOS. Further explained already in chapter 2 section 2.1.2

5.2.3 Recruitment criteria:

5.2.3.1 Inclusion criteria:

1) Women, aged 18-45 years (inclusive), with confirmed diagnosis of PCOS based on Rotterdam criteria
2) Presence of both irregular periods and biochemical hyperandrogenaemia.
3) Body mass index ≥25.
4) Negative pregnancy test during screening visit and agreed to use barrier contraception during the study period.

5.2.3.2 Exclusion criteria:

1) Non-classical 21-hydroxylase deficiency, hyperprolactinaemia, Cushing's disease and androgen-secreting tumours were excluded by appropriate tests.
2) Confirmed diagnosis of diabetes or pre-diabetes.
3) Ongoing, inadequately controlled thyroid disorder (subjects on thyroid hormone replacement therapy must be on stable dose for at least 3 months before screening day)
4) History or presence of malignant neoplasms within the last 5 years (except basal and squamous cell skin cancer and in-situ carcinoma).
5) History or plan of any form of gastrointestinal tract surgery.
6) History of pancreatitis (Acute or Chronic).
7) Any disorder which might jeopardize subject`s safety.
8) Subjects who were on any of the following medications within 3 months of recruitment:

- Metformin or other insulin-sensitizing medications (e.g. pioglitazone)
- Hormonal contraceptives (e.g. birth control pills, hormone-releasing implants, etc.)
- Anti-androgens (e.g., spironolactone, flutamide, finasteride, etc.)
- Clomiphene citrate or estrogen modulators such as letrozole
- GnRH modulators such as leuprolide
- Minoxidil

9) Female who were pregnant, breast feeding or intended to become pregnant or of child bearing potential not using adequate contraceptive methods.
10) eGFR<60 within the last 2 weeks.
11) Hypersensitivity to lactose
12) Severe hepatic impairment (ALT >3 times ULN) within the last 2 weeks.
13) Women with history of recurrent urinary tract infections.
14) Haematocrit above the upper limit of normal range within the last 2 weeks.
15) Had been involved in another medicinal trial (CTIMP) within the past four weeks.
16) Known hypersensitivity to the Investigational Medicinal Products or any of their excipients.

All participants were advised to maintain their usual dietary and lifestyle habits during the study.

5.2.4 Study visits:

This was a CTIMP that included three visits to the department of Academic Diabetes, Endocrinology and Metabolism Research Centre; Brocklehurst Building, Hull Royal Infirmary. The details for each visit are shown in Table 5.1.

Schedule of procedures	**Visit1 (Screening) Non fasting**	**Visit 2 (Week 0) (Baseline visit) Fasting**	**Telephone call (Week 6 +/- 4 days)**	**Visit 3 (Week 12 +/- 7 days) (End of study visit) Fasting**	**Telephone call (30 +/- 4 days after final visit 3)**
Consent	x				
Medical and drugs history	x				
Inclusion/Exclusion	x				
Pregnancy test	x				
Randomisation		x			
BP, Pulse rate		x		x	
Height, weight, hip & waist circumference, TANITA		x		x	
Stiffness of arteries-EndoPAT		x		x	
Fasting overnight		x		x	
Bloods for hormonal markers	x (if not done within the last 2 weeks)				
Bloods for		x		x	

hormonal, metabolic and cardiovascular risk markers					
Adipose tissue Biopsy (optional)		x		x	
Study medications dispensed		x			
Adverse event recording		x	x	x	x
Change in concomitant medication			x	x	
Study medication compliance			x	x	

Table 5.1: Empagliflozin versus metformin in PCOS study timetable
Legend: BP, Blood pressure; TANITA, Body composition analyzer; EndoPAT, Endothelial-Peripheral arterial tonometry

5.2.5 Randomization:

PCOS patients were randomized on a 1:1 ratio to receive either empagliflozin 25mg or metformin SR 1500mg daily for three months. The online web- based randomisation service https://www.sealedenvelope.com was used. Randomisation was blocked (using random

permuted blocks). Patients were randomised to empagliflozin 25mg daily to get the maximum metabolic response with comparable duration to metformin treatment group. Metformin group received Metformin SR 1500mg, which is the standard dose commonly, used in patients with PCOS in clinical practice (385). Metformin SR was used instead of immediate release metformin in view of better gastrointestinal tolerability (486).

5.3 Study measurements:

5.3.1 Anthropometric measures:

The anthropometric measures have been explained in chapter 2 section 2.3.4.

5.3.2 Blood sampling and laboratory analysis:

Already explained in chapter 2 sections 2.3.3, 2.3.4 and 2,3,6.

5.3.3 Statistical plan:

5.3.3.1 Sample size calculation:

As there were no published studies on empagliflozin or other SGLT- 2 inhibitors in women with PCOS from which to estimate sample size. Choice of numbers was pragmatic, and not based on hypothesis testing. The study was a pilot to a larger (definitive) randomized controlled trial (RCT). There was little published literature on estimating numbers for pilot studies (487-490). The authors recommended a minimum of 12 patients per group. 20 per patients group were recruited to allow for loss-to follow-up (assumed non-differential between-groups).

5.3.3.2 Statistical analysis:

The statistical analysis has already been explained in chapter 3 under section 3.4.

5.4 Results:

5.4.1 Recruitment and progress of patients in trial:

Forty-two women with PCOS were screened for the study. Two patients were excluded as they did not fulfil the inclusion and exclusion criteria. Forty patients were recruited. Twenty patients were randomized to empagliflozin 25mg and other twenty to metformin 1500mg daily for 12 weeks. Thirty nine patients completed the study period and only one patient from the empagliflozin group dropped out of the study (figure 5.1).

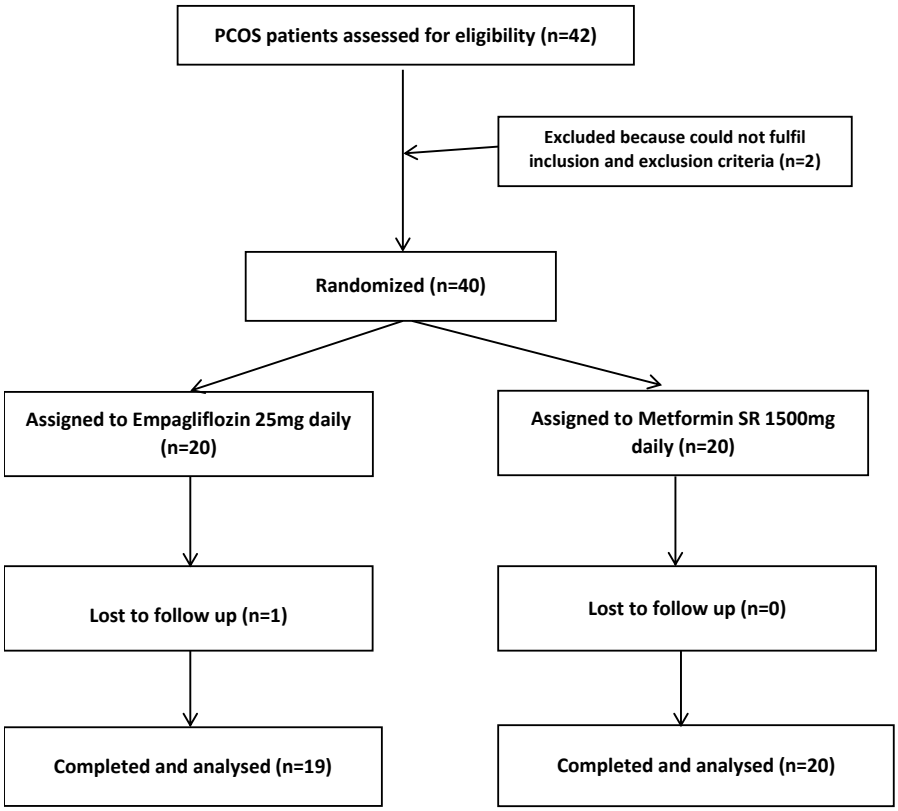

Figure 5.1: Flow chart showing the progress of patients through the trial

5.4.2 Compliance:

The compliance with the treatment was calculated by counting the returned medications. After their exclusion, compliance was 90% in empagliflozin group while it was 97% in metformin group. None of the subjects developed any significant adverse events in the course of the study.

5.4.3 Baseline characteristics of patients and changes after empagliflozin or metformin treatment:

The mean age group of patients recruited in the study was 28.6 ± 5.7 years (empagliflozin 26.5 ± 5.0 *vs.* metformin 31 ± 5.8 years). The baseline anthropometric, hormonal and biochemical parameters of the two groups are given below in table 5.2. The patients in the empagliflozin were younger than in the metformin group (p=0.02). There were no significant differences between empagliflozin and metformin groups regarding baseline values of anthropometric, hormonal and cardiovascular risk factors except fasting glucose levels (p=0.04).

Parameters	Empagliflozin group	Metformin group	P value
Age (years)	26.5 ± 5.0	31 ± 5.8	0.02*
Weight (kg)	103.1 ± 16.3	108.9 ± 25.3	0.4
BMI (kg/m^2)	37.2 ± 6.1	38.8 ± 7.8	0.5
Waist circumference (cm)	101.2 ± 9.7	106.2 ± 15.7	0.3
Hip circumference (cm)	121.6 ± 11.5	124.1 ± 17.4	0.6
SBP (mmHg)	118.1 ± 11.7	124.4 ± 15.5	0.2
DBP (mmHg)	76.4 ± 8.3	80.3 ± 10.7	0.2
RHI	1.7 ± 0.4	1.8 ± 0.5	0.5
AI	-3.3 ± 11.9	0.6 ± 8.1	0.2
FAI	10.4 ± 3.0	10.0 ± 5.9	0.1
Testosterone (nmol/L)	1.7 ± 0.4	1.8 ± 0.8	0.9
SHBG (nmol/L)	17.3 ± 6.4	21.3 ± 11.2	0.3
Androstenedione (nmol/L)	5.7 ± 1.4	5.7 ± 3.6	0.4
DHEAS (µmol/L)	6.1 ± 1.6	5.5 ± 3.3	0.5
hs-CRP (mg/L)	6.0 ± 3.6	8.1 ± 7.6	0.7
FSH (IU/L)	5.9 ± 1.6	6.7 ± 3.1	0.9
LH (IU/L)	9.7 ± 4.6	10.3 ± 7.4	0.5
Estradiol (pmol/L)	236.9 ± 163.8	310.5 ± 268.5	0.2
17OHP (nmol/L)	2.0 ± 0.9	1.8 ± 1.2	0.9
TC (mmol/L)	4.8 ± 1.0	4.7 ± 0.9	0.7
LDL (mmol/L)	2.8 ± 1.0	2.9 ± 0.8	0.5
HDL (mmol/L)	1.1 ± 0.2	1.2 ± 0.2	0.3
Triglycerides (mmol/L)	1.8 ± 1.0	1.3 ± 0.8	0.07
Cholesterol-HDL ratio	4.4 ± 1.0	3.9 ± 1.2	0.2

Non-HDL cholesterol	3.7 ± 1.0	3.5 ± 0.9	0.5
ALT (IU/L)	26.9 ± 18.4	27.6 ± 18.6	0.9
AST (IU/L)	23.0 ± 9.1	23.9 ± 13.2	0.9
Fasting glucose (nmol/L)	4.6 ± 0.4	4.9 ± 0.5	0.04*
Fasting insulin (μIU/L)	15.9 ± 9.3	16.7 ± 9.2	0.4
Basal metabolic rate (kcal)	1760.7 ± 205	1813.3 ± 273.5	0.5
Fat %	46.7 ± 3.4	46.8 ± 6.2	0.9
Fat mass (kg)	48.9 ± 11.0	52.3 ± 18.1	0.4
Fat free mass (kg)	54.8 ± 5.9	56.7 ± 7.9	0.4
Total body water (kg)	40.1 ± 4.3	41.5 ± 5.8	0.4
Whole body impedance (Ω)	592.7 ± 73.1	572.5 ± 67.6	0.3
Trunk fat %	45.0 ± 3.5	44.4 ± 6.7	0.8
Trunk fat mass (kg)	24.9 ± 4.8	26.0 ± 8.7	0.6
Trunk fat free mass (kg)	30.1 ± 3.0	31.2 ± 4.0	0.3

Table 5.2: Baseline anthropometric, hormonal and metabolic measurements between the empagliflozin group and metformin group

Legend: Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FAI, Free androgen index; SHBG, Sex hormone binding globulin; DHEAS, Dehydroepiandrosterone sulphate; 17OHP, 17-hydroxyprogesterone; PTH, Parathyroid hormone; FSH, Follicle stimulating hormone; LH, Luteinizing hormone; RHI, Reactive hyperaemic index; AI, Augmentation index; TC, total cholesterol; TG, triglycerides; LDL, low density lipoproteins; HDL, high density lipoproteins; hs-CRP, high sensitivity-C-reactive protein; ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase.

Data are presented as mean ± SD. * = $p<0.05$

	Empagliflozin group (n = 19)			Metformin group (n = 20)			% Change		
Parameter	**Baseline**	**12 week**	**P value**	**Baseline**	**12 week**	**P value**	**Empagliflozin**	**Metformin**	**P value**
Weight (kg)	103.1 ± 16.3	101.6 ± 16.3	0.07	108.9 ± 25.3	110.2 ± 25.6	0.01	-1.5 ± 3.3	1.2 ± 2.1	0.005
BMI (kg/m^2)	37.2 ± 6.1	36.6 ± 6.0	0.07	38.8 ± 7.8	39.2 ± 7.8	0.015	-1.4 ± 3.3	1.2 ± 2.1	0.005
Waist (cm)	101.2 ± 9.7	99.6 ± 9.5	0.02	106.2 ± 15.7	106.3 ± 15.4	0.7	-1.6 ± 2.8	0.2 ± 2.1	0.029
Hip (cm)	121.6 ± 11.5	119.2 ± 11.4	0.01	124.1 ± 17.4	125.3 ± 16.7	0.03	-2.0 ± 3.0	1.1 ± 1.9	0.001
SBP (mmHg)	118.1 ± 11.7	117.5 ± 14.2	0.7	124.4 ± 15.5	126.0 ± 15.8	0.3	-0.6 ± 6.7	1.4 ± 5.6	0.34
DBP (mmHg)	76.4 ± 8.3	73.8 ± 9.7	0.1	80.3 ± 10.7	80.7 ± 9.8	0.8	-3.1 ± 9.0	0.8 ± 7.1	0.14
FAI	10.4 ± 3.0	9.2 ± 3.5	0.16	10.0 ± 5.9	8.7 ± 5.3	0.1	-9.1 ± 31.8	-10.0 ± 33.0	0.8
Testosterone (nmol/L)	1.7 ± 0.4	1.6 ± 0.6	0.6	1.8 ± 0.8	1.7 ± 1.0	0.04	-2.5 ± 30.1	-5.2 ± 51.1	0.25
SHBG (nmol/L)	17.3 ± 6.4	19.1 ± 8.3	0.04	21.3 ± 11.2	22.4 ± 12.8	0.6	10.1 ± 22.0	5.7 ± 25.0	0.4
Androstenedione (nmol/L)	5.7 ± 1.4	5.6 ± 1.8	0.8	5.7 ± 3.6	5.4 ± 2.7	0.7	-0.6 ± 22.2	11.3 ± 58.6	0.8
DHEAS (µmol/L)	6.1 ± 1.6	6.1 ± 2.0	1.0	5.5 ± 3.3	5.7 ± 3.0	0.1	-0.4 ± 20.5	8.1 ± 15.0	0.2
17OHP (nmol/L)	2.0 ± 0.9	2.8 ± 1.6	0.1	1.8 ± 1.2	2.2 ± 1.7	0.6	78.3 ± 158.0	72.8 ± 190.2	0.6
FSH (IU/L)	5.9 ± 1.6	6.5 ± 2.7	0.3	6.7 ± 3.1	6.5 ± 3.7	0.2	15.7 ± 55.8	-3.8 ± 31.7	0.1
LH (IU/L)	9.7 ± 4.6	14.1 ± 14.9	0.2	10.3 ± 7.4	8.0 ± 5.3	0.1	40.4 ± 91.8	-1.1 ± 78.8	0.02
Oestradiol (pmol/L)	236.9 ± 163.8	335.3 ± 226.7	0.03	310.5 ± 268.5	295.7 ± 261.0	0.6	76.2 ± 74.5	40.6 ± 134.4	0.1
Fasting glucose (mmol/L)	4.6 ± 0.4	4.5 ± 0.4	0.47	4.9 ± 0.5	4.75 ± 0.4	0.19	-0.8 ± 5.8	-2.3 ± 8.0	0.5

Fasting insulin (µIU/ml)	15.9 ± 9.3	14.6 ± 8.1	0.5	16.7 ± 9.2	16.8 ± 11.3	1.0	1.6 ± 50.6	6.0 ± 77.0	0.8
HOMA-IR	3.5 ± 2.8	2.9 ± 2.1	0.25	3.8 ± 2.8	3.6 ± 2.6	0.8	-3.1 ± 13.9	7.2 ± 20.5	0.7
ALT (IU/L)	26.9 ± 18.4	27.5 ± 21.2	0.7	27.6 ± 18.6	29.4 ± 18.6	0.15	2.9 ± 26.3	11.1 ± 24.5	0.3
AST (IU/L)	23.0 ± 9.1	22.8 ± 12.0	0.96	23.9 ± 13.2	23.6 ± 12.7	0.59	-0.4 ± 20.4	-0.2 ± 11.9	0.97
hs-CRP (mg/L)	6.0 ± 3.6	5.5 ± 4.0	0.6	8.1 ± 7.6	8.3 ± 7.9	0.6	9.8 ± 59.1	8.4 ± 34.0	0.9
RHI	1.7 ± 0.4	1.8 ± 0.8	0.5	1.8 ± 0.5	1.7 ± 0.5	0.7	8.7 ± 38.0	5.1 ± 49.1	0.4
AI	-3.3 ± 11.9	-3.4 ± 13.3	0.9	0.6 ± 8.1	2.3 ± 10.4	0.4	-57.0 ± 169.9	-47.5 ± 145.6	0.9
TC (mmol/L)	4.8 ± 1.0	4.7 ± 1.1	0.5	4.7 ± 0.9	4.5 ± 0.9	0.17	-1.6 ± 13.7	-2.2 ± 8.5	0.9
LDL-C (mmol/L)	2.8 ± 1.0	2.8 ± 1.1	0.5	2.9 ± 0.8	2.8 ± 0.8	0.39	-4.1 ± 26.4	-1.9 ± 9.2	0.8
HDL-C (mmol/L)	1.1 ± 0.2	1.1 ± 0.2	0.8	1.2 ± 0.2	1.1 ± 0.2	0.09	-0.6 ± 9.2	-3.4 ± 9.6	0.4
TG (mmol/L)	1.8 ± 1.0	1.8 ± 1.1	1.0	1.3 ± 0.8	1.2 ± 0.6	0.6	14.3 ± 87.4	6.7 ± 39.7	0.7
Cholesterol : HDL ratio	4.4 ± 1.0	4.3 ± 1.1	0.7	3.9 ± 1.2	4.1 ± 1.0	0.3	-0.5 ± 11.2	11.6 ± 45.3	0.5
Non-HDL Cholesterol	3.7 ± 1.0	3.6 ± 1.0	0.5	3.5 ± 0.9	3.5 ± 0.9	0.8	-1.6 ± 16.5	0.4 ± 12.5	0.7
BMR (kcal)	1760.7 ± 205	1728.1 ± 200	0.016	1813.3 ± 273.5	1814.4 ± 275.8	0.9	-1.8 ± 2.9	0.05 ± 1.9	0.02
Fat %	46.7 ± 3.4	47.1 ± 3.4	0.2	46.8 ± 6.2	47.6 ± 5.9	0.015	0.7 ± 2.5	1.9 ± 3.4	0.2
Fat Mass (kg)	48.9 ± 11.0	48.6 ± 11.0	0.6	52.3 ± 18.1	53.7 ± 18.3	0.005	-0.7 ± 4.9	3.2 ± 5.0	0.02
FFM (kg)	54.8 ± 5.9	53.7 ± 5.8	0.013	56.7 ± 7.9	56.5 ± 7.9	0.6	-2.0 ± 3.2	-0.3 ± 2.2	0.056
TBW (kg)	40.1 ± 4.3	39.3 ± 4.3	0.014	41.5 ± 5.8	41.4 ± 5.8	0.6	-2.0 ± 3.2	-0.3 ± 2.2	0.06
WBI (Ω)	592.7 ± 73.1	611.3 ± 69.7	0.019	572.5 ± 67.6	554.9 ± 146.7	0.5	3.4 ± 5.3	-4.0 ± 22.6	0.1

Trunk Fat%	45.0 ± 3.5	45.7 ± 3.3	0.09	44.4 ± 6.7	45.4 ± 6.4	0.028	1.8 ± 4.1	2.6 ± 5.1	0.6
Trunk Fat Mass (kg)	24.9 ± 4.8	25.1 ± 4.9	0.47	26.0 ± 8.7	27.0 ± 8.9	0.012	1.0 ± 5.7	4.2 ± 6.9	0.1
Trunk FFM (kg)	30.1 ± 3.0	29.4 ± 3.0	0.01	31.2 ± 4.0	31.1 ± 4.1	0.5	-2.2 ± 3.4	-0.4 ± 2.6	0.08

Table 5.3: Comparison of anthropometric, metabolic and hormonal parameters before and after empagliflozin or metformin treatment

Legend:

Abbreviations: BMI, body mass index; SBP, systolic blood pressure in; DBP, diastolic blood pressure; FAI, Free androgen index; SHBG, Sex hormone binding globulin; DHEAS, Dehydroepiandrosterone sulphate; 17OHP, 17-hydroxyprogesterone; FSH, Follicle stimulating hormone; LH, Leuteinizing hormone; HOMA-IR, Homeostatic Model Assessment of Insulin resistance; ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; hs-CRP, high sensitivity-C-reactive protein; RHI, Reactive hyperaemic index; AI, Augmentation index; TC, total cholesterol; TG, triglycerides; LDL, low density lipoproteins; HDL, high density lipoproteins; BMR, Basal metabolic rate; FFM, Fat free mass; TBW, Total body water; WBI, Whole body bioelectrical impedance

Data are presented as mean ± SD. All serum results are obtained from fasting variables. Percent change is the percent difference compared with the baseline. To convert values for glucose to milligrams per deciliter, divide by 0.056. To convert values for insulin to picomoles per liter, multiply by 7. To convert values for cholesterol to milligrams per deciliter, divide by 0.0259. To convert values for triglycerides to milligrams per deciliter, divide by 0.0113.

* = $p<0.05$, **=$p<0.01$, P value = % change of the difference between Groups (95% CI)

There were significant reductions in anthropometric measures and body composition parameters especially waist circumference (WC), hip circumference (HC), basal metabolic rate (BMR), fat free mass (FFM) and total body water (TBW) in patients randomized to empagliflozin while patients on metformin had significant increase in weight, BMI, HC, body fat% and fat mass (table 5.3). In the end of trial between group comparisons, the percentage reduction in anthropometric measures in the empagliflozin group were statistically significant compared to the metformin group; weight (empagliflozin, -1.5 ± 3.3 vs. metformin, 1.2 ± 2.1; p=0.005), BMI (empagliflozin, -1.4 ± 3.3 vs. metformin, 1.2 ± 2.1; p=0.005), WC (empagliflozin, -1.6 ± 2.8 vs. metformin, 0.2 ± 2.1; p=0.029) and HC (empagliflozin, -2.0 ± 3.0 vs. metformin, 1.1 ± 1.9; p=0.001) (Figure 5.2).

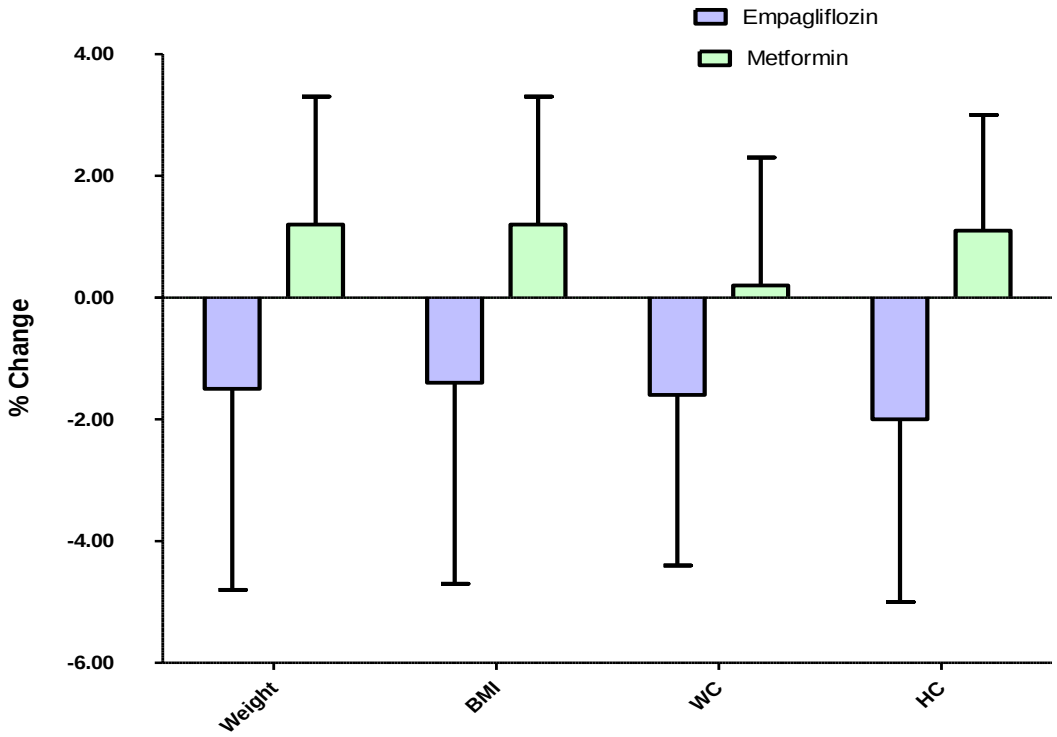

Figure 5.2: Graphical presentation of percent change in anthropometric measures after empagliflozin and metformin treatment

Legend: BMI, body mass index; WC, waist circumference; HC, hip circumference

* = p<0.05, **=p<0.01, P value = % change of the difference between Groups (95% CI)

Similarly, the percent reduction in BMR, fat mass and FFM in the empagliflozin group were statistically significant compared to the metformin group: BMR, (empagliflozin, -1.8 ± 2.9 vs. metformin, 0.05 ± 1; p=0.02), fat mass (empagliflozin, -0.7 ± 4.9 vs. metformin, 3.2 ± 5.0; p=0.02) and FFM (empagliflozin, -2.0 ± 3.2 vs. metformin, -0.3 ± 2.2; p=0.056) (figure 5.3).

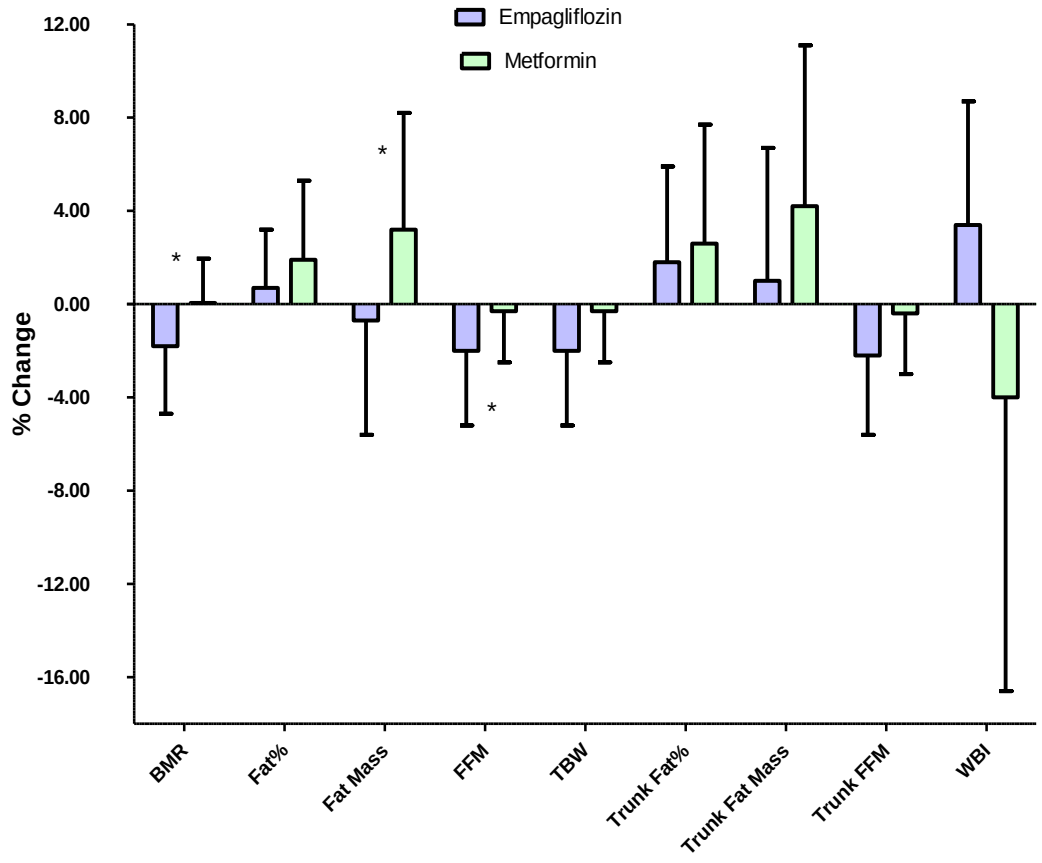

Figure 5.3: Graphical presentation of percent change in body composition parameters after empagliflozin and metformin treatment

Legend: BMR, Basal metabolic rate; FFM, Fat free mass; TBW, Total body water; WBI, Whole body bioelectrical impedance

* = p<0.05, **=p<0.01, P value = % change of the difference between Groups (95% CI)

There was a significant increase in the SHBG levels in the empagliflozin group (p=0.04). There was also a trend in decreases in free testosterone and FAI in the empagliflozin group; however, the changes were not significant. While in the metformin group there was increase

in SHBG and decrease in free testosterone and FAI levels but only testosterone decreased significantly (p=0.04). There were no significant changes in HOMA-IR, RHI, fasting lipids and other hormonal (androstenedione, DHEAS) and metabolic markers (ALT, AST) in both the groups (Table 5.3). The adipose tissue biopsy was included in the study that was optional and cosmetically unacceptable for the patients. Therefore, there were not sufficient samples available for analysis.

5.5 Discussion:

This is the first study that has shown the beneficial effects of the SGLT2 inhibitor (empagliflozin) in overweight and obese women with PCOS. After three months of supplementation with empagliflozin there was significant improvement in metabolic risk factors including weight, BMI, waist and hip circumference associated with significant improvement in body fat composition parameters including total body fat as compared to metformin that is commonly used as a treatment option in PCOS. The results obtained in this study after three months of empagliflozin therapy are comparable to the results observed after metformin treatment with an additional benefit of significant reduction in body weight and fat mass after empagliflozin treatment that suggests empagliflozin could be a potential future treatment option in obese women with PCOS.

Obesity is a complex disorder that is prevalent in women with PCOS and obesity itself is regarded as a high risk factor for future cardiovascular events. In addition, obesity increases the chance to develop metabolic syndrome and diabetes, which further multiplies the future risk of developing cardiovascular problems and therefore a decrease in quality of life and life expectancy (491). The use of BMI, WC and HC for diagnosing and assessing overweight and obesity is well documented in the literature as these parameters have good clinical reliability (492). In this trial, three months of treatment with empagliflozin resulted in a

significant improvement in future cardiovascular risk in overweight and obese women with PCOS by causing significant decrease in these parameters.

BMI cannot distinguish between body fat and lean mass and is unable to classify individuals into those who have a normal body weight with high body fat but small muscle mass and those who have high body weight with small body fat but excessive muscle mass. This differentiation is necessary as percent body fat gives more meaningful clinical information and is a better predictor of future cardiovascular risk as compared to BMI (493, 494). In the current study on overweight and obese women with PCOS, three months treatment with empagliflozin significantly improved body composition parameters (BMR, fat mass and TBW) measured by bioelectrical impedance technique thus improving their future cardiovascular risk. These findings are in accord with the recent trials on SGLT2 inhibitors that have revealed that empagliflozin decreased body weight and also improved indices of body composition in obese patients with type 2 diabetes mellitus and have suggested empagliflozin a very safe treatment option (495-499). The underlying potential mechanism by which SGLT2 inhibitors (empagliflozin) decrease obesity and fat mass has been explained in a recent trial on mice that revealed SGLT2 inhibition augments fat utilization and browning and reduces inflammation and insulin resistance via alternative or M2 macrophage activation (500, 501). Interestingly, in this study there was significant increase in anthropometric measures (weight, BMI and hip circumference) and total body fat in the metformin group. The previous data related to metformin that either it decreases weight or not is still controversial where some studies have shown improvement in weight while others have not shown any benefit (502). Secondly, metformin has shown to be more effective with higher doses and longer treatment duration and less effective as the BMI increases which could be one of the possible reasons in this study as the BMI of patients in the metformin group was higher than the patients in the empagliflozin group (503, 504).

There was a trend to decrease both the systolic (SBP) and diastolic (DBP) blood pressures in the empagliflozin group that could not reach statistical significance but no such trend was seen in the metformin group. Empagliflozin has shown previously to induce a significant decrease in both SBP and DBP in type 2 diabetic patients in the EMPA-REG BP (505, 506), however, these patients had high blood sugars that might have caused a loss of excessive body water along with the loss of glucose in the urine and secondly, they were also on anti-hypertensive drugs. PCOS patients might need longer treatment to have significant drop in blood pressures.

There was statistically significant increase in the SHBG levels in the empagliflozin group but no significant improvement was seen in FAI and free testosterone levels. The increase in SHBG levels did not differ to metformin that was also associated with an increase in SHBG. However, there was significant decrease in the free testosterone levels in the metformin group but the decrease in FAI for empagliflozin was non-significant. Similarly, the decrease in HOMA-IR in the metformin group was also non-significant. There is no previous data available looking at the effects of empagliflozin on the FAI and free testosterone levels; however, SGLT2 inhibitors including empagliflozin have shown to improve insulin resistance previously in both human and animal trials (507, 508).

This is the first trial looking at the effects of empagliflozin on endothelial function but this did not differ as evaluated by reactive hyperaemic index (RHI). Similarly, there were no significant differences in hs-CRP, androstenedione and fasting lipids in both groups. Empagliflozin has shown to potentially improve endothelial dysfunction in animal studies on diabetic rats (509, 510) but no human trial yet is available. However, recently dapagliflozin has shown to improve endothelial function in type 2 diabetic patients but not as a monotherapy but as add-on therapy to metformin for 16 weeks (511). In the clinical trials,

SGLT2 inhibitors have been reported to cause small increase in the total cholesterol, LDL-C and HDL-C levels but no such changes were observed in this study (512, 513).

There are several strengths of this study. Firstly, the compliance of the participants with the study medication was very high. Secondly, almost 98% percent of the study participants completed all the study procedures.

The limitation of this study is the duration of the study; however, the results observed after empagliflozin treatment in this study are comparable to metformin therapy that warrants further large scale and longer duration trial to explore the results further.

5.6 Conclusion:

Empagliflozin improved anthropometric and body composition indices, in overweight and obese women with PCOS, after 12 weeks of treatment and therefore, represents a potential treatment option in PCOS for improving future cardiovascular risk.

6 Chapter 6: Effect of empagliflozin versus metformin on endothelial microparticles in overweight and obese women with PCOS

6.1 Introduction:

In the previous chapter (chapter 5), we discussed that empagliflozin effects on hormonal, metabolic and cardiovascular markers are comparable to metformin and empagliflozin could potentially be used as an alternative treatment in overweight and obese women with PCOS. Further, in this chapter we tried to look more deeply into the effects of empagliflozin treatment on the vascular endothelial cells. We have already discussed in chapters 3, 4 and 5 that women with PCOS are associated with endothelial dysfunction (444) that results in abnormal activation and adhesion of platelets and leukocytes with release of cytokines resulting in atherosclerotic plaque formation (445). In PCOS, several cardiovascular risk factors contribute to endothelial dysfunction leading to an increased cardiovascular risk (446). Additionally, increased levels of EMPs in plasma reflect endothelial cell activation or apoptosis and may represent endothelial dysfunction as suggested in recent trials that EMPs promote endothelial dysfunction by suppressing the production of NO and prostacyclin and are an emerging surrogate markers of endothelial dysfunction (447, 448). The EMPs express specific surface proteins and the flow cytometry relies on the use of these markers for the identification and quantification of endothelial origin of MPs. The most common endothelial proteins expressed are CD31, CD54, CD62, CD105, CD106, CD142, CD144, and CD146 (389) (figure 1.4).

Empagliflozin, a direct inhibitor of SGLT2 is a new treatment option for T2DM (483) that has shown to induce favourable effects on arterial stiffness and vascular resistance in both

T1DM and T2DM patients (381, 382). In the *EMPA-REG OUTCOME(R) trial*, empagliflozin caused 38% relative risk reduction in cardiovascular mortality and 32% relative risk reduction in all-cause mortality in patients with T2DM (383). Furthermore, experimental models of diabetes have shown that SGLT-2 inhibition reduces oxidative stress and suppresses markers of inflammation and fibrosis (263). The suggested possible underlying mechanisms for the beneficial effects of empagliflozin, in addition to lowering HbA1c, are multiple interrelated pathways including changes in insulin resistance, plasma volume, weight/fat mass and inflammation which are also very common pathological features of PCOS (384, 514). Recently a study in Zucker diabetic fatty (ZDF) rats provided further insights into the mechanisms that may mediate the effects of empagliflozin on cardiovascular risk, which is by exhibiting anti-inflammatory effects, reducing glucotoxicity and by preventing the development of endothelial dysfunction (509). Metformin, a standard treatment in PCOS, has shown to improve endothelial dysfunction significantly in young women with PCOS in both human and animal studies (515-517). Recently, in a trial, metformin has also revealed to reduce the total number of microparticles in women with PCOS (518). However, the trials exploring the effects of SGLT2 inhibitors especially empagliflozin on endothelial function in terms of EMPs assessment in women with PCOS are lacking.

Therefore, in this study, the aim was to investigate whether empagliflozin treatment in overweight and obese women with PCOS could improve endothelial dysfunction in terms of EMPs production and compare these effects to the metformin.

6.2 Methods and material:

6.2.1 Ethical approval and informed consent:

The ethical approval and informed consent procedure have been explained in chapter 2 sections 2.1.1 and 2.1.2. The trial was registered on clinicaltrials.gov (ID: NCT03008551).

6.2.2 Subject recruitment:

This was an open-label, randomised, therapeutic exploratory study involving 40 women with PCOS. Further explained already in chapter 2 sections 2.1.2

6.2.3 Recruitment criteria

The inclusion and exclusion criteria has been explained in chapter 5 section 5.2.3

6.2.4 Study visits:

The study visits have been explained in chapter 5 section 5.2.4

6.2.5 Randomization:

The randomization process is explained in chapter 5 section 5.2.5

6.3 Study measurements:

6.3.1 Anthropometric measures:

The anthropometric measures have been explained in chapter 2 section 2.3.4.

6.3.2 Blood sampling and laboratory analysis:

Already explained in chapter 2 sections 2.3.3, 2.3.4 and 2.3.6

6.3.3 Statistical plan:

6.3.3.1 Sample size calculation:

The sample size calculation has been explained in chapter 5 section 5.3.3.1

6.3.3.2 Statistical analysis:

The statistical analysis has already been explained in chapter 3 under section 3.4.

6.4 Results:

6.4.1 Baseline characteristics and microparticles of patients:

The baseline anthropometric, hormonal and cardiovascular risk factors of both empagliflozin and metformin groups have been shown and compared in chapter 5 section 5.4.3 and table 5.2. The baseline microparticles of both groups have been shown and compared below in the table 6.1. None of the microparticles were significantly different between groups.

Parameters	Empagliflozin group	Metformin group	P value
CD31	614.4 ± 228.3	584.5 ± 184.8	0.8
CD54	1210.7 ± 623.4	1101.6 ± 575.1	0.4
CD62	841.4 ± 472.9	770.7 ± 267.3	1.0
CD105	168.1 ± 72.0	136.2 ± 28.5	0.3
CD106	258.1 ± 111.9	262.9 ± 105.3	0.5
CD142	2276.2 ± 1648.6	1740.1 ± 608.0	0.8

Table 6.1: Baseline microparticles comparison between empagliflozin and metformin group

Legend:

Abbreviations: CD31, Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1): CD54, Intercellular Adhesion Molecule-1(ICAM-1): CD62, Cell Adhesion Molecule E-selectin: CD105, Endoglin (ENG): CD106, Vascular Cell Adhesion Molecule-1 (VCAM-1): CD142, Platelet tissue factor

Data are presented as mean ± SD. * = $p<0.05$

6.4.2 Recruitment of patients:

The recruitment and progress of patients through the trial has been detailed in chapter 5 section 5.4.1

6.4.3 Compliance:

The compliance of the patients is detailed in chapter 5 section 5.4.2

6.4.4 Endothelial microparticles:

In this study, there was significant increase in CD54 and CD62 microparticles in the empagliflozin group and CD106 microparticles in both empagliflozin and metformin groups. There was no significant percentage change in CD31, CD54, CD62 CD105, CD106 and CD142 microparticles between the two groups (figure 6.1. table 6.2).

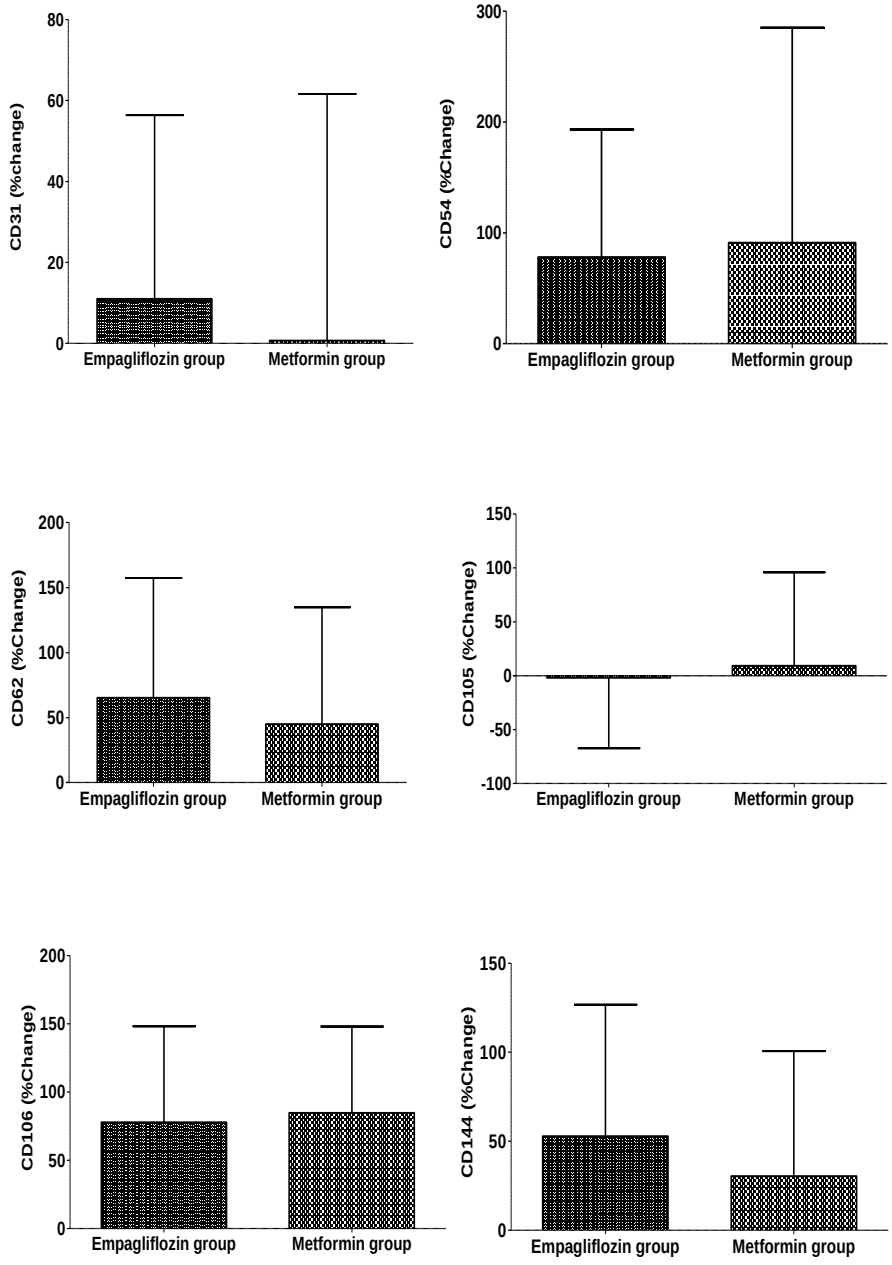

Figure 6.1: Graphical representation of effects of empagliflozin and metformin therapy on endothelial microparticles.

Legend: CD31, Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1): CD54, Intercellular Adhesion Molecule-1(ICAM-1): CD62, Cell Adhesion Molecule E-selectin: CD105, Endoglin (ENG): CD106, Vascular Cell Adhesion Molecule-1 (VCAM-1): CD142, Platelet tissue factor: % Change (Percent change is the percent difference compared with the baseline).

*States statistically significant ($p<0.05$)

	Empagliflozin group (n = 19)			Metformin group (n = 20)			% Change		
Microparticles	**Baseline**	**12 week**	**P value**	**Baseline**	**12 week**	**P value**	**Empagliflozin**	**Metformin**	**P value**
CD31	614.4 ± 228.3	643.0 ± 286.4	0.6	584.5 ± 184.8	574.9 ± 446.5	0.4	11.0 ± 45.4	0.7 ± 60.9	0.3
CD54	1210.7 ± 623.4	2076.2 ± 1884.9	0.004**	1101.6 ± 575.1	1501.7 ± 973.9	0.26	78.0 ± 115.3	91.0 ± 194.0	0.7
CD62	841.4 ± 472.9	1278.1 ± 900.7	0.009**	770.7 ± 267.3	996.4 ± 554.2	0.16	65.3 ± 92.1	44.9 ± 89.8	0.4
CD105	168.1 ± 72.0	142.5 ± 81.1	0.3	136.2 ± 28.5	141.1 ± 99.3	0.3	-1.9 ± 65.3	9.3 ± 86.5	0.8
CD106	258.1 ± 111.9	404.4 ± 98.4	0.001**	262.9 ± 105.3	471.3 ± 207.4	0.000**	77.8 ± 70.3	84.8 ± 63.2	0.8
CD142	2276.2 ± 1648.6	2953.4 ± 2252.0	0.1	1740.1 ± 608.0	1942.5 ± 767.1	0.5	52.9 ± 73.8	30.4 ± 70.2	0.4

Table 6.2: Comparison of microparticles evaluation before and after empagliflozin or metformin supplementation

Legend:

Abbreviations: CD31, Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1): CD54, Intercellular Adhesion Molecule-1(ICAM-1): CD62, Cell Adhesion Molecule E-selectin: CD105, Endoglin (ENG): CD106, Vascular Cell Adhesion Molecule-1 (VCAM-1): CD142, Platelet tissue factor

Data are presented as mean ± SD. Percent change is the percent difference compared with the baseline.

* = p<0.05, **=p<0.01, P value = % change of the difference between Groups (95% CI)

6.4.4.1 CD31: Platelet endothelial cell adhesion molecule-1 (PECAM-1):

There were no significant changes in CD31 microparticles in both empagliflozin and metformin groups as compared to their baseline values (figure 6.2). Similarly, no significant difference was observed in the empagliflozin group as compared to metformin group when percentage change was performed (11.0 ± 45.4 vs 0.7 ± 60.9; p=0.3) as shown in figure 6.1 and table 6.2.

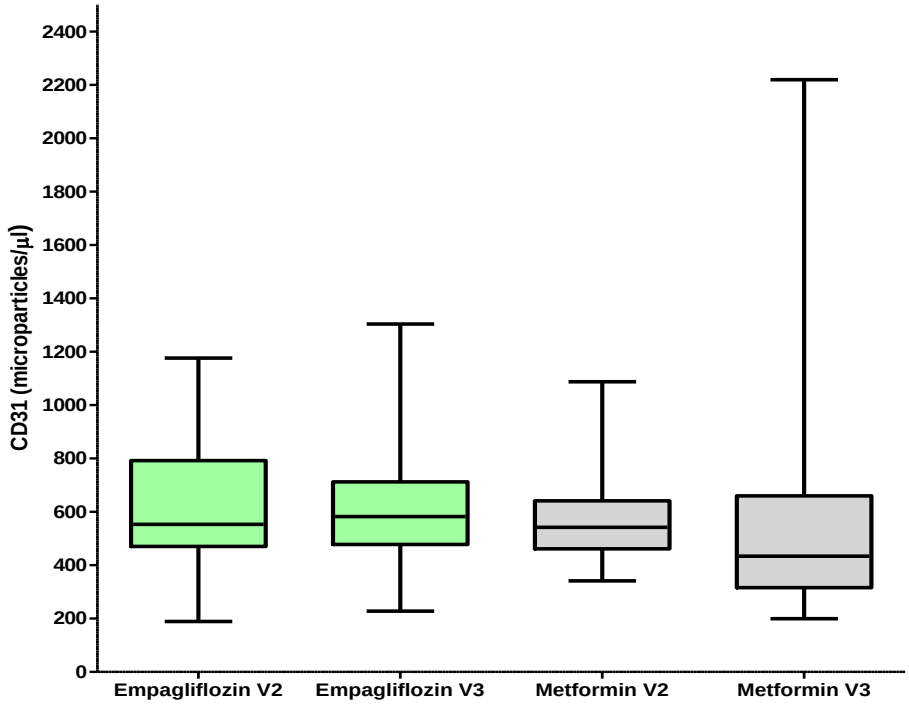

Figure 6.2: Changes in CD31 microparticles before and after empagliflozin or metformin therapy

Legend: Empagliflozin V2, Baseline levels of CD31 microparticles in empagliflozin group; Empagliflozin V3, Levels of CD31 microparticles after empagliflozin treatment; Metformin V2, Baseline levels of CD31 microparticles in metformin group; Metformin V3, Levels of CD31 microparticles after metformin treatment

6.4.4.2 CD54: Intercellular adhesion molecule-1(ICAM-1):

The CD54 microparticles were increased significantly only after empagliflozin treatment (1210.7 ± 623.4 vs 2076.2 ± 1884.9; p=0.004) but not after metformin treatment when compared to their baseline values (figure 6.3). The increase in empagliflozin group was not statistically significant when percentage change was performed as compared to metformin group as explained in figure 6.1 and table 6.2.

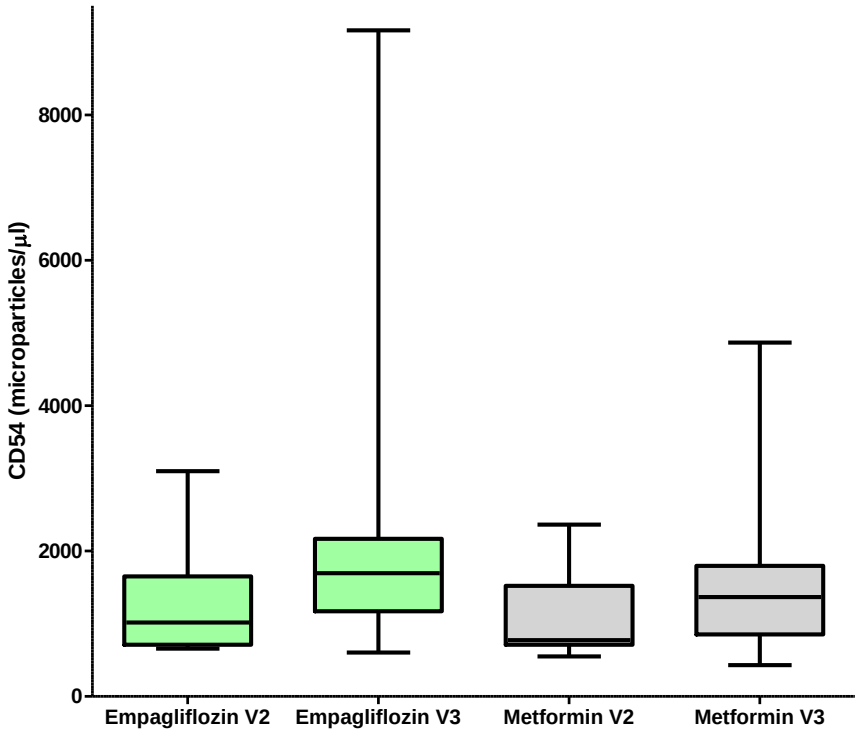

Figure 6.3: Changes in CD54 microparticles before and after empagliflozin or metformin therapy

Legend: Empagliflozin V2, Baseline levels of CD54 microparticles in empagliflozin group; Empagliflozin V3, Levels of CD54 microparticles after empagliflozin treatment; Metformin V2, Baseline levels of CD54 microparticles in metformin group; Metformin V3, Levels of CD54 microparticles after metformin treatment

6.4.4.3 CD62: Cell adhesion molecule E-selectin:

The CD62 microparticles were increased significantly only after empagliflozin treatment (841.4 ± 472.9 vs 1278.1 ± 900.7; p=0.009) but not after metformin treatment when compared to their baseline values (figure 6.4). However, the increase in the empagliflozin group was not statistically significant when percentage change was performed as compared to metformin group as explained in figure 6.1 and table 6.2.

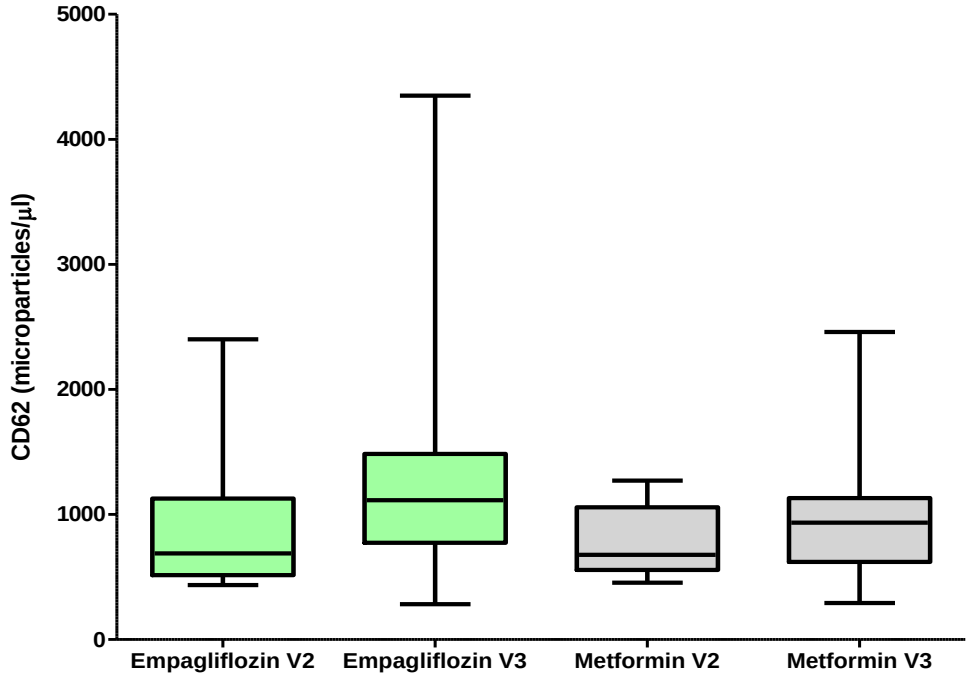

Figure 6.4: Changes in CD62 microparticles before and after empagliflozin or metformin therapy

Legend: Empagliflozin V2, Baseline levels of CD62 microparticles in empagliflozin group; Empagliflozin V3, Levels of CD62 microparticles after empagliflozin treatment; Metformin V2, Baseline levels of CD62 microparticles in metformin group; Metformin V3, Levels of CD62 microparticles after metformin treatment

6.4.4.4 CD105: Endoglin (ENG):

There were no significant changes in CD105 microparticles in both the empagliflozin and metformin groups compared to their baseline values (figure 6.5). Similarly, no significant differences were observed in percentage change in CD105 microparticles between empagliflozin and metformin group (-1.9 ± 65.3 vs 9.3 ± 86.5; p=0.8) as revealed in figure 6.1 and table 6.2.

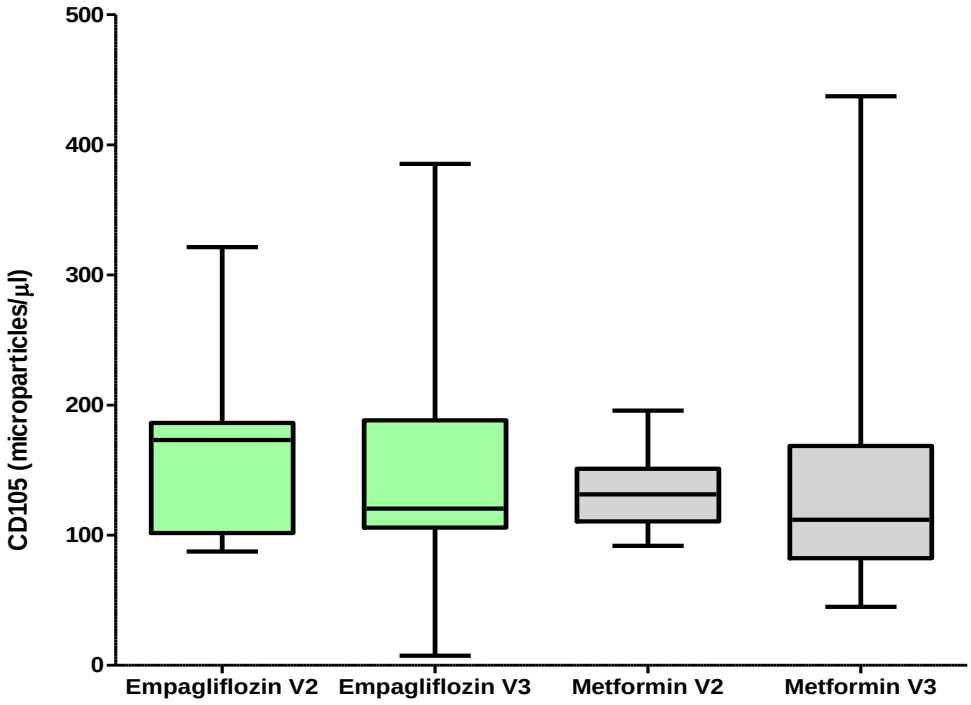

Figure 6.5: Changes in CD105 microparticles before and after empagliflozin or metformin therapy

Legend: Empagliflozin V2, Baseline levels of CD105 microparticles in empagliflozin group; Empagliflozin V3, Levels of CD105 microparticles after empagliflozin treatment; Metformin V2, Baseline levels of CD105 microparticles in metformin group; Metformin V3, Levels of CD105 microparticles after metformin treatment

6.4.4.5 CD106: Vascular cell adhesion molecule-1 (VCAM-1):

The CD106 microparticles were increased significantly after both empagliflozin (258.1 ± 111.9 vs 404.4 ± 98.4; p=0.001) and metformin treatment (262.9 ± 105.3 vs 471.3 ± 207.4; p=0.000) compared to their baseline values (figure 6.6). However, the increase in empagliflozin group was not statistically significant when percentage change was performed as compared to metformin group (p=0.8) as explained in figure 6.1 and table 6.2.

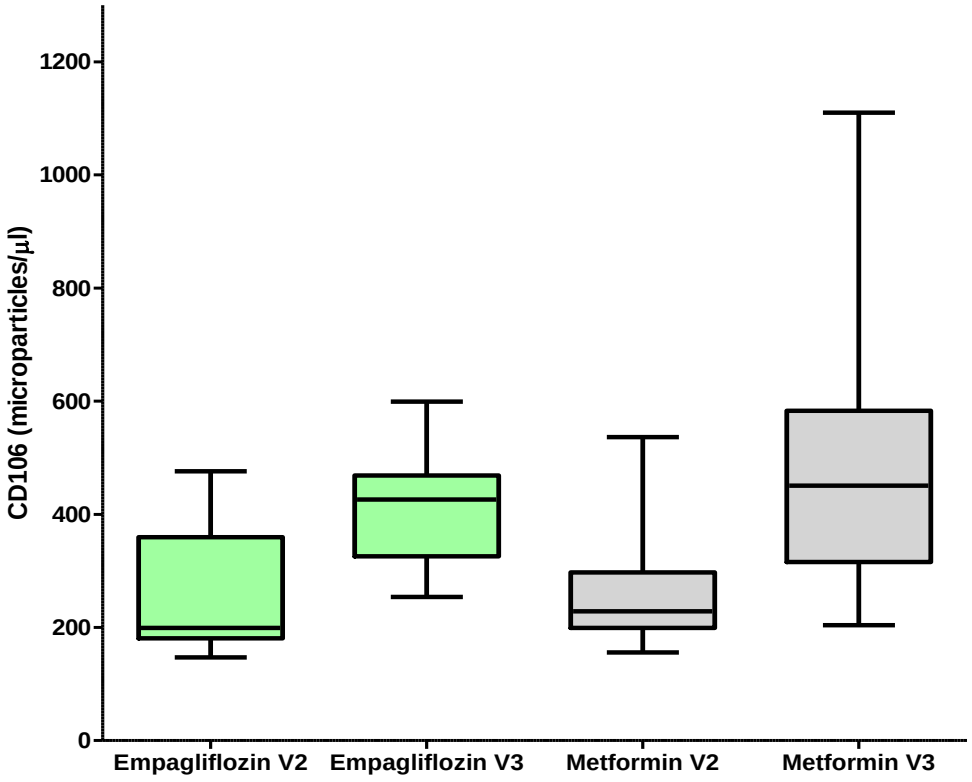

Figure 6.6: Changes in CD106 microparticles before and after empagliflozin or metformin therapy

Legend: Empagliflozin V2, Baseline levels of CD106 microparticles in empagliflozin group; Empagliflozin V3, Levels of CD106 microparticles after empagliflozin treatment; Metformin V2, Baseline levels of CD106 microparticles in metformin group; Metformin V3, Levels of CD106 microparticles after metformin treatment

6.4.4.6 CD142: Platelet tissue factor:

There were no significant changes in CD142 microparticles after both empagliflozin and metformin treatments as compared to their baseline values (figure 6.7). Similarly, no significant difference in percentage change was observed between empagliflozin and metformin groups (52.9 ± 73.8 vs 30.4 ± 70.2; p=0.4) as presented in figure 6.1 and shown in table 6.2.

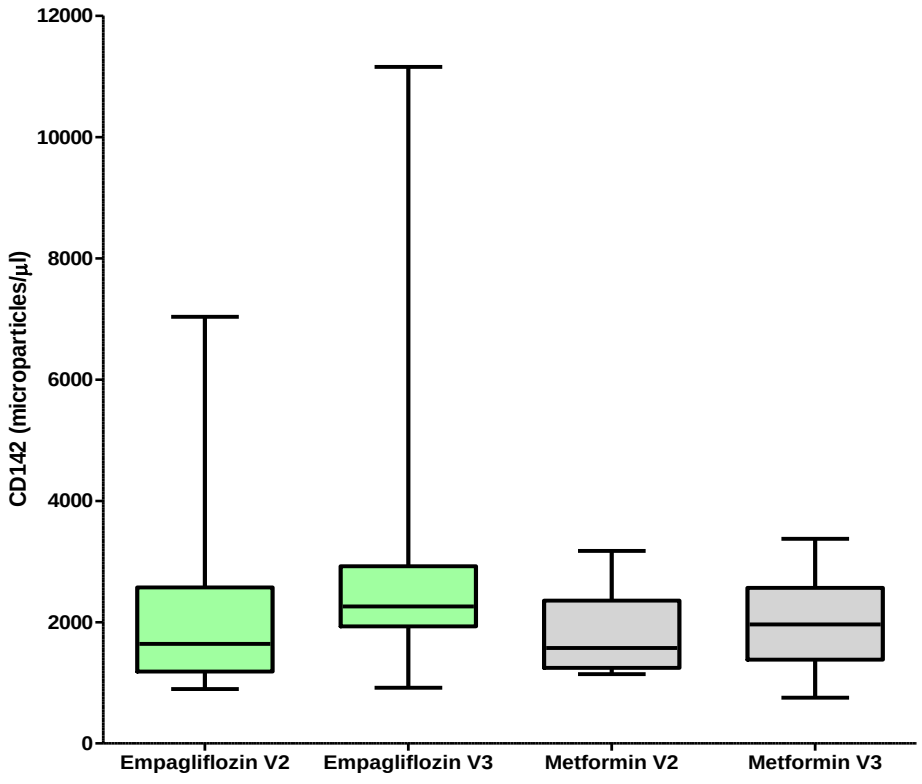

Figure 6.7: Changes in CD142 microparticles before and after empagliflozin or metformin therapy

Legend: Empagliflozin V2, Baseline levels of CD142 microparticles in empagliflozin group; Empagliflozin V3, Levels of CD142 microparticles after empagliflozin treatment; Metformin V2, Baseline levels of CD142 microparticles in metformin group; Metformin V3, Levels of CD142 microparticles after metformin treatment

6.5 Discussion:

In this study, three months of empagliflozin treatment in overweight and obese women with PCOS caused a statistically significant increase in the levels of CD54, CD62 and CD106 microparticles while metformin treatment resulted in statistically significant increase in CD106 microparticles only. There were no significant changes in CD31, CD105 and CD142 microparticles in both groups. Despite the differences in some EMPs within the groups, there were no significant differences between groups. These findings give us further understanding about the role of both empagliflozin and metformin in women with PCOS and suggest that their function could be partly mediated through the modulation of vascular endothelial function.

CD54 is a cell surface protein that is normally expressed in low amounts on the vascular endothelial cells. It is also expressed on cells of immune system such as lymphocytes and macrophages (457). During acute or chronic inflammatory processes the expression of CD54 is increased significantly and plays a vital role in promoting inflammation by helping transmigration of leukocytes across the endothelial cells into the sites of inflammation through binding with the adhesion molecule LFA-1 over the leukocytes (457). In this study, significantly increased production of CD54 microparticles after empagliflozin treatment suggests increased activation of vascular endothelial cells with increased expression of CD54 to promote inflammation which could be deleterious in obese women with PCOS as they are already associated with chronic low grade inflammation (519). It also suggests that empagliflozin worsens underlying endothelial dysfunction in women with PCOS that could worsen further endocrine and metabolic abnormalities found in these patients and increase future cardiovascular risk (324). Similarly, CD62 or E-selectins are cell adhesion molecules expressed exclusively on the activated vascular endothelial cells in response to the local release of cytokines by macrophages in the inflamed tissue (520). E-selectins are involved in

the key steps of leukocytes migration to the sites of inflammation and injury thus playing a vital role in inflammatory processes (458). Empagliflozin treatment in this study resulted in statistically significant increase in the production of E-selectin microparticles in overweight and obese women with PCOS suggesting increased underlying tissue inflammation and impaired endothelial dysfunction that could be deleterious in them.

CD106 or VCAM is a cell adhesion molecule that is expressed excessively on the vascular endothelial cells after stimulation by cytokines (465). CD106 facilitates the adhesion of inflammatory cells such as monocytes, lymphocytes, eosinophils and basophils to the vascular endothelium and, therefore, play a vital role in the development of atherosclerosis and inflammatory processes (465, 466). The significantly increased production of CD106 microparticles in this trial after empagliflozin treatment suggests further impairment of underlying endothelial dysfunction in women with PCOS that put them at high risk of having future cardiovascular risk.

The initial concept was that high microparticle levels were deleterious to health due to their association with the development of the atherothrombotic processes, sepsis and vascular dysfunction (299, 521). However, recently in vitro data have suggested the potential beneficial effects of EMPs on vascular endothelial integrity such as vascular repair, control of cellular death mechanisms, their cyto-protective activities and induction of adaptive immunity (299). Similarly, recent study on HUVECs has shown that EMPs possess functional endothelial nitric oxide synthase (eNOS) and play an important role in the feedback loop of damage and repair during homeostasis and also prevent against lipid induced endothelial damage via restoring nitric oxide production and reducing oxidative stress (522). In our study, though the CD54, CD62 and CD106 microparticles increased significantly after three months of empagliflozin treatment, endothelial function improved in this group (non-significantly) and the results were comparable to the metformin group and

did not differ significantly between groups (explained in chapter 5). Similarly, there was significant improvement in anthropometric and body composition parameters with some improvement in insulin resistance and hyperandrogenaemia after three months of empagliflozin treatment that was comparable to metformin, further suggesting that high production of EMPs in this group could be beneficial. Further work is needed to distinguish between deleterious and beneficial EMPs. There is no data currently available regarding the effects of SGLT2 inhibitors especially empagliflozin on microparticles; however, recently a study on diabetic rats suggested a beneficial role of empagliflozin through the prevention of endothelial dysfunction, diminished oxidative stress and demonstrates anti-inflammatory effects (509). The authors suggested that further in vivo studies are required to elaborate the role of SGLT2 inhibitors in modifying endothelial function.

Interestingly there was statistically significant increase in the CD106 microparticles in the metformin group that theoretically suggests worsening of underlying endothelial function, which could be related to increased weight and fat mass in this group after metformin treatment. However, there was significant improvement in free testosterone after metformin treatment associated with a trend towards improvement in FAI, EndoPAT, hormonal and metabolic parameters in this group that again suggests further exploration to elaborate the role of microparticles. Metformin has previously shown to improve endothelial function (measured using FMD, carotid intima-media thickness) in women with PCOS in several trials (523, 524). Metformin has also shown to improve the levels of endothelial microparticles and endothelial progenitor cells in patients with diabetes (525). In one of the trials in women with PCOS, metformin decreased total number of microparticles and tissue factor (CD142) related microparticles (526), however, the patients were not obese (BMI = 29.8kg/m^2) and the free testosterone level increased but in our study the PCOS women were obese (BMI = 38.88kg/m^2) and the free testosterone was significantly reduced.

When the endothelial cells are activated or stimulated, the microparticles released express specific surface proteins such as CD31, CD105 and CD142 (299). CD31 makes up a large portion of endothelial cell intercellular junctions and plays a major role in tissue inflammation (463). CD105 is a part of transforming growth factor-beta family (TGF-β) and is expressed on activated endothelial cells where it plays an important role in angiogenesis and tissue inflammation (464). CD142 or tissue factor is expressed on sub-endothelial tissue and leukocytes. It is essential for life as it has central role in haemostasis and initiating coagulation cascade but aberrant CD142 expression could initiate life-threatening thrombosis in situations such as sepsis, atherosclerosis, and cancer (527). In this study, there were no statistically significant changes in CD31, CD105 and CD142 after both empagliflozin and metformin treatment. There is no previous study regarding the effects of empagliflozin on these microparticles but tissue factor microparticles were shown to be improved by metformin in patients with diabetes associated with a significant decrease in weight and HOMA-IR, however, CD31 microparticles were unchanged as seen in this study (525).

The strengths and limitations of the study have been explained in chapter 5 under discussion section 5.5.

6.6 Conclusion:

In summary, there was significant increase in CD54 and CD62 microparticles after three months of treatment with empagliflozin and CD106 microparticles after three months of treatment with both empagliflozin and metformin suggesting the effects of empagliflozin and metformin could be partly mediated through modulation of endothelial function.

7 Chapter 7: General summary and future direction:

7.1 Summary:

PCOS is the most common endocrine disorder in women of reproductive age that is primarily characterised by hyperandrogenaemia and ovulatory dysfunction (28). Besides reproductive dysfunction, PCOS is associated with a diverse range of endocrine, metabolic and cardiovascular risk factors such as obesity, insulin resistance, type 2 diabetes, dyslipidaemia, systemic inflammation, subclinical atherosclerosis and endothelial dysfunction (149, 528). The current available treatments for PCOS are only moderately effective in controlling symptoms and preventing future cardiovascular complications (482). The use of anti-androgens along with suppression of ovarian function is often clinically unacceptable. Therefore, the development of effective treatments for PCOS still remains challenging.

Vitamin D deficiency is very prevalent in women with PCOS, with serum concentrations of <20 ng/ml have been reported. Low serum vitamin D levels in PCOS have been revealed to have positive correlation with multiple cardiovascular risk factors including obesity, insulin resistance, hyperandrogenism and DHEAS levels (368, 371, 401). Studies have shown that vitamin D replacement therapy could be a promising alternative for the prevention and treatment of PCOS and may have beneficial effects on insulin resistance, menstrual irregularities and steroidogenesis of the sex hormones - oestradiol and progesterone in obese women with PCOS but need further exploration (378, 402).

Empagliflozin, an SGLT2 inhibitor, is a new treatment option for T2DM, that has recently shown to induce favourable effects on arterial stiffness and vascular resistance (common in PCOS) in both T1DM and T2DM patients (381, 382). It improved cardiovascular risk significantly in patients with T2DM, reduces oxidative stress, and suppresses markers of inflammation (a feature of PCOS) and fibrosis (383). However, the studies looking at the

effects of SGLT2 inhibitor, empagliflozin, on hormonal, metabolic and cardiovascular risk factors in women with PCOS are lacking and therefore, need exploration. In the second study, the comparator to empagliflozin was metformin that has shown to have favourable effects on body weight, insulin sensitivity, hyperandrogenism, menstrual pattern and ovulatory function in both obese and non-obese women with PCOS (21, 159).

The emerging evidence has associated NAFLD with vitamin D deficiency and suggests they share multiple cardiometabolic risk factors (413). The data available until now supports the rationale for further interventions to restore optimal vitamin D levels as a therapeutic option for the NAFLD management, however, the results are varied and the dose of vitamin D used in the trials is either very high or below therapeutic doses (529). Further, the early recognition of NAFLD in women with PCOS is important to recognise as it can progress to advanced liver disease at a much younger age (414). The high prevalence of NAFLD and the rising costs both drive the need for devising robust screening tests to identify, monitor and treat these patients at early stage in order to prevent subsequent morbidity and mortality (530). ELF markers have has been recommended by NICE for testing and monitoring of advanced liver fibrosis in NAFLD patients as the gold standard test, liver biopsy, is not feasible in routine clinical practice (431). In this research work, three months supplementation with therapeutic dose of vitamin D 3200 IU daily in obese women with PCOS resulted in significant improvement in liver fibrosis markers (HA, PIIINP, TIMP-1) and their cumulative ELF score. This study adds up to the current evidence that overweight and obese women should be screened for underlying unrecognized NAFLD and further supports the current recommendations for using ELF markers in the routine clinical setting for early detection and monitoring of NAFLD. Additionally, this is the first study to show the beneficial effects of a therapeutic dose of vitamin D on NAFLD in women with PCOS utilising ELF markers that reflect on-going sinusoidal fibrogenesis in the liver. The changes in ELF were associated with

a significant decrease in ALT levels that is a predictor of mortality independent of liver disease and is an indicator of general health (430). The changes in ELF score and ALT were independent to changes in body weight, insulin resistance, FAI and inflammatory markers suggesting a direct beneficial effect of vitamin D on hepatic cells, perhaps reducing hepatic fibrosis directly rather than indirectly via insulin resistance. These findings are in accord to the recent data that showed no improvements in metabolic and cardiovascular risk factors after vitamin D supplementation (531). Whilst several previous trials have not shown any significant improvements in hepatic steatosis after vitamin D supplementation (529, 531), they did not focus on ELF markers in NAFLD that are thought to be more sensitive in detecting and monitoring early fibrosis as compared to previous biochemical and radiological tests in NAFLD patients (530).

There was no improvement in the endothelial function, measured by EndoPAT machine and expressed as RHI, after vitamin D supplementation; similarly, there were no significant changes in the production of endothelial microparticles measured using flow cytometry as compared to the placebo group. The dysregulation of EMPs has been suggested as an early marker of vascular dysfunction (532) and improvement in their production could subsequently reduce future cardiovascular risk in overweight and obese women with PCOS. Vitamin D supplementation did not reduce the production of microparticles; however, the increase in microparticles levels was more in the placebo group when compared to their baselines values suggesting vitamin D could modulate endothelial function by stabilizing endothelial lining. Further studies are needed to determine the optimum duration of treatment and what level of vitamin D restoration is required to maintain this benefit. Previous in-vitro and human studies have indicated the potential beneficial role of vitamin D in improving endothelial function (454, 533); however, the in-vivo data related to alterations in EMPs levels after vitamin D supplementation is lacking. This research work provided the evidence

related to underlying changes at cellular level in terms of EPMs that could be responsible for clinical benefits obtained after vitamin D supplementation and open the door for further research to explore the underlying changes related to beneficial effects of various treatments available.

In the second study, three months treatment with empagliflozin in women with PCOS was shown to have significant improvements in multiple cardio-metabolic risk factors including weight, BMI, waist and hip circumference associated with significant improvement in body fat composition parameters including total body fat as compared to metformin, that is commonly used as a treatment option in PCOS. The results obtained in this study after three months of empagliflozin therapy are comparable to the results observed after metformin treatment with an additional benefit of significant reduction in body weight and fat mass after empagliflozin treatment suggesting empagliflozin could be a potential future treatment option in obese women with PCOS. The improvement in percent body fat gives more meaningful clinical information and is a better predictor of future cardiovascular risk as compared to BMI (493, 494). There is some recent evidence from animal studies which explains that the potential underlying mechanism by which SGLT2 inhibition exerts it beneficial effects on body weight and composition is by augmenting fat utilization and browning which subsequently reduces tissue inflammation and insulin resistance (500, 501). This is the first clinical study in overweight and obese women with PCOS that further provided the evidence regarding the beneficial effects of empagliflozin on anthropometric measures and body composition parameters and warrants further exploration. Further, in this study effect of SGLT2 inhibition by empagliflozin on endothelial function was measured in overweight and obese women with PCOS that did not differ, as evaluated by RHI, in either empagliflozin or metformin groups as compared to their baselines. However, when endothelial function was explored more deeply using flow cytometry to look at the production of endothelial

microparticles there was a statistically significant increase in the levels of CD54, CD62 and CD106 microparticles after empagliflozin treatment while metformin treatment resulted in statistically significant increase in CD106 microparticles only. Despite the differences in some EMPs within the groups, there were no significant differences between groups. Although, there is no previous data in humans available to compare the effects of empagliflozin on the endothelial microparticles, these findings suggest that both empagliflozin and metformin may affect vascular endothelial function through changes in the production of endothelial microparticles. This assumption is supported by a recent study in rats showing that empagliflozin modifies endothelial function through the production of endothelial microparticles under certain environmental situations (509). This research work provides the first evidence in women with PCOS regarding the clinical effects of empagliflozin and the potential underlying mechanism by which it exerts it effects that need further exploration.

7.2 Future direction:

In the first study three months vitamin D supplementation reverted the ongoing underlying liver fibrosis in overweight and obese women with PCOS as shown by significant improvement in ELF score. Further large-scale studies would be of clinical use to look at the long term effects of vitamin D supplementation by re-assessing liver markers after 6 months and 12 months of supplementation in order to find out the required treatment duration that could benefit the patients.

The effects of vitamin D on the hepatic fibrosis were independent of any changes in body weight, hyperandrogenism, insulin resistance and inflammatory markers that suggest further studies to look into the exact mechanisms by which vitamin D may be improving the underlying fibrosis. Moreover, further studies are required to correlate the levels of vitamin D with the degree of improvement in liver function and liver markers that would be helpful to

determine the correct dose of vitamin D to give the clinical benefits, and at what level vitamin D repletion should be attained to maintain that benefit.

In the second study, the clinical benefits found after three months of empagliflozin treatment were comparable to metformin therapy with an additional benefit of loss of body weight and improvement in body composition that suggests empagliflozin as a potential treatment option in obese women with PCOS. The findings of this research work warrant further large scale trials, with duration up to six months to one year, in order to assess the long term clinical efficacy and safety of empagliflozin in obese women with PCOS.

EMPs were increased after both empagliflozin and metformin treatment for three months which has left several questions unanswered that need further experimental and clinical trials: Firstly, there is no data available that could comment on whether the production of EMPs is affected by cold or hot weather as in the second study the participants were recruited in summer and autumn while they finished the last visit in winter. Secondly, the long-term effects of both empagliflozin and metformin treatment (>3 months) on the production of EMPs need to be determined. Lastly, further evidence is required to delineate which EMPs are deleterious to the body and which one are beneficial that potentially depends upon the stimuli and probably the environmental conditions under which the production is either increased or decreased.

8 Chapter 8: References

1. Stein I LM. Amenorrhea associated with bilaterl polycystic ovaries. Am J Obstet Gynecol. 1935;29:181–5.
2. A. V. Istoria della Generazione dell'Uomo, e degli Animali, sesia da' vermicelli spermatici, o dalle uova. Venezia: Appreso Gio Gabbriel Hertz. 1721.
3. A. C. M emoires pour servir a l' etude des maladies des ovaries. Paris: Fortin, Masson & Cie. 1844.
4. Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W, et al. The Androgen Excess and PCOS Society criteria for the polycystic ovary syndrome: the complete task force report. Fertility and sterility. 2009;91(2):456-88.
5. C. R. A manual of pathological anatomy. Philadelphia, PA: Blanchard & Lea. 1855.
6. Welt CK, Duran JM. Genetics of polycystic ovary syndrome. Semin Reprod Med. 2014;32(3):177-82.
7. Waldstreicher J, Santoro NF, Hall JE, Filicori M, Crowley WF, Jr. Hyperfunction of the hypothalamic-pituitary axis in women with polycystic ovarian disease: indirect evidence for partial gonadotroph desensitization. The Journal of clinical endocrinology and metabolism. 1988;66(1):165-72.
8. Taylor AE, McCourt B, Martin KA, Anderson EJ, Adams JM, Schoenfeld D, et al. Determinants of abnormal gonadotropin secretion in clinically defined women with polycystic ovary syndrome. The Journal of clinical endocrinology and metabolism. 1997;82(7):2248-56.
9. Rebar R, Judd HL, Yen SS, Rakoff J, Vandenberg G, Naftolin F. Characterization of the inappropriate gonadotropin secretion in polycystic ovary syndrome. The Journal of clinical investigation. 1976;57(5):1320-9.
10. Chang RJ, Nakamura RM, Judd HL, Kaplan SA. Insulin resistance in nonobese patients with polycystic ovarian disease. The Journal of clinical endocrinology and metabolism. 1983;57(2):356-9.
11. Dunaif A, Segal KR, Futterweit W, Dobrjansky A. Profound peripheral insulin resistance, independent of obesity, in polycystic ovary syndrome. Diabetes. 1989;38(9):1165-74.
12. Pigny P, Merlen E, Robert Y, Cortet-Rudelli C, Decanter C, Jonard S, et al. Elevated serum level of anti-mullerian hormone in patients with polycystic ovary syndrome: relationship to the ovarian follicle excess and to the follicular arrest. The Journal of clinical endocrinology and metabolism. 2003;88(12):5957-62.
13. Adams J, Polson DW, Franks S. Prevalence of polycystic ovaries in women with anovulation and idiopathic hirsutism. Br Med J (Clin Res Ed). 1986;293(6543):355-9.
14. Adams JM, Taylor AE, Crowley WF, Jr., Hall JE. Polycystic ovarian morphology with regular ovulatory cycles: insights into the pathophysiology of polycystic ovarian syndrome. The Journal of clinical endocrinology and metabolism. 2004;89(9):4343-50.
15. Welt CK, Arason G, Gudmundsson JA, Adams J, Palsdottir H, Gudlaugsdottir G, et al. Defining constant versus variable phenotypic features of women with polycystic ovary syndrome using different ethnic groups and populations. The Journal of clinical endocrinology and metabolism. 2006;91(11):4361-8.
16. Legro RS, Chiu P, Kunselman AR, Bentley CM, Dodson WC, Dunaif A. Polycystic ovaries are common in women with hyperandrogenic chronic anovulation but do not predict metabolic or reproductive phenotype. The Journal of clinical endocrinology and metabolism. 2005;90(5):2571-9.
17. Raj SG, Thompson IE, Berger MJ, Talert LM, Taymor ML. Diagnostic value of androgen measurements in polycystic ovary syndrome. Obstetrics and gynecology. 1978;52(2):169-71.
18. Zawadzki J DA. Diagnostic criteria for polycystic ovary syndrome: towards a rational approach. In: Dunaif A, Givens HR, Haseltine FP, Merriam GR, ed. Polycystic Ovary Syndrome. Boston, MA. 1992;Blackwell Scientific:377–84.

19. Dumesic DA, Oberfield SE, Stener-Victorin E, Marshall JC, Laven JS, Legro RS. Scientific Statement on the Diagnostic Criteria, Epidemiology, Pathophysiology, and Molecular Genetics of Polycystic Ovary Syndrome. Endocrine reviews. 2015;36(5):487-525.
20. Rotterdam EA-SPcwg. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). Human reproduction. 2004;19(1):41-7.
21. Goodman NF, Cobin RH, Futterweit W, Glueck JS, Legro RS, Carmina E. American Association of Clinical Endocrinologists, American College of Endocrinology, and Androgen Excess and Pcos Society Disease State Clinical Review: Guide to the Best Practices in the Evaluation and Treatment of Polycystic Ovary Syndrome - Part 1. Endocrine practice : official journal of the American College of Endocrinology and the American Association of Clinical Endocrinologists. 2015;21(11):1291-300.
22. Dunaif A, Fauser BC. Renaming PCOS--a two-state solution. The Journal of clinical endocrinology and metabolism. 2013;98(11):4325-8.
23. Lizneva D, Suturina L, Walker W, Brakta S, Gavrilova-Jordan L, Azziz R. Criteria, prevalence, and phenotypes of polycystic ovary syndrome. Fertility and sterility. 2016.
24. Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W, et al. Positions statement: criteria for defining polycystic ovary syndrome as a predominantly hyperandrogenic syndrome: an Androgen Excess Society guideline. The Journal of clinical endocrinology and metabolism. 2006;91(11):4237-45.
25. Fruzzetti F, Campagna AM, Perini D, Carmina E. Ovarian volume in normal and hyperandrogenic adolescent women. Fertility and sterility. 2015;104(1):196-9.
26. Glueck CJ, Woo JG, Khoury PR, Morrison JA, Daniels SR, Wang P. Adolescent oligomenorrhea (age 14-19) tracks into the third decade of life (age 20-28) and predicts increased cardiovascular risk factors and metabolic syndrome. Metabolism: clinical and experimental. 2015;64(4):539-53.
27. Rosenfield RL. The Diagnosis of Polycystic Ovary Syndrome in Adolescents. Pediatrics. 2015;136(6):1154-65.
28. Legro RS, Arslanian SA, Ehrmann DA, Hoeger KM, Murad MH, Pasquali R, et al. Diagnosis and treatment of polycystic ovary syndrome: an Endocrine Society clinical practice guideline. The Journal of clinical endocrinology and metabolism. 2013;98(12):4565-92.
29. Fauser BC, Tarlatzis BC, Rebar RW, Legro RS, Balen AH, Lobo R, et al. Consensus on women's health aspects of polycystic ovary syndrome (PCOS): the Amsterdam ESHRE/ASRM-Sponsored 3rd PCOS Consensus Workshop Group. Fertility and sterility. 2012;97(1):28-38 e25.
30. Elting MW, Korsen TJ, Rekers-Mombarg LT, Schoemaker J. Women with polycystic ovary syndrome gain regular menstrual cycles when ageing. Human reproduction. 2000;15(1):24-8.
31. Elting MW, Kwee J, Korsen TJ, Rekers-Mombarg LT, Schoemaker J. Aging women with polycystic ovary syndrome who achieve regular menstrual cycles have a smaller follicle cohort than those who continue to have irregular cycles. Fertility and sterility. 2003;79(5):1154-60.
32. Pinola P, Piltonen TT, Puurunen J, Vanky E, Sundstrom-Poromaa I, Stener-Victorin E, et al. Androgen Profile Through Life in Women With Polycystic Ovary Syndrome: A Nordic Multicenter Collaboration Study. The Journal of clinical endocrinology and metabolism. 2015;100(9):3400-7.
33. Rosner W, Auchus RJ, Azziz R, Sluss PM, Raff H. Position statement: Utility, limitations, and pitfalls in measuring testosterone: an Endocrine Society position statement. The Journal of clinical endocrinology and metabolism. 2007;92(2):405-13.
34. Diamanti-Kandarakis E, Dunaif A. Insulin resistance and the polycystic ovary syndrome revisited: an update on mechanisms and implications. Endocrine reviews. 2012;33(6):981-1030.
35. Moran LJ, Misso ML, Wild RA, Norman RJ. Impaired glucose tolerance, type 2 diabetes and metabolic syndrome in polycystic ovary syndrome: a systematic review and meta-analysis. Human reproduction update. 2010;16(4):347-63.
36. Khan KA, Stas S, Kurukulasuriya LR. Polycystic ovarian syndrome. J Cardiometab Syndr. 2006;1(2):125-30; quiz 31-2.

37. Zumoff B, Freeman R, Coupey S, Saenger P, Markowitz M, Kream J. A chronobiologic abnormality in luteinizing hormone secretion in teenage girls with the polycystic-ovary syndrome. The New England journal of medicine. 1983;309(20):1206-9.
38. Legro RS. The genetics of polycystic ovary syndrome. The American journal of medicine. 1995;98(1A):9S-16S.
39. Franks S, Gharani N, Waterworth D, Batty S, White D, Williamson R, et al. The genetic basis of polycystic ovary syndrome. Human reproduction. 1997;12(12):2641-8.
40. Jones MR, Goodarzi MO. An update on the genetics of polycystic ovary syndrome: progress and future directions. Fertility and sterility. 2016.
41. Cooper HE, Spellacy WN, Prem KA, Cohen WD. Hereditary factors in the Stein-Leventhal syndrome. Am J Obstet Gynecol. 1968;100(3):371-87.
42. Li L, Baek KH. Molecular genetics of polycystic ovary syndrome: an update. Current molecular medicine. 2015;15(4):331-42.
43. Hayes MG, Urbanek M, Ehrmann DA, Armstrong LL, Lee JY, Sisk R, et al. Genome-wide association of polycystic ovary syndrome implicates alterations in gonadotropin secretion in European ancestry populations. Nat Commun. 2015;6:7502.
44. Govind A, Obhrai MS, Clayton RN. Polycystic ovaries are inherited as an autosomal dominant trait: analysis of 29 polycystic ovary syndrome and 10 control families. The Journal of clinical endocrinology and metabolism. 1999;84(1):38-43.
45. Carey AH, Chan KL, Short F, White D, Williamson R, Franks S. Evidence for a single gene effect causing polycystic ovaries and male pattern baldness. Clinical endocrinology. 1993;38(6):653-8.
46. Joharatnam J, Barber TM, Webber L, Conway GS, McCarthy MI, Franks S. Determinants of dyslipidaemia in probands with polycystic ovary syndrome and their sisters. Clinical endocrinology. 2011;74(6):714-9.
47. Sam S, Legro RS, Bentley-Lewis R, Dunaif A. Dyslipidemia and metabolic syndrome in the sisters of women with polycystic ovary syndrome. The Journal of clinical endocrinology and metabolism. 2005;90(8):4797-802.
48. Sam S, Legro RS, Essah PA, Apridonidze T, Dunaif A. Evidence for metabolic and reproductive phenotypes in mothers of women with polycystic ovary syndrome. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(18):7030-5.
49. Coviello AD, Sam S, Legro RS, Dunaif A. High prevalence of metabolic syndrome in first-degree male relatives of women with polycystic ovary syndrome is related to high rates of obesity. The Journal of clinical endocrinology and metabolism. 2009;94(11):4361-6.
50. Jahanfar S, Eden JA, Warren P, Seppala M, Nguyen TV. A twin study of polycystic ovary syndrome. Fertility and sterility. 1995;63(3):478-86.
51. Vink JM, Sadrzadeh S, Lambalk CB, Boomsma DI. Heritability of polycystic ovary syndrome in a Dutch twin-family study. The Journal of clinical endocrinology and metabolism. 2006;91(6):2100-4.
52. Kosova G, Urbanek M. Genetics of the polycystic ovary syndrome. Molecular and cellular endocrinology. 2013;373(1-2):29-38.
53. Urbanek M, Woodroffe A, Ewens KG, Diamanti-Kandarakis E, Legro RS, Strauss JF, 3rd, et al. Candidate gene region for polycystic ovary syndrome on chromosome 19p13.2. The Journal of clinical endocrinology and metabolism. 2005;90(12):6623-9.
54. Ewens KG, Stewart DR, Ankener W, Urbanek M, McAllister JM, Chen C, et al. Family-based analysis of candidate genes for polycystic ovary syndrome. The Journal of clinical endocrinology and metabolism. 2010;95(5):2306-15.
55. Villuendas G, Escobar-Morreale HF, Tosi F, Sancho J, Moghetti P, San Millan JL. Association between the D19S884 marker at the insulin receptor gene locus and polycystic ovary syndrome. Fertility and sterility. 2003;79(1):219-20.
56. Ruan Y, Ma J, Xie X. Association of IRS-1 and IRS-2 genes polymorphisms with polycystic ovary syndrome: a meta-analysis. Endocr J. 2012;59(7):601-9.

57. Wojciechowski P, Lipowska A, Rys P, Ewens KG, Franks S, Tan S, et al. Impact of FTO genotypes on BMI and weight in polycystic ovary syndrome: a systematic review and meta-analysis. Diabetologia. 2012;55(10):2636-45.
58. Louwers YV, Rayner NW, Herrera BM, Stolk L, Groves CJ, Barber TM, et al. BMI-associated alleles do not constitute risk alleles for polycystic ovary syndrome independently of BMI: a case-control study. PloS one. 2014;9(1):e87335.
59. Claussnitzer M, Dankel SN, Kim KH, Quon G, Meuleman W, Haugen C, et al. FTO Obesity Variant Circuitry and Adipocyte Browning in Humans. The New England journal of medicine. 2015;373(10):895-907.
60. Laven JS, Mulders AG, Suryandari DA, Gromoll J, Nieschlag E, Fauser BC, et al. Follicle-stimulating hormone receptor polymorphisms in women with normogonadotropic anovulatory infertility. Fertility and sterility. 2003;80(4):986-92.
61. Chen ZJ, Zhao H, He L, Shi Y, Qin Y, Shi Y, et al. Genome-wide association study identifies susceptibility loci for polycystic ovary syndrome on chromosome 2p16.3, 2p21 and 9q33.3. Nature genetics. 2011;43(1):55-9.
62. Goodarzi MO, Jones MR, Li X, Chua AK, Garcia OA, Chen YD, et al. Replication of association of DENND1A and THADA variants with polycystic ovary syndrome in European cohorts. Journal of medical genetics. 2012;49(2):90-5.
63. Welt CK, Styrkarsdottir U, Ehrmann DA, Thorleifsson G, Arason G, Gudmundsson JA, et al. Variants in DENND1A are associated with polycystic ovary syndrome in women of European ancestry. The Journal of clinical endocrinology and metabolism. 2012;97(7):E1342-7.
64. Knochenhauer ES, Key TJ, Kahsar-Miller M, Waggoner W, Boots LR, Azziz R. Prevalence of the polycystic ovary syndrome in unselected black and white women of the southeastern United States: a prospective study. The Journal of clinical endocrinology and metabolism. 1998;83(9):3078-82.
65. Azziz R, Woods KS, Reyna R, Key TJ, Knochenhauer ES, Yildiz BO. The prevalence and features of the polycystic ovary syndrome in an unselected population. The Journal of clinical endocrinology and metabolism. 2004;89(6):2745-9.
66. Yildiz BO, Bozdag G, Yapici Z, Esinler I, Yarali H. Prevalence, phenotype and cardiometabolic risk of polycystic ovary syndrome under different diagnostic criteria. Human reproduction. 2012;27(10):3067-73.
67. Musmar S, Afaneh A, Mo'alla H. Epidemiology of polycystic ovary syndrome: a cross sectional study of university students at An-Najah national university-Palestine. Reproductive biology and endocrinology : RB&E. 2013;11:47.
68. Rashidi H, Ramezani Tehrani F, Bahri Khomami M, Tohidi M, Azizi F. To what extent does the use of the Rotterdam criteria affect the prevalence of polycystic ovary syndrome? A community-based study from the Southwest of Iran. European journal of obstetrics, gynecology, and reproductive biology. 2014;174:100-5.
69. Moran C, Tena G, Moran S, Ruiz P, Reyna R, Duque X. Prevalence of polycystic ovary syndrome and related disorders in mexican women. Gynecologic and obstetric investigation. 2010;69(4):274-80.
70. Tehrani FR, Simbar M, Tohidi M, Hosseinpanah F, Azizi F. The prevalence of polycystic ovary syndrome in a community sample of Iranian population: Iranian PCOS prevalence study. Reproductive biology and endocrinology : RB&E. 2011;9:39.
71. Sanchon R, Gambineri A, Alpanes M, Martinez-Garcia MA, Pasquali R, Escobar-Morreale HF. Prevalence of functional disorders of androgen excess in unselected premenopausal women: a study in blood donors. Human reproduction. 2012;27(4):1209-16.
72. March WA, Moore VM, Willson KJ, Phillips DI, Norman RJ, Davies MJ. The prevalence of polycystic ovary syndrome in a community sample assessed under contrasting diagnostic criteria. Human reproduction. 2010;25(2):544-51.

73. Boyle JA, Cunningham J, O'Dea K, Dunbar T, Norman RJ. Prevalence of polycystic ovary syndrome in a sample of Indigenous women in Darwin, Australia. The Medical journal of Australia. 2012;196(1):62-6.
74. Ma YM, Li R, Qiao J, Zhang XW, Wang SY, Zhang QF, et al. Characteristics of abnormal menstrual cycle and polycystic ovary syndrome in community and hospital populations. Chinese medical journal. 2010;123(16):2185-9.
75. Li R, Zhang Q, Yang D, Li S, Lu S, Wu X, et al. Prevalence of polycystic ovary syndrome in women in China: a large community-based study. Human reproduction. 2013;28(9):2562-9.
76. Gabrielli L, Aquino EM. Polycystic ovary syndrome in Salvador, Brazil: a prevalence study in primary healthcare. Reproductive biology and endocrinology : RB&E. 2012;10:96.
77. Lauritsen MP, Bentzen JG, Pinborg A, Loft A, Forman JL, Thuesen LL, et al. The prevalence of polycystic ovary syndrome in a normal population according to the Rotterdam criteria versus revised criteria including anti-Mullerian hormone. Human reproduction. 2014;29(4):791-801.
78. Zhang HY, Zhu FF, Xiong J, Shi XB, Fu SX. Characteristics of different phenotypes of polycystic ovary syndrome based on the Rotterdam criteria in a large-scale Chinese population. BJOG : an international journal of obstetrics and gynaecology. 2009;116(12):1633-9.
79. Bickerton AS, Clark N, Meeking D, Shaw KM, Crook M, Lumb P, et al. Cardiovascular risk in women with polycystic ovarian syndrome (PCOS). J Clin Pathol. 2005;58(2):151-4.
80. Birdsall MA, Farquhar CM, White HD. Association between polycystic ovaries and extent of coronary artery disease in women having cardiac catheterization. Annals of internal medicine. 1997;126(1):32-5.
81. Kandaraki E, Christakou C, Diamanti-Kandarakis E. Metabolic syndrome and polycystic ovary syndrome... and vice versa. Arquivos brasileiros de endocrinologia e metabologia. 2009;53(2):227-37.
82. Sam S. Obesity and Polycystic Ovary Syndrome. Obes Manag. 2007;3(2):69-73.
83. Randeva HS, Tan BK, Weickert MO, Lois K, Nestler JE, Sattar N, et al. Cardiometabolic aspects of the polycystic ovary syndrome. Endocrine reviews. 2012;33(5):812-41.
84. Horejsi R, Moller R, Rackl S, Giuliani A, Freytag U, Crailsheim K, et al. Android subcutaneous adipose tissue topography in lean and obese women suffering from PCOS: comparison with type 2 diabetic women. Am J Phys Anthropol. 2004;124(3):275-81.
85. Escobar-Morreale HF, San Millan JL. Abdominal adiposity and the polycystic ovary syndrome. Trends in endocrinology and metabolism: TEM. 2007;18(7):266-72.
86. Moran LJ, Pasquali R, Teede HJ, Hoeger KM, Norman RJ. Treatment of obesity in polycystic ovary syndrome: a position statement of the Androgen Excess and Polycystic Ovary Syndrome Society. Fertility and sterility. 2009;92(6):1966-82.
87. Lim SS, Norman RJ, Davies MJ, Moran LJ. The effect of obesity on polycystic ovary syndrome: a systematic review and meta-analysis. Obesity reviews : an official journal of the International Association for the Study of Obesity. 2013;14(2):95-109.
88. De Frene V, Vansteelandt S, T'Sjoen G, Gerris J, Somers S, Vercruysse L, et al. A retrospective study of the pregnancy, delivery and neonatal outcome in overweight versus normal weight women with polycystic ovary syndrome. Human reproduction. 2014;29(10):2333-8.
89. Andrade VH, Mata AM, Borges RS, Costa-Silva DR, Martins LM, Ferreira PM, et al. Current aspects of polycystic ovary syndrome: A literature review. Rev Assoc Med Bras (1992). 2016;62(9):867-71.
90. Bentley-Lewis R, Seely E, Dunaif A. Ovarian hypertension: polycystic ovary syndrome. Endocrinology and metabolism clinics of North America. 2011;40(2):433-49, ix-x.
91. Kargili A, Karakurt F, Kasapoglu B, Derbent A, Koca C, Selcoki Y. Association of polycystic ovary syndrome and a non-dipping blood pressure pattern in young women. Clinics (Sao Paulo). 2010;65(5):475-9.
92. Holte J, Gennarelli G, Berne C, Bergh T, Lithell H. Elevated ambulatory day-time blood pressure in women with polycystic ovary syndrome: a sign of a pre-hypertensive state? Human reproduction. 1996;11(1):23-8.

93. Cibula D, Cifkova R, Fanta M, Poledne R, Zivny J, Skibova J. Increased risk of non-insulin dependent diabetes mellitus, arterial hypertension and coronary artery disease in perimenopausal women with a history of the polycystic ovary syndrome. Human reproduction. 2000;15(4):785-9.
94. Nandalike K, Agarwal C, Strauss T, Coupey SM, Isasi CR, Sin S, et al. Sleep and cardiometabolic function in obese adolescent girls with polycystic ovary syndrome. Sleep Med. 2012;13(10):1307-12.
95. Kahal H, Kyrou I, Tahrani AA, Randeva HS. Obstructive sleep apnoea and polycystic ovary syndrome: A comprehensive review of clinical interactions and underlying pathophysiology. Clinical endocrinology. 2017;87(4):313-9.
96. Lindberg E, Theorell-Haglow J, Svensson M, Gislason T, Berne C, Janson C. Sleep apnea and glucose metabolism: a long-term follow-up in a community-based sample. Chest. 2012;142(4):935-42.
97. Pamidi S, Wroblewski K, Broussard J, Day A, Hanlon EC, Abraham V, et al. Obstructive sleep apnea in young lean men: impact on insulin sensitivity and secretion. Diabetes care. 2012;35(11):2384-9.
98. Fogel RB, Malhotra A, Pillar G, Pittman SD, Dunaif A, White DP. Increased prevalence of obstructive sleep apnea syndrome in obese women with polycystic ovary syndrome. The Journal of clinical endocrinology and metabolism. 2001;86(3):1175-80.
99. Nitsche K, Ehrmann DA. Obstructive sleep apnea and metabolic dysfunction in polycystic ovary syndrome. Best practice & research Clinical endocrinology & metabolism. 2010;24(5):717-30.
100. Polo-Kantola P, Rauhala E, Helenius H, Erkkola R, Irjala K, Polo O. Breathing during sleep in menopause: a randomized, controlled, crossover trial with estrogen therapy. Obstetrics and gynecology. 2003;102(1):68-75.
101. Popovic RM, White DP. Upper airway muscle activity in normal women: influence of hormonal status. Journal of applied physiology. 1998;84(3):1055-62.
102. Regensteiner JG, Woodard WD, Hagerman DD, Weil JV, Pickett CK, Bender PR, et al. Combined effects of female hormones and metabolic rate on ventilatory drives in women. Journal of applied physiology. 1989;66(2):808-13.
103. Shahar E, Redline S, Young T, Boland LL, Baldwin CM, Nieto FJ, et al. Hormone replacement therapy and sleep-disordered breathing. Am J Respir Crit Care Med. 2003;167(9):1186-92.
104. Rizzo M, Rini GB, Carmina E. Androgen excess and cardiovascular risk. Minerva Endocrinol. 2007;32(1):67-71.
105. Luque-Ramirez M, Mendieta-Azcona C, Alvarez-Blasco F, Escobar-Morreale HF. Androgen excess is associated with the increased carotid intima-media thickness observed in young women with polycystic ovary syndrome. Human reproduction. 2007;22(12):3197-203.
106. Ferriman D, Gallwey JD. Clinical assessment of body hair growth in women. The Journal of clinical endocrinology and metabolism. 1961;21:1440-7.
107. Catteau-Jonard S, Dewailly D. Pathophysiology of polycystic ovary syndrome: the role of hyperandrogenism. Front Horm Res. 2013;40:22-7.
108. Burger HG. Androgen production in women. Fertility and sterility. 2002;77 Suppl 4:S3-5.
109. Pessin JE, Saltiel AR. Signaling pathways in insulin action: molecular targets of insulin resistance. The Journal of clinical investigation. 2000;106(2):165-9.
110. Uldry M, Thorens B. The SLC2 family of facilitated hexose and polyol transporters. Pflugers Arch. 2004;447(5):480-9.
111. Kido Y, Nakae J, Accili D. Clinical review 125: The insulin receptor and its cellular targets. The Journal of clinical endocrinology and metabolism. 2001;86(3):972-9.
112. Wilcox G. Insulin and insulin resistance. Clin Biochem Rev. 2005;26(2):19-39.
113. Lee J, Pilch PF. The insulin receptor: structure, function, and signaling. The American journal of physiology. 1994;266(2 Pt 1):C319-34.
114. Kahn CR. The molecular mechanism of insulin action. Annu Rev Med. 1985;36:429-51.

115. Taniguchi CM, Emanuelli B, Kahn CR. Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Biol. 2006;7(2):85-96.
116. Muniyappa R, Lee S, Chen H, Quon MJ. Current approaches for assessing insulin sensitivity and resistance in vivo: advantages, limitations, and appropriate usage. American journal of physiology Endocrinology and metabolism. 2008;294(1):E15-26.
117. Ginsberg HN. Insulin resistance and cardiovascular disease. The Journal of clinical investigation. 2000;106(4):453-8.
118. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabetic medicine : a journal of the British Diabetic Association. 1998;15(7):539-53.
119. Hosker JP, Matthews DR, Rudenski AS, Burnett MA, Darling P, Bown EG, et al. Continuous infusion of glucose with model assessment: measurement of insulin resistance and beta-cell function in man. Diabetologia. 1985;28(7):401-11.
120. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. The American journal of physiology. 1979;237(3):E214-23.
121. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia. 1985;28(7):412-9.
122. Katz A, Nambi SS, Mather K, Baron AD, Follmann DA, Sullivan G, et al. Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. The Journal of clinical endocrinology and metabolism. 2000;85(7):2402-10.
123. Legro RS, Finegood D, Dunaif A. A fasting glucose to insulin ratio is a useful measure of insulin sensitivity in women with polycystic ovary syndrome. The Journal of clinical endocrinology and metabolism. 1998;83(8):2694-8.
124. DeUgarte CM, Bartolucci AA, Azziz R. Prevalence of insulin resistance in the polycystic ovary syndrome using the homeostasis model assessment. Fertility and sterility. 2005;83(5):1454-60.
125. Coviello AD, Legro RS, Dunaif A. Adolescent girls with polycystic ovary syndrome have an increased risk of the metabolic syndrome associated with increasing androgen levels independent of obesity and insulin resistance. The Journal of clinical endocrinology and metabolism. 2006;91(2):492-7.
126. Yildiz BO, Gedik O. Insulin resistance in polycystic ovary syndrome: hyperandrogenemia versus normoandrogenemia. European journal of obstetrics, gynecology, and reproductive biology. 2001;100(1):62-6.
127. Marsden PJ, Murdoch A, Taylor R. Severe impairment of insulin action in adipocytes from amenorrheic subjects with polycystic ovary syndrome. Metabolism: clinical and experimental. 1994;43(12):1536-42.
128. Ciaraldi TP, el-Roeiy A, Madar Z, Reichart D, Olefsky JM, Yen SS. Cellular mechanisms of insulin resistance in polycystic ovarian syndrome. The Journal of clinical endocrinology and metabolism. 1992;75(2):577-83.
129. Legro RS, Bentley-Lewis R, Driscoll D, Wang SC, Dunaif A. Insulin resistance in the sisters of women with polycystic ovary syndrome: association with hyperandrogenemia rather than menstrual irregularity. The Journal of clinical endocrinology and metabolism. 2002;87(5):2128-33.
130. Goodarzi MO, Erickson S, Port SC, Jennrich RI, Korenman SG. beta-Cell function: a key pathological determinant in polycystic ovary syndrome. The Journal of clinical endocrinology and metabolism. 2005;90(1):310-5.
131. Svendsen PF, Nilas L, Norgaard K, Jensen JE, Madsbad S. Obesity, body composition and metabolic disturbances in polycystic ovary syndrome. Human reproduction. 2008;23(9):2113-21.
132. Gennarelli G, Rovei V, Novi RF, Holte J, Bongioanni F, Revelli A, et al. Preserved insulin sensitivity and {beta}-cell activity, but decreased glucose effectiveness in normal-weight women with the polycystic ovary syndrome. The Journal of clinical endocrinology and metabolism. 2005;90(6):3381-6.

133. Ciampelli M, Fulghesu AM, Cucinelli F, Pavone V, Caruso A, Mancuso S, et al. Heterogeneity in beta cell activity, hepatic insulin clearance and peripheral insulin sensitivity in women with polycystic ovary syndrome. Human reproduction. 1997;12(9):1897-901.
134. Dunaif A, Graf M. Insulin administration alters gonadal steroid metabolism independent of changes in gonadotropin secretion in insulin-resistant women with the polycystic ovary syndrome. The Journal of clinical investigation. 1989;83(1):23-9.
135. Stepto NK, Cassar S, Joham AE, Hutchison SK, Harrison CL, Goldstein RF, et al. Women with polycystic ovary syndrome have intrinsic insulin resistance on euglycaemic-hyperinsulaemic clamp. Human reproduction. 2013;28(3):777-84.
136. Kjerulff LE, Sanchez-Ramos L, Duffy D. Pregnancy outcomes in women with polycystic ovary syndrome: a metaanalysis. Am J Obstet Gynecol. 2011;204(6):558 e1-6.
137. Norman RJ, Masters L, Milner CR, Wang JX, Davies MJ. Relative risk of conversion from normoglycaemia to impaired glucose tolerance or non-insulin dependent diabetes mellitus in polycystic ovarian syndrome. Human reproduction. 2001;16(9):1995-8.
138. Karakas SE, Kim K, Duleba AJ. Determinants of impaired fasting glucose versus glucose intolerance in polycystic ovary syndrome. Diabetes care. 2010;33(4):887-93.
139. Boudreaux MY, Talbott EO, Kip KE, Brooks MM, Witchel SF. Risk of T2DM and impaired fasting glucose among PCOS subjects: results of an 8-year follow-up. Current diabetes reports. 2006;6(1):77-83.
140. Dunaif A, Segal KR, Shelley DR, Green G, Dobrjansky A, Licholai T. Evidence for distinctive and intrinsic defects in insulin action in polycystic ovary syndrome. Diabetes. 1992;41(10):1257-66.
141. Dewailly D, Catteau-Jonard S, Reyss AC, Leroy M, Pigny P. Oligoanovulation with polycystic ovaries but not overt hyperandrogenism. The Journal of clinical endocrinology and metabolism. 2006;91(10):3922-7.
142. Barber TM, Wass JA, McCarthy MI, Franks S. Metabolic characteristics of women with polycystic ovaries and oligo-amenorrhoea but normal androgen levels: implications for the management of polycystic ovary syndrome. Clinical endocrinology. 2007;66(4):513-7.
143. Boomsma CM, Eijkemans MJ, Hughes EG, Visser GH, Fauser BC, Macklon NS. A meta-analysis of pregnancy outcomes in women with polycystic ovary syndrome. Human reproduction update. 2006;12(6):673-83.
144. Koivunen RM, Juutinen J, Vauhkonen I, Morin-Papunen LC, Ruokonen A, Tapanainen JS. Metabolic and steroidogenic alterations related to increased frequency of polycystic ovaries in women with a history of gestational diabetes. The Journal of clinical endocrinology and metabolism. 2001;86(6):2591-9.
145. Lo JC, Feigenbaum SL, Escobar GJ, Yang J, Crites YM, Ferrara A. Increased prevalence of gestational diabetes mellitus among women with diagnosed polycystic ovary syndrome: a population-based study. Diabetes care. 2006;29(8):1915-7.
146. Hoffman LK, Ehrmann DA. Cardiometabolic features of polycystic ovary syndrome. Nat Clin Pract Endocrinol Metab. 2008;4(4):215-22.
147. Diamanti-Kandarakis E, Papavassiliou AG, Kandarakis SA, Chrousos GP. Pathophysiology and types of dyslipidemia in PCOS. Trends in endocrinology and metabolism: TEM. 2007;18(7):280-5.
148. Rizzo M, Berneis K, Hersberger M, Pepe I, Di Fede G, Rini GB, et al. Milder forms of atherogenic dyslipidemia in ovulatory versus anovulatory polycystic ovary syndrome phenotype. Human reproduction. 2009;24(9):2286-92.
149. Wild RA, Carmina E, Diamanti-Kandarakis E, Dokras A, Escobar-Morreale HF, Futterweit W, et al. Assessment of cardiovascular risk and prevention of cardiovascular disease in women with the polycystic ovary syndrome: a consensus statement by the Androgen Excess and Polycystic Ovary Syndrome (AE-PCOS) Society. The Journal of clinical endocrinology and metabolism. 2010;95(5):2038-49.
150. Wild RA, Rizzo M, Clifton S, Carmina E. Lipid levels in polycystic ovary syndrome: systematic review and meta-analysis. Fertility and sterility. 2011;95(3):1073-9 e1-11.

151. Westerveld HE, Hoogendoorn M, de Jong AW, Goverde AJ, Fauser BC, Dallinga-Thie GM. Cardiometabolic abnormalities in the polycystic ovary syndrome: pharmacotherapeutic insights. Pharmacol Ther. 2008;119(3):223-41.
152. Panidis D, Tziomalos K, Papadakis E, Vosnakis C, Chatzis P, Katsikis I. Lifestyle intervention and anti-obesity therapies in the polycystic ovary syndrome: impact on metabolism and fertility. Endocrine. 2013;44(3):583-90.
153. Deanfield J, Donald A, Ferri C, Giannattasio C, Halcox J, Halligan S, et al. Endothelial function and dysfunction. Part I: Methodological issues for assessment in the different vascular beds: a statement by the Working Group on Endothelin and Endothelial Factors of the European Society of Hypertension. Journal of hypertension. 2005;23(1):7-17.
154. Ridker PM, Hennekens CH, Buring JE, Rifai N. C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. The New England journal of medicine. 2000;342(12):836-43.
155. Rifai N, Buring JE, Lee IM, Manson JE, Ridker PM. Is C-reactive protein specific for vascular disease in women? Annals of internal medicine. 2002;136(7):529-33.
156. Ridker PM, Rifai N, Rose L, Buring JE, Cook NR. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. The New England journal of medicine. 2002;347(20):1557-65.
157. Ross R. Atherosclerosis--an inflammatory disease. The New England journal of medicine. 1999;340(2):115-26.
158. Pasceri V, Willerson JT, Yeh ET. Direct proinflammatory effect of C-reactive protein on human endothelial cells. Circulation. 2000;102(18):2165-8.
159. Diamanti-Kandarakis E, Paterakis T, Alexandraki K, Piperi C, Aessopos A, Katsikis I, et al. Indices of low-grade chronic inflammation in polycystic ovary syndrome and the beneficial effect of metformin. Human reproduction. 2006;21(6):1426-31.
160. Boulman N, Levy Y, Leiba R, Shachar S, Linn R, Zinder O, et al. Increased C-reactive protein levels in the polycystic ovary syndrome: a marker of cardiovascular disease. The Journal of clinical endocrinology and metabolism. 2004;89(5):2160-5.
161. Kelly CC, Lyall H, Petrie JR, Gould GW, Connell JM, Sattar N. Low grade chronic inflammation in women with polycystic ovarian syndrome. The Journal of clinical endocrinology and metabolism. 2001;86(6):2453-5.
162. Morin-Papunen L, Rautio K, Ruokonen A, Hedberg P, Puukka M, Tapanainen JS. Metformin reduces serum C-reactive protein levels in women with polycystic ovary syndrome. The Journal of clinical endocrinology and metabolism. 2003;88(10):4649-54.
163. Escobar-Morreale HF, Botella-Carretero JI, Villuendas G, Sancho J, San Millan JL. Serum interleukin-18 concentrations are increased in the polycystic ovary syndrome: relationship to insulin resistance and to obesity. The Journal of clinical endocrinology and metabolism. 2004;89(2):806-11.
164. van den Oord SC, Sijbrands EJ, ten Kate GL, van Klaveren D, van Domburg RT, van der Steen AF, et al. Carotid intima-media thickness for cardiovascular risk assessment: systematic review and meta-analysis. Atherosclerosis. 2013;228(1):1-11.
165. Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, et al. Heart disease and stroke statistics--2012 update: a report from the American Heart Association. Circulation. 2012;125(1):e2-e220.
166. De Michele M, Panico S, Iannuzzi A, Celentano E, Ciardullo AV, Galasso R, et al. Association of obesity and central fat distribution with carotid artery wall thickening in middle-aged women. Stroke. 2002;33(12):2923-8.
167. Orio F, Jr., Palomba S, Cascella T, De Simone B, Di Biase S, Russo T, et al. Early impairment of endothelial structure and function in young normal-weight women with polycystic ovary syndrome. The Journal of clinical endocrinology and metabolism. 2004;89(9):4588-93.

168. Talbott EO, Guzick DS, Sutton-Tyrrell K, McHugh-Pemu KP, Zborowski JV, Remsberg KE, et al. Evidence for association between polycystic ovary syndrome and premature carotid atherosclerosis in middle-aged women. Arteriosclerosis, thrombosis, and vascular biology. 2000;20(11):2414-21.
169. Talbott EO, Zborowski JV, Rager JR, Boudreaux MY, Edmundowicz DA, Guzick DS. Evidence for an association between metabolic cardiovascular syndrome and coronary and aortic calcification among women with polycystic ovary syndrome. The Journal of clinical endocrinology and metabolism. 2004;89(11):5454-61.
170. Christian RC, Dumesic DA, Behrenbeck T, Oberg AL, Sheedy PF, 2nd, Fitzpatrick LA. Prevalence and predictors of coronary artery calcification in women with polycystic ovary syndrome. The Journal of clinical endocrinology and metabolism. 2003;88(6):2562-8.
171. Yarali H, Yildirir A, Aybar F, Kabakci G, Bukulmez O, Akgul E, et al. Diastolic dysfunction and increased serum homocysteine concentrations may contribute to increased cardiovascular risk in patients with polycystic ovary syndrome. Fertility and sterility. 2001;76(3):511-6.
172. Poonawala A, Nair SP, Thuluvath PJ. Prevalence of obesity and diabetes in patients with cryptogenic cirrhosis: a case-control study. Hepatology. 2000;32(4 Pt 1):689-92.
173. Matteoni CA, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. Gastroenterology. 1999;116(6):1413-9.
174. Kelley CE, Brown AJ, Diehl AM, Setji TL. Review of nonalcoholic fatty liver disease in women with polycystic ovary syndrome. World J Gastroenterol. 2014;20(39):14172-84.
175. Clark JM, Diehl AM. Nonalcoholic fatty liver disease: an underrecognized cause of cryptogenic cirrhosis. Jama. 2003;289(22):3000-4.
176. Syn WK, Choi SS, Diehl AM. Apoptosis and cytokines in non-alcoholic steatohepatitis. Clin Liver Dis. 2009;13(4):565-80.
177. Harrison SA, Torgerson S, Hayashi PH. The natural history of nonalcoholic fatty liver disease: a clinical histopathological study. Am J Gastroenterol. 2003;98(9):2042-7.
178. Adams LA, Lymp JF, St Sauver J, Sanderson SO, Lindor KD, Feldstein A, et al. The natural history of nonalcoholic fatty liver disease: a population-based cohort study. Gastroenterology. 2005;129(1):113-21.
179. Vernon G, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. Alimentary pharmacology & therapeutics. 2011;34(3):274-85.
180. Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, et al. The diagnosis and management of non-alcoholic fatty liver disease: Practice guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. Am J Gastroenterol. 2012;107(6):811-26.
181. Angulo P. Nonalcoholic fatty liver disease. The New England journal of medicine. 2002;346(16):1221-31.
182. Ong JP, Pitts A, Younossi ZM. Increased overall mortality and liver-related mortality in non-alcoholic fatty liver disease. J Hepatol. 2008;49(4):608-12.
183. Fracanzani AL, Valenti L, Bugianesi E, Andreoletti M, Colli A, Vanni E, et al. Risk of severe liver disease in nonalcoholic fatty liver disease with normal aminotransferase levels: a role for insulin resistance and diabetes. Hepatology. 2008;48(3):792-8.
184. Cerda C, Perez-Ayuso RM, Riquelme A, Soza A, Villaseca P, Sir-Petermann T, et al. Nonalcoholic fatty liver disease in women with polycystic ovary syndrome. J Hepatol. 2007;47(3):412-7.
185. Setji TL, Holland ND, Sanders LL, Pereira KC, Diehl AM, Brown AJ. Nonalcoholic steatohepatitis and nonalcoholic Fatty liver disease in young women with polycystic ovary syndrome. The Journal of clinical endocrinology and metabolism. 2006;91(5):1741-7.
186. Michaliszyn SF, Lee S, Tfayli H, Arslanian S. Polycystic ovary syndrome and nonalcoholic fatty liver in obese adolescents: association with metabolic risk profile. Fertility and sterility. 2013;100(6):1745-51.

187. Economou F, Xyrafis X, Livadas S, Androulakis, II, Argyrakopoulou G, Christakou CD, et al. In overweight/obese but not in normal-weight women, polycystic ovary syndrome is associated with elevated liver enzymes compared to controls. Hormones. 2009;8(3):199-206.
188. Kumar R, Mohan S. Non-alcoholic Fatty Liver Disease in Lean Subjects: Characteristics and Implications. J Clin Transl Hepatol. 2017;5(3):216-23.
189. Schwimmer JB, Khorram O, Chiu V, Schwimmer WB. Abnormal aminotransferase activity in women with polycystic ovary syndrome. Fertility and sterility. 2005;83(2):494-7.
190. Targher G, Rossini M, Lonardo A. Evidence that non-alcoholic fatty liver disease and polycystic ovary syndrome are associated by necessity rather than chance: a novel hepato-ovarian axis? Endocrine. 2016;51(2):211-21.
191. Hossain N, Stepanova M, Afendy A, Nader F, Younossi Y, Rafiq N, et al. Non-alcoholic steatohepatitis (NASH) in patients with polycystic ovarian syndrome (PCOS). Scand J Gastroenterol. 2011;46(4):479-84.
192. Ramezani-Binabaj M, Motalebi M, Karimi-Sari H, Rezaee-Zavareh MS, Alavian SM. Are women with polycystic ovarian syndrome at a high risk of non-alcoholic Fatty liver disease; a meta-analysis. Hepat Mon. 2014;14(11):e23235.
193. Vassilatou E. Nonalcoholic fatty liver disease and polycystic ovary syndrome. World J Gastroenterol. 2014;20(26):8351-63.
194. Abruzzese GA, Motta AB. Nonalcoholic fatty liver disease in children and adolescents - Relationship with Polycystic Ovary Syndrome. Curr Pharm Des. 2015;21(35):5144-50.
195. Lerchbaum E, Gruber HJ, Schwetz V, Giuliani A, Moller R, Pieber TR, et al. Fatty liver index in polycystic ovary syndrome. European journal of endocrinology / European Federation of Endocrine Societies. 2011;165(6):935-43.
196. Chen MJ, Chiu HM, Chen CL, Yang WS, Yang YS, Ho HN. Hyperandrogenemia is independently associated with elevated alanine aminotransferase activity in young women with polycystic ovary syndrome. The Journal of clinical endocrinology and metabolism. 2010;95(7):3332-41.
197. Zueff LF, Martins WP, Vieira CS, Ferriani RA. Ultrasonographic and laboratory markers of metabolic and cardiovascular disease risk in obese women with polycystic ovary syndrome. Ultrasound Obstet Gynecol. 2012;39(3):341-7.
198. Chen MJ, Ho HN. Hepatic manifestations of women with polycystic ovary syndrome. Best Pract Res Clin Obstet Gynaecol. 2016;37:119-28.
199. Barber TM, Franks S. Genetics of polycystic ovary syndrome. Front Horm Res. 2013;40:28-39.
200. Baranova A, Tran TP, Afendy A, Wang L, Shamsaddini A, Mehta R, et al. Molecular signature of adipose tissue in patients with both non-alcoholic fatty liver disease (NAFLD) and polycystic ovarian syndrome (PCOS). J Transl Med. 2013;11:133.
201. Targher G, Marra F, Marchesini G. Increased risk of cardiovascular disease in non-alcoholic fatty liver disease: causal effect or epiphenomenon? Diabetologia. 2008;51(11):1947-53.
202. Francque SM, van der Graaff D, Kwanten WJ. Non-alcoholic fatty liver disease and cardiovascular risk: Pathophysiological mechanisms and implications. J Hepatol. 2016;65(2):425-43.
203. Dawson AJ, Sathyapalan T, Smithson JA, Vince RV, Coady AM, Ajjan R, et al. A comparison of cardiovascular risk indices in patients with polycystic ovary syndrome with and without coexisting nonalcoholic fatty liver disease. Clinical endocrinology. 2014;80(6):843-9.
204. Brown AJ, Tendler DA, McMurray RG, Setji TL. Polycystic ovary syndrome and severe nonalcoholic steatohepatitis: beneficial effect of modest weight loss and exercise on liver biopsy findings. Endocrine practice : official journal of the American College of Endocrinology and the American Association of Clinical Endocrinologists. 2005;11(5):319-24.
205. Clark JM. The epidemiology of nonalcoholic fatty liver disease in adults. J Clin Gastroenterol. 2006;40 Suppl 1:S5-10.
206. Vassilatou E, Lafoyianni S, Vryonidou A, Ioannidis D, Kosma L, Katsoulis K, et al. Increased androgen bioavailability is associated with non-alcoholic fatty liver disease in women with polycystic ovary syndrome. Human reproduction. 2010;25(1):212-20.

207. Tan S, Bechmann LP, Benson S, Dietz T, Eichner S, Hahn S, et al. Apoptotic markers indicate nonalcoholic steatohepatitis in polycystic ovary syndrome. The Journal of clinical endocrinology and metabolism. 2010;95(1):343-8.
208. Bedogni G, Bellentani S, Miglioli L, Masutti F, Passalacqua M, Castiglione A, et al. The Fatty Liver Index: a simple and accurate predictor of hepatic steatosis in the general population. BMC Gastroenterol. 2006;6:33.
209. Festi D, Schiumerini R, Marzi L, Di Biase AR, Mandolesi D, Montrone L, et al. Review article: the diagnosis of non-alcoholic fatty liver disease -- availability and accuracy of non-invasive methods. Alimentary pharmacology & therapeutics. 2013;37(4):392-400.
210. Huang X, Xu M, Chen Y, Peng K, Huang Y, Wang P, et al. Validation of the Fatty Liver Index for Nonalcoholic Fatty Liver Disease in Middle-Aged and Elderly Chinese. Medicine (Baltimore). 2015;94(40):e1682.
211. Pinzani M. The ELF panel: a new crystal ball in hepatology? Gut. 2010;59(9):1165-7.
212. Guha IN, Parkes J, Roderick P, Chattopadhyay D, Cross R, Harris S, et al. Noninvasive markers of fibrosis in nonalcoholic fatty liver disease: Validating the European Liver Fibrosis Panel and exploring simple markers. Hepatology. 2008;47(2):455-60.
213. Nobili V, Parkes J, Bottazzo G, Marcellini M, Cross R, Newman D, et al. Performance of ELF serum markers in predicting fibrosis stage in pediatric non-alcoholic fatty liver disease. Gastroenterology. 2009;136(1):160-7.
214. Macut D, Bozic-Antic I, Bjekic-Macut J, Tziomalos K. MANAGEMENT OF ENDOCRINE DISEASE: Polycystic ovary syndrome and nonalcoholic fatty liver disease. European journal of endocrinology / European Federation of Endocrine Societies. 2017;177(3):R145-R58.
215. Hernaez R, Lazo M, Bonekamp S, Kamel I, Brancati FL, Guallar E, et al. Diagnostic accuracy and reliability of ultrasonography for the detection of fatty liver: a meta-analysis. Hepatology. 2011;54(3):1082-90.
216. Papagianni M, Sofogianni A, Tziomalos K. Non-invasive methods for the diagnosis of nonalcoholic fatty liver disease. World J Hepatol. 2015;7(4):638-48.
217. Loria P, Adinolfi LE, Bellentani S, Bugianesi E, Grieco A, Fargion S, et al. Practice guidelines for the diagnosis and management of nonalcoholic fatty liver disease. A decalogue from the Italian Association for the Study of the Liver (AISF) Expert Committee. Dig Liver Dis. 2010;42(4):272-82.
218. Permutt Z, Le TA, Peterson MR, Seki E, Brenner DA, Sirlin C, et al. Correlation between liver histology and novel magnetic resonance imaging in adult patients with non-alcoholic fatty liver disease - MRI accurately quantifies hepatic steatosis in NAFLD. Alimentary pharmacology & therapeutics. 2012;36(1):22-9.
219. Hashemi SA, Alavian SM, Gholami-Fesharaki M. Assessment of transient elastography (FibroScan) for diagnosis of fibrosis in non-alcoholic fatty liver disease: A systematic review and meta-analysis. Caspian J Intern Med. 2016;7(4):242-52.
220. Lupsor M, Badea R, Stefanescu H, Grigorescu M, Serban A, Radu C, et al. Performance of unidimensional transient elastography in staging non-alcoholic steatohepatitis. J Gastrointestin Liver Dis. 2010;19(1):53-60.
221. Kwok R, Tse YK, Wong GL, Ha Y, Lee AU, Ngu MC, et al. Systematic review with meta-analysis: non-invasive assessment of non-alcoholic fatty liver disease--the role of transient elastography and plasma cytokeratin-18 fragments. Alimentary pharmacology & therapeutics. 2014;39(3):254-69.
222. Castera L, Foucher J, Bernard PH, Carvalho F, Allaix D, Merrouche W, et al. Pitfalls of liver stiffness measurement: a 5-year prospective study of 13,369 examinations. Hepatology. 2010;51(3):828-35.
223. Kim D, Kim WR, Talwalkar JA, Kim HJ, Ehman RL. Advanced fibrosis in nonalcoholic fatty liver disease: noninvasive assessment with MR elastography. Radiology. 2013;268(2):411-9.
224. Poynard T, Munteanu M, Luckina E, Perazzo H, Ngo Y, Royer L, et al. Liver fibrosis evaluation using real-time shear wave elastography: applicability and diagnostic performance using methods without a gold standard. J Hepatol. 2013;58(5):928-35.

225. Boursier J, Isselin G, Fouchard-Hubert I, Oberti F, Dib N, Lebigot J, et al. Acoustic radiation force impulse: a new ultrasonographic technology for the widespread noninvasive diagnosis of liver fibrosis. Eur J Gastroenterol Hepatol. 2010;22(9):1074-84.
226. Lovren F, Verma S. Evolving role of microparticles in the pathophysiology of endothelial dysfunction. Clin Chem. 2013;59(8):1166-74.
227. Rajendran P, Rengarajan T, Thangavel J, Nishigaki Y, Sakthisekaran D, Sethi G, et al. The vascular endothelium and human diseases. Int J Biol Sci. 2013;9(10):1057-69.
228. Sena CM, Pereira AM, Seica R. Endothelial dysfunction - a major mediator of diabetic vascular disease. Biochimica et biophysica acta. 2013;1832(12):2216-31.
229. Deanfield JE, Halcox JP, Rabelink TJ. Endothelial function and dysfunction: testing and clinical relevance. Circulation. 2007;115(10):1285-95.
230. Schechter AN, Gladwin MT. Hemoglobin and the paracrine and endocrine functions of nitric oxide. The New England journal of medicine. 2003;348(15):1483-5.
231. Vita JA, Keaney JF, Jr. Endothelial function: a barometer for cardiovascular risk? Circulation. 2002;106(6):640-2.
232. Corson MA, James NL, Latta SE, Nerem RM, Berk BC, Harrison DG. Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. Circ Res. 1996;79(5):984-91.
233. Forstermann U, Munzel T. Endothelial nitric oxide synthase in vascular disease: from marvel to menace. Circulation. 2006;113(13):1708-14.
234. Busse R, Edwards G, Feletou M, Fleming I, Vanhoutte PM, Weston AH. EDHF: bringing the concepts together. Trends Pharmacol Sci. 2002;23(8):374-80.
235. Halcox JP, Narayanan S, Cramer-Joyce L, Mincemoyer R, Quyyumi AA. Characterization of endothelium-derived hyperpolarizing factor in the human forearm microcirculation. American journal of physiology Heart and circulatory physiology. 2001;280(6):H2470-7.
236. Ghosh S, Karin M. Missing pieces in the NF-kappaB puzzle. Cell. 2002;109 Suppl:S81-96.
237. Stamler JS, Lamas S, Fang FC. Nitrosylation. the prototypic redox-based signaling mechanism. Cell. 2001;106(6):675-83.
238. Moncada S, Higgs EA, Vane JR. Human arterial and venous tissues generate prostacyclin (prostaglandin x), a potent inhibitor of platelet aggregation. Lancet. 1977;1(8001):18-20.
239. Arehart E, Gleim S, Kasza Z, Fetalvero KM, Martin KA, Hwa J. Prostacyclin, atherothrombosis, and cardiovascular disease. Curr Med Chem. 2007;14(20):2161-9.
240. Bohm F, Pernow J. The importance of endothelin-1 for vascular dysfunction in cardiovascular disease. Cardiovascular research. 2007;76(1):8-18.
241. Ozerlat I. Reproductive endocrinology: Endothelial dysfunction in women with polycystic ovary syndrome--an inherent effect? Nature reviews Endocrinology. 2012;8(10):566.
242. Sprung VS, Atkinson G, Cuthbertson DJ, Pugh CJ, Aziz N, Green DJ, et al. Endothelial function measured using flow-mediated dilation in polycystic ovary syndrome: a meta-analysis of the observational studies. Clinical endocrinology. 2013;78(3):438-46.
243. Moran LJ, Cameron JD, Strauss BJ, Teede HJ. Vascular function in the diagnostic categories of polycystic ovary syndrome. Human reproduction. 2011;26(8):2192-9.
244. Sorensen MB, Franks S, Robertson C, Pennell DJ, Collins P. Severe endothelial dysfunction in young women with polycystic ovary syndrome is only partially explained by known cardiovascular risk factors. Clinical endocrinology. 2006;65(5):655-9.
245. Toulis KA, Goulis DG, Mintziori G, Kintiraki E, Eukarpidis E, Mouratoglou SA, et al. Meta-analysis of cardiovascular disease risk markers in women with polycystic ovary syndrome. Human reproduction update. 2011;17(6):741-60.
246. Ludmer PL, Selwyn AP, Shook TL, Wayne RR, Mudge GH, Alexander RW, et al. Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. The New England journal of medicine. 1986;315(17):1046-51.

247. Juonala M, Viikari JS, Laitinen T, Marniemi J, Helenius H, Ronnemaa T, et al. Interrelations between brachial endothelial function and carotid intima-media thickness in young adults: the cardiovascular risk in young Finns study. Circulation. 2004;110(18):2918-23.
248. Linder L, Kiowski W, Buhler FR, Luscher TF. Indirect evidence for release of endothelium-derived relaxing factor in human forearm circulation in vivo. Blunted response in essential hypertension. Circulation. 1990;81(6):1762-7.
249. Celermajer DS, Sorensen KE, Gooch VM, Spiegelhalter DJ, Miller OI, Sullivan ID, et al. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. Lancet. 1992;340(8828):1111-5.
250. Tousoulis D, Antoniades C, Stefanadis C. Evaluating endothelial function in humans: a guide to invasive and non-invasive techniques. Heart. 2005;91(4):553-8.
251. Flammer AJ, Luscher TF. Human endothelial dysfunction: EDRFs. Pflugers Arch. 2010;459(6):1005-13.
252. Bonetti PO, Lerman LO, Lerman A. Endothelial dysfunction: a marker of atherosclerotic risk. Arteriosclerosis, thrombosis, and vascular biology. 2003;23(2):168-75.
253. Berry C, L'Allier PL, Gregoire J, Lesperance J, Levesque S, Ibrahim R, et al. Comparison of intravascular ultrasound and quantitative coronary angiography for the assessment of coronary artery disease progression. Circulation. 2007;115(14):1851-7.
254. Virdis A, Ghiadoni L, Taddei S. Human endothelial dysfunction: EDCFs. Pflugers Arch. 2010;459(6):1015-23.
255. Nabel EG, Selwyn AP, Ganz P. Paradoxical narrowing of atherosclerotic coronary arteries induced by increases in heart rate. Circulation. 1990;81(3):850-9.
256. Egashira K, Katsuda Y, Mohri M, Kuga T, Tagawa T, Kubota T, et al. Role of endothelium-derived nitric oxide in coronary vasodilatation induced by pacing tachycardia in humans. Circ Res. 1996;79(2):331-5.
257. Trochu JN, Bouhour JB, Kaley G, Hintze TH. Role of endothelium-derived nitric oxide in the regulation of cardiac oxygen metabolism: implications in health and disease. Circ Res. 2000;87(12):1108-17.
258. AlBadri A, Wei J, Mehta PK, Landes S, Petersen JW, Anderson RD, et al. Acetylcholine versus cold pressor testing for evaluation of coronary endothelial function. PloS one. 2017;12(2):e0172538.
259. Camici PG, Crea F. Coronary microvascular dysfunction. The New England journal of medicine. 2007;356(8):830-40.
260. Leung DY, Leung M. Non-invasive/invasive imaging: significance and assessment of coronary microvascular dysfunction. Heart. 2011;97(7):587-95.
261. Schindler TH, Schelbert HR, Quercioli A, Dilsizian V. Cardiac PET imaging for the detection and monitoring of coronary artery disease and microvascular health. JACC Cardiovasc Imaging. 2010;3(6):623-40.
262. Doyle M, Fuisz A, Kortright E, Biederman RW, Walsh EG, Martin ET, et al. The impact of myocardial flow reserve on the detection of coronary artery disease by perfusion imaging methods: an NHLBI WISE study. J Cardiovasc Magn Reson. 2003;5(3):475-85.
263. Bonetti PO, Pumper GM, Higano ST, Holmes DR, Jr., Kuvin JT, Lerman A. Noninvasive identification of patients with early coronary atherosclerosis by assessment of digital reactive hyperemia. Journal of the American College of Cardiology. 2004;44(11):2137-41.
264. Takase B, Uehata A, Akima T, Nagai T, Nishioka T, Hamabe A, et al. Endothelium-dependent flow-mediated vasodilation in coronary and brachial arteries in suspected coronary artery disease. Am J Cardiol. 1998;82(12):1535-9, A7-8.
265. Chatzizisis YS, Coskun AU, Jonas M, Edelman ER, Feldman CL, Stone PH. Role of endothelial shear stress in the natural history of coronary atherosclerosis and vascular remodeling: molecular, cellular, and vascular behavior. Journal of the American College of Cardiology. 2007;49(25):2379-93.

266. Lavi S, Bae JH, Rihal CS, Prasad A, Barsness GW, Lennon RJ, et al. Segmental coronary endothelial dysfunction in patients with minimal atherosclerosis is associated with necrotic core plaques. Heart. 2009;95(18):1525-30.
267. Chuah SS, Woolfson PI, Pullan BR, Lewis PS. Plethysmography without venous occlusion for measuring forearm blood flow: comparison with venous occlusive method. Clin Physiol Funct Imaging. 2004;24(5):296-303.
268. Walker HA, Jackson G, Ritter JM, Chowienczyk PJ. Assessment of forearm vasodilator responses to acetylcholine and albuterol by strain gauge plethysmography: reproducibility and influence of strain gauge placement. British journal of clinical pharmacology. 2001;51(3):225-9.
269. Petrie JR, Ueda S, Morris AD, Murray LS, Elliott HL, Connell JM. How reproducible is bilateral forearm plethysmography? British journal of clinical pharmacology. 1998;45(2):131-9.
270. Joannides R, Haefeli WE, Linder L, Richard V, Bakkali EH, Thuillez C, et al. Nitric oxide is responsible for flow-dependent dilatation of human peripheral conduit arteries in vivo. Circulation. 1995;91(5):1314-9.
271. Taddei S, Versari D, Cipriano A, Ghiadoni L, Galetta F, Franzoni F, et al. Identification of a cytochrome P450 2C9-derived endothelium-derived hyperpolarizing factor in essential hypertensive patients. Journal of the American College of Cardiology. 2006;48(3):508-15.
272. Giannarelli C, Virdis A, De Negri F, Magagna A, Duranti E, Salvetti A, et al. Effect of sulfaphenazole on tissue plasminogen activator release in normotensive subjects and hypertensive patients. Circulation. 2009;119(12):1625-33.
273. Rahman AM, Murrow JR, Ozkor MA, Kavtaradze N, Lin J, De Staercke C, et al. Endothelium-derived hyperpolarizing factor mediates bradykinin-stimulated tissue plasminogen activator release in humans. J Vasc Res. 2014;51(3):200-8.
274. Benjamin N, Calver A, Collier J, Robinson B, Vallance P, Webb D. Measuring forearm blood flow and interpreting the responses to drugs and mediators. Hypertension. 1995;25(5):918-23.
275. Parker BA, Tschakovsky ME, Augeri AL, Polk DM, Thompson PD, Kiernan FJ. Heterogenous vasodilator pathways underlie flow-mediated dilation in men and women. American journal of physiology Heart and circulatory physiology. 2011;301(3):H1118-26.
276. Lieberman EH, Gerhard MD, Uehata A, Selwyn AP, Ganz P, Yeung AC, et al. Flow-induced vasodilation of the human brachial artery is impaired in patients <40 years of age with coronary artery disease. Am J Cardiol. 1996;78(11):1210-4.
277. Joannides R, Richard V, Haefeli WE, Linder L, Luscher TF, Thuillez C. Role of basal and stimulated release of nitric oxide in the regulation of radial artery caliber in humans. Hypertension. 1995;26(2):327-31.
278. Thijssen DH, Black MA, Pyke KE, Padilla J, Atkinson G, Harris RA, et al. Assessment of flow-mediated dilation in humans: a methodological and physiological guideline. American journal of physiology Heart and circulatory physiology. 2011;300(1):H2-12.
279. Charakida M, Masi S, Luscher TF, Kastelein JJ, Deanfield JE. Assessment of atherosclerosis: the role of flow-mediated dilatation. Eur Heart J. 2010;31(23):2854-61.
280. Harris RA, Nishiyama SK, Wray DW, Richardson RS. Ultrasound assessment of flow-mediated dilation. Hypertension. 2010;55(5):1075-85.
281. Mitchell GF, Parise H, Vita JA, Larson MG, Warner E, Keaney JF, Jr., et al. Local shear stress and brachial artery flow-mediated dilation: the Framingham Heart Study. Hypertension. 2004;44(2):134-9.
282. Mitchell GF, Vita JA, Larson MG, Parise H, Keyes MJ, Warner E, et al. Cross-sectional relations of peripheral microvascular function, cardiovascular disease risk factors, and aortic stiffness: the Framingham Heart Study. Circulation. 2005;112(24):3722-8.
283. Anderson TJ, Charbonneau F, Title LM, Buithieu J, Rose MS, Conradson H, et al. Microvascular function predicts cardiovascular events in primary prevention: long-term results from the Firefighters and Their Endothelium (FATE) study. Circulation. 2011;123(2):163-9.

284. Philpott AC, Lonn E, Title LM, Verma S, Buithieu J, Charbonneau F, et al. Comparison of new measures of vascular function to flow mediated dilatation as a measure of cardiovascular risk factors. Am J Cardiol. 2009;103(11):1610-5.
285. Yeboah J, Folsom AR, Burke GL, Johnson C, Polak JF, Post W, et al. Predictive value of brachial flow-mediated dilation for incident cardiovascular events in a population-based study: the multi-ethnic study of atherosclerosis. Circulation. 2009;120(6):502-9.
286. Pyke KE, Dwyer EM, Tschakovsky ME. Impact of controlling shear rate on flow-mediated dilation responses in the brachial artery of humans. Journal of applied physiology. 2004;97(2):499-508.
287. Thijssen DH, Dawson EA, Black MA, Hopman MT, Cable NT, Green DJ. Heterogeneity in conduit artery function in humans: impact of arterial size. American journal of physiology Heart and circulatory physiology. 2008;295(5):H1927-34.
288. Gori T, Dragoni S, Lisi M, Di Stolfo G, Sonnati S, Fineschi M, et al. Conduit artery constriction mediated by low flow a novel noninvasive method for the assessment of vascular function. Journal of the American College of Cardiology. 2008;51(20):1953-8.
289. Gori T, Grotti S, Dragoni S, Lisi M, Di Stolfo G, Sonnati S, et al. Assessment of vascular function: flow-mediated constriction complements the information of flow-mediated dilatation. Heart. 2010;96(2):141-7.
290. Gori T, Parker JD, Munzel T. Flow-mediated constriction: further insight into a new measure of vascular function. Eur Heart J. 2011;32(7):784-7.
291. Gori T, Muxel S, Damaske A, Radmacher MC, Fasola F, Schaefer S, et al. Endothelial function assessment: flow-mediated dilation and constriction provide different and complementary information on the presence of coronary artery disease. Eur Heart J. 2012;33(3):363-71.
292. Kuvin JT, Patel AR, Sliney KA, Pandian NG, Sheffy J, Schnall RP, et al. Assessment of peripheral vascular endothelial function with finger arterial pulse wave amplitude. Am Heart J. 2003;146(1):168-74.
293. Hamburg NM, Keyes MJ, Larson MG, Vasan RS, Schnabel R, Pryde MM, et al. Cross-sectional relations of digital vascular function to cardiovascular risk factors in the Framingham Heart Study. Circulation. 2008;117(19):2467-74.
294. Nohria A, Gerhard-Herman M, Creager MA, Hurley S, Mitra D, Ganz P. Role of nitric oxide in the regulation of digital pulse volume amplitude in humans. Journal of applied physiology. 2006;101(2):545-8.
295. Rubinshtein R, Kuvin JT, Soffler M, Lennon RJ, Lavi S, Nelson RE, et al. Assessment of endothelial function by non-invasive peripheral arterial tonometry predicts late cardiovascular adverse events. Eur Heart J. 2010;31(9):1142-8.
296. Schnabel RB, Schulz A, Wild PS, Sinning CR, Wilde S, Eleftheriadis M, et al. Noninvasive vascular function measurement in the community: cross-sectional relations and comparison of methods. Circ Cardiovasc Imaging. 2011;4(4):371-80.
297. Hamburg NM, Palmisano J, Larson MG, Sullivan LM, Lehman BT, Vasan RS, et al. Relation of brachial and digital measures of vascular function in the community: the Framingham heart study. Hypertension. 2011;57(3):390-6.
298. Paudel KR, Panth N, Kim DW. Circulating Endothelial Microparticles: A Key Hallmark of Atherosclerosis Progression. Scientifica (Cairo). 2016;2016:8514056.
299. Dignat-George F, Boulanger CM. The many faces of endothelial microparticles. Arteriosclerosis, thrombosis, and vascular biology. 2011;31(1):27-33.
300. Thomson RL, Brinkworth GD, Noakes M, Clifton PM, Norman RJ, Buckley JD. The effect of diet and exercise on markers of endothelial function in overweight and obese women with polycystic ovary syndrome. Human reproduction. 2012;27(7):2169-76.
301. Kravariti M, Naka KK, Kalantaridou SN, Kazakos N, Katsouras CS, Makrigiannakis A, et al. Predictors of endothelial dysfunction in young women with polycystic ovary syndrome. The Journal of clinical endocrinology and metabolism. 2005;90(9):5088-95.

302. Orio F, Jr., Palomba S, Cascella T, De Simone B, Manguso F, Savastano S, et al. Improvement in endothelial structure and function after metformin treatment in young normal-weight women with polycystic ovary syndrome: results of a 6-month study. The Journal of clinical endocrinology and metabolism. 2005;90(11):6072-6.
303. Charitidou C, Farmakiotis D, Zournatzi V, Pidonia I, Pegiou T, Karamanis N, et al. The administration of estrogens, combined with anti-androgens, has beneficial effects on the hormonal features and asymmetric dimethyl-arginine levels, in women with the polycystic ovary syndrome. Atherosclerosis. 2008;196(2):958-65.
304. Heutling D, Schulz H, Nickel I, Kleinstein J, Kaltwasser P, Westphal S, et al. Asymmetrical dimethylarginine, inflammatory and metabolic parameters in women with polycystic ovary syndrome before and after metformin treatment. The Journal of clinical endocrinology and metabolism. 2008;93(1):82-90.
305. Rajendran S, Willoughby SR, Chan WP, Liberts EA, Heresztyn T, Saha M, et al. Polycystic ovary syndrome is associated with severe platelet and endothelial dysfunction in both obese and lean subjects. Atherosclerosis. 2009;204(2):509-14.
306. Teede HJ, Meyer C, Hutchison SK, Zoungas S, McGrath BP, Moran LJ. Endothelial function and insulin resistance in polycystic ovary syndrome: the effects of medical therapy. Fertility and sterility. 2010;93(1):184-91.
307. Moran LJ, Hutchison SK, Meyer C, Zoungas S, Teede HJ. A comprehensive assessment of endothelial function in overweight women with and without polycystic ovary syndrome. Clin Sci (Lond). 2009;116(10):761-70.
308. Ozgurtas T, Oktenli C, Dede M, Tapan S, Kenar L, Sanisoglu SY, et al. Metformin and oral contraceptive treatments reduced circulating asymmetric dimethylarginine (ADMA) levels in patients with polycystic ovary syndrome (PCOS). Atherosclerosis. 2008;200(2):336-44.
309. Jezovnik MK, Jensterle Sever M, Janez A, Pfeifer M, Spirkoska A, Kravos NA, et al. Comparison of different methods investigating functional and morphological markers of early atherogenesis in obese women with polycystic ovary syndrome. Int Angiol. 2016;35(5):446-54.
310. Yilmaz MI, Sonmez A, Qureshi AR, Saglam M, Stenvinkel P, Yaman H, et al. Endogenous testosterone, endothelial dysfunction, and cardiovascular events in men with nondialysis chronic kidney disease. Clin J Am Soc Nephrol. 2011;6(7):1617-25.
311. Akishita M, Hashimoto M, Ohike Y, Ogawa S, Iijima K, Eto M, et al. Low testosterone level is an independent determinant of endothelial dysfunction in men. Hypertens Res. 2007;30(11):1029-34.
312. Empen K, Lorbeer R, Dorr M, Haring R, Nauck M, Glaser S, et al. Association of testosterone levels with endothelial function in men: results from a population-based study. Arteriosclerosis, thrombosis, and vascular biology. 2012;32(2):481-6.
313. Lopes RA, Neves KB, Carneiro FS, Tostes RC. Testosterone and vascular function in aging. Front Physiol. 2012;3:89.
314. Paradisi G, Steinberg HO, Hempfling A, Cronin J, Hook G, Shepard MK, et al. Polycystic ovary syndrome is associated with endothelial dysfunction. Circulation. 2001;103(10):1410-5.
315. Pepene CE. Evidence for visfatin as an independent predictor of endothelial dysfunction in polycystic ovary syndrome. Clinical endocrinology. 2012;76(1):119-25.
316. Brinkworth GD, Noakes M, Moran LJ, Norman R, Clifton PM. Flow-mediated dilatation in overweight and obese women with polycystic ovary syndrome. BJOG : an international journal of obstetrics and gynaecology. 2006;113(11):1308-14.
317. Studen KB, Sebestjen M, Pfeifer M, Prezelj J. Influence of spironolactone treatment on endothelial function in non-obese women with polycystic ovary syndrome. European journal of endocrinology / European Federation of Endocrine Societies. 2011;164(3):389-95.
318. Diamanti-Kandarakis E, Economou FN, Livadas S, Tantalaki E, Piperi C, Papavassiliou AG, et al. Hyperreninemia characterizing women with polycystic ovary syndrome improves after metformin therapy. Kidney Blood Press Res. 2009;32(1):24-31.

319. Farquharson CA, Struthers AD. Spironolactone increases nitric oxide bioactivity, improves endothelial vasodilator dysfunction, and suppresses vascular angiotensin I/angiotensin II conversion in patients with chronic heart failure. Circulation. 2000;101(6):594-7.
320. Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A, et al. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. The New England journal of medicine. 1999;341(10):709-17.
321. Treasure CB, Klein JL, Weintraub WS, Talley JD, Stillabower ME, Kosinski AS, et al. Beneficial effects of cholesterol-lowering therapy on the coronary endothelium in patients with coronary artery disease. The New England journal of medicine. 1995;332(8):481-7.
322. Sader MA, Celermajer DS. Endothelial function, vascular reactivity and gender differences in the cardiovascular system. Cardiovascular research. 2002;53(3):597-604.
323. Carrero JJ, Kyriazis J, Sonmez A, Tzanakis I, Qureshi AR, Stenvinkel P, et al. Prolactin levels, endothelial dysfunction, and the risk of cardiovascular events and mortality in patients with CKD. Clin J Am Soc Nephrol. 2012;7(2):207-15.
324. Tarkun I, Arslan BC, Canturk Z, Turemen E, Sahin T, Duman C. Endothelial dysfunction in young women with polycystic ovary syndrome: relationship with insulin resistance and low-grade chronic inflammation. The Journal of clinical endocrinology and metabolism. 2004;89(11):5592-6.
325. Carmassi F, De Negri F, Fioriti R, De Giorgi A, Giannarelli C, Fruzzetti F, et al. Insulin resistance causes impaired vasodilation and hypofibrinolysis in young women with polycystic ovary syndrome. Thrombosis research. 2005;116(3):207-14.
326. Victor VM, Rovira-Llopis S, Banuls C, Diaz-Morales N, Lopez-Domenech S, Escribano-Lopez I, et al. Metformin modulates human leukocyte/endothelial cell interactions and proinflammatory cytokines in polycystic ovary syndrome patients. Atherosclerosis. 2015;242(1):167-73.
327. Merhi Z, McGee EA, Buyuk E. Role of advanced glycation end-products in obesity-related ovarian dysfunction. Minerva Endocrinol. 2014;39(3):167-74.
328. Diamanti-Kandarakis E, Katsikis I, Piperi C, Kandaraki E, Piouka A, Papavassiliou AG, et al. Increased serum advanced glycation end-products is a distinct finding in lean women with polycystic ovary syndrome (PCOS). Clinical endocrinology. 2008;69(4):634-41.
329. Pertynska-Marczewska M, Diamanti-Kandarakis E, Zhang J, Merhi Z. Advanced glycation end products: A link between metabolic and endothelial dysfunction in polycystic ovary syndrome? Metabolism: clinical and experimental. 2015;64(11):1564-73.
330. Diamanti-Kandarakis E, Piperi C, Patsouris E, Korkolopoulou P, Panidis D, Pawelczyk L, et al. Immunohistochemical localization of advanced glycation end-products (AGEs) and their receptor (RAGE) in polycystic and normal ovaries. Histochem Cell Biol. 2007;127(6):581-9.
331. Mather KJ, Verma S, Corenblum B, Anderson TJ. Normal endothelial function despite insulin resistance in healthy women with the polycystic ovary syndrome. The Journal of clinical endocrinology and metabolism. 2000;85(5):1851-6.
332. Lambert EA, Teede H, Sari CI, Jona E, Shorakae S, Woodington K, et al. Sympathetic activation and endothelial dysfunction in polycystic ovary syndrome are not explained by either obesity or insulin resistance. Clinical endocrinology. 2015;83(6):812-9.
333. Rasouli N, Kern PA. Adipocytokines and the metabolic complications of obesity. The Journal of clinical endocrinology and metabolism. 2008;93(11 Suppl 1):S64-73.
334. Ozegowska KE, Pawelczyk LA. The role of insulin and selected adipocytokines in patients with polycystic ovary syndrome (PCOS) - a literature review. Ginekol Pol. 2015;86(4):300-4.
335. Mansfield R, Galea R, Brincat M, Hole D, Mason H. Metformin has direct effects on human ovarian steroidogenesis. Fertility and sterility. 2003;79(4):956-62.
336. Moghetti P, Castello R, Negri C, Tosi F, Perrone F, Caputo M, et al. Metformin effects on clinical features, endocrine and metabolic profiles, and insulin sensitivity in polycystic ovary syndrome: a randomized, double-blind, placebo-controlled 6-month trial, followed by open, long-term clinical evaluation. The Journal of clinical endocrinology and metabolism. 2000;85(1):139-46.

337. Balfour JA, McClellan K. Topical eflornithine. American journal of clinical dermatology. 2001;2(3):197-201; discussion 2.
338. Jayasena CN, Franks S. The management of patients with polycystic ovary syndrome. Nature reviews Endocrinology. 2014;10(10):624-36.
339. Neumann F, Berswordt-Wallrabe RV, Elger W, Steinbeck H, Hahn JD, Kramer M. Aspects of androgen-dependent events as studied by antiandrogens. Recent Prog Horm Res. 1970;26:337-410.
340. Menard RH, Guenthner TM, Kon H, Gillette JR. Studies on the destruction of adrenal and testicular cytochrome P-450 by spironolactone. Requirement for the 7alpha-thio group and evidence for the loss of the heme and apoproteins of cytochrome P-450. The Journal of biological chemistry. 1979;254(5):1726-33.
341. Tolino A, Petrone A, Sarnacchiaro F, Cirillo D, Ronsini S, Lombardi G, et al. Finasteride in the treatment of hirsutism: new therapeutic perspectives. Fertility and sterility. 1996;66(1):61-5.
342. Moghetti P, Tosi F, Tosti A, Negri C, Misciali C, Perrone F, et al. Comparison of spironolactone, flutamide, and finasteride efficacy in the treatment of hirsutism: a randomized, double blind, placebo-controlled trial. The Journal of clinical endocrinology and metabolism. 2000;85(1):89-94.
343. Panidis D, Tziomalos K, Papadakis E, Katsikis I. Infertility treatment in polycystic ovary syndrome: lifestyle interventions, medications and surgery. Front Horm Res. 2013;40:128-41.
344. Thessaloniki EA-SPCWG. Consensus on infertility treatment related to polycystic ovary syndrome. Human reproduction. 2008;23(3):462-77.
345. Diamanti-Kandarakis E. Role of obesity and adiposity in polycystic ovary syndrome. International journal of obesity. 2007;31 Suppl 2:S8-13; discussion S31-2.
346. Escobar-Morreale HF, Botella-Carretero JI, Alvarez-Blasco F, Sancho J, San Millan JL. The polycystic ovary syndrome associated with morbid obesity may resolve after weight loss induced by bariatric surgery. The Journal of clinical endocrinology and metabolism. 2005;90(12):6364-9.
347. Panidis D, Tziomalos K, Papadakis E, Chatzis P, Kandaraki EA, Tsourdi EA, et al. The role of orlistat combined with lifestyle changes in the management of overweight and obese patients with polycystic ovary syndrome. Clinical endocrinology. 2014;80(3):432-8.
348. Kahal H, Abouda G, Rigby AS, Coady AM, Kilpatrick ES, Atkin SL. Glucagon-like peptide-1 analogue, liraglutide, improves liver fibrosis markers in obese women with polycystic ovary syndrome and nonalcoholic fatty liver disease. Clinical endocrinology. 2014;81(4):523-8.
349. Gambineri A, Patton L, De Iasio R, Cantelli B, Cognini GE, Filicori M, et al. Efficacy of octreotide-LAR in dieting women with abdominal obesity and polycystic ovary syndrome. The Journal of clinical endocrinology and metabolism. 2005;90(7):3854-62.
350. Diabetes Prevention Program Research G. Long-term effects of lifestyle intervention or metformin on diabetes development and microvascular complications over 15-year follow-up: the Diabetes Prevention Program Outcomes Study. The lancet Diabetes & endocrinology. 2015;3(11):866-75.
351. Valkenburg O, Steegers-Theunissen RP, Smedts HP, Dallinga-Thie GM, Fauser BC, Westerveld EH, et al. A more atherogenic serum lipoprotein profile is present in women with polycystic ovary syndrome: a case-control study. The Journal of clinical endocrinology and metabolism. 2008;93(2):470-6.
352. Duleba AJ, Banaszewska B, Spaczynski RZ, Pawelczyk L. Simvastatin improves biochemical parameters in women with polycystic ovary syndrome: results of a prospective, randomized trial. Fertility and sterility. 2006;85(4):996-1001.
353. Gao L, Zhao FL, Li SC. Statin is a reasonable treatment option for patients with Polycystic Ovary Syndrome: a meta-analysis of randomized controlled trials. Exp Clin Endocrinol Diabetes. 2012;120(6):367-75.
354. Tang T, Glanville J, Hayden CJ, White D, Barth JH, Balen AH. Combined lifestyle modification and metformin in obese patients with polycystic ovary syndrome. A randomized, placebo-controlled, double-blind multicentre study. Human reproduction. 2006;21(1):80-9.

355. Lord JM, Flight IH, Norman RJ. Metformin in polycystic ovary syndrome: systematic review and meta-analysis. Bmj. 2003;327(7421):951-3.
356. Legro RS, Barnhart HX, Schlaff WD, Carr BR, Diamond MP, Carson SA, et al. Clomiphene, metformin, or both for infertility in the polycystic ovary syndrome. The New England journal of medicine. 2007;356(6):551-66.
357. Misso ML, Costello MF, Garrubba M, Wong J, Hart R, Rombauts L, et al. Metformin versus clomiphene citrate for infertility in non-obese women with polycystic ovary syndrome: a systematic review and meta-analysis. Human reproduction update. 2013;19(1):2-11.
358. Meyer ML, Malek AM, Wild RA, Korytkowski MT, Talbott EO. Carotid artery intima-media thickness in polycystic ovary syndrome: a systematic review and meta-analysis. Human reproduction update. 2012;18(2):112-26.
359. Carmina E, Chu MC, Longo RA, Rini GB, Lobo RA. Phenotypic variation in hyperandrogenic women influences the findings of abnormal metabolic and cardiovascular risk parameters. The Journal of clinical endocrinology and metabolism. 2005;90(5):2545-9.
360. Jones G, Strugnell SA, DeLuca HF. Current understanding of the molecular actions of vitamin D. Physiological reviews. 1998;78(4):1193-231.
361. Panidis D, Balaris C, Farmakiotis D, Rousso D, Kourtis A, Balaris V, et al. Serum parathyroid hormone concentrations are increased in women with polycystic ovary syndrome. Clin Chem. 2005;51(9):1691-7.
362. Thys-Jacobs S, Donovan D, Papadopoulos A, Sarrel P, Bilezikian JP. Vitamin D and calcium dysregulation in the polycystic ovarian syndrome. Steroids. 1999;64(6):430-5.
363. Steingrimsdottir L, Gunnarsson O, Indridason OS, Franzson L, Sigurdsson G. Relationship between serum parathyroid hormone levels, vitamin D sufficiency, and calcium intake. Jama. 2005;294(18):2336-41.
364. Kinuta K, Tanaka H, Moriwake T, Aya K, Kato S, Seino Y. Vitamin D is an important factor in estrogen biosynthesis of both female and male gonads. Endocrinology. 2000;141(4):1317-24.
365. Danilenko KV, Sergeeva OY, Verevkin EG. Menstrual cycles are influenced by sunshine. Gynecological endocrinology : the official journal of the International Society of Gynecological Endocrinology. 2011;27(9):711-6.
366. Thomson RL, Spedding S, Buckley JD. Vitamin D in the aetiology and management of polycystic ovary syndrome. Clinical endocrinology. 2012;77(3):343-50.
367. Wehr E, Pilz S, Schweighofer N, Giuliani A, Kopera D, Pieber TR, et al. Association of hypovitaminosis D with metabolic disturbances in polycystic ovary syndrome. European journal of endocrinology / European Federation of Endocrine Societies. 2009;161(4):575-82.
368. Muscogiuri G, Policola C, Prioletta A, Sorice G, Mezza T, Lassandro A, et al. Low levels of 25(OH)D and insulin-resistance: 2 unrelated features or a cause-effect in PCOS? Clin Nutr. 2012;31(4):476-80.
369. Ngo DT, Chan WP, Rajendran S, Heresztyn T, Amarasekera A, Sverdlov AL, et al. Determinants of insulin responsiveness in young women: Impact of polycystic ovarian syndrome, nitric oxide, and vitamin D. Nitric Oxide. 2011;25(3):326-30.
370. Teegarden D, Donkin SS. Vitamin D: emerging new roles in insulin sensitivity. Nutr Res Rev. 2009;22(1):82-92.
371. Hahn S, Haselhorst U, Tan S, Quadbeck B, Schmidt M, Roesler S, et al. Low serum 25-hydroxyvitamin D concentrations are associated with insulin resistance and obesity in women with polycystic ovary syndrome. Exp Clin Endocrinol Diabetes. 2006;114(10):577-83.
372. Li HW, Brereton RE, Anderson RA, Wallace AM, Ho CK. Vitamin D deficiency is common and associated with metabolic risk factors in patients with polycystic ovary syndrome. Metabolism: clinical and experimental. 2011;60(10):1475-81.
373. Wehr E, Pieber TR, Obermayer-Pietsch B. Effect of vitamin D3 treatment on glucose metabolism and menstrual frequency in polycystic ovary syndrome women: a pilot study. Journal of endocrinological investigation. 2011;34(10):757-63.

374. Ozkan S, Jindal S, Greenseid K, Shu J, Zeitlian G, Hickmon C, et al. Replete vitamin D stores predict reproductive success following in vitro fertilization. Fertility and sterility. 2010;94(4):1314-9.
375. Ott J, Wattar L, Kurz C, Seemann R, Huber JC, Mayerhofer K, et al. Parameters for calcium metabolism in women with polycystic ovary syndrome who undergo clomiphene citrate stimulation: a prospective cohort study. European journal of endocrinology / European Federation of Endocrine Societies. 2012;166(5):897-902.
376. He C, Lin Z, Robb SW, Ezeamama AE. Serum Vitamin D Levels and Polycystic Ovary syndrome: A Systematic Review and Meta-Analysis. Nutrients. 2015;7(6):4555-77.
377. Yildizhan R, Kurdoglu M, Adali E, Kolusari A, Yildizhan B, Sahin HG, et al. Serum 25-hydroxyvitamin D concentrations in obese and non-obese women with polycystic ovary syndrome. Archives of gynecology and obstetrics. 2009;280(4):559-63.
378. Selimoglu H, Duran C, Kiyici S, Ersoy C, Guclu M, Ozkaya G, et al. The effect of vitamin D replacement therapy on insulin resistance and androgen levels in women with polycystic ovary syndrome. Journal of endocrinological investigation. 2010;33(4):234-8.
379. Ginde AA, Scragg R, Schwartz RS, Camargo CA, Jr. Prospective study of serum 25-hydroxyvitamin D level, cardiovascular disease mortality, and all-cause mortality in older U.S. adults. J Am Geriatr Soc. 2009;57(9):1595-603.
380. Sathyapalan T, Shepherd J, Arnett C, Coady AM, Kilpatrick ES, Atkin SL. Atorvastatin increases 25-hydroxy vitamin D concentrations in patients with polycystic ovary syndrome. Clin Chem. 2010;56(11):1696-700.
381. Zinman B, Wanner C, Lachin JM, Fitchett D, Bluhmki E, Hantel S, et al. Empagliflozin, Cardiovascular Outcomes, and Mortality in Type 2 Diabetes. The New England journal of medicine. 2015;373(22):2117-28.
382. Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, et al. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. Journal of the American College of Cardiology. 2002;39(2):257-65.
383. Fitchett D, Zinman B, Wanner C, Lachin JM, Hantel S, Salsali A, et al. Heart failure outcomes with empagliflozin in patients with type 2 diabetes at high cardiovascular risk: results of the EMPA-REG OUTCOME(R) trial. Eur Heart J. 2016;37(19):1526-34.
384. Scheen AJ. Pharmacokinetic and pharmacodynamic profile of empagliflozin, a sodium glucose co-transporter 2 inhibitor. Clin Pharmacokinet. 2014;53(3):213-25.
385. Lashen H. Role of metformin in the management of polycystic ovary syndrome. Therapeutic advances in endocrinology and metabolism. 2010;1(3):117-28.
386. Wu C, Qiu S, Zhu X, Li L. Vitamin D supplementation and glycemic control in type 2 diabetes patients: A systematic review and meta-analysis. Metabolism: clinical and experimental. 2017;73:67-76.
387. Brant LC, Barreto SM, Passos VM, Ribeiro AL. Reproducibility of peripheral arterial tonometry for the assessment of endothelial function in adults. Journal of hypertension. 2013;31(10):1984-90.
388. Selamet Tierney ES, Newburger JW, Gauvreau K, Geva J, Coogan E, Colan SD, et al. Endothelial pulse amplitude testing: feasibility and reproducibility in adolescents. J Pediatr. 2009;154(6):901-5.
389. Adan A, Alizada G, Kiraz Y, Baran Y, Nalbant A. Flow cytometry: basic principles and applications. Crit Rev Biotechnol. 2017;37(2):163-76.
390. MGe o. Flow Cytometry A Basic Introduction. Oxford University Press, Oxford, UK. 2000.
391. VanWijk MJ, VanBavel E, Sturk A, Nieuwland R. Microparticles in cardiovascular diseases. Cardiovascular research. 2003;59(2):277-87.
392. Mariani M, Colombo F, Assennato SM, Frugoni C, Cattaneo A, Trombetta E, et al. Evaluation of an easy and affordable flow cytometer for volumetric haematopoietic stem cell counting. Blood Transfus. 2014;12(3):416-20.

393. Hu SS, Zhang HG, Zhang QJ, Xiu RJ. Small-size circulating endothelial microparticles in coronary artery disease. PLoS One. 2014;9(8):e104528.
394. Hu SS, Zhang HG, Zhang QJ, Xiu RJ. CD144(+)EMPs/CD62E(+)EMPs: a couple of new biomarkers to monitor endothelial function in hypertension with hyperlipidemia involved. Int J Cardiol. 2014;175(1):203.
395. Guzick DS. Cardiovascular risk in PCOS. The Journal of clinical endocrinology and metabolism. 2004;89(8):3694-5.
396. Schmidt J, Landin-Wilhelmsen K, Brannstrom M, Dahlgren E. Cardiovascular disease and risk factors in PCOS women of postmenopausal age: a 21-year controlled follow-up study. The Journal of clinical endocrinology and metabolism. 2011;96(12):3794-803.
397. Melamed ML, Michos ED, Post W, Astor B. 25-hydroxyvitamin D levels and the risk of mortality in the general population. Arch Intern Med. 2008;168(15):1629-37.
398. Giovannucci E, Liu Y, Hollis BW, Rimm EB. 25-hydroxyvitamin D and risk of myocardial infarction in men: a prospective study. Arch Intern Med. 2008;168(11):1174-80.
399. Forouhi NG, Luan J, Cooper A, Boucher BJ, Wareham NJ. Baseline serum 25-hydroxy vitamin d is predictive of future glycemic status and insulin resistance: the Medical Research Council Ely Prospective Study 1990-2000. Diabetes. 2008;57(10):2619-25.
400. Gagnon C, Lu ZX, Magliano DJ, Dunstan DW, Shaw JE, Zimmet PZ, et al. Low serum 25-hydroxyvitamin D is associated with increased risk of the development of the metabolic syndrome at five years: results from a national, population-based prospective study (The Australian Diabetes, Obesity and Lifestyle Study: AusDiab). The Journal of clinical endocrinology and metabolism. 2012;97(6):1953-61.
401. Irani M, Seifer DB, Grazi RV, Julka N, Bhatt D, Kalgi B, et al. Vitamin D Supplementation Decreases TGF-beta1 Bioavailability in PCOS: A Randomized Placebo-Controlled Trial. The Journal of clinical endocrinology and metabolism. 2015;100(11):4307-14.
402. Lerchbaum E, Obermayer-Pietsch B. Vitamin D and fertility: a systematic review. European journal of endocrinology / European Federation of Endocrine Societies. 2012;166(5):765-78.
403. Holick MF. Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease. The American journal of clinical nutrition. 2004;80(6 Suppl):1678S-88S.
404. Kromhout D, Bosschieter EB, de Lezenne Coulander C. The inverse relation between fish consumption and 20-year mortality from coronary heart disease. The New England journal of medicine. 1985;312(19):1205-9.
405. Burr ML, Fehily AM, Gilbert JF, Rogers S, Holliday RM, Sweetnam PM, et al. Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). Lancet. 1989;2(8666):757-61.
406. Sathyapalan T, Shepherd J, Coady AM, Kilpatrick ES, Atkin SL. Atorvastatin reduces malondialdehyde concentrations in patients with polycystic ovary syndrome. The Journal of clinical endocrinology and metabolism. 2012;97(11):3951-5.
407. Raja-Khan N, Shah J, Stetter CM, Lott ME, Kunselman AR, Dodson WC, et al. High-dose vitamin D supplementation and measures of insulin sensitivity in polycystic ovary syndrome: a randomized, controlled pilot trial. Fertility and sterility. 2014;101(6):1740-6.
408. Ardabili HR, Gargari BP, Farzadi L. Vitamin D supplementation has no effect on insulin resistance assessment in women with polycystic ovary syndrome and vitamin D deficiency. Nutrition research. 2012;32(3):195-201.
409. Krul-Poel YH, Snackey C, Louwers Y, Lips P, Lambalk CB, Laven JS, et al. The role of vitamin D in metabolic disturbances in polycystic ovary syndrome: a systematic review. European journal of endocrinology / European Federation of Endocrine Societies. 2013;169(6):853-65.
410. Gambarin-Gelwan M, Kinkhabwala SV, Schiano TD, Bodian C, Yeh HC, Futterweit W. Prevalence of nonalcoholic fatty liver disease in women with polycystic ovary syndrome. Clin Gastroenterol Hepatol. 2007;5(4):496-501.

411. Targher G, Byrne CD. Clinical Review: Nonalcoholic fatty liver disease: a novel cardiometabolic risk factor for type 2 diabetes and its complications. The Journal of clinical endocrinology and metabolism. 2013;98(2):483-95.
412. Karoli R, Fatima J, Chandra A, Gupta U, Islam FU, Singh G. Prevalence of hepatic steatosis in women with polycystic ovary syndrome. J Hum Reprod Sci. 2013;6(1):9-14.
413. Eliades M, Spyrou E. Vitamin D: a new player in non-alcoholic fatty liver disease? World J Gastroenterol. 2015;21(6):1718-27.
414. Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, et al. The diagnosis and management of non-alcoholic fatty liver disease: practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. Hepatology. 2012;55(6):2005-23.
415. European Association for Study of L, Asociacion Latinoamericana para el Estudio del H. EASL-ALEH Clinical Practice Guidelines: Non-invasive tests for evaluation of liver disease severity and prognosis. J Hepatol. 2015;63(1):237-64.
416. Lichtinghagen R, Pietsch D, Bantel H, Manns MP, Brand K, Bahr MJ. The Enhanced Liver Fibrosis (ELF) score: normal values, influence factors and proposed cut-off values. J Hepatol. 2013;59(2):236-42.
417. Rosenberg WM, Voelker M, Thiel R, Becka M, Burt A, Schuppan D, et al. Serum markers detect the presence of liver fibrosis: a cohort study. Gastroenterology. 2004;127(6):1704-13.
418. Parkes J, Roderick P, Harris S, Day C, Mutimer D, Collier J, et al. Enhanced liver fibrosis test can predict clinical outcomes in patients with chronic liver disease. Gut. 2010;59(9):1245-51.
419. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. The Journal of clinical endocrinology and metabolism. 2011;96(7):1911-30.
420. Martins D, Wolf M, Pan D, Zadshir A, Tareen N, Thadhani R, et al. Prevalence of cardiovascular risk factors and the serum levels of 25-hydroxyvitamin D in the United States: data from the Third National Health and Nutrition Examination Survey. Archives of internal medicine. 2007;167(11):1159-65.
421. Wang TJ, Pencina MJ, Booth SL, Jacques PF, Ingelsson E, Lanier K, et al. Vitamin D deficiency and risk of cardiovascular disease. Circulation. 2008;117(4):503-11.
422. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). Hum Reprod. 2004;19(1):41-7.
423. Aspray TJ, Bowring C, Fraser W, Gittoes N, Javaid MK, Macdonald H, et al. National Osteoporosis Society vitamin D guideline summary. Age Ageing. 2014;43(5):592-5.
424. Li R, Guo E, Yang J, Li A, Yang Y, Liu S, et al. 1,25(OH)2 D3 attenuates hepatic steatosis by inducing autophagy in mice. Obesity. 2017;25(3):561-71.
425. Ding N, Yu RT, Subramaniam N, Sherman MH, Wilson C, Rao R, et al. A vitamin D receptor/SMAD genomic circuit gates hepatic fibrotic response. Cell. 2013;153(3):601-13.
426. Papapostoli I, Lammert F, Stokes CS. Effect of Short-Term Vitamin D Correction on Hepatic Steatosis as Quantified by Controlled Attenuation Parameter (CAP). J Gastrointestin Liver Dis. 2016;25(2):175-81.
427. Potter JJ, Liu X, Koteish A, Mezey E. 1,25-dihydroxyvitamin D3 and its nuclear receptor repress human alpha1 (I) collagen expression and type I collagen formation. Liver Int. 2013;33(5):677-86.
428. Sharifi N, Amani R, Hajiani E, Cheraghian B. Does vitamin D improve liver enzymes, oxidative stress, and inflammatory biomarkers in adults with non-alcoholic fatty liver disease? A randomized clinical trial. Endocrine. 2014;47(1):70-80.
429. Castera L, Vilgrain V, Angulo P. Noninvasive evaluation of NAFLD. Nature reviews Gastroenterology & hepatology. 2013;10(11):666-75.

430. Kim WR, Flamm SL, Di Bisceglie AM, Bodenheimer HC, Public Policy Committee of the American Association for the Study of Liver D. Serum activity of alanine aminotransferase (ALT) as an indicator of health and disease. Hepatology. 2008;47(4):1363-70.
431. NICE. Non-alcoholic fatty liver disease (NAFLD): assessment and management. NICE guideline [NG49]. National Institute for Health and Clinical Excellence. (2016).
432. Martinez SM, Fernandez-Varo G, Gonzalez P, Sampson E, Bruguera M, Navasa M, et al. Assessment of liver fibrosis before and after antiviral therapy by different serum marker panels in patients with chronic hepatitis C. Aliment Pharmacol Ther. 2011;33(1):138-48.
433. Abramovitch S, Dahan-Bachar L, Sharvit E, Weisman Y, Ben Tov A, Brazowski E, et al. Vitamin D inhibits proliferation and profibrotic marker expression in hepatic stellate cells and decreases thioacetamide-induced liver fibrosis in rats. Gut. 2011;60(12):1728-37.
434. Garg G, Kachhawa G, Ramot R, Khadgawat R, Tandon N, Sreenivas V, et al. Effect of vitamin D supplementation on insulin kinetics and cardiovascular risk factors in polycystic ovarian syndrome: a pilot study. Endocr Connect. 2015;4(2):108-16.
435. Pergialiotis V, Karampetsou N, Panagopoulos P, Trakakis E, Papantoniou N. The effect of Vitamin D supplementation on hormonal and glycaemic profile of patients with PCOS: A meta-analysis of randomised trials. Int J Clin Pract. 2017;71(6).
436. Dalan R, Liew H, Assam PN, Chan ES, Siddiqui FJ, Tan AW, et al. A randomised controlled trial evaluating the impact of targeted vitamin D supplementation on endothelial function in type 2 diabetes mellitus: The DIMENSION trial. Diabetes & vascular disease research. 2016;13(3):192-200.
437. Borgi L, McMullan C, Wohlhueter A, Curhan GC, Fisher ND, Forman JP. Effect of Vitamin D on Endothelial Function: A Randomized, Double-Blind, Placebo-Controlled Trial. Am J Hypertens. 2017;30(2):124-9.
438. Patel P, Poretsky L, Liao E. Lack of effect of subtherapeutic vitamin D treatment on glycemic and lipid parameters in Type 2 diabetes: A pilot prospective randomized trial. J Diabetes. 2010;2(1):36-40.
439. Wang Y, Si S, Liu J, Wang Z, Jia H, Feng K, et al. The Associations of Serum Lipids with Vitamin D Status. PloS one. 2016;11(10):e0165157.
440. Forney LA, Earnest CP, Henagan TM, Johnson LE, Castleberry TJ, Stewart LK. Vitamin D status, body composition, and fitness measures in college-aged students. J Strength Cond Res. 2014;28(3):814-24.
441. Zhong W, Gu B, Gu Y, Groome LJ, Sun J, Wang Y. Activation of vitamin D receptor promotes VEGF and CuZn-SOD expression in endothelial cells. J Steroid Biochem Mol Biol. 2014;140:56-62.
442. Said A, Akhter A. Meta-Analysis of Randomized Controlled Trials of Pharmacologic Agents in Non-alcoholic Steatohepatitis. Ann Hepatol. 2017;16(4):538-47.
443. Rouabhia S, Milic N, Abenavoli L. Metformin in the treatment of non-alcoholic fatty liver disease: safety, efficacy and mechanism. Expert Rev Gastroenterol Hepatol. 2014;8(4):343-9.
444. Matsuzawa Y, Kwon TG, Lennon RJ, Lerman LO, Lerman A. Prognostic Value of Flow-Mediated Vasodilation in Brachial Artery and Fingertip Artery for Cardiovascular Events: A Systematic Review and Meta-Analysis. J Am Heart Assoc. 2015;4(11).
445. Carmina E, Orio F, Palomba S, Longo RA, Cascella T, Colao A, et al. Endothelial dysfunction in PCOS: role of obesity and adipose hormones. The American journal of medicine. 2006;119(4):356 e1-6.
446. Widlansky ME, Gokce N, Keaney JF, Jr., Vita JA. The clinical implications of endothelial dysfunction. Journal of the American College of Cardiology. 2003;42(7):1149-60.
447. Carvalho LM, Ferreira CN, Soter MO, Sales MF, Rodrigues KF, Martins SR, et al. Microparticles: Inflammatory and haemostatic biomarkers in Polycystic Ovary Syndrome. Molecular and cellular endocrinology. 2017;443:155-62.
448. Carvalho LML, Ferreira CN, Soter MO, Sales MF, Rodrigues KF, Martins SR, et al. Microparticles: Inflammatory and haemostatic biomarkers in Polycystic Ovary Syndrome. Molecular and cellular endocrinology. 2017;443:155-62.

449. Lekakis J, Abraham P, Balbarini A, Blann A, Boulanger CM, Cockcroft J, et al. Methods for evaluating endothelial function: a position statement from the European Society of Cardiology Working Group on Peripheral Circulation. Eur J Cardiovasc Prev Rehabil. 2011;18(6):775-89.
450. Koga H, Sugiyama S, Kugiyama K, Watanabe K, Fukushima H, Tanaka T, et al. Elevated levels of VE-cadherin-positive endothelial microparticles in patients with type 2 diabetes mellitus and coronary artery disease. Journal of the American College of Cardiology. 2005;45(10):1622-30.
451. Chowdhury R, Kunutsor S, Vitezova A, Oliver-Williams C, Chowdhury S, Kiefte-de-Jong JC, et al. Vitamin D and risk of cause specific death: systematic review and meta-analysis of observational cohort and randomised intervention studies. Bmj. 2014;348:g1903.
452. Rodrigues KF, Pietrani NT, Fernandes AP, Bosco AA, de Sousa MCR, de Fatima Oliveira Silva I, et al. Circulating microparticles levels are increased in patients with diabetic kidney disease: A case-control research. Clinica chimica acta; international journal of clinical chemistry. 2018;479:48-55.
453. Reynolds J, Ray D, Alexander MY, Bruce I. Role of vitamin D in endothelial function and endothelial repair in clinically stable systemic lupus erythematosus. Lancet. 2015;385 Suppl 1:S83.
454. Jia X, Xu J, Gu Y, Gu X, Li W, Wang Y. Vitamin D suppresses oxidative stress-induced microparticle release by human umbilical vein endothelial cells. Biology of reproduction. 2017;96(1):199-210.
455. Irani M, Seifer DB, Grazi RV, Irani S, Rosenwaks Z, Tal R. Vitamin D Decreases Serum VEGF Correlating with Clinical Improvement in Vitamin D-Deficient Women with PCOS: A Randomized Placebo-Controlled Trial. Nutrients. 2017;9(4).
456. Irani M, Minkoff H, Seifer DB, Merhi Z. Vitamin D increases serum levels of the soluble receptor for advanced glycation end products in women with PCOS. The Journal of clinical endocrinology and metabolism. 2014;99(5):E886-90.
457. Yang L, Froio RM, Sciuto TE, Dvorak AM, Alon R, Luscinskas FW. ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha-activated vascular endothelium under flow. Blood. 2005;106(2):584-92.
458. Ley K. The role of selectins in inflammation and disease. Trends Mol Med. 2003;9(6):263-8.
459. Martinesi M, Bruni S, Stio M, Treves C. 1,25-Dihydroxyvitamin D3 inhibits tumor necrosis factor-alpha-induced adhesion molecule expression in endothelial cells. Cell Biol Int. 2006;30(4):365-75.
460. Stach K, Kalsch AI, Nguyen XD, Elmas E, Kralev S, Lang S, et al. 1alpha,25-dihydroxyvitamin D3 attenuates platelet activation and the expression of VCAM-1 and MT1-MMP in human endothelial cells. Cardiology. 2011;118(2):107-15.
461. Corada M, Liao F, Lindgren M, Lampugnani MG, Breviario F, Frank R, et al. Monoclonal antibodies directed to different regions of vascular endothelial cadherin extracellular domain affect adhesion and clustering of the protein and modulate endothelial permeability. Blood. 2001;97(6):1679-84.
462. Xing S, Luo Y, Liu Z, Bu P, Duan H, Liu D, et al. Targeting endothelial CD146 attenuates colitis and prevents colitis-associated carcinogenesis. Am J Pathol. 2014;184(5):1604-16.
463. Douaisi M, Resop RS, Nagasawa M, Craft J, Jamieson BD, Blom B, et al. CD31, a Valuable Marker to Identify Early and Late Stages of T Cell Differentiation in the Human Thymus. J Immunol. 2017;198(6):2310-9.
464. Li C, Guo B, Ding S, Rius C, Langa C, Kumar P, et al. TNF alpha down-regulates CD105 expression in vascular endothelial cells: a comparative study with TGF beta 1. Anticancer Res. 2003;23(2B):1189-96.
465. Carter RA, Wicks IP. Vascular cell adhesion molecule 1 (CD106): a multifaceted regulator of joint inflammation. Arthritis and rheumatism. 2001;44(5):985-94.
466. Wu TC. The role of vascular cell adhesion molecule-1 in tumor immune evasion. Cancer Res. 2007;67(13):6003-6.
467. Wong MS, Leisegang MS, Kruse C, Vogel J, Schurmann C, Dehne N, et al. Vitamin D promotes vascular regeneration. Circulation. 2014;130(12):976-86.

468. Al Mheid I, Patel R, Murrow J, Morris A, Rahman A, Fike L, et al. Vitamin D status is associated with arterial stiffness and vascular dysfunction in healthy humans. Journal of the American College of Cardiology. 2011;58(2):186-92.
469. Witham MD, Adams F, Kabir G, Kennedy G, Belch JJ, Khan F. Effect of short-term vitamin D supplementation on markers of vascular health in South Asian women living in the UK--a randomised controlled trial. Atherosclerosis. 2013;230(2):293-9.
470. Yiu YF, Yiu KH, Siu CW, Chan YH, Li SW, Wong LY, et al. Randomized controlled trial of vitamin D supplement on endothelial function in patients with type 2 diabetes. Atherosclerosis. 2013;227(1):140-6.
471. Witham MD, Price RJ, Struthers AD, Donnan PT, Messow CM, Ford I, et al. Cholecalciferol treatment to reduce blood pressure in older patients with isolated systolic hypertension: the VitDISH randomized controlled trial. JAMA Intern Med. 2013;173(18):1672-9.
472. Stricker H, Tosi Bianda F, Guidicelli-Nicolosi S, Limoni C, Colucci G. Effect of a single, oral, high-dose vitamin D supplementation on endothelial function in patients with peripheral arterial disease: a randomised controlled pilot study. Eur J Vasc Endovasc Surg. 2012;44(3):307-12.
473. Harris RA, Pedersen-White J, Guo DH, Stallmann-Jorgensen IS, Keeton D, Huang Y, et al. Vitamin D3 supplementation for 16 weeks improves flow-mediated dilation in overweight African-American adults. Am J Hypertens. 2011;24(5):557-62.
474. Tarcin O, Yavuz DG, Ozben B, Telli A, Ogunc AV, Yuksel M, et al. Effect of vitamin D deficiency and replacement on endothelial function in asymptomatic subjects. The Journal of clinical endocrinology and metabolism. 2009;94(10):4023-30.
475. Sokol SI, Srinivas V, Crandall JP, Kim M, Tellides G, Lebastchi AH, et al. The effects of vitamin D repletion on endothelial function and inflammation in patients with coronary artery disease. Vasc Med. 2012;17(6):394-404.
476. Cipriani C, Romagnoli E, Scillitani A, Chiodini I, Clerico R, Carnevale V, et al. Effect of a single oral dose of 600,000 IU of cholecalciferol on serum calciotropic hormones in young subjects with vitamin D deficiency: a prospective intervention study. The Journal of clinical endocrinology and metabolism. 2010;95(10):4771-7.
477. Petrofsky J, Alshammari F, Khowailed IA, Rodrigues S, Potnis P, Akerkar S, et al. The effect of acute administration of vitamin D on micro vascular endothelial function in Caucasians and South Asian Indians. Med Sci Monit. 2013;19:641-7.
478. Legro RS. Evaluation and Treatment of Polycystic Ovary Syndrome. In: De Groot LJ, Chrousos G, Dungan K, Feingold KR, Grossman A, Hershman JM, et al., editors. Endotext. South Dartmouth (MA)2000.
479. Yildiz BO. Approach to the patient: contraception in women with polycystic ovary syndrome. The Journal of clinical endocrinology and metabolism. 2015;100(3):794-802.
480. Cosma M, Swiglo BA, Flynn DN, Kurtz DM, Labella ML, Mullan RJ, et al. Clinical review: Insulin sensitizers for the treatment of hirsutism: a systematic review and metaanalyses of randomized controlled trials. The Journal of clinical endocrinology and metabolism. 2008;93(4):1135-42.
481. Lamos EM, Malek R, Davis SN. GLP-1 receptor agonists in the treatment of polycystic ovary syndrome. Expert Rev Clin Pharmacol. 2017;10(4):401-8.
482. Raja-Khan N, Stener-Victorin E, Wu X, Legro RS. The physiological basis of complementary and alternative medicines for polycystic ovary syndrome. American journal of physiology Endocrinology and metabolism. 2011;301(1):E1-E10.
483. Dixit D, Yoon Y, Volino LR, Mansukhani RP. Empagliflozin: a sodium-glucose cotransporter 2 inhibitor for treatment of type 2 diabetes. Am J Health Syst Pharm. 2015;72(22):1943-54.
484. Goodman NF, Cobin RH, Futterweit W, Glueck JS, Legro RS, Carmina E, et al. American Association of Clinical Endocrinologists, American College of Endocrinology, and Androgen Excess and Pcos Society Disease State Clinical Review: Guide to the Best Practices in the Evaluation and Treatment of Polycystic Ovary Syndrome--Part 1. Endocrine practice : official journal of the American

College of Endocrinology and the American Association of Clinical Endocrinologists. 2015;21(11):1291-300.
485. Conway G, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Franks S, Gambineri A, et al. The polycystic ovary syndrome: a position statement from the European Society of Endocrinology. European journal of endocrinology / European Federation of Endocrine Societies. 2014;171(4):P1-29.
486. Blonde L, Dailey GE, Jabbour SA, Reasner CA, Mills DJ. Gastrointestinal tolerability of extended-release metformin tablets compared to immediate-release metformin tablets: results of a retrospective cohort study. Current medical research and opinion. 2004;20(4):565-72.
487. Thabane L, Ma J, Chu R, Cheng J, Ismaila A, Rios LP, et al. A tutorial on pilot studies: the what, why and how. BMC Med Res Methodol. 2010;10:1.
488. Eldridge SM, Lancaster GA, Campbell MJ, Thabane L, Hopewell S, Coleman CL, et al. Defining Feasibility and Pilot Studies in Preparation for Randomised Controlled Trials: Development of a Conceptual Framework. PloS one. 2016;11(3):e0150205.
489. Birkett MA, Day SJ. Internal pilot studies for estimating sample size. Statistics in medicine. 1994;13(23-24):2455-63.
490. Julious SA. Sample size of 12 per group rule of thumb for a pilot study. Pharm Stat. 2005(4 (4) (2005)):pp. 287–91.
491. James WP. The epidemiology of obesity: the size of the problem. Journal of internal medicine. 2008;263(4):336-52.
492. Mooney SJ, Baecker A, Rundle AG. Comparison of anthropometric and body composition measures as predictors of components of the metabolic syndrome in a clinical setting. Obes Res Clin Pract. 2013;7(1):e55-66.
493. Sun G, French CR, Martin GR, Younghusband B, Green RC, Xie YG, et al. Comparison of multifrequency bioelectrical impedance analysis with dual-energy X-ray absorptiometry for assessment of percentage body fat in a large, healthy population. The American journal of clinical nutrition. 2005;81(1):74-8.
494. Zeng Q, Sun XN, Fan L, Ye P. Correlation of body composition with cardiac function and arterial compliance. Clin Exp Pharmacol Physiol. 2008;35(1):78-82.
495. Haring HU, Merker L, Seewaldt-Becker E, Weimer M, Meinicke T, Broedl UC, et al. Empagliflozin as add-on to metformin in patients with type 2 diabetes: a 24-week, randomized, double-blind, placebo-controlled trial. Diabetes care. 2014;37(6):1650-9.
496. Scott LJ. Empagliflozin: a review of its use in patients with type 2 diabetes mellitus. Drugs. 2014;74(15):1769-84.
497. Neeland IJ, McGuire DK, Chilton R, Crowe S, Lund SS, Woerle HJ, et al. Empagliflozin reduces body weight and indices of adipose distribution in patients with type 2 diabetes mellitus. Diabetes & vascular disease research. 2016;13(2):119-26.
498. Kovacs CS, Seshiah V, Swallow R, Jones R, Rattunde H, Woerle HJ, et al. Empagliflozin improves glycaemic and weight control as add-on therapy to pioglitazone or pioglitazone plus metformin in patients with type 2 diabetes: a 24-week, randomized, placebo-controlled trial. Diabetes, obesity & metabolism. 2014;16(2):147-58.
499. Liakos A, Karagiannis T, Athanasiadou E, Sarigianni M, Mainou M, Papatheodorou K, et al. Efficacy and safety of empagliflozin for type 2 diabetes: a systematic review and meta-analysis. Diabetes, obesity & metabolism. 2014;16(10):984-93.
500. Xu L, Nagata N, Nagashimada M, Zhuge F, Ni Y, Chen G, et al. SGLT2 Inhibition by Empagliflozin Promotes Fat Utilization and Browning and Attenuates Inflammation and Insulin Resistance by Polarizing M2 Macrophages in Diet-induced Obese Mice. EBioMedicine. 2017;20:137-49.
501. Xu L, Ota T. Emerging roles of SGLT2 inhibitors in obesity and insulin resistance: Focus on fat browning and macrophage polarization. Adipocyte. 2017:1-8.

502. Seifarth C, Schehler B, Schneider HJ. Effectiveness of metformin on weight loss in non-diabetic individuals with obesity. Exp Clin Endocrinol Diabetes. 2013;121(1):27-31.
503. Harborne LR, Sattar N, Norman JE, Fleming R. Metformin and weight loss in obese women with polycystic ovary syndrome: comparison of doses. The Journal of clinical endocrinology and metabolism. 2005;90(8):4593-8.
504. Naderpoor N, Shorakae S, de Courten B, Misso ML, Moran LJ, Teede HJ. Metformin and lifestyle modification in polycystic ovary syndrome: systematic review and meta-analysis. Human reproduction update. 2015;21(5):560-74.
505. Mancia G, Cannon CP, Tikkanen I, Zeller C, Ley L, Woerle HJ, et al. Impact of Empagliflozin on Blood Pressure in Patients With Type 2 Diabetes Mellitus and Hypertension by Background Antihypertensive Medication. Hypertension. 2016;68(6):1355-64.
506. Tikkanen I, Narko K, Zeller C, Green A, Salsali A, Broedl UC, et al. Empagliflozin reduces blood pressure in patients with type 2 diabetes and hypertension. Diabetes care. 2015;38(3):420-8.
507. Kern M, Kloting N, Mark M, Mayoux E, Klein T, Bluher M. The SGLT2 inhibitor empagliflozin improves insulin sensitivity in db/db mice both as monotherapy and in combination with linagliptin. Metabolism: clinical and experimental. 2016;65(2):114-23.
508. Merovci A, Solis-Herrera C, Daniele G, Eldor R, Fiorentino TV, Tripathy D, et al. Dapagliflozin improves muscle insulin sensitivity but enhances endogenous glucose production. The Journal of clinical investigation. 2014;124(2):509-14.
509. Steven S, Oelze M, Hanf A, Kroller-Schon S, Kashani F, Roohani S, et al. The SGLT2 inhibitor empagliflozin improves the primary diabetic complications in ZDF rats. Redox Biol. 2017;13:370-85.
510. Oelze M, Kroller-Schon S, Welschof P, Jansen T, Hausding M, Mikhed Y, et al. The sodium-glucose co-transporter 2 inhibitor empagliflozin improves diabetes-induced vascular dysfunction in the streptozotocin diabetes rat model by interfering with oxidative stress and glucotoxicity. PloS one. 2014;9(11):e112394.
511. Shigiyama F, Kumashiro N, Miyagi M, Ikehara K, Kanda E, Uchino H, et al. Effectiveness of dapagliflozin on vascular endothelial function and glycemic control in patients with early-stage type 2 diabetes mellitus: DEFENCE study. Cardiovascular diabetology. 2017;16(1):84.
512. Briand F, Mayoux E, Brousseau E, Burr N, Urbain I, Costard C, et al. Empagliflozin, via Switching Metabolism Toward Lipid Utilization, Moderately Increases LDL Cholesterol Levels Through Reduced LDL Catabolism. Diabetes. 2016;65(7):2032-8.
513. Kohler S, Zeller C, Iliev H, Kaspers S. Safety and Tolerability of Empagliflozin in Patients with Type 2 Diabetes: Pooled Analysis of Phase I-III Clinical Trials. Adv Ther. 2017;34(7):1707-26.
514. Abdul-Ghani M, Del Prato S, Chilton R, DeFronzo RA. SGLT2 Inhibitors and Cardiovascular Risk: Lessons Learned From the EMPA-REG OUTCOME Study. Diabetes care. 2016;39(5):717-25.
515. Mohiyiddeen L, Watson AJ, Apostolopoulos NV, Berry R, Alexandraki KI, Jude EB. Effects of low-dose metformin and rosiglitazone on biochemical, clinical, metabolic and biophysical outcomes in polycystic ovary syndrome. J Obstet Gynaecol. 2013;33(2):165-70.
516. Jensterle M, Sebestjen M, Janez A, Prezelj J, Kocjan T, Keber I, et al. Improvement of endothelial function with metformin and rosiglitazone treatment in women with polycystic ovary syndrome. European journal of endocrinology / European Federation of Endocrine Societies. 2008;159(4):399-406.
517. Hurliman AK, Osol G, Casson PR, Maille N. Metformin Treatment Prevents the Development of Endothelial Dysfunction in a Rat Model of Polycystic Ovary Syndrome (Pcos). Fertility and sterility. 2012;98(3):S60-S.
518. Carvalho LML, Ferreira CN, Candido AL, Reis FM, Soter MO, Sales MF, et al. Metformin reduces total microparticles and microparticles-expressing tissue factor in women with polycystic ovary syndrome. Archives of gynecology and obstetrics. 2017;296(4):617-21.
519. Repaci A, Gambineri A, Pasquali R. The role of low-grade inflammation in the polycystic ovary syndrome. Molecular and cellular endocrinology. 2011;335(1):30-41.

520. Chiu JJ, Chen LJ, Lee CI, Lee PL, Lee DY, Tsai MC, et al. Mechanisms of induction of endothelial cell E-selectin expression by smooth muscle cells and its inhibition by shear stress. Blood. 2007;110(2):519-28.
521. Ferraris VA. Microparticles: the good, the bad, and the ugly. J Thorac Cardiovasc Surg. 2015;149(1):312-3.
522. Mahmoud AM, Wilkinson FL, McCarthy EM, Moreno-Martinez D, Langford-Smith A, Romero M, et al. Endothelial microparticles prevent lipid-induced endothelial damage via Akt/eNOS signaling and reduced oxidative stress. FASEB J. 2017;31(10):4636-48.
523. Romualdi D, Costantini B, Selvaggi L, Giuliani M, Cristello F, Macri F, et al. Metformin improves endothelial function in normoinsulinemic PCOS patients: a new prospective. Human reproduction. 2008;23(9):2127-33.
524. Kaya MG, Yildirim S, Calapkorur B, Akpek M, Unluhizarci K, Kelestimur F. Metformin improves endothelial function and carotid intima media thickness in patients with PCOS. Gynecological Endocrinology. 2015;31(5):401-5.
525. Esposito K, Maiorino MI, Di Palo C, Gicchino M, Petrizzo M, Bellastella G, et al. Effects of pioglitazone versus metformin on circulating endothelial microparticles and progenitor cells in patients with newly diagnosed type 2 diabetes-a randomized controlled trial. Diabetes Obesity & Metabolism. 2011;13(5):439-45.
526. Carvalho LML, Ferreira CN, Candido AL, Reis FM, Ster MO, Sales MF, et al. Metformin reduces total microparticles and microparticles-expressing tissue factor in women with polycystic ovary syndrome. Archives of gynecology and obstetrics. 2017;296(4):617-21.
527. Mackman N. Role of tissue factor in hemostasis, thrombosis, and vascular development. Arterioscl Throm Vas. 2004;24(6):1015-22.
528. Diamanti-Kandarakis E, Alexandraki K, Piperi C, Protogerou A, Katsikis I, Paterakis T, et al. Inflammatory and endothelial markers in women with polycystic ovary syndrome. European journal of clinical investigation. 2006;36(10):691-7.
529. Barchetta I, Cimini FA, Cavallo MG. Vitamin D Supplementation and Non-Alcoholic Fatty Liver Disease: Present and Future. Nutrients. 2017;9(9).
530. Miele L, De Michele T, Marrone G, Antonietta Isgro M, Basile U, Cefalo C, et al. Enhanced liver fibrosis test as a reliable tool for assessing fibrosis in nonalcoholic fatty liver disease in a clinical setting. Int J Biol Markers. 2017;32(4):e397-e402.
531. Barchetta I, Del Ben M, Angelico F, Di Martino M, Fraioli A, La Torre G, et al. No effects of oral vitamin D supplementation on non-alcoholic fatty liver disease in patients with type 2 diabetes: a randomized, double-blind, placebo-controlled trial. BMC medicine. 2016;14:92.
532. Curtis AM, Edelberg J, Jonas R, Rogers WT, Moore JS, Syed W, et al. Endothelial microparticles: sophisticated vesicles modulating vascular function. Vasc Med. 2013;18(4):204-14.
533. Zhang Q, Zhang M, Wang H, Sun C, Feng Y, Zhu W, et al. Vitamin D supplementation improves endothelial dysfunction in patients with non-dialysis chronic kidney disease. Int Urol Nephrol. 2018.

9 Chapter 9: Appendix

9.1 Patient information sheet and consent form for study 1:

Hull and East Yorkshire Hospitals NHS Trust

Vitamin D & PCOS

Participant Information Sheet and Consent Form

(Version 3.0 – 11/09/2014)

1. Study Title:

The effect of colecalciferol (Vitamin D) on hormonal, metabolic and cardiovascular risk factors in patients with polycystic ovary syndrome (PCOS)

Short title: Vitamin D & PCOS

2. Invitation

We would like to invite you to take part in a research study. Before you decide, we would like you to understand why we are doing this research and what it would involve. Please, take your time to read the following information carefully and discuss it with your friends, relatives and your GP if you wish. Ask us if there is anything that is not clear to you or if you want more information. Take time to decide whether or not you wish to take part.

3. What is the purpose of the study?

Polycystic ovary syndrome (PCOS) is a very common condition in women which could present with irregular periods, excessive hair growth on body, acne and cysts in the ovaries.

PCOS is also associated with increased risk of problems later in life like diabetes, high cholesterol levels and heart disease. One of the risk factors for having increased incidence of such problems in PCOS patients could be low vitamin D levels as many women with polycystic ovary syndrome (PCOS) are vitamin D deficient. Vitamin D supplementation may have a beneficial effect on insulin levels and fat around the abdomen. It has been seen in previous research studies that low level of vitamin D is related to a greater risk of diabetes and heart disease. Low vitamin D levels are also associated with fat in the liver. The amount of fat in the liver is a sign of early liver disease. So, in this study we want to supplement women having PCOS and vitamin D deficiency with vitamin D (3,200 IU) and examine the effects on hormones related to PCOS and risk factors for diabetes and heart disease in them.

4. Why have I been chosen?

You have been invited to take part in this study because you are a woman, aged 18-45, having PCOS. You cannot take part in this study if you have a history of diabetes, kidney problems, high level of calcium in urine or blood, allergic to soya or peanut

and planning to conceive or having any form of hormonal contraceptives. If you will be interested in taking part, you will be advised to stop hormonal contraceptives and use barrier methods of contraception while on this study.

5. Do I have to take part?

No, the choice to take part is entirely yours. Only when you feel satisfied that you have been given enough information about the study and you would like to participate, will you be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. You will not be penalized if you choose to withdraw and it will not compromise your medical care. For independent advice on taking part in clinical research you can visit NIHR website www.nihr.ac.uk

6. What will happen to me if I take part?

Please have a look at the study timetable (appendix 1). This will give you information about what the study involves. For example how often you will have to come and see us and what tests will be performed.

The study includes total of 3 visits to the Diabetes Centre, Hull Royal Infirmary, Anlaby road, Hull HU3 2RW.

Visit 1:

Visit 1 is the screening visit to identify your eligibility to take part in the study. The length of this visit will be 1.5 hours. The screening includes:

1) Answering any questions you may have before asking you to sign the consent form.
2) Medical history including your previous and current medical conditions and your list of medications, if you are taking any.
3) Physical examination. It will include listening to heart & lung sounds with the stethoscope, brief examination of the stomach and assessing the nerves

of the body by checking the sensations and strength of muscles of arms and legs. If requested, a chaperone will be provided.

4) Measuring your heart rate and blood pressure.

5) Routine blood tests to assess your blood count, kidney function and vitamin D levels. Total amount of blood taken during this visit is around 15 millilitres (mls), the same as 3 teaspoons.

If your vitamin D levels are low and you meet all the study criteria, we will invite you on to the study. But if your vitamin D levels are normal, you will not be able to participate in the study.

Visit 2:

Visit 2 will be within 1-2 weeks after the screening visit. You will be asked to fast for 10 hours except drinking water before attending this visit. This visit will last for 2.5 hours. You will be randomly allocated to either one capsule of vitamin D (active preparation) or a dummy capsule (placebo) for 3 months. You have equal chances of receiving the active preparation or placebo. This visit includes:

1) Measurement of height, weight, hip & waist circumference and body fat percentage with a special instrument called TANITA (shown below). You will be asked to stand on this scale to measure percentage of your body fat. This procedure will take only 5 minutes.

2) Measuring your heart rate and blood pressure

3) **EndoPAT 2000 test**. This is a non-invasive way of looking at early signs of heart disease by looking at the health of the walls of the blood vessels. A probe is placed on one finger of each hand and a blood pressure cuff is placed on one arm. The blood pressure cuff is then inflated (to a level above your blood pressure) for 5 minutes. This may cause some tingling in the arm but it should not cause pain. Occasionally it can cause pain, if this happens the procedure will be stopped immediately. After 5 minutes the cuff is deflated and the blood flow through the fingers is analysed.

4) We will take **small sample of fat** (about as much as that can fit on your small finger nail) from your tummy using a small needle. To do this we inject a small amount of local anaesthetic which temporarily numbs the area of fat whilst we get the fat, so it should be painless. **But this procedure is optional**. You can take part in this study even if you don't consent for this procedure.

5) Bloods to measure levels of hormones, glucose levels, blood fats, vitamin D levels and risk markers for heart disease. Total amount of blood taken during this visit will be around 50 millilitres (mls), the same as 3.5 tablespoons.

6) Urine sample to measure calcium levels.

7) Dispensing of study medications.

8) Both the blood and urine samples will be processed in the correct manner and stored in freezers at -80 degrees. All samples will be stored in anonymised form (appropriately labelled with a study number and study visit number which will be identifiable only by the researchers involved in the study). All samples will be stored for 2 years upon completing the study after which any remaining samples will be disposed as clinical waste.

Visit 3:

Visit 3 will be 12 weeks after visit 2. You will be asked to fast for 10 hours except drinking water before attending this visit. This visit will last for 2.5 hours. This visit includes:

1) Measurement of height, weight, hip & waist circumference and body fat percentage with a special instrument called TANITA

2) Measuring you heart rate and blood pressure.

3) EndoPAT 2000 test
4) Small sample of fat (about as much as that can fit on your small finger nail) from your tummy using a small needle **(optional)**.
5) Bloods to measure levels of hormones, glucose levels, blood fats, vitamin D levels and risk markers for heart disease. Total amount of blood taken during this visit will be around 50 millilitres (mls), the same as 3.5 tablespoons.
6) Urine sample to measure calcium levels.

At the end of the study you will receive treatment with vitamin D if indicated in your blood tests.

7. What do I have to do?

If you would like to participate in this study, please contact Dr Zeeshan Javed Tel: 01482 675314 or any members of the clinical trial team on Tel: 01482 675372 or 01482 675387.

If you decide to participate, you will be asked to sign the consent form as proof that you agree to participate in the study. This information sheet and a copy of the signed consent form will be given to you as a record. You will receive a number of appointments to attend the departments according to the protocol of the study.

You must tell your study doctor immediately if you have:

1) Any new symptoms or illnesses
2) Any new medical treatment, including surgery that you have from your GP or any other doctor

8. What are the possible benefits of taking part?

The study will not have any direct benefits to you apart from increasing your blood vitamin D levels if you are in active preparation group. But if at the end of the study you were not in active preparation group you will receive treatment with vitamin D. The information we get from this study will also determine whether this dose of

vitamin D is effective at improving a number of risk factors associated with polycystic ovary syndrome. If there are benefits then this could alter treatments available for those with the condition. Increasing vitamin D, irrespective of the direct impact on PCOS is regarded as protective for a number of health conditions.

9. Are there any risks to taking part in the study?

While taking blood samples and fat sample from the tummy may cause discomfort and risks of inflammation/ infection/ bruising at the needle site. The procedures will be explained to you before they are done to minimise the anxiety. The fat sampling will be done under local anaesthesia by fully trained doctor to make it pain free and blood sampling will be performed by the experienced nursing staff or study doctor in accordance with the local guideline to minimise the risks. Please, let us know if you have had a problem in the past.

10. What happens if new information becomes available?

Sometimes during the course of a trial, new information becomes available about the treatment that is being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue with the study. If you decide to continue in the study you will be asked to sign an updated consent form.

We may decide that it is best for you to stop taking part in the study. We will explain the reason to you. If the study is stopped for any other reason, we will tell you.

11. Expenses and payments

Volunteers will be compensated for all reasonable travel and parking costs. There will be no formal payment for volunteering in the study.

12. Will my taking part in the study be kept confidential?

Yes. Unless you tell anyone that you are taking part, only your GP will know. The blood samples and test results that are collected will be anonymous when the results are studied. All information will be confidential and treated in accordance with the Data Protection Act 1998. Authorised personnel such as researchers, regulatory authorities and people monitoring the quality of the research will have access to the data with your consent.

13. What will happen to the results of the study?

The results will be published in appropriate medical journals. However, individual people will not be identified and complete anonymity will be maintained in line with Trust policy and the Data Protection Act 1998.

14. What will happen if you don't want to carry on with the study?

Your participation in this study is strictly voluntary. You have the right to leave this study at any time. If you withdraw from the study, we will destroy all your identifiable samples, but we will need to use the data collected up to your withdrawal. You will not be penalized if you choose to withdraw.

15. What if something goes wrong?

We do not anticipate any problems with the study. If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (01482 675314/5372). However, in the unlikely event that this occurs you will be covered under the NHS compensation scheme, and details on our complaint procedure can be obtained on our trust website (http://www.hey.nhs.uk).

16. Who is organising and funding the research?

The research is organised by Dr Thozhukat Sathyapalan and sponsored by:

Hull and East Yorkshire Hospitals NHS Trust,

R & D department, Office 6, 2nd Floor Daisy Building,

Castle Hill Hospital, Castle road,

Cottingham, East Yorkshire HU16 5JQ

17. Who has reviewed the study?

The study has been reviewed in the UK by NRES Committee Yorkshire & The Humber and has been given a favourable ethical opinion for conduct in the NHS.

18. Contact for further information

General issues about research

For independent advice on taking part in clinical research you can visit NIHR website.

www.nihr.ac.uk

Specific information about this research project

Information can be discussed with Dr Zeeshan Javed or any members of the clinical trial team, please telephone 01482 675314/675372, 8am to 4pm weekdays and 01482 328541 out of hours.

19. What if there is a complaint?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions 01482 675372. If you remain unhappy contact Patient Advice and Liaison Service (PALS), if necessary, they will activate formal complaint procedure. Details can be obtained from Hull Royal Infirmary.

Thank you for taking time to read the details of our research project.

This information sheet and a copy of the consent form that you have signed are for you to keep for future reference.

Table 1: Study matrix showing tests that will be conducted and at what time-points.

	Visit1 (Screening)	**Visit 2 (Week 0)**	**Visit 3 (Week 12)**
Consent	x		
Medical history	x		
Inclusion/Exclusion	x		
Full physical examination	x		
BP & pulse	x	x	x
Height, weight, hip & waist circumference, TANITA		x	x
Stiffness of arteries-EndoPAT 2000		x	x
Fasting overnight		x	x
Blood tests	x	x	x
Urine sample		x	x
Adipose tissue biopsy (Optional)		x	x
Study medications dispensed		x	
Adverse event recording			x

Hull and East Yorkshire Hospitals NHS Trust

Consent Form for Vitamin D & PCOS Study (Version 3.0 – 11/09/2014)

Study Title: The effect of colecalciferol (Vitamin D) on hormonal, metabolic and cardiovascular risk factors in patients with polycystic ovary syndrome (PCOS)

Names of Researchers: Dr T Sathyapalan, Dr Zeeshan Javed

Participant Initials:

Participant identification number:

Please Initial Box

1	I confirm that I have read and understand the information sheet dated 11/09/2014 (version 3.0) for the above study. I have had the opportunity to consider the information, ask questions and have had those answered satisfactorily.	
2	I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	
3	I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from regulatory authorities or from the NHS Trust, where relevant to my taking part in the research. I give permission to these individuals to have access to my records.	
4	I agree to my general practitioner being informed about my participation in the study.	
5	I agree to have the adipose tissue biopsy under local anaesthesia (you can opt out of this aspect of the study but still participate in the study)	

6	I agree for the storage and use of blood and adipose tissue biopsy samples for future research in PCOS patients and various related conditions including obesity and type 2 diabetes mellitus. Samples will be stored anonymised for up to 2 years	
7	I give permission to the research team to be able to access my medical records.	
8	I agree to take part in the study.	

Name of the Participant **Signature** **Date and time**

……..... …….....

……...

Name of Researcher **Signature** **Date and time**

……..... …….....

……...

One for patient, one for researcher, one to be kept with source documents

9.2 Patient information sheet and consent form study 2:

Hull and East Yorkshire Hospitals NHS
NHS Trust

Empagliflozin vs metformin in PCOS

Participant Information Sheet and Consent Form

Study title: The effect of empagliflozin versus metformin on hormonal, metabolic and cardiovascular risk factors in patients with polycystic ovary syndrome (PCOS) – a randomised, open-label, parallel therapeutic exploratory pilot study

Short title: Empagliflozin vs **met**formin in PCOS (EMMET)

Invitation

We would like to invite you to take part in a research study. Before you decide, we would like you to understand why we are doing this research and what it would involve.

Please, take your time to read the following information carefully and discuss it with your friends, relatives and your GP if you wish.

Ask us if there is anything that is not clear to you or if you want more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

Polycystic ovary syndrome (PCOS) is a very common health problem that affects one in 10 women of child bearing age. Women with PCOS have a hormonal imbalance and metabolism problems that may affect their overall health and appearance. Some of the symptoms of PCOS include:

- **Irregular periods**. Women with PCOS may have fewer periods or miss periods. On the other hand, their periods may come every 21 days or more often. Some women with PCOS stop having menstrual periods.
- Too much hair on the face, chin, or other parts of the body where men usually have hair. This is called "**hirsutism**." It affects up to 70 percent of women with PCOS.
- **Acne** on the face, chest, and upper back
- **Hair loss** or thinning of hair on the scalp; male pattern baldness
- **Weight gain** or difficulty in losing weight
- **Darkening of skin**, mainly along neck creases, in the groin, and underneath breasts
- **Skin tags**, which are small flaps of excess skin in the armpits or neck area

Furthermore, PCOS is also associated with increased risk of problems later in life like diabetes, high cholesterol levels and heart disease.

There is no cure for PCOS but you can manage the symptoms of PCOS. Many women need a combination of treatments in addition to weight loss depending on the symptoms you have. The treatment options are very limited, so efforts are being made to develop new medicines for PCOS patients to help in improving their quality of life.

Therefore, we are running a study in which a medication named "Empagliflozin" will be tested. Empagliflozin belongs to a class of medicines known as SGLT-2 inhibitors. SGLT-2 stands for sodium glucose co-transporter 2. Empagliflozin, in simple terms, increases the amount of glucose passed in your urine. This medicine is usually used in patients with type 2 diabetes and has led to improved blood pressure and weight loss in addition to improving blood glucose levels.

We want to give Empagliflozin to women with PCOS to see its effect on hormones related to PCOS and the risk factors for diabetes and heart disease. We will be comparing its

effects to metformin (another drug for diabetes) which has already been used in PCOS with very good results.

This is a feasibility study. This means we are looking to understand the effects of empagliflozin in PCOS women, and other aspects of conducting other studies like this. This information can be used in designing a bigger trial in the future using Empagliflozin as one of the treatments we would be studying.

Why have I been chosen?

You have been invited to take part in this study because you are aged 18-45 with PCOS. You cannot take part in this study if you have a history of diabetes, kidney or liver problems, recurrent urinary tract infections (infections that can affect the bladder, the kidneys and the tubes connected to them), an allergy to lactulose, are planning to conceive or taking any form of hormonal contraceptives. However, if you are taking a hormonal contraceptive and are interested in taking part in the study, you will be advised to stop your hormonal contraceptive for 3 months prior to starting the study and use barrier methods of contraception while on this study.

Do I have to take part?

No, the choice to take part is entirely yours. Only when you feel satisfied that you have been given enough information about the study and you would like to participate, will you be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. If you chose not to take part or withdraw from the study it will not affect your planned treatment and care in any way. For independent advice on taking part in clinical research you can visit the NIHR website www.nihr.ac.uk

What will happen to me if I take part?

If you decide to participate, you will be asked to sign a consent form as proof that you agree to participate in the study.

The study includes a total of 3 visits to the Academic Diabetes, Endocrinology & Metabolism Research Centre, Brocklehurst Building, Hull Royal Infirmary, Anlaby Road, Hull HU3 2RW. The 3 visits will occur over a maximum of 17 weeks. The table further on in this information sheet shows the visits and the procedures done at each visit.

You have to refrain from any form of hormonal contraception while participating in this study. If you fall pregnant whilst participating in the trial, you will be withdrawn from the study drug. You will then be followed up by visits/telephone contacts once every 3 months during pregnancy and at birth and until 3 months after the birth of the baby to monitor for any adverse effects.

Visit 1

Visit 1 is the screening visit to identify whether you meet all the study criteria needed to be able to take part in the study. The length of this visit will be approximately 1 hour. The screening includes:

1) Answering any questions, you may have before asking you to sign the consent form.

2) Review of your medical history including your previous and current medical conditions and your list of medications, if you are taking any.

3) Measuring your heart rate and blood pressure.

4) Taking routine blood tests to assess your hormone levels (if you have had these blood tests done in the previous 2 weeks no further blood will be

taken at this visit). Total amount of blood taken during this visit is around 10 millilitres (mls), the same as 2 teaspoons.

If you meet all the study criteria, we will invite you to join the study.

Visit 2

Visit 2 will be within 3 weeks after the screening visit. You will be asked not to eat any food for 10 hours before attending this visit, although you will be allowed to drink water. This visit will last for approximately 2.5 hours. You will be randomly allocated to receive either empagliflozin 25mg daily for 3 months or metformin SR (Slow Release) 1500mg daily for 3 months. You have an equal chance of receiving either the empagliflozin or metformin.

This visit includes:

1) Review of any changes in your health or medications since the previous visit. You must tell your study doctor if you have:
    - Any new symptoms or illnesses
    - Any new medical treatment, including surgery that you have from your GP or any other doctor

2) Measurement of height, weight, hip & waist measurements as well as a body fat percentage measured with a special instrument called TANITA (shown below). You will be asked to stand on this scale to measure percentage of your body fat. This procedure will take only 5 minutes.

3) Measuring your heart rate and blood pressure

4) A urine pregnancy test will be performed.

5) **EndoPAT 2000 test**. This is a non-invasive way of looking at early signs of heart disease by looking at the health of the walls of the blood vessels. A probe is placed on one finger of each hand and a blood pressure cuff is placed on one arm. The blood pressure cuff is then inflated (to a level above your blood pressure) for 5 minutes. This may cause some tingling in the arm but it should not cause pain, but should pain occur the procedure will be stopped immediately. After 5 minutes the cuff is deflated and the blood flow through the fingers is analysed.

6) We would like to take a **small sample of fat** (about as much as that can fit on your small finger nail) from your tummy using a small needle. To do this we inject a small amount of local anaesthetic that temporarily numbs the area of fat whilst we remove it, so it will be painless. **This procedure is optional** and you can take part in this study even if you don't consent for this procedure.

7) Bloods to measure levels of hormones, glucose levels, blood fats, and risk indicators for heart disease. Total amount of blood taken during this visit will be around 50 millilitres (mls), the same as 3.5 tablespoons.

8) You will be asked to fill out 3 health questionnaires. These questionnaires are approved and are designed in a way to help us better understand the effects of the study drug on your health especially PCOS symptoms, your daily life activities and mood.
9) You will be given your study medication.

Telephone call at Week 6

We will telephone you at this point to check that you are managing to take your medication as instructed and to ask if you have had any changes in your health and if you are taking any new medication.

Visit 3

Visit 3 will be 12 – 13 weeks after visit 2. You will be asked not to eat any food for 10 hours before attending this visit, although you will be allowed to drink water. This visit will last for approximately 2.5 hours.

This visit includes:

1) Review of any changes in your health or medications since the previous visit. You must tell your study doctor if you have:
    - Any new symptoms or illnesses
    - Any new medical treatment, including surgery that you have from your GP or any other doctor.

2) Measurement of height, weight, hip & waist circumference and body fat percentage with the TANITA instrument mentioned in Visit 2.

3) Measuring you heart rate and blood pressure.

4) EndoPAT 2000 test as detailed in Visit 2.

5) Small sample of fat (about as much as that can fit on your small finger nail) from your tummy using a small needle (optional) as detailed in Visit 2.

6) Bloods to measure levels of hormones, glucose levels, blood fats, and risk indicators for heart disease. Total amount of blood taken during this visit will be around 50 millilitres (mls), the same as 3.5 tablespoons. You will be asked to fill out 3 health questionnaires as in Visit 2.

Telephone call 30 days after your last study visit (Visit 3)

We will telephone you at this point to check if you have had any changes in your health since your final study visit.

Table of visits and procedures

	Visit 1 (Screening) Non fasting (within 3 weeks of visit 2)	**Visit 2 (Week 0) (Baseline visit) Fasting**	**Telephone call (Week 6) (+/- 4 days)**	**Visit 3 (Week 12 +/- 7 days) (End of study visit) Fasting**	**Telephone call (30 days after final visit 3) (+/- 4 days)**
Consent	x				
Medical and drugs history	x				
Inclusion/Exclusion	x				
Pregnancy test	x				
Randomisation		x			
BP, pulse		x		x	
Height, weight, hip & waist circumference, TANITA		x		x	
Stiffness of arteries- EndoPAT		x		x	
Fasting overnight		x		x	
Bloods for hormonal markers	x (if not done within the last 2 weeks)				
Bloods for hormonal, metabolic and cardiovascular risk markers		x		x	
MPCOSQ		x		x	
PHQ-4		x		x	

WPAI:GH		x		x	
Fat Biopsy (optional)		x		x	
Study medications dispensed		x			
Adverse event recording		x	x	x	x
Change in concomitant medication			x	x	
Study medication compliance			x	x	

What do I have to do?

If you would like to participate in this study, please contact Dr Zeeshan Javed Tel: 01482 675314 or any members of the clinical trial team on Tel: 01482 675372 or 01482 675387.

What will happen to the samples I give?

The blood samples will be processed and stored in freezers at -80 degrees. All samples will be stored in an anonymised form (appropriately labelled with a study number and study visit number which will be identifiable only by the researchers involved in the study).

The fat samples will be analysed within one year of the end of the study and then destroyed. All blood samples will be stored for future research in PCOS patients and various related conditions including obesity and type 2 diabetes mellitus. The anonymised samples will be stored for up to 2 years and then destroyed.

What are the possible benefits of taking part?

You may benefit as a result of your participation in this trial, there is, however, no guarantee that you will. Results from this trial may benefit others in the future as the data collected will provide the medical and scientific community with information about treatment for PCOS. The information we get from this study will also determine whether this dose of empagliflozin is effective at improving a number of risk factors associated with polycystic ovary syndrome. If there are benefits, then this could alter treatments available for those with the condition.

Are there any risks to taking part in the study?

Taking blood samples and fat samples from the tummy may cause discomfort and risks of inflammation/ infection/ bruising at the needle site. The procedures will be explained to you before they are done to minimise any anxiety. The fat sampling will be done under local anaesthesia by a fully trained doctor to make it pain free and blood sampling will be performed by the experienced nursing staff or study doctor in accordance with the local guideline to minimise the risks. Please, let us know if you have had a problem in the past.

What are the side effects of treatment?

Treatment with any medicine can cause side effects. As mentioned, you will be treated with either empagliflozin or metformin in this trial. In this section the potential side effects for empagliflozin when used to treat Type 2 diabetes are listed by frequency. Frequencies are defined as:

- very common (affects 1 to 10 patients in 10)
- common (affects 1 to 9 patients in 100)
- uncommon (affects 1 to 9 patients in 1000)
- rare (affects 1 to 9 patients in 10,000)
- very rare (<1/10,000; affects less than 1 patient in 10,000)
- frequency not known (cannot be estimated from the available data)

If you suffer from any of the side effects mentioned below, you should tell your trial doctor the next time you meet, regardless of whether or not you feel they are related to, or caused by, the trial medication. If you are worried about any of these side affects you can contact your trial doctor whenever you want.

As with all drugs, empagliflozin may cause an allergic reaction that could become serious and require immediate treatment in a hospital.

Empagliflozin side effects

In research studies carried out so far, empagliflozin has been given as a single dose up to 800 mg to healthy people, as multiple doses up to 100 mg for up to 28 days to people with Type 2 diabetes, and as multiple doses up to 25 mg for up to 56 days to people with Type 1 diabetes.

Approximately 8,700 patients with Type 2 diabetes mellitus have been treated with empagliflozin in research studies, about 4,900 of them for at least 52 weeks, and about 2,800 of them for at least 76 weeks. At the end of 2014 approximately 8,000 patients were still participating in ongoing long-term studies. The side effects observed with empagliflozin in Type 2 diabetes are as shown below. Similar side effects are anticipated in this study.

- Increase in urination frequency or amount of urine (common)
- Painful urination, also known as dysuria (uncommon)
- Urinary tract infections (common)
- Genital infections (common)
- Symptoms and signs of volume depletion (decrease of water in the body) such as hypotension (low blood pressure), syncope (fainting) (uncommon)
- Itching, also known as pruritus (common)
- Diabetic ketoacidosis (DKA) is a serious problem that happens to people with diabetes when chemicals called “ketones” build up in their blood. As you do not have diabetes then this will not happen (uncommon)
- Increased cholesterol in the blood (common)

- Increased amount of red cells in the blood (uncommon).

In recent clinical trials, empagliflozin has shown beneficial effects, especially has resulted in blood pressure decrease up to 4.8 mmHg and body weight decrease up to 2.2 kg. So, your blood pressure and body weight will be monitored throughout the trial.

Metformin side effects

Metformin therapy is usually well tolerated and safe. The most common side effects are nausea, vomiting, diarrhoea, tummy pain, flatulence and loss of appetite. These can occur in around 1 in 10 people but usually only in the first two weeks of treatment.

Metformin therapy may lead to lactic acidosis, the build-up of lactic acid in the blood which is potentially dangerous, in people who have moderately or severely abnormal kidney or liver blood tests. We will make sure that your kidney and liver tests are adequate before proceeding with the study. **You should not exceed standard recommendations for safe limits of alcohol consumption while you are on the study medication and you should refrain from drinking more than 7 units per week.**

What happens if new information becomes available?

Sometimes during the course of a trial, new information becomes available about the treatment that is being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue with the study. If you decide to continue in the study you will be asked to sign an updated consent form.

We may decide that it is best for you to stop taking part in the study. We will explain the reason to you. If the study is stopped for any other reason, we will tell you.

What happens when the research study stops?

At the end of the study (after Visit 3) you will stop taking the study medication and return to your normal routine treatment.

What happens if you become pregnant whilst on the study?

You will be advised to use adequate contraception whilst on the study; however, if you become pregnant we will ask you to stop the trial medication immediately. We will take expert advice from the obstetrician and the research team will follow you up by visits or telephone contacts once every 3 months during pregnancy and at birth and until 3 months after the birth of the baby.

Expenses and payments

Volunteers will be compensated for all reasonable travel and parking costs. There will be no formal payment for volunteering in the study.

Will my taking part in the study be kept confidential?

Your records of being in this study will be kept private unless law requires other people to see them. The following people may or will need to have access to your study records:

- Clinical research team
- Hull and East Yorkshire Hospitals NHS Trust R&D Department (Sponsor)
- The Medicines and Healthcare Products Regulatory Agency (MHRA)
- The Research Ethics Committee (REC)

With your permission on the consent form, representatives from the Sponsor may review some or all of your medical records to verify that you actually participated in the study and that the information is accurate. All those who view your records will have a duty of confidentiality to you as a research participant that nothing which could reveal your identity will be disclosed outside the research site.

All information which is collected about you during the course of the research will be kept strictly confidential in the same way that your medical records are. No data identifying you will be sent out of the hospital without your permission. Your full name will not appear on any trial data or results, only your initials and study number. Your full name will only appear on the consent form, prescription and the patient identification list that we have to keep. All these documents will be kept in our study files which will be kept in secure locked areas. Access to the study files is only given to authorised staff.

What will happen to the results of the study?

When the study has finished, the results from all patients who took part in the study will be analysed by the research team and checked by a statistician. The results will be made available on a public website and published in appropriate medical journals. Your doctor can provide you with a copy of these results if you are interested. You will not be identified in any report, publication or presentation.

What will happen if you don't want to carry on with the study?

Your participation in this study is strictly voluntary. You have the right to leave this study at any time. If you withdraw from the study, we will use the data collected up to your withdrawal unless instructed otherwise

What if something goes wrong?

We do not anticipate any problems with the study. If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (01482 675314/5387). If you remain unhappy and wish to complain, you can do so via the NHS Complaints Procedure. Details can be obtained from:

Patients Advisory Liaison Service (PALS) Officers

Tel: 01482 623065

Insurance and indemnity?

In the unlikely event that something does go wrong and you are harmed during the research due to someone's negligence, you may have grounds for legal action for compensation against Hull and East Yorkshire Hospitals NHS Trust. You may have to pay your legal costs. The normal National Health Service complaints mechanisms will be available to you.

Who is organising and funding the research?

The research is organised by Professor Thozhukat Sathyapalan, Honorary Consultant Endocrinologist. The University of Hull will fund all the study related costs.

The study is sponsored by:

Hull and East Yorkshire Hospitals NHS Trust,

R & D department, Office 6, 2nd Floor Daisy Building,

Castle Hill Hospital, Castle road,

Cottingham, East Yorkshire HU16 5JQ

Who has reviewed the study?

This study has been reviewed and approved by a Research Ethics Committee (REC), the Health Research Authority (HRA) and the Medicines for Health Care Products Regulatory Agency (MHRA). The REC is made up of a group of healthcare professionals and non-medical people who are completely independent from anyone organising the trial. The REC checks that the study protects your safety, rights, wellbeing and dignity. The HRA check that

the study complies with legal aspects regarding clinical research. The MHRA check that the study medication is safe to be taken by patients.

Contact for further information

General issues about research

For independent advice on taking part in clinical research you can visit the NIHR website www.nihr.ac.uk .

Specific information about this research project

This information can be discussed with Dr Zeeshan Javed, Clinical Research Fellow or any members of the clinical trial team, please telephone 01482 675314/675387, 8am to 4pm weekdays and 01482 328541 out of hours.

Thank you for considering participation in this study.

You will be given a copy of this information sheet and your signed consent form to keep if you decide that you wish to take part in the study.

Study title: The effect of empagliflozin versus metformin on hormonal, metabolic and cardiovascular risk factors in patients with polycystic ovary syndrome (PCOS) – a randomised, open-label, parallel therapeutic exploratory pilot study.

Principal Investigator: Professor Thozhukat Sathyapalan

Short title: Empagliflozin vs metformin in PCOS study	**Participants initials**
1) I confirm that I have read and understand the information sheet dated 05/09/2017, version 6 for the above study. I have had the opportunity to consider the information, ask questions and have had those answered satisfactorily.	
2) I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	
3) Should I choose to withdraw consent, I agree that information obtained from me in this study up to that point may still be used.	
4) I understand that relevant sections of my medical notes and data collected during the study may be looked at in confidence by individuals from the research team, representatives from the Sponsor (Hull and East Yorkshire Hospitals NHS Trust) and from regulatory authorities, in order to check that the trial is being carried out correctly. I give permission to these individuals to have access to my records to view sections that are relevant to my taking part in this research.	
5) I agree to my General Practitioner being informed of my participation in the study.	
Optional 6) I agree to have the fat tissue biopsy under local anaesthesia (you can opt out of this aspect of the study but still participate in the study)	
7) I agree for the storage and use of blood samples for future research in PCOS patients and various related conditions including obesity and type 2 diabetes mellitus. The anonymised samples will be stored for up to 2 years.	
8) I agree to take part in the study.	

Participant identification number: _____

* **Please do not complete the name or date for the patient.**

When completed: 1 copy for patient, 1 copy for medical records, 1 original for investigator site file

	Name	**Signature**	**Date** dd.mm.yy	**Time** hh:mm
Participant				
Investigator* On Delegation Log				