Effect of non-esterified fatty acids on insulin resistance and cardiovascular risk in polycystic ovary syndrome

Myint Myint Aye M.B.B.S, MRCP

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ABSTRACT

Non-esterified fatty acids, insulin resistance and polycystic ovary syndrome

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Introduction: Insulin resistance (IR) and obesity coexist in polycystic ovary syndrome (PCOS) and contribute to increased risk of diabetes and cardiovascular disease. An intrinsic insulin signalling defect is present in skeletal muscle of PCOS and it affects insulin mediated glucose transport in the presence of lipid *in vitro* studies.

Methods: The effect of non-esterified fatty acids (NEFA) on IR, postprandial lipids and cardiovascular risk in obese women with PCOS compared to controls was examined by lowering NEFA levels with acute overnight acipimox and chronic 12 week tredaptive therapy. Additional studies included elevating NEFA by lipid infusions and improving NEFA metabolism by moderate intensity exercise.

Results: Effective lowering of NEFA with overnight acipimox therapy improved fasting and postprandial IR in PCOS. It enhanced chylomicron clearance with reduced overnight VLDL production. A rebound rise in NEFA following chronic tredaptive therapy worsened fasting and postprandial IR. However, despite this, tredaptive had the counterintuitive effect of lowering fasting and postprandial triglycerides without effecting endothelial function and hsCRP.

PCOS women were found to be less tolerant to acutely induced lipaemia than controls with an exaggerated fall in their rate of glucose disposal during a hyperinsulinaemic euglycaemic clamp. Exercise improved cardiovascular fitness and cardiovascular risk in PCOS. Exercise enhanced fasting insulin sensitivity and the rate of glucose disposal during the saline and hyperlipidaemia. Unlike controls, the platelets from PCOS subjects were more susceptible to platelet agonists and less responsive to platelet antagonists in induced hyperlipidaemia, triggering platelet hyper-activation that was not corrected by a supraphysiological dose of insulin.

Conclusions: These studies demonstrate the definite role of NEFA in the pathophysiology of IR in PCOS and support the in vitro findings of high NEFA reducing insulin mediated glucose transport. This work also supports the concept that platelet insulin resistance in PCOS during lipaemia might increase cardiovascular risk in these patients.

LIST OF PUBLICATIONS FROM THIS RESEARCH WORK

Publications

1) **Aye MM**, Kilpatrick ES, Afolabi P, Wootton SA, Rigby AS, Coady AM, Sandeman DD, Atkin SL: Postprandial effects of long-term niacin/laropiprant use on glucose and lipid metabolism and on cardiovascular risk in patients with polycystic ovary syndrome: Diabetes Obes Metab. 2014 Jan 8. doi: 10.1111/dom.1225 (IF 5.18)

2) **Aye MM**, Kilpatrick ES, Aburima A, Wraith KS, Spurgeon B, Magwenzi S, Rigby AS, Sandeman DD, Naseem KM, Atkin SL: Acute hypertriglyceridemia induces platelet hyperactivity that is not attenuated by insulin in polycystic ovary syndrome (Journal of the American Heart Association Cardiovascular and Cerebrovascular disease(Elec): doi: 10.1161/JAHA.113.000706

3) **Aye MM**, Kahal H, Hooson F, Sathyapalan T, Atkin SL, Kilpatrick ES. HaemoglobinA1c in diagnosis of impaired glucose regulation in polycystic ovary syndrome: Endocrine Abstracts 2012; 28: P217

4) **Aye MM**, Kahal H, Smith H, Sandeman D, Kilpatrick ES, Atkin SL. Role of metabolic dyslipidaemia in screening for impaired glucose regulation in polycystic ovary syndrome; Endocrine Abstracts 2012; 28: P192

5) **Aye MM**, Afolabi P, Sandeman D, Wootton SA, Kilpatrick ES, Atkin SL. Postprandial hypertriglyceridaemia in patients with polycystic ovary syndrome; Endocrine Abstracts 2011;25: P146

Conference presentation

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αIIbβ3	Integrin alpha IIb beta3 (GPIIbIIIa)
ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
ADP	Adenosine 5'-diphosphate
AIRg	Acute insulin response to glucose
ALT	Alanine aminotransferase
Apo	Apolipoproteins
ASP	Acylation stimulating protein
ATGL	Adipose tissue triglyceride lipase
AUC	Area Under the Curve
BMI	Body mass index
Ca^{+2}	Calcium ion
CAD	Coronary artery disease
cAMP	Cyclic adenosine 3',5'-monophosphate
CD62P	CD62 P selectin
CETP	Cholesterol ester transfer protein
COX	Cyclooxygenase
8-CPT-6-Phe-cAMP	8- (4- Chlorophenylthio)- N6- phenyladenosine- 3', 5'- cyclic monophosphate
CPT I	Carnitinepalmitoyl-transferase I
CV	Cardiovascular
CVD	Cardiovascular disease
DAG	Diacylglycerol
DGAT-2	Diacylglycerol acyltransferase-2
EMTG	Extramyocellular triglycerides

FA	Fatty acids
FABP	Fatty acid-binding proteins
FABPpm	Fatty-acid binding protein plasma membrane
FAI	Free androgen index
FAT	Fatty acid translocase
FATP	Fatty acid transporter protein
FFA	Free fatty acids
FITC	Fluorescein isothiocyanate
FMD	Flow mediated dilatation
FSH	Follicular stimulating hormone
GLUT	Glucose transporter
GP	glycoprotein
H	hour
HbA1c	Haemoglobin A1c
HDL-c	High density lipoprotein- cholesterol
HOMA-IR	Homeostasis model assessment –insulin resistance
hsCRP	High sensitivity C -reactive protein
HSL	Hormone sensitive lipase
IDL	Intermediate density lipoproteins
IFG	Impaired fasting glucose
IGR	Impaired glucose regulation
IGT	Impaired glucose tolerance
IMT	Intima media thickness
IMCL	Intramyocellular lipids
IMTG	Intramyocellular triglycerides
IR	Insulin resistance

IRS	Insulin receptor substrates
ISI	Insulin Sensitivity Index
IVGTT	Intravenous glucose tolerance test
LCAT	Lecithin: cholesterol acyltransferase
LCFA	Long chain fatty acids
LDL-c	Low density lipoprotein- cholesterol
LH	Luteinizing hormone
LPL	Lipoprotein lipase
MAG	Monoacylglycerol
Min	Minutes
NEFA	Non-esterified Fatty Acids
NIH	National Institute of Health
NO	Nitric oxide
ODI	Oral disposition index
OGTT	Oral glucose tolerance test
PAI-1	Plasminogen activator inhibitor-1
PCOS	Polycystic ovary syndrome
PDE	Phosphodiesterase 3
PE	R-Phycoerythrin
PGI_2	Prostacyclin (Prostaglandin I_2)
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB	Protein Kinase B
PKC	Protein kinase C
PLRP	phospholipid rich lipoproteins
RHI	Reactive hyperaemic index

SH2	Src Homology 2 domain
SHBG	Sex hormone binding globulin
sICAM-1	Soluble intra-cellular adhesion molecule-1
sVCAM-1	Soluble vascular cell adhesion molecule-1
T2DM	Type2 diabetes mellitus
TAG	Triacylglycerol
TAGRP	Triacylglycerol rich protein
TG	Triglycerides
TNFα	Tissue necrotic factor α
TRL	Triglyceride rich lipoproteins
VLDL	Very low density lipoprotein
VO_2 max	Maximal oxygen consumption
vWF	von Willebrand factor
WHR	Waist Hip Ratio
Wk	Week

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AUTHOR'S DECLARATION

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Chapter 1 Introduction

1.1 The polycystic ovary syndrome

1.1.1 Overview

The history of PCOS probably began with its description made by the Italian scientist Antonio Vallisneri in 1721 (1). *"Giovane rustica, maritata, modicamente pingue, et infeconda, con due ovaie più grandi del normale, come uova di colomba, bernoccolute, lucenti et biancastre..."* ("Young rustic, married, moderately stout, barren, with two larger than normal ovaries, as a dove eggs, bumpy, shiny whitish"). In 1844, Chereau et al. described sclerocystic changes in the human ovary (2).

In 1935, Stein and Leventhal, the American gynaecologists, first reported the link between bilateral polycystic ovaries and clinical symptoms consisting of "menstrual irregularity featuring amenorrhea, a history of infertility, masculine type hirsutism and less consistently, obesity" (3). Since then, the condition was, for a long time, called the Stein-Leventhal syndrome.

In 1958, McArthur et al. described that anovulatory PCOS was associated with a relative lowering of urinary interstitial cell stimulating hormone (ICSH), now known as luteinizing hormone (LH), when compared to follicular stimulating hormone (FSH) secretion (4). Of interest, she and her colleagues discovered that the normal menstrual cycle is related to a mid-cycle peak period of FSH and ICSH followed by ovulation and menstruation (4, 5).

In 1961, Ferriman and Gallwey developed a method for the clinical assessment of body hair in women using 5 grades based on densities and areas involved, in 11 defined sites on the body. This score is currently used for the assessment of clinical hyperandrogenism in PCOS (6).

In 1970, Yen et al. measured serum LH and FSH levels using radioimmunoassay methods. A disproportionately high LH with a consistently low FSH level was found in PCOS in their study (7). However, changes in LH and FSH secretion are not due to an inherent defect of hypothalamic-pituitary gonadotrophin secretion but are related to increased sensitivity of FSH to preferential negative inhibitory feedback action of

oestrogen and its relative insensitivity to stimulatory Luteinising Hormone Releasing Factor compared to LH (8).

The next milestone was the discovery, in 1980, of an association between insulin resistance (IR) and PCOS. Fasting plasma insulin, androstenedione and testosterone levels are higher in obese PCOS compared to obese controls. Furthermore, hyperinsulinaemia is well correlated with hyperandrogenism in PCOS (9).

In 1981, Swanson et al. first described polycystic ovaries as usually symmetrical with numerous tiny cysts ranging in diameter from 2 to 6mm in the periphery of an ovary or throughout the parenchyma. In Swanson's study, 50% of the women with polycystic ovaries showed classic signs and symptoms of PCOS, 25% had variants of the syndrome and the remaining 25% were asymptomatic (10).

PCOS is a dysmetabolic condition although it was primarily thought of as an ovarian dysfunction. PCOS is associated with metabolic disturbances such as fasting hyperinsulinaemia (11), insulin resistance (12-15) and metabolic dyslipidaemia (16, 17). As a result, impaired glucose regulation (18-25), gestational diabetes (26, 27) and metabolic syndrome (16, 17, 28-32) are more prevalent in PCOS compared to healthy women. Therefore, women with PCOS carry higher cardiovascular (CV) risk profiles.

Premenopausal women with PCOS were found to have a 2-fold increased risk of Non-Alcoholic Fatty Liver Disease (NAFLD) compared to controls. Moreover, elevated alanine aminotransferase (ALT) serum levels were associated with a marked decrease in insulin sensitivity (33). The prevalence of both PCOS and NAFLD rises proportionately with the degree of IR and an increase in the mass of adipose tissue (34).

There is a significantly increased prevalence of mental health problems such as depression, anxiety, binge eating disorders and low health related quality of life in PCOS (35, 36). A systematic review in 2012, showed that the prevalence of generalized anxiety symptoms is significantly greater in PCOS women (20.4%) compared to controls (3.9%) (37).

PCOS may carry an increased risk for endometrial carcinoma in women of child bearing age. In an Australian study, which included 156 PCOS and 398 without PCOS

women, those with PCOS had a fourfold increased risk of endometrial cancer compared to those without PCOS. Features of PCOS such as obesity, hirsutism and irregular periods were significantly associated with endometrial cancer risk (38).

Obstructive sleep apnoea and daytime sleepiness are more common in PCOS compared to age and weight matched healthy controls. Those features seem to be related to the severity of obesity and IR (39-41).

1.1.2 Diagnosis of PCOS

The diagnosis of PCOS was first standardized by the National Institute of Health (NIH) criteria in 1990, the diagnostic criteria of which are (in order of importance) 1) hyperandrogenism and/or hyperandrogenaemia, 2) oligomenorrhoea and/or anovulation and 3) exclusion of other endocrine disorders, such as Cushing's syndrome, hyperprolactinaemia and congenital adrenal hyperplasia.

The second set of diagnostic criteria was the Rotterdam Criteria published in 2003 by experts in a PCOS consensus workshop sponsored, in part, by the European Society for Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM) (42, 43). According to these criteria, PCOS can be diagnosed, after the exclusion of related disorders, by the presence of two out of three features: 1) oligomenorrhoea or anovulation 2) clinical and/or biochemical hyperandrogenism and 3) polycystic ovaries. In comparison with the NIH 1990 criteria, it has expanded the definition of PCOS by adding two new phenotypes, one with ovulatory dysfunction and polycystic ovaries with no hyperandrogenism, and the other with hyperandrogenism and polycystic ovaries but no ovulatory dysfunction. Although there has been debate about the strengths and weaknesses of these definitions, both the NIH 1990 and the Rotterdam 2003 criteria are currently in use to identify PCOS for research as well as clinical purposes.

1.1.3 Prevalence of PCOS

PCOS is a dysmetabolic condition and seems to be highly prevalent across the world similar to type 2 diabetes mellitus (T2DM). Of the 277 unselected Afro-Caribbean and Caucasian women from the south eastern United States, PCOS was diagnosed based on NIH 1990 criteria in 4.7% of the latter and 3.4% of the former in 1998 (44). The

prevalence of PCOS was 4.8% in Caucasian and 8% in Afro-Caribbean women in 400 unselected, consecutive, premenopausal women aged between 18 and 45 years attending pre-employment health checks at the University of Alabama in 2004 (45). This data is comparable with a prevalence of 4.8% found in a Swedish study which involved 147 premenopausal women (46) and 6.3% in a population study in Spain (47). March WA et al. examined the prevalence of PCOS in 728 Australian women aged 27-34 years collected using a birth cohort study. It was reported that the prevalence was 8.7% using NIH 1990 criteria and 11.9% using the Rotterdam 2003 criteria. Of the women with PCOS, 68-69% were not diagnosed PCOS before the study suggesting that this condition was markedly under diagnosed in the community (48). PCOS is also found in Asian countries. For example, the prevalence was 6.3% in Sri Lanka in a population study of 2,915 premenopausal women (49) and 2.2% in Southern China among 915 women of child bearing age (50). Interestingly, the prevalence was similar between female to male transsexuals (11.5%) and controls (9.6%) in a study carried out in the United States (51).

1.1.4 Obesity and PCOS

Obesity is a major health concern in the modern world. It is associated with increased relative and population attributable risk for hypercholesterolaemia, diabetes, hypertension and cardiovascular diseases (CVD) according to the Framingham study (52).

Obesity appears to be closely associated with PCOS. The incidence of obesity has increased dramatically worldwide (53). In England, 61% of the total population in 2009 was recorded as overweight or obese. The proportion of women with a normal Body Mass Index (BMI) in England decreased from 49% to 41% and the proportion with obesity increased from 16% to 24% between 1993 and 2009 according to published statistics on obesity, physical activity and diet (Statistics on obesity, physical activity and diet: England, Feb 2011, The NHS Information Centre, Lifestyles Statistics.page18-19).

It is interesting to examine whether the incidence of PCOS can be correlated with the increasing incidence of obesity. Yildiz B et al. studied the prevalence of PCOS according to BMI in 675 unselected women and then compared BMI of newly diagnosed PCOS with the BMI of 746 women with PCOS diagnosed in 1987-1990 over

a time period of 15 years (54). Among 675 unselected women, the prevalence rates of PCOS in underweight, normal-weight, overweight, and obese women were 8.2, 9.8, 9.9, and 9.0%, respectively. In subgroups of obesity, prevalence rates reached 12.4 and 11.5% in women with BMI 35–40 kg/m^2 and greater than 40 kg/m^2 respectively. The mean BMI of PCOS patients at diagnosis increased from 31.3 kg/m^2 in 1987-1990 to 37.3 kg/m^2 in 2000–2002, paralleling the change in BMI of the surrounding population (10–14% obesity rate in 1987 and 25% or greater in 2002). Alternatively, 51% of PCOS women diagnosed in 1987-1990 were obese and 74% in 2002. Although the degree of obesity of PCOS patients had increased similarly to that observed in the general population, the overall risk of PCOS was only minimally increased with obesity. In contrast, an increasing trend in prevalence of PCOS from 6.5% in 2000 (47) to 28.3% in 2006 (55) has been detected in Spanish studies.

1.2 Cardiovascular risk in PCOS

1.2.1 Factors influencing cardiovascular risk in PCOS

A crucial factor in assessing cardiovascular (CV) risk for a woman with PCOS depends on the definition of PCOS itself. Amongst women with PCOS diagnosed with non-NIH criteria, only 75% met the classic NIH criteria and the remainder had either ovulatory or normo-androgenic phenotypes (56). Women with classic PCOS had greater menstrual irregularity, hyperandrogenism, total and abdominal obesity, IR and had more severe risk factors for T2DM and CVD than PCOS patients diagnosed using non-NIH criteria (57-60). When comparing ovulatory PCOS with non-hyperandrogenic PCOS, the latter have a more metabolically favorable risk profile, often indistinguishable from normal women (61, 62).

Women with PCOS, particularly those who are obese, hyperandrogenic and hyperinsulinaemic, (63) are strongly associated with CV risk markers (64), dyslipidaemia (65), metabolic syndrome (66), impaired glucose regulation, (19, 21, 67) depression, anxiety and reduced quality of life (68, 69).

Increased CV risk factors in PCOS cannot be exclusively attributed to individuals with preponderant centripetal obesity. Comparison of CV risk factors were made between 488 patients with well-defined PCOS and 261 controls (free-living population women

with regular menses from the Princeton Follow-up Study) and it was found that those with PCOS had higher risk factors (BMI, waist circumference, total and low density lipoprotein cholesterol, triglycerides, systolic blood pressure, diastolic blood pressure, insulin, glucose, and homeostatic model assessment- insulin resistance) compared to controls. After adjusting for age and BMI, women with PCOS had lower high density lipoprotein cholesterol (HDL-c) and higher systolic blood pressure and insulin secretion compared with the healthy women (70).

1.2.2 Endothelial dysfunction in PCOS

Circulating markers of endothelial dysfunction are elevated in PCOS. These markers include asymmetric dimethylarginine (ADMA), an endogenous competitive inhibitor of nitric oxide (NO) synthase; plasminogen activator inhibitor-1 (PAI-1), a prothrombotic factor that inhibits fibrinolysis; intra-cellular adhesion molecule-1 (sICAM-1) and vascular cell adhesion molecule-1 (sVCAM-1) (71-76). These markers, as well as lowered brachial artery flow mediated dilatation (FMD), are interrelated with hyperandrogenaemia (77). However, the effect of PCOS on brachial artery FMD is not consistent: Tarkun et al. and Meyer et al. found lowered FMD (78, 79) whilst Mather et al. and Brinkworth et al. found no changes (80, 81). In a recent meta-analysis of twenty-one published studies (PCOS, n=908; controls, n=566, there was a reduction in FMD in PCOS than controls but with high heterogeneity between studies (82).

1.2.3 Platelet dysfunction in PCOS

PCOS is also known to be associated with platelet dysfunction with markedly impaired platelet responsiveness to NO, hyperaggregability and endothelial dysfunction (83, 84).

1.2.4 Atherosclerosis in PCOS

When evidence of atherosclerosis was studied, carotid intima-media thickness (IMT) was increased, and brachial artery FMD was decreased in 50 young women with PCOS compared to age and BMI matched ovulatory controls. Changes in carotid IMT or FMD did not correlate with BMI or waist hip ratio (WHR) but were positively correlated with insulin resistance (85). Subsequent studies showed that carotid IMT is significantly higher in women with PCOS than controls (86-89).

1.2.5 Cardiovascular disease in PCOS

Talbott et al. reported that women with PCOS have a higher prevalence of coronary artery calcification (CAC) and aortic calcification (AC) than controls. After adjustment for age and BMI, PCOS was a significant predictor of CAC. Low HDL-c and high insulin levels mediated risk for CAC in PCOS, and total testosterone for AC in all subjects after controlling for PCOS, age, and BMI (88). Therefore, PCOS status and hyperandrogenaemia are associated with endothelial dysfunction and atherosclerosis. Other studies supported the finding that PCOS has higher prevalence of CAC compared to controls (88-91).

In The Nurses' Health Study, 82,439 female nurses were followed up for 14 years. Those usually reporting irregular or very irregular cycles had an increased risk for non-fatal or fatal coronary artery disease (CAD). This remained significant after adjustment for age, BMI and several potential confounders. Menstrual cycle irregularity, a feature of PCOS, may be a marker of metabolic abnormalities predisposing to increased risk for CVD (92). Similar association of previous menstrual irregularity with arterial hypertension, hypercholesterolaemia, hypertriglyceridaemia and coronary angioplasty was found in a population study of 414 postmenopausal women with a mean age of 60 years (93).

A Women's Ischemia Syndrome Evaluation study evaluated the risk of cardiovascular events in 104 postmenopausal women with clinical features of PCOS defined by a premenopausal history of irregular menses and current biochemical evidence of hyperandrogenaemia, compared with 286 women without PCOS. Those with clinical features of PCOS were more often diabetic, obese, had the metabolic syndrome, and had more angiographic coronary artery disease and lowered cumulative 5 year CV event-free survival compared to those without clinical features of PCOS (94).

PCOS status appeared to be associated with an increased CV risk. Krentz et al. studied the effect of putative PCOS phenotype in 713 postmenopausal women with atherosclerotic CVD. Putative PCOS phenotype was defined as the presence of three or more of the following features: (1) recalled history of irregular menses, (2) symptomatic premenopausal hyperandrogenism or biochemical evidence of current hyperandrogenism, (3) history of infertility or miscarriage, (4) central obesity or (5) IR.

This study found that prevalent atherosclerotic CVD is associated with features of a putative PCOS phenotype among non-diabetic postmenopausal women with intact ovaries (95). In addition, CVD is more common and develops at earlier age in PCOS mothers of women with PCOS compared with those who do not have PCOS (96). The increased CV risk was not entirely related to obesity. For example, lean PCOS have a 2.5 times higher prevalence of hypertension than those without PCOS according to a Dutch population study involving 346 women with PCOS (97).

Wild et al. reported that epidemiological data suggested more frequent CVD in classic PCOS than non-classic cases, mostly mediated through increased total and abdominal adiposity and perhaps interacting with PCOS-related hyperandrogenism (98).

1.2.6 Cardiovascular morbidity and mortality in PCOS

Although there is cumulative evidence of CV risk and early makers of CVD in PCOS, the actual effect of PCOS on CVD morbidity and mortality is still elusive. In a cohort of 319 women with PCOS and 1060 age-matched controls with a follow up of 30 years, a history of non-fatal cerebrovascular disease and CV risk factors including diabetes was more prevalent among women with PCOS (99). However, there was no increase in all-cause mortality and coronary artery disease morbidity or mortality in PCOS compared with controls (64). Similarly, there was no increase in CVD mortality although a higher prevalence of hypertension and higher triglyceride levels were found in PCOS than controls when 25 women with PCOS were followed up for 21 years (100).

Lifelong metabolic dysfunction such as IR, metabolic dyslipidaemia and perhaps hyperandrogenaemia in women with PCOS leads to an exaggerated risk for CVD independent of, and additive with obesity. However, data on long-term risk for CV morbidity and mortality are inconsistent.

1.2.7 Assessment and reduction of CV risk in PCOS

A panel appointed by the Androgen Excess and Polycystic Ovary Syndrome (AE-PCOS) Society reviewed all published evidence assessing CV risk in PCOS *vs.* non-PCOS women and recommended the following PCOS-related guidelines for CVD prevention. Women with PCOS with obesity, cigarette smoking, dyslipidaemia,

hypertension, impaired glucose tolerance (IGT), and sub-clinical vascular disease are at risk, whereas those with metabolic syndrome and/or T2DM are at high risk for CVD. It was recommended to assess and monitor BMI, waist circumference, serum lipid/glucose, and blood pressure for all women with PCOS. Oral glucose tolerance testing was recommended in those with obesity, advanced age, personal history of gestational diabetes or family history of T2DM. Mood disorder assessment was suggested in all PCOS patients. Lifestyle management was recommended for primary CVD prevention, targeting HDL-c and LDL-c and adding insulin-sensitizing and other drugs if dyslipidaemia or other risk factors persist (98).

1.3 Insulin resistance

1.3.1 Insulin and its action

Insulin is the most potent anabolic hormone known and promotes glucose oxidation, inhibits lipid oxidation, enhances lipogenesis and glycogenesis. Insulin exerts its action via insulin receptors present in virtually all vertebrate tissues although their concentration varies from as few as 40 on circulating erythrocytes to more than 200,000 on adipocytes and hepatocytes (101). The receptor gene is located on the short arm of human chromosome 19 (102). The insulin receptor is composed of two α-subunits that are each linked to a β-subunit and to each other by disulphide bonds.

The binding of insulin, following its release from the beta cells of the pancreas, to cell surface insulin receptors initiates a complex series of intracellular signaling events that leads to increased glucose uptake, glycogen synthesis, and fat storage (103). The insulin receptor is a trans-membrane glycoprotein with intrinsic protein tyrosine kinase activity (Figure 1-1). Tyrosine kinase mediates the insulin response through tyrosine phosphorylation of endogenous substrates, such as insulin receptor substrates (IRS)-1 and -2. This in turn acts as docking sites for many SH2 domain–containing proteins, including the p85α regulatory subunit of phosphatidylinositol 3-kinase (PI3K) which is activated when binding to this site (104). PI3K then activates downstream protein kinases including Akt/PKB (protein kinase B) (105). Ultimately, these signalling events lead to stimulation of insulin-mediated glucose uptake into cells via enhanced translocation of glucose transporter 4 (GLUT4) molecules to the cell membrane, leading to an increase in insulin-mediated glucose transport. The activation of PI3K

stimulates phosphodiesterase-3 (PDE) so that more cyclic adenosine 3', 5'-monophosphates (cAMP) is metabolized. Reduction in cAMP decreases phosphorylation of hormone-sensitive lipase (HSL) leading to reduction in lipolysis in fat cells in the presence of insulin. Phosphorylation of PKB deactivates glycogen synthase kinase3 (GSK3) which fails to deactivate glycogen synthase (GS), and increases glycogen synthesis. In addition, insulin mediates an effect on adipocyte differentiation, inducing genes involved in lipogenesis, and repressing those involved in fatty acids (104).

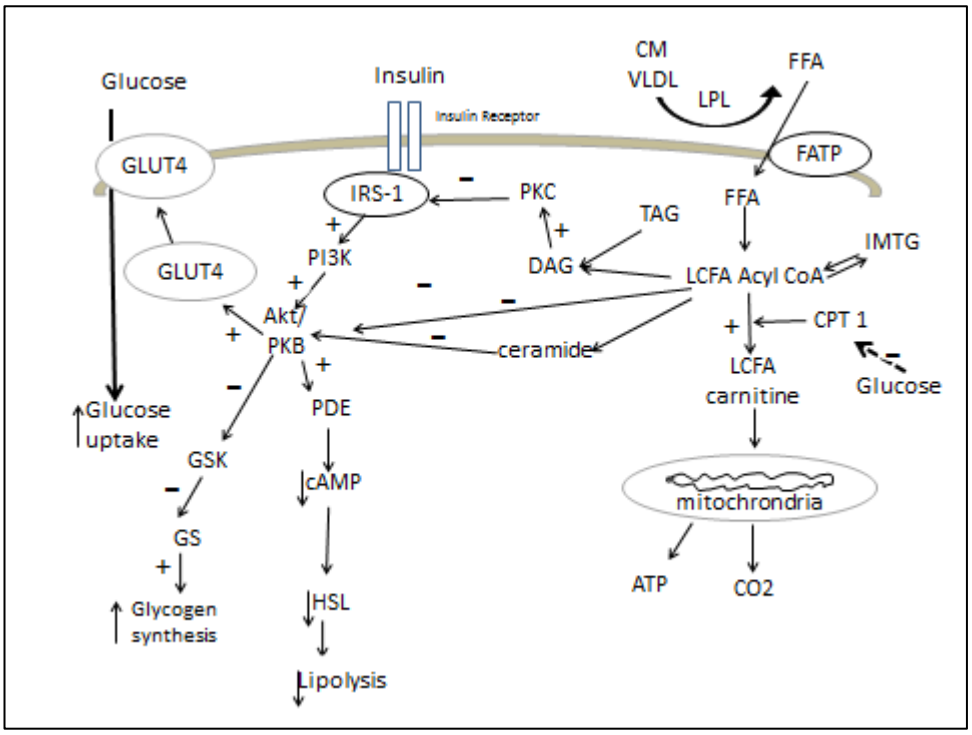

Figure 1-1 Mode of insulin action and its interaction with intramyocellular lipid metabolites

Insulin increases glucose uptake in skeletal muscle, promotes glycogen synthesis in liver and inhibits lipolysis in adipocytes. Its action is disturbed by intramyocellular lipid metabolites (DAG, LCFA acyl CoA, ceramides)

1.3.2 Effect of insulin on glucose metabolism

Insulin mediates its anabolic action and glucose homeostasis by acting on target organs such as adipose tissue, liver and skeletal muscle. Glucose homeostasis is tightly controlled by balancing glucose absorption from the intestine, hepatic glucose production, and glucose uptake in peripheral tissues. Insulin plays an essential role in maintaining glucose homeostasis and energy storage. Glucose regulates itself and energy homeostasis by gene transcription, enzyme activity, hormone secretion, and the activity of gluco-regulatory neurons. In pancreatic beta cells, glucose is the principal regulator of insulin secretion. In the brain, groups of glucose-sensitive neurons are activated or inhibited by changes in glucose levels. These neurons are involved in the control of feeding, energy expenditure and glucose homeostasis (106).

1.3.2.1 Glucose transporters

In most tissues, except hepatocytes, intestinal cells, renal tubular cells and pancreatic beta cells, glucose uptake is controlled by glucose transporter expression at the cell surface. In 1948, Le Fevre first postulated that a specific component within the cellular plasma membrane was required for the transfer of glucose across lipid bi-layers (107). In the early 1950s, Widdas et al. proposed a mobile carrier mechanism to explain the observed kinetics of glucose transport across sheep placenta (108). Glucose transporter 1 (GLUT1) was first discovered as an erythrocyte glucose transporter in 1985 (109) and 13 related members of the SLC2A (GLUT) protein family have subsequently been identified in humans (110). Among the fourteen GLUT proteins, GLUT 1-4 are well-established glucose transporter isoforms, have distinct kinetic properties and regulate cell surface expression to provide fine tuning of glucose uptake, metabolism, and signal generation in order to preserve cellular and whole body metabolic integrity (111).

GLUT1 catalyses the rate-limiting step in supplying brain cells with glucose, an essential fuel for these cells (112).

GLUT2 is a bidirectional transporter, allowing glucose to flow in two directions. It is expressed at very high level in pancreatic beta cells and in the basolateral membranes of renal tubular cells, small intestinal epithelial cells and hepatocytes (113).

GLUT3 is a major neuronal glucose transporter, present in both dendrites and axons, and its level of expression in different regions of the brain correlates with regional cerebral glucose utilization (114) . GLUT3 has a high affinity for glucose, allowing it to transport even in times of low glucose concentrations.

GLUT4 is mainly expressed in adipose tissues and striated muscle (skeletal muscle and cardiac muscle) and is an insulin-regulated glucose transporter (115). Knowledge of the acute and chronic regulation of GLUT4 in intact skeletal muscle, the major site of insulin-stimulated whole body glucose disposal, is still relatively poor (116).

1.3.3 Effect of insulin on lipid metabolism

In the postprandial state, insulin switches energy metabolism from non-esterified fatty acids (NEFA) to glucose in peripheral tissues. It increases glucose uptake in muscle and adipose tissue and inhibits hepatic glucose production, thus serving as the primary regulator of blood glucose concentration. At the same time, insulin inhibits HSL and decreases the release of NEFA from adipose tissue. Insulin also stimulates lipoprotein lipase (LPL) in adipose tissue and to a lesser degree in skeletal muscle to enhance the uptake of NEFA during the postprandial period.

Glucose and lipid metabolism seem to be interrelated in the presence of insulin. Adipocyte- specific insulin receptor gene knockout mice have low fat mass, and are protected against age-related and hypothalamic lesion-induced obesity, and obesity-related glucose intolerance. Muscle-specific insulin receptor knockout mice displayed elevated fat mass, serum triglycerides, and NEFA, but blood glucose, serum insulin, and glucose tolerance were normal (117).

1.3.4 Definition of insulin resistance

Steady-state, basal plasma glucose and insulin levels are determined by their interaction in a feedback loop. Insulin resistance (IR) is traditionally defined as a subnormal glucose response to normal insulin concentrations. Alternatively, IR can be defined as a state that requires high levels of insulin to maintain glucose homeostasis as a result of decreased insulin sensitivity (118). Up to 75% of insulin-stimulated glucose uptake occurs in skeletal muscle. Adipose tissue is responsible for a relatively small proportion (<10%) of the peripheral glucose utilization in response to insulin.

However, adipocytes are also highly sensitive to insulin and may play a significant role in IR through their endocrine function and involvement in lipid metabolism. Therefore, IR in skeletal muscle and adipose tissue has an important implication for glucose and lipid metabolism. IR can be caused by inherited abnormalities of insulin receptors or their signalling. IR is commonly associated with obesity, excess hormones such as growth hormone and steroids, inactivity, pregnancy, stress and infection.

1.3.5 Assessment of insulin resistance in PCOS

The hyperinsulinaemic euglycaemic clamp technique (119) and the frequently sampled intravenous glucose tolerance test (IVGTT) are considered the least compromised means available for quantifying insulin sensitivity. In hyperinsulinaemic euglycaemic clamping, insulin sensitivity is measured as M value, a rate of insulin-stimulated glucose disposal i.e. rate of 20% dextrose infusion to maintain euglycaemia whilst infusing a fixed supra-physiological dose of intravenous insulin. The insulin sensitivity index (ISI) is calculated by dividing average M value by average plasma insulin concentration after a defined period usually the last 20-30 min of an euglycaemic insulin clamp. The disposition index, an alternative way of expressing IR, is calculated by multiplying ISI and acute insulin response to glucose (AIRg). Although these tests are expensive, time consuming, and labour intensive, they are regarded as gold standards for measuring IR.

In contrast to dynamic methods, steady-state assessments of insulin sensitivity are used to express IR such as measurement of fasting insulin concentration, fasting glucose to insulin ratio, the HOMA-IR (defined as insulin pmol/Lx glucose mmol/L /22.5) (120, 121), and a recently developed method, the quick insulin sensitivity check index, termed QUICKI, (defined as 1/(log [insulin]) _ log [glucose]) (122).

The best method of insulin sensitivity assessment in PCOS is still diverse because IR is associated with ethnic and genetic variability and the presence of other factors influencing IR such as obesity, stress and ageing. Whilst the hyperinsulinaemic euglycaemic clamp is a gold standard method for measuring IR, a fasting glucose (mg/dL) to insulin (μU/mL) ratio >4.5 provided a sensitivity of 95% and specificity of 84% and was suggested as a screening test for predicting IR (123). In addition, studies have shown that the next best single predictor of IR is fasting insulin level. HOMA-IR is a reliable and easy way of accessing IR and an oral glucose tolerance test (OGTT) is

the best simple, office-based method to assess both IR and glucose intolerance in women with PCOS.

1.3.6 Insulin resistance in PCOS

Insulin resistance appears to play a central role in the development of PCOS. Approximately 50-80% of all women with PCOS have some degree of IR (29). Fasting hyperinsulinaemia in PCOS was first reported in 1980 (9), and subsequently confirmed in numerous studies (124-128). IR is more severe in older and obese patients with PCOS (30, 129). In age and BMI matched women, fasting hyperinsulinaemia and IR is more common in those with biochemical hyperandrogenism and family history of diabetes mellitus (11, 130). Although there is an association between IR and obese women with PCOS, data for normal weight women with PCOS are conflicting.

A decrease in insulin-mediated glucose disposal rate in women with PCOS was observed when compared to age and BMI matched controls (14, 131-133). Twenty-eight women (15 obese and 13 non-obese) with PCOS and 29 (14 obese and 15 non-obese) age and weight matched controls were examined using a modified, frequently sampled IVGTT (134). There was a significant decrease in insulin sensitivity and disposition index in PCOS. There was also a negative trend for decreased insulin sensitivity from lean controls, to lean PCOS, and obese controls, and finally to obese PCOS. According to these studies, PCOS is associated with IR independent of their weight. However, some other studies did not confirm higher IR in lean PCOS compared to their counterparts. Holte et al. measured insulin sensitivity in 41 women with PCOS and 39 controls using an euglycaemic clamp (135). The significant difference in insulin sensitivity index between both groups was found only in subjects with higher BMI. Interestingly, the insulin sensitivity in this study was largely determined by truncal- abdominal skin fold thickness. After a significant weight loss, IR was significantly improved becoming comparable to BMI matched controls (136).

When IR of 15 lean controls was compared to 53 lean and 30 obese PCOS in a European study, the fasting insulin level was significantly increased in both lean and obese PCOS compared to lean controls. In contrast, glucose disposal was comparable between lean controls and lean PCOS but significantly increased in obese PCOS. In a study including 17 lean and 17 obese controls and 15 lean and 28 obese PCOS, a trend

towards hyperinsulinaemia and impairment of insulin sensitivity was observed in lean PCOS but significant only in obese PCOS subjects (137). Although increased IR in the lean PCOS group is not yet universally confirmed, there is no doubt that central obesity in particular, is a major determinant of IR in PCOS.

1.3.7 Pathophysiology of insulin resistance in PCOS

The pathophysiology of IR in PCOS is complex and multifactorial in nature. It has been implicated so far that the defect would lie in insulin secretion i.e. beta cell function, its action i.e. skeletal muscle insulin resistance or hepatic insulin resistance and its clearance.

1.3.7.1 Insulin signalling defect

Selective resistance to the effects of insulin on glucose metabolism in skeletal muscle and adipose tissue is a key feature of PCOS. Insulin action cascade, receptor binding, tyrosine kinase activity, and glucose transport activity were examined in isolated adipocytes prepared from subjects with PCOS to localise and specify the defect. There was no abnormality in the number of insulin receptors or their affinity and receptor kinase activity, but there was a large rightward shift in the insulin dose-response curve for glucose transport stimulation with a decrease in maximal insulin- mediated glucose transport in PCOS women compared to controls (138, 139). This suggested that IR in PCOS is due to a post-receptor defect in the insulin signal transduction chain between the receptor kinase and glucose transport. This defect is independent of obesity, metabolic derangement, body fat or sex hormone levels. Thus it appears to be related to intrinsic abnormalities in PCOS. There was a decreased suppression of hepatic glucose production in obese PCOS, suggesting changes in hepatic insulin sensitivity may be acquired with obesity (131).

In skeletal muscle, the major site of insulin-mediated glucose uptake, IRS-1-associated PI3K activity is decreased in women with PCOS *in vivo* when compared to age, weight, and ethnicity matched control women. It was therefore consistent with a defect in IRS-1-mediated signalling independent of obesity (140). IR clustered in PCOS families (141) and defects in insulin action persisted in cultured skin fibroblasts from women with PCOS (142) suggesting a genetic propensity for these abnormalities.

Corbould et al. examined insulin action on glucose metabolism and insulin signalling in cultured skeletal muscle from women with PCOS to determine whether the defects detected in acutely isolated skeletal muscle were intrinsic or not. Despite defects in insulin signalling via IRS-1 and IRS-2, cultured myotubes from women with PCOS showed normal insulin responsiveness, consistent with a major role for the metabolic/hormonal environment in the pathogenesis of *in vivo* IR in this syndrome (143). This finding highlights the important role of environmental factors in the pathogenesis of a decrease in insulin mediated glucose transport in PCOS. Unlike skin fibroblasts and skeletal muscle, neither a decrease in basal and insulin stimulated glucose transport nor insulin signalling defect was found when adipocytes from women with PCOS were either repeatedly cultured alone or co-cultured with insulin resistant PCOS fibroblasts (144).

In summary, PCOS has an intrinsic post-receptor insulin signalling defect in skeletal muscle but the defect exerts a negative impact on insulin stimulated glucose uptake only in the presence of chronic environmental influences such as obesity in *in vitro* studies.

1.3.7.2 Pancreatic beta cell function

The relationship between IR, beta cell function, obesity and androgen levels were examined in 60 PCOS women using HOMA-IR. It was found that beta cell function was an independent predictor of IR and bio-available testosterone. In clamp studies, a significant reduction in the disposition index and rate of glucose disposal in PCOS confirmed peripheral IR. Furthermore, failure to increase acute insulin response to infused glucose during clamps was suggested a defect in beta cell compensation for the degree of IR postprandially (134, 145-147). The National Health and Nutrition Examination Study *NHANES III (1988-1994) revealed a significantly stronger relationship between beta cell function and IR in PCOS compared with controls suggesting an intrinsic beta cell defect (148). However, in other studies, beta cell adaptation and compensation (AIRg) was appropriately intact in all PCOS patients (149) (135, 150).

1.3.7.3 Metabolic clearance of insulin

It is not clear yet if women with PCOS have abnormal metabolic clearance of insulin because the findings are inconsistent so far. Metabolic clearance of insulin decreased in hyperinsulinaemic PCOS independent of BMI in one study (151) but had no change in another study (152).

1.3.8 Relationship of IR and hyperandrogenism in PCOS

Women with PCOS suffer from hirsutism and menstrual irregularity related to hyperandrogenaemia. These symptoms were among the most serious concerns followed by psychological problems in women with PCOS whereas weight and infertility were the least concerns in the assessment of health related quality of life (HRQOL) questionnaires (69). Hyperandrogenism was present in 75% of PCOS (153). Free testosterone levels were raised in 60%, total testosterone in 33% and dihydroepiandrosterone sulphate (DHEAS) in 32.7% of patients with PCOS (154). Hyperandrogenaemia is most commonly assessed by measurement of serum total testosterone and sex hormone binding globulin (SHBG) followed by calculation of free androgen index (FAI) (155-157). Both free testosterone and FAI are accepted as the most sensitive methods of assessing hyperandrogenaemia (155, 156, 158).

Hyperandrogenaemia is strongly associated with hyperinsulinaemia (130) and metabolic syndrome, and independent of obesity in PCOS (29). Improving IR by metformin reduces testosterone level and improves menstrual irregularities (159, 160). However, correcting hyperandrogenism either by GnRH analogue (gonadotrophin releasing hormone analogue) (161) or by laparoscopic ovarian cauterisation to normal levels did not improve IR (162). This suggested that hyperinsulinaemia stimulates androgen production rather than the reverse. Interestingly, hyperandrogenaemia is associated with elevated ALT levels in women with PCOS independent of obesity, IR and dyslipidaemia (163).

1.4 Impaired glucose regulation and PCOS

1.4.1 Diagnosis of impaired glucose regulation

According to the World Health Organisation (WHO) diabetes diagnostic criteria, normal glucose tolerance is defined as fasting plasma glucose (FPG) ≤6mmol/L and 2 h postprandial plasma glucose (PPG) <7.8mmol/L of 2 h 75g oral glucose tolerance test. FPG > 6mmol/L but ≤ 6.9mmol/L is defined as impaired fasting glucose (IFG), and PPG ≥7.8mmol/L but < 11.1mmol/L as impaired glucose tolerance (IGT). Diabetes (DM) is diagnosed when either FPG is ≥7mmol/L or PPG ≥11.1mmol/L. Impaired glucose regulation (IGR) is defined as the presence of IFG and/or IGT, or DM.

1.4.2 Impaired glucose regulation and CV risk

The risk of CVD was examined in 1314 subjects who were initially free from CVD and followed up for 16 years in the Framingham Study. Diabetic subjects were predisposed to all of the major CVD outcomes and had higher levels of fibrinogen, hypertension, hypertriglyceridaemia, and obesity, but lower HDL-c values. There was a rise in fibrinogen values throughout the range of blood glucose levels suggesting thrombogenic involvement is a unique diabetic effect. However, multivariate analysis indicated that glucose intolerance had a residual effect on CVD after all of the standard risk factors and fibrinogen had been taken into account (164).

In a prospective cohort study, a total of 57% of patients who presented with acute coronary event had IGR (165). Postprandial glucose (PPG) showed a linear relationship with CV death, and treatment targeted at PPG has been shown to reduce progression of atherosclerosis and CV events according to the report of the international prandial glucose regulation study group (166).

1.4.3 Prevalence of impaired glucose regulation in PCOS

As women with PCOS are insulin resistant, they are at higher risk of IGR compared to women without PCOS. Obesity increases the risk of IGR in PCOS. The prevalence of IGT and T2DM classified according to the WHO criteria was 31-35% and 7.5-10% respectively in 2 large studies (21, 24). Moreover, IGT (29.6%) and T2DM (7.4%) are significantly prevalent in adolescent girls with PCOS (25). The increased prevalence of

IGR in women with PCOS is not only in western countries but also in Chinese (IGT 20.5%, T2DM 1.9%) (20) and Thai (IFG 3.2%,13.6% IGT, DM 5.6%) populations (167).

Moran LJ et al. reviewed 2192 studies, and included 35 studies for a systematic review to compare women with PCOS (835 subjects) with those without PCOS (538 subjects) for the end point of prevalence or incidence of IGT, T2DM and metabolic syndrome, and 30 studies (BMI matched 347 PCOS and 319 control subjects) for meta-analysis. PCOS had an elevated prevalence of IGT, T2DM and metabolic syndrome than controls in both BMI and non-BMI matched studies (168).

1.4.4 Prevalence of PCOS in women with T2DM

Up to one third of all incident cases of T2DM were estimated to be attributable to PCOS in Caucasian women when the percentage risk of attributable population was calculated using Levin's formula (169). The prevalence of PCOS, using NIH 1990 criteria, was high at 8.3% in 157 Iranian women with T2DM. The age of onset of diabetes was lower, but BMI and waist circumference were higher in women who also had PCOS than those without (170).

1.4.5 Progression to T2DM in PCOS

The rate of conversion from normal glucose tolerance (NGT) to IGT and to T2DM in PCOS women is substantial. Fifty-four PCOS women with NGT and 13 with IGT at baseline were followed up for 6.2 years. Nine percent of normoglycaemic women developed IGT and 8% developed T2DM. More than 50% of women with IGT at baseline progressed to T2DM. However, this was an uncontrolled study (171). Legro et al. followed up 39 PCOS women and 23 healthy subjects who had had NGT at baseline for a mean duration of 2-3 years. For PCOS women, the progression rates were 16%, and 2% per year for NGT to IGT, and IGT to T2DM respectively. Progression rates were less prominent in controls. The progression to IGR was associated with significantly decreased insulin sensitivity (23).

1.5 Lipids and fatty acids metabolism

Fatty acids (FA) are straight chain carbon compounds with a variable number of carbon atoms and double bonds. Fatty acids are derived from hydrolysis of triglyceride rich

lipoproteins (TRL); triglycerides rich exogenous lipoproteins, chylomicrons, and endogenous lipoproteins, very low density lipoproteins (VLDL), by LPL during postprandial period and from triglycerides of adipose tissue by hormone sensitive lipase (HSL) during post-absorptive period. Those with one double bond are defined as monounsaturated free fatty acids (FFA), those with more than one double bond are referred to as polyunsaturated and those with no double bond known as saturated fatty acids. FA binds to albumin and circulates as non-esterified fatty acids (NEFA).

1.5.1 Types of lipids

There are four main forms of lipids namely triglycerides, cholesterol, cholesterol ester and phospholipids. Since they are poorly soluble in water, lipids bind to apolipoproteins to form lipoproteins enabling circulation in plasma.

Triglycerides (TG), also known as triacylglycerol (TAG), are made up of three fatty acids esterified with glycerol. The vast majority of TAG (>95%) in the body is found in adipose-tissue stores. Cholesterol is a steroid alcohol present in all cells and body fluids in animals and is a precursor for the synthesis of bile acids and steroid hormones. Cholesterol is synthesized from arachidonic acid by hydroxymethylglutaryl-coenzyme A (HMG-CoA) in the liver where HMG-CoA reductase acts as a rate-limiting enzyme. About 70% of plasma cholesterol is incorporated as low density lipoproteins cholesterol (LDL-c) and 20% as HDL-c. Two thirds of plasma cholesterol are esterified with fatty acids to form cholesterol esters.

Phospholipids are complex lipids containing phosphate and a nitrogenous base. They provide an important structural role in cell membranes and confer solubility on non-polar lipids and cholesterol in lipoproteins.

1.5.2 Lipoproteins

A lipoprotein consists of a core of triglycerides, cholesteryl ester and an outer monolayer of phospholipids, unesterified cholesterol and apolipoproteins. There are five major classes of lipoproteins based on their density that reflect the relative proportion of lipid and protein content. The greater the lipid: protein ratio, the larger their size and the lower the density. These are called as chylomicrons, VLDL, intermediate density lipoproteins (IDL), LDL-c and HDL-c. The first three of these are

triglycerides rich and larger in size and the last two contain mainly cholesterol and are smaller in size. Triglycerides for example, form 90% of chylomicron mass whereas they form only 5% of HDL-c mass. Chylomicrons are the largest and least dense lipoproteins, and transport exogenous lipids from the intestine to all cells. VLDL transports endogenous lipids from liver to cells. LDL-c is the major carrier of cholesterol in human plasma and is well known to be involved in the process of atherosclerosis (172). HDL-c removes cholesterol from cells and transports it back to the liver in the process known as reverse cholesterol transport (173).

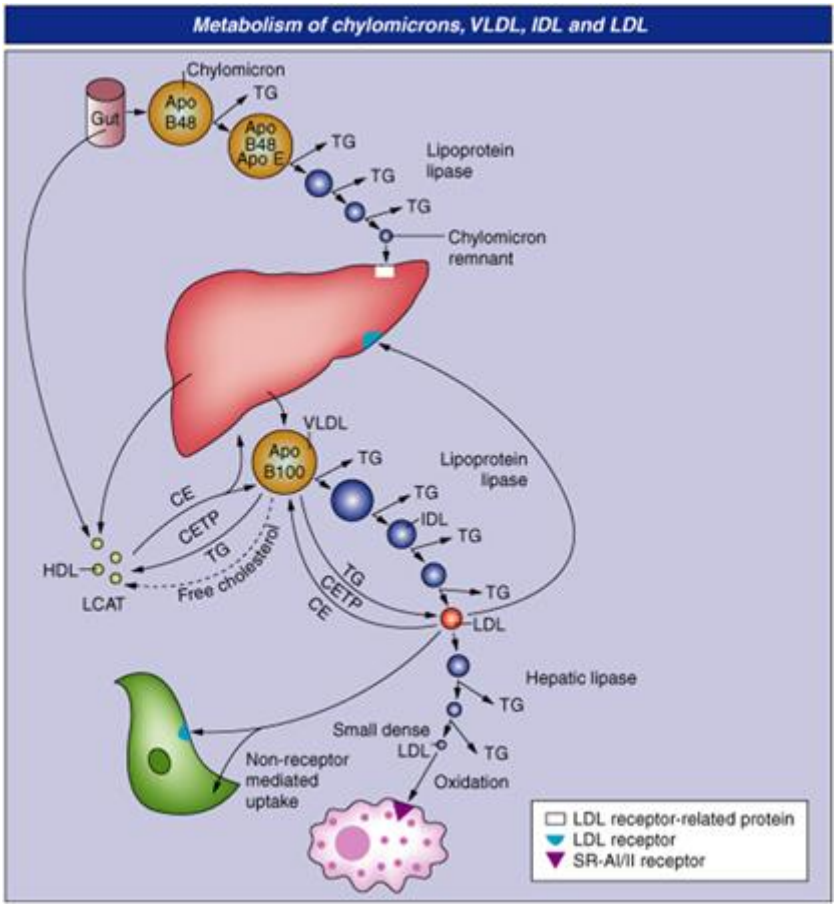

Figure 1-2 An outline of the major metabolic pathways of the major lipoproteins (Durrington, 2007)(174)

1.5.3 Lipid Metabolism

Lipoprotein metabolism can be examined in exogenous and endogenous pathways.

1.5.3.1 Exogenous pathway

During the postprandial period, cholesterol and fatty acids released from dietary fat by the action of pancreatic lipase, and cholesterol from bile derived from enterohepatic circulation are absorbed into intestinal mucosal cells where they are re-esterified to form cholesterol esters and triglycerides. These, together with phospholipids, intestinal cell derived apolipoproteins Apo B48 and Apo A, are taken up into the lymphatic system then to the systemic circulation as chylomicrons. The assembly of chylomicrons depends upon on synthesis of Apo B48. Apolipoproteins Apo C and Apo E both derived from HDL-c are added to the chylomicrons in the lymph and plasma (175). The enzyme LPL in the vascular surface of the capillary endothelium of skeletal muscle and adipose tissue is activated by Apo CII and inhibited by Apo CIII. LPL activity in adipose tissue is increased six fold postprandially but down-regulated in skeletal muscle. LPL activity hydrolyses triglycerides from chylomicrons into fatty acids and glycerol (176, 177). Chylomicrons and VLDL compete for LPL for hydrolysis in a common lipolytic pathway (178, 179). In the postprandial period, 80% of the increase in plasma triglyceride concentration is contributed by chylomicrons and their remnants as those contain a large amount of triglycerides (180). Ninety percent of the rise of cholesterol in the TRL fraction is a result of accumulation of large endogenous VLDL particles secondary to preferential lipolysis of chylomicrons (181).

Fatty acids are taken up by the skeletal muscle for beta oxidation and by adipose tissue where they are re-esterified and stored as triglycerides or simply bound to albumin and circulate in the plasma. The glycerol component enters the hepatic glycolytic pathway. During this process, chylomicrons become smaller and release Apo A and Apo C along with phospholipids that are incorporated into HDL particles. The chylomicron remnants enriched in Apo B, Apo E and cholesterol bind to LDL receptors and LDL receptor related proteins mediated by Apo E as a ligand in the liver (182). Within hepatic cells, cholesterol is recycled and apolipoproteins catalyzed. During the post-absorptive period, the flux of NEFA from adipose tissue to the liver

promotes endogenous VLDL synthesis and their release. Those are hydrolysed by LPL in skeletal muscle and supply NEFA for energy metabolism.

1.5.3.2 Endogenous pathway

Liver is the main source of endogenous lipids. Triglycerides are synthesized using fatty acids derived from adipose tissue by the action of adipose triglyceride lipase (183) and HSL during fasting (184), from hepatic de novo synthesis, and from hydrolysis of circulating chylomicrons by LPL and glycerol derived from glucose metabolism. Cholesterol from local synthesis in the liver and from hepatic uptake of chylomicron remnants and HDL-c along with triglycerides are assembled with Apo B100 in hepatocytes and released into the circulation as VLDL. These are enriched with Apo C and Apo E after interaction with circulating HDL-c. Like chylomicrons, VLDL particles become smaller and denser after the release of triglycerides through hydrolysis by LPL. VLDL is transformed into IDL in the process.

The majority of IDL are modified by hepatic lipase and cholesteryl ester transfer protein (CETP) and transformed to LDL-c. The remainder are cleared directly by the liver via LDL receptor mediated uptake (185). Therefore, IDL are transient or not at all present in plasma. The number of LDL particles in the circulation depends upon the rate of production of VLDL as an end product of VLDL, and their rate of clearance via LDL receptors in hepatocytes. LDL-c transports cholesterol from the liver to cells. LDL-c can be taken up by most cells but mainly in liver via LDL receptors. The rate of synthesis of LDL receptors is controlled by a negative feedback mechanism to avoid excessive accumulation of intracellular cholesterol.

HDL-c is synthesized and secreted from hepatic and intestinal cells in the form of nascent particles consisting of free cholesterol, phospholipids and Apolipoproteins A-I (Apo A-I). Nascent HDL-c interacts with peripheral cells, such as macrophages, to facilitate the efflux of excess free cholesterol, a highly regulated process facilitated by specific transport proteins including ATP-binding cassette (ABC) transporters (ABCA1 and ABCG1) (186-188). Excess free cholesterol in HDL-c is esterified to cholesterol esters by Apo A- I activated lecithin: cholesterol acyltransferase (LCAT) (189). Most cholesterol esters are exchanged with triglycerides from chylomicrons, VLDL and LDL-c through cholesterol ester transfer protein (CETP) and therefore ultimately

reach the liver. The remaining cholesterol esters in the HDL-c are selectively removed by the scavenger receptor class-B, type I (SR-BI) expressed in the liver (190) and secreted in bile.

1.5.4 Effect of adipose derived proteins in lipid partitioning

Adipose tissue secretes a large variety of proteins, including cytokines, chemokines and hormone-like factors, such as leptin, adiponectin and resistin. Many of these proteins take a significant role in regulating lipid and glucose metabolism and inflammation.

Hotamisligil and colleagues (191), and Karasik and colleagues (192) first reported the ability of tissue necrotic factor alpha (TNFα) to induce IR in adipose tissue. TNF-α down regulates GLUT4 and induces IR in adipose tissue (193). It stimulates lipolysis and increases delivery of FFA from adipose tissue in obese subjects (194). TNF-α down regulates adipose tissue LPL activity (195, 196) and protein expression (197-199), reduces the expression of FFA transporters such as fatty acid transporter protein (FATP) and fatty acid translocase(FAT) (200), decreases the expression of key enzymes involved in lipogenesis namely acetyl-CoA carboxylase (201) and fatty acid synthase (202) leading to a decreased triglyceride accumulation in adipocytes. Thus, the net effect of TNF-α is to decrease lipogenesis and to increase lipolysis (203).

Plasma adiponectin and adipose tissue adiponectin mRNA levels were highly correlated and had a negative correlation with obesity and IR. Their expression was inversely and significantly related to TNF-α mRNA expression (204).

Leptin, secreted from adipose tissue, appears to play a major role in the control of body fat stores through coordinated regulation of feeding behaviour, metabolism, autonomic nervous system and body energy balance.

Acylation stimulating protein (ASP), a lipogenic hormone, secreted by white adipose tissue (205) facilitates energy storage by stimulating TG synthesis through activation of diacylglycerol (DAG) acyltransferase and increases glucose transport in *in vitro* studies (206-209). ASP production is stimulated by insulin up to 2 fold and by chylomicrons up to 150 fold in human differentiated adipocytes (209) and correlates with postprandial chylomicron triglyceride clearance (210, 211). ASP indirectly increases LPL activity by upregulating TG storage (212, 213) in white adipose tissue but reduces

LPL activity in skeletal muscle, similar to the effects of insulin (214, 215). Furthermore, ASP inhibits adipose tissue HSL activity, decreases lipid hydrolysis and increases re-esterification of FFA (216). Overall, ASP promotes fat uptake and storage in adipose tissue.

1.5.5 Adipose tissue in lipid metabolism

White adipose tissue is traditionally regarded as an energy storage depot, fatty acids being released when fuel is required. It is now recognised as a highly active metabolic and endocrine organ. It plays a critical role in maintaining the homeostasis of energy metabolism and coordinates the storage and mobilization of TG using NEFA as a vehicle. Adipose tissue functions are regulated by multiple external influences such as autonomic nervous system activity, the rate of blood flow and the delivery of a complex mix of substrates and hormones in the plasma (217).

Adipose tissue can be divided into two major compartments – subcutaneous and visceral which vary both in their distribution and metabolism. Subcutaneous and visceral compartments constitute 80% and 10% of total body fat respectively (218), the remainder is contributed from other depots, such as retroperitoneal, peri-renal and orbital fat. Although both subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) are correlated with metabolic risk factors, VAT remains strongly associated with an adverse metabolic risk profile suggesting it as a unique, pathogenic fat depot (219).

1.5.6 Adipose tissue function in lipid partitioning

Fatty acids released from adipose tissue after the hydrolysis of stored triglycerides is a major fuel for energy in the postprandial period. Fat storage and mobilization during normal daily life are controlled by coordinated function of lipolytic and anti-lipolytic enzymes in white adipose tissue. The enzyme LPL is involved in fat storage and HSL in fat mobilization. The fat storage and mass in white adipose tissue appears to be tightly regulated by coordinated function of LPL, HSL and fatty acid esterification where the latter governs fatty acid mobilization, deposition in adipose tissue and possibly de novo lipogenesis (220, 221).

In the fed state, therefore, HSL is suppressed and esterification stimulated, and fatty acids generated by the increased rate of LPL action are drawn into adipocytes along a concentration gradient. Entrapment of fatty acids is influenced by ASP. In the fasted state HSL is active, LPL less so, and the esterification pathway is not activated. Fatty acids then flow in a net sense from adipocytes into the capillaries for distribution to other tissues via the circulation.

1.5.6.1 Lipid mobilization

Adipose-tissue lipolysis is the major regulator of the supply of lipid energy because it controls the release of fatty acids into plasma. Lipolysis refers to the hydrolysis of TAG, via diacylglycerol (DAG) and monoacylglycerol (MAG) intermediates, to fatty acids and glycerol. This lipolysis is mediated by HSL and other lipases such as adipose tissue triglyceride lipase (ATGL). Whilst HSL and ATGL both have the capacity to hydrolyse triglycerides (183), only HSL holds diacylglycerol lipase activity that hydrolyses DAG to MAG (222). MAG lipase is required to complete hydrolysis of MAG to glycerol and FFA (223).

HSL promotes the hydrolysis of DAG under both stimulated and basal conditions. Non-HSL lipases such as ATGL contribute to the hydrolysis of triglycerides into DAG under basal conditions (224). Resistance to catecholamine-induced lipolysis in subcutaneous adipose tissue has been demonstrated in obese adults and children (201, 202) and is most likely due to impaired HSL expression in mature adipocytes (224).

1.5.6.2 Factors influencing lipid mobilization

The rate-limiting step of adipose-tissue lipolysis is the hydrolysis of TAG by HSL (224, 225) and is therefore one of the enzymes determining whole-body lipid fuel availability during the post-absorptive state. HSL is hormone dependent and mediates the hydrolysis of triglycerides when stimulated by catecholamine and natriuretic peptides (199) and inhibited by insulin (226). Catecholamine activates HSL and promotes release of fatty acids and glycerol up to 100 fold via binding to β1 and β2 adrenergic receptors (206), then activating adenylate cyclase via stimulatory G_s proteins causing a rise in cAMP levels and increased cAMP-dependent protein kinase-A (PKA) activity (227, 228).

1.5.6.3 Lipid storage

Lipoprotein lipase is a key enzyme regulating the disposal of lipid fuels in the body. LPL is expressed in a number of tissues and its regulation is tissue specific in a way based on the need for, and uptake of lipid fuels. LPL is also found in the circulation, but is often lipolytically inactive. The preferential action of LPL is the hydrolysis of chylomicron particles. LPL is synthesized within parenchymal cells and presented on the capillary endothelium allowing TG in TRL particles passing through the capillary to be hydrolysed and so that the fatty acids generated are taken up by parenchymal cells. This movement into cells seems to follow concentration gradients across the endothelium (229). LPL is expressed to different extents in different tissues. In the fed state, LPL activity is increased by six-fold in white adipose tissue and down regulated in skeletal muscle and heart so that fatty acids will be directed to adipose tissue for storage as triglycerides (113). In the postabsorptive state, however, this is reversed with upregulation in muscle and suppression in adipose tissue to enhance the delivery of fatty acids to the tissue in which they are needed as an oxidative fuel (113).

1.5.6.4 Factors influencing lipid storage

Several antilipolytic factors have been recognized as acting through inhibitory G_i protein-coupled receptors (230). These factors include catecholamines acting via α_2-adrenergic receptors (206), and other receptors including those for adenosine (A1-adenosine receptor) (231), prostaglandin E_2 (232) and nicotinic acid (GPR109A receptor) (233). Insulin and insulin-like growth factor represent the most potent inhibitory hormones in lipolysis (207, 234). Their effects are primarily communicated through insulin receptors, polyphosphorylation of insulin receptor substrates 1–4 (IRS1–4), activation of PI3K, the induction of Akt/protein kinase B, and activation of phosphodiesterase-3 (PDE) which degrades cAMP and suppresses HSL activity.

1.5.7 Insulin resistance in adipose tissue

Adipose tissue is responsible only for a relatively small proportion (<10%) of peripheral glucose utilization in response to insulin. Circulating factors such as TNF-α and FFA released from adipocytes appear to play a role in the development of adipocyte insulin resistance by inhibiting insulin signalling. Adipocytes are highly

sensitive to insulin and their resistance to it may have an important implication for glucose and lipid metabolism.

In insulin-sensitive adipocytes, insulin mediates glucose uptake by phosphorylation/activation of IRS proteins and stimulation of PI3K (104). In turn, PI3K activates downstream protein kinases, including Akt/PKB (235) and stimulates PDE so that more cAMP is metabolized in fat cells resulting in reduced lipolysis by HSL. In addition, insulin mediates effects on adipocyte differentiation, inducing genes involved in lipogenesis and repressing those involved in fatty acids release (104). In insulin-resistant adipocytes, the resistance to glucose metabolism is more marked than resistance to lipolysis.

In T2DM, adipocytes revealed a significant reduction in the cellular expression of the docking protein IRS 1 and as a result showed markedly lower insulin-stimulated PI3K activity in adipose tissue. Consequently, there was a marked reduction in downstream activation and serine phosphorylation of Akt/PKB by insulin (236). Similarly, impaired IRS-1 expression and attenuated activation of PI3K in response to insulin in adipocytes was found in the non-diabetic insulin resistant state (236, 237). A low expression of IRS-1 with low mRNA level in adipose tissue predicts IR and T2DM (237). Subjects with a family history of T2DM had impaired suppression of adipose tissue lipolysis and glucose uptake by insulin compared with healthy controls (238).

1.5.8 Adipose tissue and systemic insulin resistance

Adipose tissue is a major site of energy storage and an important determinant of overall insulin sensitivity. In the postprandial state, the increase in plasma insulin concentration promotes glucose uptake, glycogen synthesis, fatty acid synthesis and de novo triglyceride synthesis in adipocytes, whilst potently suppressing FFA release, in part, by inhibiting the activity of HSL. Insulin also activates LPL in adipose tissue and results in increased clearance of triglyceride-rich plasma lipoproteins. This process is facilitated by the simultaneous stimulatory effects of insulin on cellular triglyceride synthesis and on glycolysis, which generates glycerol-3-phosphate, a three-carbon molecule essential for incorporation of FFA into cellular triglycerides. Therefore, with the postprandial rise of insulin level, adipose tissue contributes to the maintenance of

normal plasma concentrations of glucose and FFA by storing excess fuel molecules in the form of triglycerides.

During the post-absorptive state, lipolysis with FFA release from adipose tissue is triggered by a fall in plasma insulin concentration together with the rise in levels of insulin counteracting hormones, such as glucagon, and increased sympathetic nervous system activity. FFA oxidation becomes a major source for energy in muscle, and FFA serves as a substrate for hepatic gluconeogenesis. If adipose tissue becomes insulin resistant and no longer fulfils its role as fat storage, other tissues will be exposed to excess levels of plasma glucose and FFA, and will have to store these fuel molecules as triglycerides in order to compensate for the adipose tissue dysfunction. The shift of triglyceride synthesis from adipose tissue to other tissue sites will ultimately reduce insulin sensitivity in insulin-target tissues, most notably liver and skeletal muscle.

1.6 Metabolic dyslipidaemia

In a large European cohort, fasting triglycerides were significantly correlated with fasting glucose and insulin levels after adjustment for the effects of age, sex, obesity and inter-centre variability in healthy subjects (239). It has been long recognized that fasting plasma triglyceride concentrations predict the magnitude and duration of postprandial lipaemia (240).

1.6.1 Physiological postprandial lipaemia

Physiological postprandial lipaemia is a transitory alteration in lipoprotein metabolism lasting from 6 to 12 h after ingesting a fatty meal. In normolipidaemic subjects, plasma triglycerides typically increase from a fasting level of 1.0 -2.0 mmol/L at 2–4 h after a meal (241). The postprandial rise of triglycerides reflects the accumulation TRLs in the circulation, intestinal derived Apo B48 containing chylomicrons and their remnants, and liver derived Apo B100 VLDL and VLDL remnants.

1.6.2 Postprandial dyslipidaemia

Postprandial dyslipidaemia refers to an increase in magnitude and duration of these lipoprotein responses, and involves quantitative and qualitative changes not only in chylomicrons and their remnants, but also endogenously synthesized lipoproteins.

TRLs accumulate in plasma and exchange triglycerides with cholesteryl esters from LDL-c and HDL-c. Under the action of lipolytic enzymes, triglyceride-enriched LDL-c and HDL-c particles become smaller, denser, more atherogenic and more susceptible to oxidation. These changes also include an increase in lipoprotein(a) with TRL (242). Either overproduction of intestinal and/or hepatic TRL or ineffective TRL clearance results in postprandial dyslipidaemia.

1.6.3 Factors contributing postprandial dyslipidaemia

1.6.3.1 Non-metabolic factors

Constitutional, environmental, hormonal and genetic factors can perturb postprandial lipoprotein composition and transport. Male sex (243), increasing age (244), obesity (245) and body fat distribution (246, 247) all affect the clearance of TRL indicating the importance of visceral fat and IR in postprandial hypertriglyceridaemia. The fat content of a meal influences postprandial lipaemia. For example, a meal rich in saturated fat impairs the clearance of postprandial TRL (248) and whilst others, such as polyunsaturated n-3 fatty acids enhances their clearance (249, 250).

Trained athletes cleared postprandial chylomicrons better than sedentary people (251). Endurance exercise decreased fasting and postprandial triglycerides by increasing catabolism of TRL and decreasing VLDL production (252). The effect of exercise on TG metabolism usually lasts for 48 h (253). Therefore, a 12 week endurance exercise did not improve lipaemic response to consumption of a high-fat mixed meal when these responses are determined 48 h after the last exercise bout (254).

Thyroxine therapy in subjects with hypothyroidism (255) and hormone replacement therapy in menopausal women (256) improve chylomicron clearance. Genetic factors have been shown to play a role in the regulation of postprandial lipoprotein metabolism including mutations involving variants of LPL (257), Apo B100 signal peptide (258) and Apo CIII (259).

1.6.3.2 Metabolic factors

Postprandial dyslipidaemia results from either overproduction of intestinal and/or hepatic TRL, ineffective TRL clearance due to decreased LPL activity, or defects in hepatic LDL receptors and LPL receptor like proteins.

Increased hepatic VLDL production is a fundamental defect in T2DM (260, 261) and visceral obesity (262-264), and is probably due to increased availability of substrates in the liver (263, 264). It is positively correlated with insulin, plasma glucose and FFA levels (265). Microsomal triglyceride transfer protein (MTP), which is essential for the synthesis of the chylomicron particles in the intestine, is increased in T2DM suggesting overproduction of intestinal chylomicrons (266).

Decreased VLDL catabolism towards IDL and LDL could increase postprandial TG in T2DM (260, 267, 268). Defective LDL receptor function could also lead to postprandial dyslipidaemia as LDL receptors are considered to be a primary route of remnant clearance. Insulin seems to enhance hepatic LDL receptor related proteins (269) and consequently insulin resistance interferes with hepatic clearance of lipids (270). Delayed and ineffective removal of chylomicron remnants by LDL receptors or LDL receptor related protein in the liver was found in T2DM (271) and postmenopausal women (272). A significantly low LDL-receptor binding of lipoproteins was found in viscerally obese men when compared with lean healthy controls (273).

Apo CIII inhibits LPL activity that hydrolyses chylomicrons and VLDL (274) and regulates TRL remnant uptake by hepatic lipoprotein receptors and increased plasma Apo CIII levels in obese subjects contributed to postprandial hypertriglyceridaemia (264). Increased hepatic VLDL production is recognised in both familial hypercholesterolaemia (275) and familial combined dyslipidaemia (276, 277).

1.6.4 Metabolic dyslipidaemia in PCOS

Dyslipidaemia is a common metabolic abnormality in PCOS (278) and prevalence has been reported to be different in PCOS sufferers from various ethnic and geographic backgrounds (279). According to National Cholesterol Education Program (NCEP) guidelines, approximately 70% of PCOS patients exhibit abnormal serum lipid levels

(280). The most common lipid abnormalities in PCOS are low HDL-c and high triglycerides (66). It has been reported that almost all women who have both PCOS and metabolic syndrome, demonstrate decreased HDL-c whereas more than half have increased TG levels (281). Both obese and lean women with PCOS are associated with higher total cholesterol and LDL-c than weight matched controls (65).

The prevalence of metabolic dyslipidaemia increases two-fold in insulin resistant PCOS and is strongly correlated with HOMA-IR (282), central obesity (283) and hyperandrogenism (66) independent of BMI. Women with PCOS show significantly higher values of the TG/HDL-c ratio that is closely related with waist circumference and IR (284). Postprandial dyslipidaemia is also common in obese women with PCOS (285).

1.6.5 Metabolic syndrome in PCOS

PCOS shares several traits with the metabolic syndrome, including IR (12), obesity (54), hypertension (70), IGT (21) and dyslipidaemia (286). The prevalence of the metabolic syndrome in PCOS is approximately 43-47%, a rate 2-fold higher than that for women in the general population (287) but this can vary from 3% to 50% depending on BMI (288) and geographic background (28).

Ehrmann DA et al. analysed the prevalence and predictors of metabolic syndrome in 394 PCOS women. More than two thirds met the NCEP criterion of WHR and low HDL-c levels, and one third met the high triglyceride levels. Overall, 33% of PCOS had metabolic syndrome in their study (66).

In an adolescent study, metabolic syndrome was present in 11% of overweight, and 63% of obese PCOS compared with 0% and 32% respectively for those age-matched girls without PCOS. The incidence of metabolic syndrome was still 4.5 times higher in PCOS than controls after adjusting for BMI. None of the girls with normal BMI had metabolic syndrome. Hyperandrogenaemia was found to be a risk factor for metabolic syndrome independent of obesity and IR (29). Metabolic syndrome is associated with increased CV morbidity and mortality (289). Being high prevalence of metabolic syndrome in PCOS may increase the risk of diabetes and CVD.

1.6.6 Metabolic dyslipidaemia and CV risk

Hypertriglyceridaemia is an independent risk factor for CVD for both men and women in the general population (290, 291). A 1-mmol/L increase in triglycerides is associated with a relative risk increase of 14% in men and of 37% in women for the incidence of CVD (292). A triglyceride level as low as 2.25 mmol/L was shown to be associated with increased CVD mortality in a 14 year follow up study (293). The association between triglycerides and risk of CVD is intact in women at all ages (294). However, the nature of the link between hypertriglyceridaemia and CVD is not well understood. Hypertriglyceridaemia may play an indirect role in atherosclerosis (295, 296) as a central component of the "atherogenic dyslipidaemia triad" together with low HDL-c (297, 298), a preponderance of small dense LDL-c (299) and association with central obesity and IR (300, 301).

Fasting triglycerides are preferred as they are reproducible but this is only representative of a small part of the lipid profile since for most of the time an individual would be in the postprandial state. In 1979, Zilversmit first proposed that TRL seen in postprandial hypertriglyceridaemia plays an independent role in atherosclerosis (302). Postprandial hypertriglyceridaemia contributes to endothelial dysfunction (303-306), and suggests a role for triglycerides in the initiation and progression of atherosclerosis and CAD (242, 303, 307, 308). The Physicians Health Study and the Multiple Risk Factor Intervention Trial reported that elevated postprandial triglyceride levels independently increase the incidence of myocardial infarction by 40% per 1.1 mmol/L increase (309). Postprandial triglyceride levels appear to be independent but highly correlated with the angiographic progression of coronary atherosclerosis and carotid IMT in T2DM (310). Patients with coronary artery disease often have increased postprandial triglyceride levels compared with healthy control subjects, and it has been demonstrated that postprandial hypertriglyceridaemia is an independent predictor of coronary artery disease (311, 312).

1.7 Non Esterified Fatty Acids (NEFA) and insulin resistance

1.7.1 Non-esterified fatty acids

NEFA circulate in the plasma bound to plasma albumin. In 1956, Vincent Dole first demonstrated a relationship between NEFA and the metabolism of glucose. Insulin and glucose drastically lower NEFA levels postprandially but little effect is seen with a fat only meal (313). Later in the year, Gordon RS Jr reported that plasma levels of NEFA fluctuate rapidly and markedly from time to time in normal individuals. Levels tended to rise after the ingestion of fat, but rise similarly on fasting so that lipaemia per se could not be correlated with an increase in NEFA. Dietary carbohydrate and insulin production lead to a uniform, dramatic fall in NEFA levels but dietary proteins have a lesser effect (314). It was suggested at that time that adipose tissue maintains calorific homeostasis by liberating more or less NEFA into the circulation. Since then, NEFA is recognised to be acting as a vehicle for the transport fatty acids from adipose tissue to myocardium, skeletal muscle and viscera (315).

1.7.2 Mobilization of NEFA

Adipose tissue is the only significant site for the secretion of NEFA into plasma. Although intra-abdominal adipose tissue is the most lipolytically active tissue depot (316), it contributes very little to whole-body fatty acid flux (317) as it mainly delivers NEFA to the liver for VLDL synthesis. Abdominal subcutaneous fat is the dominant source of NEFA whilst considerably less comes from adipose tissue in the leg or abdomen (196, 318).

Details of FFA mobilization and storage have been mentioned in section 1.5.6.1, 1.5.6.3. Briefly, plasma NEFA arises almost entirely from hydrolysis of triglycerides within adipocytes via HSL during the post-absorptive period. In the postprandial state, LPL hydrolyses circulating chylomicrons and VLDL, and the released are taken up into adipocytes for storage, and skeletal muscle for oxidation. However, some FFA joins the plasma NEFA pool in a process referred as spill-over (319, 320). Spill-over FFA may constitute 40–50% of the total plasma NEFA pool in the postprandial period (321).

1.7.3 Circulating NEFA levels

NEFA turnover is rapid, with a plasma half-life approximately 2-4 min (318). Circulating NEFA levels vary with time, physical activity and fed state being high in the morning after overnight fast or even higher after prolonged fasting (314). Moderately intense exercise (322, 323) or stress (324, 325) can lead to a rise in NEFA. Women have higher NEFA levels than men (326, 327). In a large European cohort, NEFA at steady state was positively correlated with fasting plasma glucose, plasma insulin and triglycerides, and negatively correlated with the rate of glucose disposal in healthy subjects (239).

Increased fasting NEFA observed in T2DM is highly correlated with fasting insulin, fasting glucose, hepatic glucose production and lipid oxidation. Insulin infusion and oral hypoglycaemic agents improve NEFA levels along with other parameters during the post-absorptive state (328-335). However, a higher insulin level is required to suppress NEFA, lipid oxidation and hepatic glucose production in T2DM compared to controls (336-338).

The association of high NEFA levels with obesity is still controversial. Some studies have reported that obese subjects had high NEFA levels despite hyperinsulinaemia (339, 340). In other studies, ambient plasma NEFA levels have not been different between obese and non-obese women (341). In a recent review, ambient plasma NEFA levels do not correlate with an increased fat mass (342). This is probably due to down regulation of rate of delivery of NEFA in response to expansion of adipose tissue in obesity (321).

1.7.4 Effect of NEFA on glucose metabolism

In 1963, Randle et al. first described the glucose-fatty acid cycle that introduces the pathophysiological effect of NEFA on glucose metabolism. The authors suggested that increased availability of NEFA enhances fat oxidation and decreases glucose uptake and its utilization in striated muscle (343-346). In 1964, it was discovered that an intravenous injection of heparin enhances the release of circulating NEFA and consequently increases glucose levels in healthy controls during an oral glucose tolerance test but the effect of heparin is very brief (347). Felber and Vannotti et al.

first reported that continuous fat infusion causes glucose intolerance in normal subjects (348). Subsequent studies investigated the effect of an elevated level of NEFA on glucose metabolism using lipid and heparin infusion with hyperinsulinaemic euglycaemic clamps. However, such studies should be interpreted with caution since pharmacological rather than physiological concentrations of insulin were used that might have had an effect glucose homeostasis.

Acute elevation of NEFA either by lipid infusion or by a high fat meal induces IR within 2–4 h which disappears within 4 h after normalization of FFA levels (349). Induction of NEFA by these methods decreases skeletal muscle glucose uptake (350, 351), inhibits insulin suppression of hepatic glucose production (HGP) (352-354), increases total body fat oxidation and decreases insulin stimulated glucose oxidation as reported following a 2 h lipid infusion test in healthy subjects (355-362)

1.7.5 Effect of elevated NEFA on beta cell function

Acute exposure to elevated NEFA enhances glucose and non-glucose stimulated insulin secretion in vitro (363), in animal (364) and human studies (365-368). Boden et al. showed that a significant rise in NEFA increases levels of beta-hydroxybutyrate and C-peptide. When compensatory insulin release is prevented by concurrent use of somatostatin, a rise in NEFA is associated with an increase in plasma glucose secondary to increased hepatic glucose output and a decrease in glucose disposal. Therefore, lipids have an adverse effect on carbohydrate metabolism unless there is a compensatory increase of beta cell function (369).

Prolonged exposure of more than 24 h to elevated NEFA leads to a reduced glucose stimulated insulin secretion *in vitro* associated with increased triglyceride content in pancreatic islet cells (370). However, absolute glucose stimulated insulin secretion was increased (371-373), unchanged (365, 374-377) or decreased (368, 378) in response to prolonged exposure of elevated NEFA in human studies. This inconsistency is probably related to 1) variability in study subjects such as obesity and genetic predisposition to diabetes such as the presence of IGT, T2DM and family history of DM and 2) decreased insulin sensitivity. A consistent finding in all studies is that prolonged exposure to NEFA significantly decreases insulin sensitivity and impairs the

disposition index suggesting inadequate beta cell compensation to lipid induced hepatic and skeletal muscle IR.

In summary, NEFA/lipid and glucose metabolism are interrelated with excess NEFA promoting lipid oxidation, enhancing basal hepatic glucose production, inhibiting insulin mediated and non-insulin mediated glucose uptake and oxidation in the skeletal muscles. These effects result in hyperglycaemia if the beta cells fail to compensate by increased insulin secretion. Obese people and those with a family history of diabetes are more susceptible to lipid induced beta cell dysfunction and IR than healthy subjects. Lipid induced impairment of beta cell function might not be significant in frankly diabetic patients with little remaining islet cell reserves (379).

1.7.6 Effect of lowering NEFA on glucose metabolism

Fasting NEFA level is strongly related to the degree of fasting hyperglycaemia in patients with T2DM (331, 380, 381). Lowering NEFA in diabetic rats significantly improved fasting glucose by enhanced insulin stimulated glucose uptake (382). Although there is strong evidence that elevated NEFA interferes with glucose metabolism, lowering NEFA by acipimox has not consistently improved plasma glucose level in human studies. It is probably related to a difference in dose or timing of acipimox treatment. In subjects with T2DM, either a single dose, or three day treatment of acipimox did not improve plasma glucose or insulin levels although it effectively lowered plasma NEFA and increased insulin stimulated oxidative and non-oxidative glucose disposal and reduced lipid oxidation (383, 384). In contrast, effective lowering of NEFA has improved glucose disposal and plasma glucose level in GH deficient adults (385). The use of acipimox every 2 h with 6 doses, lowered NEFA by 20% during a 24 h fast which was followed by an increase in disposition index of 31% in healthy subjects (386).

1.8 Overview of exercise and fatty acids metabolism

1.8.1 Exercise and fatty acids metabolism

Adipose tissue triglycerides are the main source of fuel to meet energy demands during exercise. A coordinated system is required to regulate lipolysis, blood flow and fatty acid transport in skeletal muscle and enhance the delivery of released fatty acids

from adipose tissue to the mitochondria of working muscle thus providing a sustained energy supply during exercise.

1.8.2 Substrate utilization during exercise

During exercise, skeletal muscle utilizes energy derived from adipose tissue triglycerides, plasma glucose, muscle glycogen and intramyocellular triglycerides (IMTG) and possibly plasma triglycerides. Utilization of substrate sources as a fuel depends upon the intensity and duration of exercise. Romijn et al. studied the effect of exercise intensities with 25% (mild), 65% (moderate), and 85% (high) of maximal oxygen consumption (VO_2 max) on fat and glucose oxidation. Plasma glucose tissue uptake and muscle glycogen oxidation increased in relation to exercise intensity. In contrast, plasma FA derived from adipose tissue lipolysis was at maximum use at the lowest and moderate exercise intensities but not in high intensity exercise. Instead, muscle triglycerides were used mainly in high intensity exercise (322). Even during low-intensity exercise, adipose tissue lipolysis (measured as the rate of appearance of glycerol) increased 2 - 5 fold above resting levels (322, 387-389). The rate of appearance of fatty acids increased starting at the low intensity exercise and became progressively higher throughout exercise at 65% VO_2 max but declined in high intensity exercise. At the same time, the rate of re-esterification of released fatty acids fell and therefore, a greater proportion of released fatty acids were delivered to the skeletal muscle for oxidation (388).

1.8.3 Source of fatty acids supply during exercise

Abdominal subcutaneous fat contributes fatty acids to the skeletal muscle mainly during exercise (196). Lipid sources other than triglycerides stored in adipose tissue also contribute to fatty acid oxidation during endurance exercise. Skeletal muscle contains significant quantities of lipids stored as triglyceride droplets within the muscle, known as intra- or extra-myocellular triglycerides (IMTG or EMTG respectively). Some previous studies suggested that IMTG provides as much as 10–50% of total fat oxidation during exercise (390, 391). Endurance exercise training promotes total whole body fat oxidation as well as increased lipolysis of IMTG (392). Moreover, in aerobically trained individuals, IMTG is thought to serve as an important fuel source

during prolonged moderate-intensity exercise (322, 389). Plasma triglycerides are another potential source of fuel particularly during aerobic exercise (393).

1.8.4 Lipolysis during exercise

During exercise decreased plasma insulin increases adipose tissue lipolysis by enhancing HSL activity (394, 395). Exercise also increases circulating catecholamines, in particular β-adrenoceptor stimulation, which overrides α_2-mediated inhibition and increases the lipolytic rate (196).

1.8.5 Fatty acids transport for beta oxidation

Transport of fatty acids across the plasma and mitochondrial membranes is essential for fatty acid oxidation in skeletal muscle. Since intramuscular storage sites are a limited source, skeletal muscle relies heavily upon a continuous supply of exogenous long chain fatty acids (LCFA) derived mainly from adipose tissue particularly in the post-absorptive state and during exercise. Because of their hydrophobic nature, LCFA can enter cells by passive diffusion along a concentration gradient at the sarcolemma. However, recent studies have shown that LCFA transfer between intracellular membranes is facilitated by binding to soluble fatty acid-binding proteins (FABP)(396). Once LCFA passes from the sarcolemma through the cytoplasm to the outer mitochondrial membrane, the site of acyl-CoA synthetase, this enzyme converts LCFAs into acyl-CoA to make it available for TG synthesis and mitochondrial beta oxidation. Acyl-CoA crosses the mitochondrial outer and inner membranes mediated by carnitine palmitoyl-transferase I (CPT I) for beta oxidation (397).

Three, putative LCFA transporters all expressed in skeletal muscle are fatty-acid translocase (FAT/CD36), plasma membrane-associated fatty-acid binding protein (FABPpm) and fatty-acid transport proteins (FATP). However, only FABPpm and FAT/CD36, but not FATP1, correlate with vesicular LCFA transport across the plasma membrane. LCFA transport can be increased by increased amount of FAT/CD36 protein in muscle (chronic adaptation) or via the translocation of FAT/CD36 from an intracellular pool to the plasma membrane during muscle contraction (acute adaptation) (193). The transport of LCFA across mitochondrial membranes is regulated mainly by CPT I activity. FAT/CD36 influences not only LCFA transport

across the plasma membrane, but also LCFA transport into mitochondria (398).

High intensity exercise training increases FAT/CD36 at the whole muscle plasma membrane and in mitochondria and fatty acid binding proteins in the muscle plasma membrane and subsequently increases skeletal muscle fatty acid oxidation (399). Similarly, endurance cycling at VO_2 max 60% induced an increase in plasma membrane FAT/CD36 and FABPpm content in human skeletal muscle, which is predicted to increase fatty acid transport (195).

1.8.6 Moderate intensity exercise

During moderately intense exercise, lipolysis increases approximately three fold in response to an increased β adrenergic stimulation. Furthermore, blood flow to adipose tissue is doubled and the rate of re-esterification is halved (322, 388). There is also a dramatic increase in blood flow to skeletal muscle that enhances fatty acid delivery to muscle. During the first 15 min of exercise, plasma fatty acid concentrations usually decrease because their rate of uptake by muscle exceeds their rate of production from lipolysis. Thereafter, their rate of production is in excess of their use by muscle, and then levels increase. The rise in plasma fatty acids depends on the intensity and the duration of exercise. During moderate exercise, the levels may reach 1 mmol/L within 60 min. Although a large variation is observed between individuals, fat oxidation is high over a wide range of exercise intensities, being highest at 64% VO_2 max but declines rapidly at high intensities (>80% VO_2 max) (400). Elevated fatty acid levels after lipid infusion during low- and moderate-intensity exercise results in a 4-fold increase in leg fatty acid uptake and a 23% reduction in glycogenolysis (401). However, there was no concomitant increase in fatty acid even oxidation during high intensity exercise when increased by lipid infusion (217).

1.8.7 Endurance exercise training

Endurance exercise training increases the use of fat as a fuel (402). However, this increase in fat oxidation is not the result of increased lipolysis of adipose tissue triglycerides. Lipolytic rates are similar in endurance-trained athletes and untrained volunteers during exercise performed at the same absolute intensity (389). In a prospective study, endurance training for 12 weeks did not increase adipose tissue

lipolysis although there was an increase in whole body fat oxidation (403). Moreover, endurance training did not affect whole-body lipolytic sensitivity to a physiological range of catecholamine concentrations (404, 405). In fact, this increase in fat oxidation without an increased lipolytic rate improved the coordination between fatty acid availability and oxidation, limiting the amount of fatty acids that are released into the circulation but not oxidized.

1.9 Skeletal muscle insulin resistance

1.9.1 Competition between fatty acids and glucose

In 1963, Randle et al. introduced the concept of substrate competition, whereby, enhanced availability and oxidation of FFA leads to impairment of glucose oxidation in isolated rat hearts and hemi-diaphragms. It was subsequently proved by the same group that increased availability of FFA enhances fat oxidation and decreases glucose uptake and utilization in striated muscle via an increase in mitochondrial acetyl-CoA/CoA ratio and inhibition of pyruvate dehydrogenase (343-346). During a hyperinsulinaemic euglycaemic clamp in healthy subjects, lipid infusion increases insulin inhibited fat oxidation by 40% and decreases insulin stimulated glucose oxidation by 63% within an hour and these effects are reversible. These changes are followed by a reduction in glucose infusion rate along with a decrease in glucose disappearance 2 h after initiation of lipid infusion. The reduction in glucose disposal is associated with a decreased muscle glycogen synthase fractional velocity and increased muscle acetyl CoA and acetyl-CoA/free CoA-SH ratio. This suggests that lipid promptly replaces carbohydrate as fuel for oxidation in muscle and hours later inhibits glucose uptake, presumably by interfering with muscle glycogen formation (349).

1.9.2 Effect of fatty acids on skeletal muscle insulin signaling

In 1999, lowered glucose oxidation and muscle glycogen synthesis induced by lipid infusion was associated with decreased intramuscular glucose-6-phosphate concentration and insulin-stimulated PI3K activity. These findings, in humans, implied that high levels of plasma FFA induce IR by inhibiting glucose transport activity probably via decreased IRS-1-associated PI3K activity (406, 407), but not by inhibition of pyruvate dehydrogenase activity (346).

Subsequent studies confirmed that acutely raised NEFA levels are associated with a significant reduction in (IRS)-1-associated PI3K activity, blunted insulin-stimulated IRS-1 tyrosine and produced a four-fold increase in membrane-bound, or active, protein kinase C (PKC) theta (408, 409). Activated PI3K is required to activate downstream protein kinases, including Akt/PKB (235) which in turn enhances GLUT4 for insulin stimulated glucose uptake into muscle and deactivates glycogen synthase kinase3 (GSK3). Therefore, lowered PI3K activity eventually interferes with glucose transport, and deactivates glycogen synthase resulting in reduced glycogen synthesis. By this mechanism high NEFA induces skeletal muscle IR.

1.9.3 Effect of intramyocellular lipids on insulin resistance

1.9.3.1 IMTG and obesity

Lipids are stored not only in adipocytes but also 'ectopically' in tissues such as muscle, liver, beta cells and other sites. Factors determining FFA uptake, lipolysis and lipogenesis influence IMTG stores in skeletal muscle. These are an accessible form of energy that may decrease skeletal muscle glucose uptake, thereby contributing to impaired glucose metabolism.

IMTG is associated with a percentage of body fat and FFA levels in all subjects. In obese adolescents, an increase in total body fat and central adiposity is accompanied by higher IMTG and EMTG stores when compared to non-obese adolescents suggesting ectopic lipid deposition is not age related. IMTG content is positively correlated with the degree of visceral fat, and both are inversely related to insulin sensitivity (410, 411). Patients with PCOS had a similar total and trunk fat mass but increased central abdominal fat when compared with weight-matched controls (412). Therefore, PCOS women may well have high IMTG content although this has not been studied so far.

1.9.3.2 IMTG levels and insulin resistance

High levels of IMTG in skeletal muscle are associated with IR. IMTG levels are correlated positively with measures of obesity but negatively with insulin sensitivity (413). In addition, intra-lipid infusion increases IMTG content and is accompanied by a significant increase in IR in both controls and subjects with IGT (414, 415). Chronically elevated circulating FFA levels coupled with reduced lipid oxidation in obese and

T2DM patients (416) or in obese skeletal muscle (417) result in excessive IMTG content. However, increased IMTG content is not only prevalent in insulin resistant obese subjects with or without diabetes, but also in insulin-sensitive endurance-trained subjects. Similarly, whole body as well as leg insulin sensitivity in women is found to be higher than in age matched men despite having higher IMTG levels than men (418). Therefore, increased IMTG content alone does not explain IR in skeletal muscle.

1.9.3.3 IMTG turnover and insulin resistance

Turnover rather than amount of IMTG plays a major role in IR. IMTG turnover is a composite measure of the dynamic balance between lipolysis and lipid synthesis; both are influenced by mitochondrial fat oxidation and plasma FFA availability. A higher oxidative, lipolytic, and lipid storage capacity in muscle of endurance trained subjects reflects a higher fractional turnover of the IMTG pool. This high turnover rate of the IMTG pool is considered to reduce accumulation of lipotoxic intermediates interfering with insulin signalling in athletes. Thus, the co-localization of inter-myofibrillar lipid droplets and mitochondria allows for a fine coupling of lipolysis of the IMTG pool with mitochondrial β-oxidation. Conversely, reduced oxidative capacity and a mismatch between IMTG lipolysis and mitochondrial β-oxidation might be detrimental to insulin sensitivity by generating several lipotoxic intermediates in sedentary populations including obese/T2DM subjects (419).

1.9.3.4 Intra-myocellular lipid metabolites and insulin resistance

Accumulation of lipid metabolites rather than increased IMTG content plays a significant role in skeletal muscle IR by interfering with insulin signalling and glucose uptake (420, 421). Fatty acids accumulate intra-myocellularly as LCFA acyl CoA that is either used for energy production via β-oxidation or converted to various lipid metabolites namely, MAG, DAG, phosphatidic acid, TAG and ceramides, by de novo synthesis, through phospholipase C activation or phospholipid hydrolysis (422). Among these fatty acid derivatives, high intra-myocellular levels of DAG, TAG and ceramides are directly associated with IR (423-425). The mechanism is illustrated in **Figure 1-1**. Schenk et al. demonstrated that one session of exercise completely decreased the accumulation of highly bioactive fatty acid metabolites and then reversed fatty acid-induced IR in healthy subjects (426).

1.9.3.5 IMTG and endurance exercise

Aerobic exercise training decreases the amount of lipid products and increases lipid oxidative capacity in skeletal muscle cells. Thus, aerobic exercise training may prevent IR by correcting a mismatch between FFA uptake and FFA oxidation in skeletal muscle. A single session of aerobic exercise increases glucose uptake by skeletal muscle during exercise, increases the ability of insulin to promote glucose uptake, and increases glycogen accumulation after exercise, all of which are important for blood glucose control (427). Eight weeks of moderate intensity exercise reduced IMTG and IR in subjects with T2DM (428).

1.9.4 Mitochondrial fatty acid oxidation

Impairment of fatty acid oxidation/utilization may lead to the accumulation of fatty acid metabolites and contribute to the aetiology of skeletal muscle IR. Therefore, normal mitochondrial oxidative phosphorylation is required in order to prevent excessive accumulation of IMTG and their metabolites. In women with PCOS, the impaired insulin-stimulated total oxidative and non-oxidative glucose disposal are associated with a consistent down regulation of OXPHOS (mitochondrial oxidative phosphorylation) gene expression in skeletal muscle that couples with reduced levels of peroxisome proliferator-activated receptor, gamma co-activator alpha (PGC-1alpha) that cannot be ascribed to obesity and diabetes (429). Bruce et al. illustrated that moderate intensity exercise increases mitochondrial FA oxidation, decreases DAG and ceramides content of skeletal muscle but had no effect on IMTG content in obese subjects (430).

1.9.5 Endurance exercise and skeletal muscle insulin resistance

Endurance exercise improves skeletal muscle insulin sensitivity by increasing fatty acids oxidation. Endurance exercise improves skeletal muscle insulin signal modulation by increasing GLUT4 protein concentration and increasing the activities of glycogen synthase and hexokinase, the enzyme that phosphorylates glucose (431, 432). Reductions in lipid metabolite concentrations may partly explain the improvements in GLUT4 translocation and activities of hexokinase and glycogen synthase. Endurance training for 12 weeks improves mitochondrial biogenesis and electron transport chain

activity in older persons (433). Endurance training may increase the resistance of skeletal muscle to injuries caused by lipid peroxidation (434).

1.9.6 Exercise and cardiovascular risk modification

In 1953, Morris J et al. first reported that exercise training is associated with decreased incidence of CAD in London bus driver teams (435). Several studies have demonstrated powerful positive associations between sedentary lifestyle/low cardio-respiratory fitness and poor health outcomes such as T2DM, CVD mortality and all-cause mortality (436-438). In a systematic review and meta-analysis of 33 cohort studies consisting of 883,372 participants, Nocon et al. (439) reported that physical activity was associated with a risk reduction of 35% for CVD mortality and 33% for all-cause mortality. In their analysis, studies that objectively measured physical fitness was associated with a larger risk reduction compared with self-reported physical activity because participants overestimated their physical activity levels in self-reports. In addition, inverse dose–response relations have been found between the volume of physical activity behaviour and all-cause mortality (440, 441), CVD mortality (441, 442) and risk of CAD (443, 444). Similarly, light-to-moderate activity is associated with lower CAD rates in women. For walking paces of less than 2.0 mph (mile per hour), 2.0-2.9 mph, and 3.0 mph or more, compared with no regular walking, relative risk for CVD is significantly reduced by more than 50%. When analysed simultaneously, time spent walking rather than walking pace predicted lower risk. At least 1 hour of walking per week predicted lower risk in women (445).

In addition, previous studies strongly support the role of lifestyle intervention involving physical activity to improve glucose and insulin homeostasis and subsequent risk of diabetes and CVD (218, 446). The Diabetes Prevention Program Research Group demonstrated that a lifestyle modification program with goals of ≥7% weight loss and ≥150 min per week of physical activity in overweight patients with impaired fasting glucose, resulted in a 58% reduction in the incidence of T2DM, whereas there was a 31% reduction with metformin (850 mg twice daily) compared with placebo (447). On the basis of these findings, the 2009 AHA (American Heart Association) Scientific Statement on exercise training in T2DM recommends 150 min per week of moderate intensity exercise combined with resistance training (448).

The relation of physical activity and cardiovascular events was examined in a 24 year follow up of 1166 men in the Framingham study. In the study, increased level of physical activity except physical demands of the jobs improved overall, cardiovascular and coronary mortality at all ages including elderly. The effect was sustained with a more pronounced effect with the passage of time, despite presumed decrease in level of activity (449). Women who were more active live longer but this effect as not related with decreased CVD (450).

Exercise training improves FMD in obese subjects with IR and in menopausal women (451, 452). It also reduces sICAM-I and PAI-1, markers of endothelial dysfunction in obese subjects with IR syndrome (452).

In summary, lack of physical activity is associated with poor CV morbidity and mortality, and all-cause mortality. Moderate intensity exercise improves risk of diabetes and CV outcomes.

1.10 Platelet and cardio-metabolic diseases

Platelets are produced by megakaryocytes as anucleate cells and their half-life in the circulation is 10 days. Platelets play a central role in coagulation, in maintenance of haemostasis, and in the pathophysiology of thrombotic diseases.

1.10.1 Platelet Activation

In response to a blood vessel injury, platelets accumulate at the site, recruit other platelets, promote clotting, and form a haemostatic plug to stop bleeding. Activation of platelets has long been recognized as one of the essential steps in the genesis and propagation of atherothrombosis. On activation, platelets release dense granules that contain the nucleotide adenosine 5'-diphosphate (ADP), which activates other platelets. They also possess alpha granules, which contain proteins such as P selectin, and protein mediators such as platelet-derived growth factor (platelet factor 4) that are involved in inflammatory processes (453). Platelets possess an affinity for adherence, especially to injured vessel walls, where they release their granule contents and then aggregate. These properties promote platelets' involvement in many vascular processes, including CAD.

1.10.2 Mechanism of platelet activation

Platelets exist in a non-activated state and are drawn passively into areas of vascular injury. The initial tethering of platelets at sites of vascular injury is mediated by glycoprotein Ib/V/IX, a structurally unique receptor complex expressed in platelets. Von Willebrand factor (vWF) which is large extracellular matrix protein produced by endothelial cells is the major ligand for one component of this complex, glycoprotein Ib (454). VWF is essential for initial tethering and translocation of platelets at the site of vascular injury. After the initial adhesion of platelets to exposed endothelium via vWF, the vWF exposed on the surface of immobilised platelets binds to the glycoprotein Ibα on the surface of free-flowing platelets (455). The subsequent recruitment of additional platelets into a growing platelet thrombus requires mediators such as ADP, thromboxane A_2 (TXA_2) or thrombin, which act through G protein-coupled receptors. Platelet activation via G protein-coupled receptors involves 3 major G protein-mediated signalling pathways that are initiated by the activation of the G proteins G(q), G(13), and G(i) (456). The final pathway for all agonists is the activation of the platelet integrin, glycoprotein IIb/IIIa (αIIbβ3), the main receptor for adhesion and aggregation (455).

1.10.3 Factors influencing platelet activation

Platelet activity is normally tightly controlled by a balance between platelet activation mediators such as TXA_2, thrombin and ADP, and endothelial and platelet derived inhibitors such as prostacyclin (PGI_2) and nitric oxide (NO) (457, 458). When this regulation is disrupted, it can lead to inappropriate platelet activation and subsequent expansion of thrombus and cardiovascular events.

1.10.4 Platelet activators

TXA_2 is synthesized by activated platelets from arachidonic acid (AA) through the cyclooxygenase (COX) pathway. Once formed, TXA_2 binds to G protein coupled receptors, TPα and TPβ, on the platelet surface and then leads to increase cytosolic levels of Ca^{2+}, which is released from the endoplasmic reticulum.

Thrombin is rapidly generated at sites of vascular injury from circulating prothrombin and, besides mediating fibrin generation, represents the most potent platelet activator. Platelet responses to thrombin are largely mediated through G-protein–linked protease-activated receptors (PARs), leading to increased intracellular Ca^{2+} and decreased cAMP.

ADP is released from platelets and red cells. Platelets express at least two ADP receptors, P_2Y_1 and $P_2Y_{12,}$ which couple to Gq and Gi, respectively. The P_2Y_{12} receptor is the major receptor able to amplify and sustain platelet activation in response to ADP. The activation of P_2Y_{12} inhibits adenylate cyclase, causing a decrease in the cAMP level, and the activation of P_2Y_1 causes an increase in the intracellular Ca^{2+}level.

The effects of agonists mediated by the decrease in cAMP levels and increase in intracellular Ca^{2+} levels lead to platelet activation through the conformational change in the ligand-binding properties of the glycoprotein IIb/IIIa (αIIbβ3), which acquires the ability to bind soluble adhesive proteins such as fibrinogen and vWF (457).

1.10.5 Platelet Inhibitors

The vascular endothelium controls platelet reactivity by means of three pathways: the arachidonic acid–prostacyclin pathway, the l-arginine–nitric oxide pathway, and the endothelial ectoadenosinediphosphatase (ecto-ADPase) pathway (459).

Vascular endothelial cells, under basal conditions and in response to various vasoactive agents, synthesize and release PGI_2 and endothelial derived relaxing factor, NO, two of the most important platelet inhibitors. PGI_2 in endothelial cells is synthesized from arachidonic acid released from membrane-bound lipids via the calcium dependent enzymatic actions of phospholipase A_2 (460). Arachidonic acid is metabolised by cyclooxygenase (COX) into prostaglandin endoperoxides PGG_2 and PGH_2. COX-1 is constitutively expressed and predominant isoform in healthy endothelial cells, whilst COX- 2 isoform is largely associated with pathological functions (461). Endothelial cells are enriched in COX-1 and PGI_2 synthase, which is why, when phospholipase A_2 is activated, prostacyclin is the predominant metabolite made. By way of a comparison, it is important to note that in platelets, which also express predominantly COX-1, thromboxane is the principal product made. This is because platelets express mainly

thromboxane synthase (462) with negligible levels of PGI_2 synthase.

The inhibition of platelet activation caused by PGI_2 and NO is mediated by cAMP and cyclic guanosine 3', 5'-monophospate (cGMP) dependent protein kinases (PK), respectively. Human platelets contain high concentrations of cAMP-PK and cGMP-PK. Elevation of cAMP by cGMP inhibition of the phosphodiesterase-3 contributes to the well-known synergism between cGMP and cAMP elevating platelet inhibitors. The protein kinases cause a decrease in intracellular Ca^{2+} flux by which suppresses the conformational change in glycoprotein IIb/IIIa that is required for binding of the integrin to fibrinogen, thereby decreasing the number and affinity of fibrinogen binding sites on the platelet's surface (463). The physiological effects of cyclic nucleotide elevating platelet inhibitors are terminated by cyclic nucleotide degrading phosphodiesterase and protein phosphatases.

1.10.6 Platelet aggregation

Platelet aggregation serves complementary to activation of the coagulation cascade. As a result of initiating the coagulation cascade, thrombin is produced, which cleaves fibrinogen to fibrin and results in the formation of stable complex thrombi. Additionally, thrombin, by interacting with PAR receptors, amplifies platelet activation and smooth muscle cells proliferation, recruitment of inflammatory cells, macrophages. Therefore, thrombin favours the development of atherothrombosis.

Once activated, platelet release chemokines, cytokines and immunomodulatory ligands contribute to endothelial activation, mediate the inflammatory responses, and exert immunomodulatory activity. These substances act in together to mediate a wide range of functions including cell adhesion, activation, aggregation, chemotaxis, cell survival, proliferation and coagulation (464). Platelet factor4, CD40 ligand, and interleukin 1alpha are some of the key molecules released from activated platelets that promote endothelial inflammation (465).

1.10.7 Effect of platelet dysfunction

Activated platelets appear to be the common thread that links inflammation, thrombosis and atherogenesis (464). Platelets are involved both in the early stages of atherosclerosis namely vascular smooth muscle cells chemotaxis, migration to the

intima and proliferation, and in the advanced lesions: after plaque formation, they contribute to vessel occlusion by promoting vasoconstriction, activation of the coagulation system and intravascular thrombus formation (466).

1.10.7.1 Atherosclerosis and platelet activation

Increased platelet activation in the presence of endothelial dysfunction enhances atherosclerosis. The discontinuity of the endothelial surface is not an essential prerequisite for functionally relevant interactions of platelets with vascular endothelial cells. For example, higher shear stress induces the exposure of platelet receptors and the triggering of the aggregation cascade (467). In addition, chronic exposure to risk factors such as smoking, hyperlipidaemia, insulin resistance, hypertension and diabetes also induces endothelial dysfunction, down regulates NO and PGI_2 and promotes expression of adhesive molecules such as fibronectin, ICAM-1, P selectin, E selectin and vWF (468). These enhance subsequent platelet adhesion and activation. At low shear rates, platelet adhesion occurs mainly through collagen receptor binding to platelet receptors. These stimuli induce a limited platelet deposition that facilitates the progression of atherosclerosis.

1.10.7.2 Thrombosis and platelet activation

In the context of endothelial denudation (erosion) and atherosclerotic plaque rupture, exposure of components of the vascular matrix to the bloodstream triggers extensive platelet adhesion and activation that eventually leads to aggregation and thrombus formation (464). Regardless of trigger, the final pathway of platelet aggregation is regulated by activation of platelet GPIIb/IIIa receptor which is the most abundant platelet surface protein and their binding with fibrinogen of plasma or platelet origin. Fibrinogen is converted to fibrin, providing stabilization of platelet aggregates. Besides the recruitment of more platelets, mediators released by activated platelets recruit other blood cells including leukocytes and red blood cells (469, 470). This process leads to accelerated thrombosis and cardiovascular events.

1.10.8 Metabolic factors influencing platelet dysfunction

1.10.8.1 Dyslipidaemia

1.10.8.1.1 Triglycerides and platelets

Hypertriglyceridaemia appears to be associated with coagulation factors such as increased factor VII (471, 472), fibrinogen and thrombin induced by lipid peroxidase (473, 474). However, the effect of hypertriglyceridaemia on platelet function remains controversial. Patients with familial hypertriglyceridaemia have a lower threshold for aggregation in response to ADP and collagen compared to normal controls (475). Other studies have found no difference in platelet reactivity between controls and hypertriglyceridaemic subjects (476). Platelet function has not been studied in patients with PCOS in the presence of hypertriglyceridaemia.

1.10.8.1.2 LDL and platelets

Early studies demonstrated that subjects with familial hypercholesterolaemia had higher platelet aggregation compared to healthy controls in response to a range of platelet agonists or elevated levels of β-thromboglobulin, a marker of platelet activation (477). Aoki et al. demonstrated that platelet-dependent thrombin generation was increased in patients with hypercholesterolemia, and with combined hypercholesterolemia compared with patients with hypertriglyceridaemia, and control subjects (478). Following a 5 min pre-incubation of platelets with LDL, a dose-dependent increase in platelet fibrinogen binding was found after optimal stimulation with α-thrombin (479). The increased activation was not corrected by platelet antagonists (479, 480). Under the same conditions, HDL did not change fibrinogen binding or aggregation (479). Following prior incubation of platelets with LDL for 2h, the platelet modified LDL caused a greater increase in collagen induced *in vitro* platelet aggregation than control LDL and interacted more readily with the LDL receptor and induced macrophage cholesterol accumulation (481). Zhao B et al. demonstrated that incubation of platelets with oxidised LDL causes changes in shape and pseudopodium formation, and advances acceleration of their adhesion. Thus, oxidised LDL may contribute to pathological thrombosis and arteriosclerosis (482). Even mildly oxidised

LDL enhances platelet shape change and increases sensitivity to platelet agonist (483-485).

1.10.8.2 Insulin resistance

1.10.8.2.1 Insulin and platelets

In 1979, Hayek et al. found insulin receptors in human platelets which have a concentration per surface area similar to that described in other cells(486). Insulin at physiological concentrations reduced platelet response to aggregating agents such as ADP, thrombin, collagen and adrenaline, and increased antagonist-induced platelet anti-aggregation in *in vitro* studies (487). However, insulin at supra-physiological doses induced a pro-aggregation effect (488) which might be related to enhanced phosphorylation and activation of the cGMP inhibited, cAMP phosphodiesterase (cGI-PDE) in human platelets and may decrease cAMP/cAMP dependent protein kinase (cAMP-PK) activity (489).

1.10.8.2.2 Obesity and platelets

Obese women had significantly higher platelet activation than non-obese women as measured by 11-dehydro-thromboxane B_2 which was negatively correlated with insulin sensitivity and WHR (490). Platelets from obese subjects are resistant to physiological anti-aggregating agents, i.e., NO and PGI_2, and to their effectors, i.e., the cyclic nucleotides cGMP and cAMP (491, 492). Platelet resistance to physiological anti-aggregating agents in obese subjects is independently correlated with insulin resistance (492, 493).

1.10.8.2.3 Insulin resistance and platelets

Patients with T2DM have fewer platelet insulin receptors with lesser affinity compared to controls (494). They have increased expression of GPIIb/IIIa platelet receptors without stimulation by platelet agonists compared to controls (495). They show significantly higher levels of 8-iso-PGF_2alpha, 11-dehydro-TXB_2 and sCD40L, markers of platelet activation, than controls (496). When compared with obese subjects, the anti-aggregating effect of insulin is dramatically lowered in diabetic obese patients (497). However, obesity in diabetes plays a significant role in platelet activation as

described by Anfossi et al. who found that platelet sensitivity to NO was preserved in lean T2DM but reduced in insulin-resistant states of obesity and obese T2DM (491). When platelets were incubated with a high concentration of glucose *in vitro*, platelet response to ADP was exaggerated (498). Postprandial hyperglycaemia was associated with enhanced lipid peroxidation and platelet activation in subjects with T2DM (499).

1.11 Aims of this thesis

The aim of this thesis was to examine the hypothesis: non esterified free fatty acids induce insulin resistance that underpins hyperandrogenism, metabolic dyslipidaemia and cardiovascular risk in PCOS.

The specific questions I wished to answer were:

1) Does acute reduction of NEFA by overnight acipimox improve insulin resistance in PCOS?
2) Does acute reduction of NEFA by overnight acipimox improve postprandial hypertriglyceridaemia in PCOS?
3) Does chronic reduction of NEFA with a 12 week tredaptive therapy improve insulin resistance in PCOS?
4) Does chronic reduction of NEFA with a 12 week tredaptive therapy improve androgen profile, metabolic dyslipidaemia and cardiovascular risk markers in PCOS?
5) Do women with PCOS tolerate acutely raised NEFA as equal as healthy women?
6) Does endurance exercise reverse lipid induced insulin resistance in PCOS by promoting fatty acid oxidation?
7) Does endurance exercise improve androgen profile and cardiovascular risk in PCOS?
8) Does acute lipaemia and acute insulin resistance aggravate platelet activation in PCOS?

1.12 Summary of chapters

Data are presented in Chapters 3 to 6.

Chapter 2 describes methodology common to all studies.

Chapter 3 describes the effect of acute suppression of NEFA by acipimox on insulin resistance and postprandial hypertriglyceridaemia in PCOS

Chapter 4 describes the effect of chronic suppression of NEFA by slow release niacin/ laropiprant on insulin resistance, postprandial hypertriglyceridaemia and endothelial function in PCOS

Chapter 5 describes the role of moderate intensity exercise that promotes fat oxidation in modifying metabolic incompetence in PCOS

Chapter 6 describes the effect of acutely raised triglycerides and free fatty acids on platelet activation in PCOS.

Chapter 7 describes summary of the research work and suggestions for future direction.

Chapter 8 includes list of references.

Chapter 2 Overview of methods and materials

2.1 Study design and protocols

2.1.1 Study approvals

All studies were sponsored by the Research and Development Department, Hull and East Yorkshire Hospitals NHS trust. The Acipimox study was a hypothesis generating, case control study approved by the Hull and East Riding Ethics Committee (09/H1304/72). The Tredaptive study was a double blind, randomized, placebo controlled trial conducted at the Hull and East Yorkshire Hospitals NHS trust after receiving an ethical approval from Leeds Research Ethics Committee (LREC Ref No. 09/H1306/103). The Medicines and Healthcare Product Regulatory Agency (MHRA) has given authorisation to conduct the Clinical Trial of an Investigational Medicinal Product (CT-IMP) study (Eudra CT number: 2009- 015729-35). The exercise study was a case control interventional study approved by the Leeds (Central) Research Ethics Committee (ref: 10/H1313/44).

2.1.2 Recruitment methods

PCOS patients were recruited from endocrine clinics with permission from their clinicians and general practitioners. Healthy volunteers were recruited through advertisements on the intranet at the University of Hull, and the Hull and East Yorkshire Hospitals. All study subjects who met inclusion and exclusion criteria for their respective studies were included after obtaining their informed consent.

2.1.3 Recruiting criteria

For all studies, pre-menopausal women aged between 18 and 40 years with or without PCOS were invited for the studies. PCOS was diagnosed by the presence of two of three diagnostic criteria, oligomenorrhoea, clinical and/or biochemical hyperandrogenism and polycystic ovaries on ultrasound according to the Rotterdam diagnostic criteria. These criteria were met after exclusion of other endocrine causes of hyperandrogenism such as non-classical 21-hydroxylase deficiency, hyperprolactinaemia, Cushing's disease, and androgen-secreting tumours (500). Healthy controls had screening tests for the exclusion of PCOS. At the screening visit,

fasting blood samples were taken for pregnancy testing, full blood count, coagulation screening, liver function, renal function, cholesterol, triglycerides and androgen profile. An oral glucose tolerance test was also performed using 75 g of anhydrous glucose. Subjects were excluded if they were found to have abnormal glucose metabolism as defined by WHO criteria. Healthy subjects who had a first- degree relative with diabetes were not included.

All the participants were non-smokers. Participants had no concurrent illness, were not on any prescription or over-the-counter medication that was likely to affect insulin sensitivity or lipids including hormonal contraceptives for the preceding three months. None were breast feeding or planning to conceive and all were using barrier contraception. They were advised not to change their lifestyle, including physical activity or dietary habits, during the study period. All subjects undergoing platelet study were asked not to use drugs containing acetylsalicylic acid during the week preceding the experiments. For the acipimox and tredaptive study, subjects who had food allergy and known allergies to acipimox and tredaptive were excluded. For the exercise study, subjects who had a family history of sudden death, history of regular exercise three times a week for the last three months or took weight reduction medications were excluded.

2.1.4 Sample size calculations

For the tredaptive study, 36 patients with PCOS were randomised into two groups, a tredaptive group and a placebo group. A sample size of 36 with 1:1 randomisation was calculated to show a one standard deviation difference in the primary outcome measure of fasting HDL-c between the two groups (80% power, 5% significance, two-tailed) with an assumption of 10% loss to follow-up.

The acipimox and exercise studies are hypothesis generating studies so a formal sample size calculation was not feasible (501).

2.1.5 Study protocols

2.1.5.1 Acipimox study

All participants have attended three visits detailed in Table 2-1.

2.1.5.2 Tredaptive study

This tredaptive study involved 7 visits in total and detailed schedule is listed in Table 2-2. The visit 2A, 3A and 4A were telephone calls to check tolerance.

2.1.5.3 Exercise study

All subjects attended 7 visits to the diabetes centre for the study and three times a week for 8 weeks to the exercise laboratory for supervised exercise program. Details of the study visits are illustrated in Table 2-3.

Table 2-1 Acipimox study timetable

Visits	Interval	Requirements	Duration	Procedures
1	NA	Fasting since 19:00h except water	3 h	For consent Measurement of weight, height, waist and hip circumference, Blood Pressure Oral Glucose tolerance test (OGTT)
2	0-1 wk	Fasting since 19:00h except water	6 h	Mixed Meal test
3	1 wk	Fasting since 19:00h except water To take oral acipimox; one tablet at 20:00h, 23:00h and 06:00h	6 h	Mixed Meal test

Table 2-2 Tredaptive study timetable

Visits	Interval	Requirements	Duration	Procedures
1	NA	Overnight fasting 12 h except water	3 h	For consent Baseline anthropometric measurement Baseline blood tests ECG & Blood Pressure
2	0-2 wk	Overnight fasting 12 h except water	6 h	EndoPAT 2000 Meal test Prescription of study medicine either niacin/laropiprant or placebo
2A	1-2 wk	No	10 min	To check tolerability
3	2-3 wk	No	20 min	Prescription of study medicine either niacin/laropiprant or placebo To check tolerability and compliance and safety, blood tests
3A	1-2 wk		10 min	To check tolerability
4	2-3 wk	No	20 min	Prescription of study medicine either niacin/laropiprant or placebo To check tolerability and compliance and safety, blood tests
4A	1-2 wk		10 min	To check tolerability
5	2-3 wk	Overnight fasting 12 h except water	3 h	Anthropometric measurement Blood Pressure, Blood tests EndoPAT 2000
6	0-1wk	Overnight fasting 12 h except water	6 h	Meal test
7	4wk	No	30 min	Review and further management

Table 2-3 Exercise study timetable

Visits	Interval	Special requirements	Duration	Procedures
1	NA	Overnight fasting 12 h except water	2.5 h	Informed consent To measure weight, height, hip and waist Baseline screening blood tests Oral Glucose tolerance test Body Volume Index 3D scanning
2	0-1wk	Overnight fasting 12 h except water	6 h	Hyperinsulinaemic euglycaemic clamp with saline trial
3	0-1wk	Overnight fasting 12h except water	6 h	Hyperinsulinaemic euglycaemic clamp with intralipid trial
4	0 wk	No fasting	1 h	To measure exercise capacity and enter the exercise program
5	4 wk	Fasting	30 min	Review Anthropometry Interim blood tests
6	4 wk	Overnight fasting 12h except water	6 h	Hyperinsulinaemic euglycaemic clamp with saline trial
7	0-1wk	Overnight fasting 12h except water	6 h	Hyperinsulinaemic euglycaemic clamp with intralipid trial Anthropometry
8	4 wk	No special requirement	30 min	Review

2.2 Interventions

2.2.1 Acipimox

Acipimox (5-methylpyrazinecarboxylic acid 4-oxide) is a lipolysis inhibitor that has a distant chemical relationship with nicotinic acid. It is rapidly absorbed, reaches peak plasma level after 2 h and is eliminated almost unchanged in urine. Its plasma level has been correlated with inhibition of lipolysis (502). Acipimox inhibits the release of fatty acids and glycerol from adipose tissue stimulated *in vitro* by the β1 agonist isoprenaline but has no effect on cholesterol synthesis. Acipimox appears to exert its main hypolipidaemic effect by reducing lipolysis and free fatty acid flux to the liver, thereby reducing the precursor pool size of very low density lipoprotein (VLDL)-triglyceride and VLDL synthesis (503). Acipimox decreases glycerol release from abdominal subcutaneous tissue in obese subjects (504).

2.2.1.1 Dosage and administration

The pharmaceutical form of acipimox is a red-brown/ dark pink hard gelatin capsule containing a white/cream coloured powder. The recommended dosage is one 250 mg capsule 2 or 3 times daily with or after meals. Acipimox is contra-indicated in patients with peptic ulceration and those who are hypersensitive to the active substance or its excipients. Acipimox was not given to patients with severe renal impairment (creatinine clearance < 30 ml/min).

2.2.1.2 Adverse effects

Flushing, dyspepsia, urticaria and headache are common adverse effects of acipimox whilst abdominal pain, pruritus, rash, erythema, myositis, myalgia, arthralgia, nausea and bronchospasm are very uncommon.

2.2.2 Tredaptive

Niacin also known as vitamin B3 or nicotinic acid is a water-soluble vitamin and one of the essential nutrients. Niacin has been in clinical use for more than 50 years.

2.2.2.1 Cholesterol lowering effect

Niacin receptors (G protein-coupled receptors GPR109A) are found in adipose tissue and immune cells such as neutrophils and monocytes (505, 506). Niacin inhibits the hydrolysis of triglycerides in adipose tissue, reducing the release of FFA into the circulation (507) and subsequently reducing the hepatic synthesis and release of VLDL and LDL-c (508). Niacin also seems to enhance degradation of intracellular Apo B proteins in the liver by inhibiting hepatocellular diacylglycerol acyltransferase-2 (DGAT-2) (506, 509, 510). In humans, niacin initially lowers plasma concentrations of NEFA by inhibiting lipolysis but this reduction in FFA is followed by a rebound increase of 40-50% from baseline 9 h after ingestion (511).

2.2.2.2 Non lipid effect

Niacin has been demonstrated to induce a number of non-lipid or pleiotropic effects. It enhances adiponectin secretion from adipose tissue (512-515) and has been shown to lower plasma CRP levels (516, 517). Lipoprotein associated, phospholipase A2, an independent risk predictor for cardiovascular disease, was lowered after 3 months of niacin administration. *In vitro* studies have shown that niacin inhibits vascular inflammation by decreasing endothelial reactive oxygen species production, LDL oxidation, vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1 expression, resulting in decreased monocyte and macrophage adhesion and accumulation (518).

2.2.2.3 Dosage and administration

Each modified-release tablet contained 1000 mg of nicotinic acid and 20 mg of laropiprant. Tablets were taken orally with a maximum daily dose of 2 tablets per day with food.

2.2.2.4 Pharmacokinetics

Following a 2000 mg dose of nicotinic acid administered orally as two modified-release tablets of nicotinic acid/laropiprant with food, nicotinic acid was absorbed with a median time to peak plasma concentration (T_{max}) of 4 h. Laropiprant is rapidly absorbed with a median T_{max} of 1h. Bioavailability of nicotinic acid with or without food

is at least 72% and laropiprant approximately 71%. Less than 20% of nicotinic acid is bound to serum proteins. Laropiprant is highly bound (> 99%) to plasma proteins, with binding independent upon concentration. Nicotinic acid is metabolised in the liver and predominantly excreted in the urine as metabolites.

2.2.2.5 Adverse effects

One of the common adverse effects of niacin is flushing due to niacin-induced release of prostaglandin D_2 from Langerhans cells in skin (519). The severity of flushing is related to dosing and tends to improve with time on treatment. Laropiprant is a selective antagonist of the prostaglandin D_2 receptor subtype 1 (DP1) and there is study evidence to support the assertion that combining laropiprant with niacin reduces flushing and improves tolerance (520, 521).

Less common adverse effects of niacin are dizziness, headache, abnormal sensation, loose motion, indigestion, nausea, vomiting, itchiness, rashes, urticaria, feeling hot, elevated liver enzymes, raised glucose and uric acid.

2.2.3 Intralipid

Intralipid is a soluble lipid emulsion with a high energy to fluid volume ratio, neutral pH and iso-osmolarity with plasma. It also provides essential fatty acids and is usually used for parenteral nutritional therapy. Intralipid 20% is composed of 20% soybean oil, 1.2% egg yolk phospholipids, and 2.2% glycerol (Kabi Fresenius Pharmacia, Clayton, NC).

2.2.3.1 Intralipid as true chylomicron counterparts

When parenteral lipid emulsion is introduced into the general circulation, it becomes enriched in cholesterol (free and esterified) and apolipoproteins-Cs, -As, and -E, obtained essentially from HDL lipoprotein particles (522). Subsequently, the Triacylglycerol Rich Protein (TAGRP) undergoes catabolism by LPL with release of FFA and monoglycerides. The hydrolysis of part of their TAG transforms them into smaller particles called TAGRP-remnants. The redundant surface material, which is rich in phospholipid, forms intermediate vesicular particles, or rather small, disk-shaped, bi-layer particles that are incorporated into the HDL pool (523, 524). At the liver, hepatic lipase (HL) finishes the process (525, 526) by hydrolysing especially TAG

but also the phospholipid of the remnants. The final uptake of these particles occurs principally in hepatocytes through endocytosis mediated by Apo B/E and/or Apo E receptors, and likewise in macrophages (524) through scavenger receptors. It is because this metabolism mimics that of the chylomicrons that the TAGRP of parenteral lipid emulsion are usually considered as true chylomicron counterparts (527). Intralipid induced elevation of NEFA levels require at least 120 min to induce insulin resistance, 270 min to cause a significant effect with a peak effect at 360 min (528).

2.2.3.2 Effect of intralipid on lipoprotein cholesterol

Following an intralipid infusion there is an increase of TG, phospholipids, free cholesterol and cholesterol ester but an overall decrease of LDL-c and HDL-c (529). Triglyceride content was increased in VLDL and HDL-c and phospholipid content increased in VLDL but not HDL-c. LDL-c was unchanged. Within HDL-c sub-fractions, HDL2 was increased by 11% and HDL3 decreased by 27%. These changes are reversed within a few hours of stopping the infusion (530, 531).

Salonen et al. studied the association between cholesterol levels in serum HDL-c and its sub-fractions HDL2 and HDL3 and the risk of acute myocardial infarction in 1,799 randomly selected men. After adjustment of confounding risk factors, the study confirmed that both total HDL-c and HDL2 levels have inverse associations with the risk of acute myocardial infarction and may thus be protective factors in ischemic heart disease, whereas the role of HDL3 remains equivocal (532).

Granot et al. confirmed that artificial triacylglycerol from intralipid emulsion can act as a triglyceride rich VLDL, as an acceptor for cholesterol ester and as a donor of triacylglycerol in the absence of apolipoproteins. As a result of exchange of cholesterol ester from the HDL and LDL with TG from intralipid, HDL and LDL became initially larger with increased TG content and decreased cholesterol content These were then hydrolysed resulting in small HDL-c particles, and small and dense LDL-c particles (533). Graffin et al. examined the role of small and dense LDL-c particles (LDL-III) in CAD and their relation with TG. It was found that LDL-III was independently associated with the presence of CAD and myocardial infarction, and had a strong positive association with TG levels at and above 1.5 mmol/L (534).

2.2.3.3 Intralipid and Lipoprotein X

The flux of cholesterol ester out of cells starts as soon as the phospholipid rich lipoproteins (PLRP) enter the bloodstream. Membrane cholesterol loss was compensated for by hydrolysis of intracellular stores of cholesterol esters (535) and by stimulating de novo cholesterol synthesis as observed in muscle, adipose tissue (536, 537), macrophages, liver and intestine (538). This continues until the molar ratio of free cholesterol/phospholipid reaches 1:1 and exogenous phospholipid leads to the progressive transformation of PLRP into lipoprotein-X. It is this efflux of free cholesterol to the vascular compartment as lipoprotein-X that is the principal cause of the rise of cholesterolaemia, or at least of the dyslipoproteinaemia observed in any total parenteral nutrition including intralipid fat emulsion.

2.2.4 Exercise

All subjects gave informed consent and answered the pre-exercise medical questionnaires. All exercise sessions and measurements of VO_2 max (maximal oxygen consumption) were undertaken at the exercise laboratory in the Department of Sports, Health and Exercise Science, University of Hull. VO_2 max was measured at baseline, at the end of 4 weeks and at completion of 8 weeks exercise. VO_2 max measurement was performed on a motorised treadmill starting with a warm up, then speeding to walking pace then increasing every minute until the subject could not keep pace with the treadmill or became too tired to continue. Inhaled and exhaled air and heart rate (HR) were continuously monitored and recorded. Each subject was asked to score the difficulty of the exercise using a scale of 0 to 10 (0 being very easy and 10 being very intense).

The exercise program consisted of 3 sessions (60 min in each session) per week, of moderate intensity for 8 weeks. All supervised exercise sessions were performed on a motorized treadmill and achieved a target heart rate equivalent to 60% of their baseline VO_2 max. The moderate exercise intensity was recalculated at the end of 4 weeks by repeating the VO_2 max test to account for any improvement in fitness during the first half of the program.

Performance and attendance was supervised by a sports specialist from the University of Hull. All participants had body shape and measurements assessed using a body volume scanner pre and post exercise intervention.

2.3 Procedures

2.3.1 Meal test

2.3.1.1 Ingredients of the test meal

In the acipimox study, the study meal consisted of a cheese sandwich, cereal and milk and an emulsion freshly made of olive oil, sunflower oil, Mavel milk powder, double cream, glucose, sugar and strawberry nesquick powder. The meal was 900 Kcl consisting of 45 g lipid, 98 g carbohydrate, and 31 g protein. The tracer (10 mg per kilogram body weight [1, 1, 1-^{13}C] tripalmitin 99 atom percent excess; Masstrace, Woburn, MA) was added to measure whole body fat oxidation. The same test meal but without tracer was used in the tredaptive study. The ingredients of the study meal and their nutritional values are listed in Table 2-4 and Table 2-5 respectively. All nutritional data was obtained from suppliers branded food packets.

Table 2-4 Ingredients of the study meal

Food	Amount g	Nutritional Data from	Protein g /100g	Protein g in recipes	Fat g /100g	Fat g in recipes	CHO g /100g	CHO g in recipes	Calc Kcal /100g	Calc Kcal in recipes
Kellogg's rice krispies	20	Packet	6	1.2	1	0.2	86	17.2	380	76
Semi-skimmed milk	100	Packet	3.4	3.4	1.6	1.6	4.9	4.9	47	47
Kingmill's bread	80	Packet	9	7.2	2	1.6	44.6	35.68	238	190.4
Flora	20	Packet	0	0	70	14	0	0	630	126
Cheddar cheese (Tesco)	40	Packet	28	11.2	34.4	13.76	0	0	420	168
Olive oil	3.5	packet	0	0	100	3.5	0	0	900	31.5
Sunflower oil	3	packet	0	0	100	3	1.5	0.04	900	27
Double cream(Elmlea)	22	packet	2.5	0.55	36	7.92	3.5	0.77	350	77
Marvel Milk Powder	20	packet	36.1	7.22	0.6	0.12	52.9	10.58	360	72
D (+)Glucose	9	packet	0	0	0	0	100	9	400	36
Beet sugar	4.5	packet	0	0	0	0	100	4.5	400	18
Nesquik	10	packet	0	0	0	0	98.1	9.81	393	39.3
Total nutritional value in the test meal				30.77		45.7		92.48		908.2

Table 2-5 Nutritional value of meal ingredients

Food	Source	Protein g /100g	Fat g /100g	CHO g /100g	Calc Kcal /100g
Olive oil	Tesco	0	100	0	900
Sunflower oil	Tesco	0	100	0	900
Double cream	Elmlea	1.5	50.5	1.6	467
Milk powder	Marvel	36.1	0.6	52.9	348
D (+) Glucose	Lab	0	0	100	400
Beet sugar	Tesco	0	0	100	400
Nesquik	Nestle	0	0	98.1	393
Rice krispies	Kellogg's	6	1	86	380
Bread	Kingsmill	9	2	44.6	265.7
Flora 20g	Flora	0	70	0	630
Cheddar cheese	Tesco	0	34.4	0	420
Semi- skimmed milk	Tesco	3.4	1.6	4.9	47

2.3.1.2 Preparation of the emulsion

Lipid: glucose: protein emulsion was freshly made using 4.5 g of olive oil, 3 g sunflower oil, 4.5 g beet sugar, 9 g glucose, 22 g double cream, 25 g of marvel milk powder and 10 g of Nesquik powder. These ingredients were measured in separate weighing boats. Ten mg per kg body weight of ^{13}Ctripalmitic acid was measured to the nearest 0.001g.

The tripalmitic acid powder, olive oil and sunflower oil were put together into an insulated plastic cup in a hot water bath, with a thermometer and clamp stand, heated to 85-90°C. Once the fat powder was dissolved in the oil, the double cream, milk powder, beet sugar, glucose and 70 mL of hot water were added then blended using a hand held blender for 2 min. Nesquik powder was added and blended for further 30 seconds. A further 50 mL of boiled hot water was used to rinse the blender into the cup. The emulsion and the rinsed water of the plastic cup using 20 mL of water were then transferred into a serving cup. The emulsion was allowed to cool to 70°C before use.

2.3.1.3 Meal test protocol

In the acipimox interventional study, all subjects underwent two meal tests, one at baseline and one following overnight acipimox therapy. Subjects were asked to avoid foods naturally enriched with ^{13}C, for example, maize products, pineapple and cane sugar, and to avoid alcohol and strenuous exercise for two days prior to each study day. They were asked to consume a low fat (<10g of fat) meal before 19:00hr on the evening before the study day and then to fast overnight (except water) until the study commenced. They refrained from taking any medicine from the evening before the study day. The same protocol was followed in the evening of each meal test.

On the study day, subjects attended our diabetes research centre at 08:00h. Body weight and height were measured. A specimen of expired air was collected to measure baseline ^{13}C- tripalmitin excretion in exhaled breath. An indwelling cannula was sited in a forearm vein, and fasting blood samples withdrawn. Subjects then consumed the test meal breakfast cereal and milk, bread, butter and cheese together with a lipid: glucose: protein emulsion including tracer. The participants were asked to consume the emulsion first whilst hot immediately prior to the rest of the breakfast. The participants remained seated or supine for the duration of the study period. No additional foods or liquid, other than water were allowed during the study. Venous blood samples were taken half-hourly for 3 h and then hourly until 6 h after the meal when the study was completed. The exhaled breath samples were taken half an hourly throughout the 6 h period. Subjects were then instructed to collect four further breath samples at home at 10 h, 12 h, 14 h and 24 h after the meal.

In the tredaptive interventional study, the meal tests were conducted at the beginning and at the end of the study. The same protocol was used but the test meal did not include tracer and therefore breath samples were not taken.

2.3.2 EndoPAT

2.3.2.1 EndoPAT 2000

EndoPAT (Figure 2-1) is a medical device for non-invasive endothelial function assessment. It is FDA-cleared, CE-marked and used in pre-eminent clinical and research institutions for major epidemiological population-based studies (such as the

Framingham Heart Study). It is a product of Itamar Medical Ltd, medical device manufacturer of diagnostics products based on the PAT™ (Peripheral Arterial Tone) signal technology. It measures endothelium-mediated changes in vascular tone using unique bio-sensors placed on the fingertips. These changes are elicited by creating a down-stream hyperaemic response induced by a standard 5-minute occlusion of the feeding artery (using a standard blood pressure cuff). When the cuff is released, the surge of blood flow causes an endothelium-dependent Flow Mediated Dilatation (FMD). The dilatation, manifested as Reactive Hyperaemia, is captured by EndoPAT as an increase in the PAT Signal amplitude. A post-occlusion to pre-occlusion ratio is automatically calculated by the EndoPAT software, thus providing an EndoScore. Measurements from the opposite arm are used to control for concurrent non-endothelial dependent changes in vascular tone. The test takes 15 min to complete, is simple to perform, and both operator and interpreter independent.

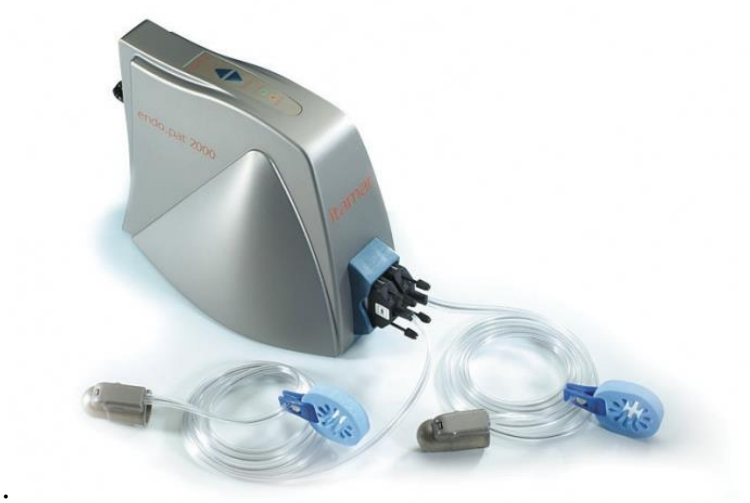

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Figure 2-1 EndoPAT machine

2.3.2.2 EndoPAT protocol

Reactive hyperaemic index (RHI) was measured using EndoPAT 2000 technology at the beginning and at the end of the intervention in the tredaptive study to examine its

effect on cardiovascular risk modification.

Subjects attended the research centre at 08:00h. The subject having an EndoPAT test was comfortably seated for at least 15 min to reach a relaxed cardiovascular steady-state in the study room where temperature was maintained at 21°C to 24°C. The fingers of subjects were inspected for deformations or injuries that could affect the study. Blood pressure was measured from the subject's control arm at least 5 min before the commencement of the EndoPAT baseline recording. Then the subject was advised to be seated comfortably with the hands supported at approximately heart level. Firstly, a blood pressure cuff was placed on the upper arm of the designated test arm. Then, the PAT probes were placed inside the appropriate sockets of the arm supports. Both index fingers of the subject were inserted into the appropriate probes and connected via pneumoelectric tubes to the EndoPAT 2000 device. The subject was asked to refrain from talking. The probes were inflated and the PAT signals recorded per the manufacturer protocol. Briefly, the PAT signals were recorded as a baseline for 5 min at relaxed state, followed by an occluded phase for 5 min and finally a 5 min post-occluded/recovery phase. Arterial occlusion in the test arm was achieved by inflating the blood pressure cuff to a supra-systolic level (the recommended pressure is at least 60 mmHg above systolic blood pressure and no less than 200 mmHg). After 5 min (the occlusion is complete), the pressure cuff was deflated as quickly as possible. At the end of final recording of 5 min recovery time, the subject was freed after deflation of the probes and removal of attached tubes.

Endothelial dysfunction is associated to cardiovascular risk factors and predicts cardiovascular events. Peripheral arterial tonometry (PAT) is a novel non-invasive method to assess endothelial function. Since 2003, the Framingham Heart study has included endothelial function measurements with EndoPAT. Hamburg et al. established a significant inverse relation between RHI and multiple CV risk factors including mal sex, total/HDL cholesterol, diabetes, smoking, and lipid lowering treatment in the cross-sectional analysis of 1957 3rd generation subjects from Framingham Heart Study (539).

When the feasibility and reproducibility of PAT were assessed in 123 adult participants, the coefficient of variation was 18.0% (540). When compared the reliability and reproducibility of FMD and PAT to assess endothelial function, the within-day

variability was lower for the FMD measurements than for the PAT measurements (10% versus 18%) and the between-day variability was similar (11%). RHI from PAT and FMD were strongly correlated (541). RHI is a significant predictor of high-risk status among men with CAD and therefore it may be useful in risk stratification in CVD (542).

2.3.3 Body Volume Index 3D Scanner

The Body Volume Index 3D scanner is a non- invasive device that uses visual light to calculate a person's body volume and detect obesity independently of their shape. This scanner was used in this study to calculate accurate waist and hip circumference. A validation study compared the use of the 3D Body Volume Index scanner against manual measurements for estimating obesity in 80 participants. It reported that the 3D Body Volume Index scanner provides a valid, reliable and reproducible method for measuring waist and hip circumferences (543). To have a scan, a person puts on flesh coloured underwear and stepped into a booth where they are illuminated with white light. The light reflected is picked up by a series of sensors and computer controlled cameras which create a 3D image o the body within 7 seconds. The software detects predetermined body landmarks, including measuring waist circumference at 55% of height and hip circumference at its widest diameter.

2.3.4 Hyperinsulinaemic euglycaemic clamp

2.3.4.1 The euglycaemic clamp

The hyperinsulinaemic euglycaemic clamp is a gold standard test for the measurement of insulin resistance. It consists of an insulin infusion at a predetermined fixed dose and a variable rate glucose infusion to measure the rate of whole body glucose disposal whilst maintaining blood glucose at a predetermined level. In the exercise study, the test was performed in the last 2 h of 5 h of either saline or intralipid infusion following an overnight fast. Two cannula were inserted into contralateral arms, one used for test infusions (insulin/dextrose) and one at the dorsum of the hand which was heated in a heat box at temperature 60°C to provide the arterial blood for glucose reading.

A primary insulin infusion at 80 mU/m^2surface area/minute was administered for the first 20 min to raise plasma insulin above physiological level. It was assumed that

endogenous glucose production is more than 90% suppressed by an acute rise of insulin level with primed insulin infusion (544). This was followed by insulin infusion at a constant rate of 40 mU/m^2 surface area/minute for the last 100 min. During the euglycaemic clamp procedure, the glucose infusion rate (GIR) was adjusted to maintain blood glucose level at 5 mmol/L.

2.3.4.2 Insulin infusion

The dose of insulin infusion is calculated for individual based on her body surface area. Body surface area (BSA) was calculated using the DuBois and DuBois Formula, as detailed below:

$$BSA = W^{0.425} \times H^{0.725} \times 0.007184$$

For example a Subject with a body weight (W) of 70 kg and Height (H) of 180 cm

$$BSA = 70^{0.425} \times 180^{0.725} \times 0.007184 = 1.886\ m^2$$

The amount of insulin required to deliver the intended dose in 50ml of saline infusate is calculated as follows:

Dose (U/h) = 40 mU x 1.886 m^2 x 60 min = 4527.05 mU/h = 4.527 U/h

Dose rate = 15 mL/h

Concentration of insulin = 4.527 (U/h) / 15 (mL/h) = 0.3018U/mL

Thus, the amount of insulin to add in the 50 mL saline infusate = 0.3018x60 = 15.09U/50 mL

The insulin infusate was prepared in isotonic saline to which 2 mL of the subject's blood per 50 mL infusate was added in order to prevent adsorption of insulin to glassware and plastic surfaces. Then the calculated amount of insulin (Humulin S; 100 U/mL) was added to the infusate to provide a subject-specific insulin concentration. The insulin infusate is intravenously infused initially at a constant rate of 30 mL/h to deliver insulin at a rate of 80 mU/m^2 body surface area/min for 20 min and it was followed by a constant rate of 15 mL/h (40 mU/m^2 body surface area/min) for the last 100 min.

2.3.4.3 Glucose infusion

The intravenous glucose infusion contained 20% dextrose solution (20 g/100 mL; 200 mg/mL) and the infusion commenced 4 min after the start of the insulin infusion at an initial rate of 2.0 mg/ kg/ min and increased to a rate of 2.5 mg/ kg/ min from 10 to 15 min, after which infusion rate was variable. The initial infusion rates were calculated as detailed below:

Initial dose (g/h) = weight (kg) x 2 mg/min x 60 min

$$\text{Initial Infusion Rate (mL/h)} = \frac{\text{Initial dose (g/h)} \times 100\ \text{mL}}{20\ \text{g}}$$

Increased dose (g/h) = Weight (kg) x 2.5 mg/min x 60 min

$$\text{Increased Infusion Rate (mL/h)} = \frac{\text{Increased dose (g/h)} \times 100\ \text{mL}}{20\ \text{g}}$$

For example: Subject weight 70 kg

Initial dose = 140 mg/min = 8.4 g/h.

Initial Infusion Rate = 42 mL/h

Increased dose will be 175 mg/min = 10.5 g/h.

Increased Infusion Rate = 52.2 mL/h

The glucose infusion would continue for 110 min with blood glucose levels checked every 5 min using HemoCue® glucose 201$^+$ with plasma glucose conversion (Angelhom, Sweden) for instant results (545, 546). The rate of glucose infusion was altered continuously to maintain glucose levels as close as possible to the targeted 5 mmol/L throughout the clamp. Adjustments to the infusion rate were made by means of the algorithm published by DeFronzo et al. (119).

Glycaemia values were calculated every 5 min and used to adjust the glucose infusion rate by the following formula:

$$IR = \frac{(Gd - Gi) \times 10 \times (0.19 \times weight)}{G\inf \times 15} + (SM_i)$$

$$SM_i = SM_{i-2} \times FM_i \times FM_{i-1}$$

$$FMi = \frac{Gd}{Gi}$$

Where:

i- The number of the evaluation in sequence (iteration)

IR- Infusion rate in millilitres per minute

Gd- Target glucose concentration in mg/dL

Gi- Measured glucose in mg/dL in the evaluation i

Weight - Weight of the subject in kilograms

Ginf- Concentration of glucose in the infusate in mg per mL (200 for 20% Dextrose)

FM- Dimensionless correction factor for the distance from the goal

SM- Metabolic factor

2.3.4.4 Calculations for rate of glucose disposal

The rate of glucose disposal (mg/kg/min) (M), a measure of insulin sensitivity, is the glucose infusion rate. The total M is calculated using the mean of five 20 min periods from 20-120min during the clamp using the Defronzo method (119).

2.3.5 Measurement of platelet activation

2.3.5.1 Flow cytometry

Whole blood flow cytometry is a physiological assay of platelet function that determines the activation state of circulating platelets and the reactivity of circulating

platelets to an exogenous agonist/antagonist. When platelets are activated, changes occur at the platelet surface such as conformational changes of platelet membrane glycoproteins IIb and IIIa into competent receptors for fibrinogen. Activated platelets also secrete stored proteins such as P selectin from intracellular alpha granules to the surface. The two most widely studied types of activation-dependent monoclonal antibodies are those directed against conformational changes in αIIbβ3 and those against granule membrane proteins.

In this study, platelet activation in response to agonist, ADP, and antagonists, PGI_2 or 8-CPT-6-Phe-cAMP, were studied using a monoclonal antibody specific for the fibrinogen receptor on activated platelets and an antibody specific for P selectin that associates with platelet surface during secretion. Platelet bound antibodies were detected using antibodies conjugated to fluorescein isothiocyanate (FITC) or Phycoerythrin (PE). The method involves minimal handling of platelet as it does not require centrifugation or washing steps. This method can detect as few as 0.8% activated platelets when mixed with unstimulated platelets (547).

2.3.5.2 Materials

- HEPES buffer (NaCl (Sodium chloride) 150mM, KCl (Potassium Chloride) 5mM, $MgSO_4$ (Magnesium Sulphate), 1mM, HEPES 10mM, pH adjusted to 7.4 using 1M HCl (Hydrochloric acid)
- 3.8% Tri- sodium citrate
- 0.2% formaldehyde
- FITC (fluorescein isothiocyanate) Mouse IgG1 k Isotope Control Antibody (Becton Dickinson, Oxford, England)
- FTIC mouse Anti-Human CD42b antibody (Becton Dickinson, Oxford, England)
- R-Phycoerythin (PE)-conjugated anti-CD62P monoclonal antibody (Becton Dickinson, Oxford, England)
- FITC rabbit Anti-Fibrinogen Antibody (Dako, Denmark)
- Adenosine 5' Diphosphate (ADP) (Sigma, Poole UK)
- Prostacyclin (PGI_2) (Sigma, Poole UK)

- 8-(4- Chlorophenylthio)-N^6-phenyladenosine-3', 5'-cyclic monophosphate (8-CPT-6-Phe-cAMP) (Sigma, Poole UK)

2.3.5.3 Blood sampling

At 2 h after commencing saline or intralipid infusion (before the insulin clamp was initiated), whole blood (4.5 mL) was collected from the cannula sited in the ante-cubital vein into a syringe filled with 0.5 mL of 3.8% tri-sodium citrate (1:9 ratio), the initial 5mL of blood was discarded to avoid artificial platelet activation. Then the blood was decanted into a falcon tube and gently mixed to avoid platelet activation. The blood was processed for flow cytometric analysis as per protocol within 10 min of sampling. A second set of blood samples was taken (as per same protocol) at the end of the 5 h saline or intralipid infusion.

2.3.5.4 Preparation of assay tubes

Whole blood is added to each test tube containing 50 µL of HEPES, either 2 µL of FITC Mouse IgG1 k Isotope Control Antibody to detect background activation, or 2 µL of FTIC mouse Anti-Human CD42b antibody, an activation independent platelet specific antibody which ensures that >95% of the gated cell population for study are platelets. For assessment of platelet fibrinogen binding, citrated whole blood was immediately added to tubes containing 50µL of HEPES buffer and 2 µL of FITC rabbit Anti-Fibrinogen Antibody, 2 µL of PE-conjugated anti-CD62P monoclonal antibody for measurement of P-selectin. To study the sensitivity of platelets to activation, some tubes were supplemented with ADP (0.1, 1, 10 µM). Tube contents were mixed gently and left to incubate at room temperature for 10 min before fixing with 500 µL of 0.2% (v/v) formaldehyde. For the assessment of platelet inhibition PGI_2 (0.01, 0.05, 0.1µM) or to 8-CPT-6-Phe-cAMP (50, 100, 200 µM) was added to blood samples and incubated for 1 min before adding ADP (1 µM) and a further incubation for 10 min prior to fixation with 0.2% formaldehyde. All samples were prepared in duplicate.

2.3.5.5 Flow cytometric analysis and data collection

Immunostaining was completed within 10 min of venepuncture and flow cytometric analysis carried out within 2 h of fixation. All antibodies were optimised for maximum fluorescence with minimal non-specific binding. Non-specific binding was also checked

using isotype controls for all antibodies. The platelet population in whole blood was identified by its characteristic forward- and side-scatter profile and by FITC-conjugated anti-CD42b monoclonal antibody which stained positive >95% of the gated platelet population (548). Ten thousand cells from this population were analysed on a FACS Aria flow cytometer (Becton Dickinson, San Diego, USA) using BD FACS Diva™ analytical Software. A compensation method was performed to compensate for overlapped fluorescence activity during analyses of those assay tubes with both FTIC and PE antibodies. Platelet activation was measured as percentage of platelets expressing either fibrinogen binding or P selectin receptor per 10,000 gated platelet populations. The data were presented as mean of results from duplicated samples.

2.3.5.6 Sample handling and analysis

All venous blood samples as per protocols were collected into either heparinised or serum gel or fluoride oxalate containing vacutainers. Samples were separated by centrifugation at 1500 G for 15 min at 4°C, and plasma and serum were aliqouted and immediately frozen at –80°C.

2.4 Biochemical Analyses

Total cholesterol (TC), TG, and HDL-c were measured enzymatically using a Synchron LX20 analyser (Beckman-Coulter, High Wycombe, UK). LDL-c was calculated using the Friedewald equation (549). Non-esterified fatty acids (NEFA) were analysed using enzymatic colorimetric methods (Wako NEFA-HR2) on a Konelab20 autoanalyzer with an inter-assay coefficient of variation of 1.4%. Serum insulin was assayed using a competitive chemiluminescent immunoassay performed on the manufacturer's DPC Immulite 2000 analyzer (Euro/DPC, Llanberis, UK). The analytical sensitivity of the assay was 2 μU/mL, the coefficient of variation was 6%, and there was no stated cross-reactivity with pro-insulin. Plasma glucose was measured using a Synchron LX 20 analyzer (Beckman-Coulter, High Wycombe, UK), according to the manufacturer's recommended protocol. The coefficient of variation for the assay was 1.2% at a mean glucose value of 94.6 mg/dl (5.3mmol/L) during the study period.

Serum testosterone was measured by high pressure liquid chromatography linked to tandem mass spectrometry (Waters Corporation, Manchester, UK) and sex hormone

binding globulin (SHBG) measured by immunometric assay with fluorescence detection on the DPC Immulite 2000 analyser. The free androgen index (FAI) was obtained as the quotient 100* Testosterone/SHBG. High-sensitivity C -reactive protein (hsCRP) was measured turbidimetrically using a Beckman SYNCHRON® System.

[^{13}C]-Enrichment of CO_2 excreted in the breath was measured by continuous-flow isotope ratio mass spectrometry (IRMS) using a 20/20 stable isotope analyzer equipped with a gas/solid/liquid interface (PDZ-Europa, Crewe, UK) (550). The proportion of administered labeled tripalmitic acid converted to $^{13}CO_2$ over 24 h was calculated from the [^{13}C]-enrichment and whole-body CO_2 excretion.

2.5 Statistical Analysis

Statistical analysis was performed using SPSS for Windows NT, version 19.0 (SPSS Inc., Chicago, IL). Comparison before and after intervention within the group was made using the Wilcoxon signed rank test for variables that violated the assumptions of normality when tested using the Kolmogorov-Smirnov test, and the t tests for normally distributed variables. Mann- Whitney t test and the independent sample t test were used respectively for comparison between groups. Relative changes (percentage) of variables from baseline were compared between groups using unpaired t tests for normally distributed variables and the Kruskal Wallis test for skewed variables. Results are expressed as means ± SD for normally distributed data and medians (25^{th}, 75^{th} centiles) for skewed variables. For all analyses, a two-tailed $P < 0.05$ was considered to indicate statistical significance.

2.6 Calculations

2.6.1 Area under the curve

Total and incremental TG area under the response curve (AUC), and insulin, glucose and NEFA AUC were calculated using a trapezoidal rule (514, 551).

When n+ 1 measurements yi at times t, (i = 0,…..n) are performed, then the area under the curve (AUC) is calculated as

$$AUC = \frac{1}{2}\sum_{t=0}^{n-1}(t_{i+1} + t_i)\,(y_i + y_{i+1})$$

2.6.2 Homeostatic model assessment of insulin resistance (HOMA-IR)

HOMA-IR was calculated using the formula: HOMA-IR = fasting plasma insulin (μU/mL) x fasting plasma glucose (mmol/L)/22.5 (120). Fasting beta cell function (HOMA-B%) was calculated using the HOMA calculator and the HOMA 2 model (552).

2.6.3 Disposition index

On the basis of the hyperbolic relationship between insulin response and insulin sensitivity (SI), the oral disposition index (DI_O) was calculated from the acute insulin response to glucose (AIRg) multiplied by Insulin Sensitivity (553, 554). The early insulin response was determined as the ratio of mean increments in insulin and glucose from 0 to 30 min following the mixed meal test (ΔI0–30/ΔG0–30). Insulin sensitivity (SI) was calculated from 1/ fasting insulin. The DI_O was used as a measure of compensatory beta cell function in response to insulin resistance (555). The hyperbolic relation relationship between acute insulin response to glucose (AIRg) and insulin sensitivity (SI) were illustrated in normal control Figure 2-2 and in individuals with a beta cell defect Figure 2-3.

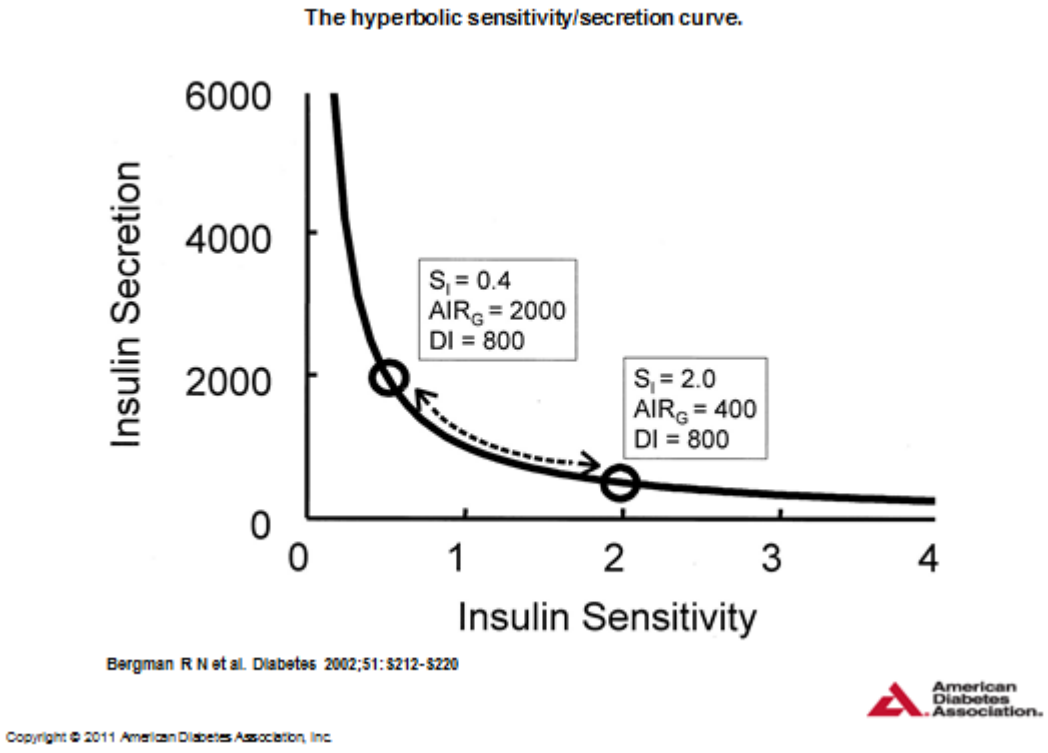


Figure 2-2 The hyperbolic sensitivity/secretion curve

It is envisioned that, in the course of environmentally induced reductions in insulin sensitivity, "normal" pancreatic islets would respond by upregulation of the β-cells' sensitivity to glucose. In the example shown, individuals with an SI of 2.0 × 10−5 min−1 per pmol/l and first-phase insulin response (AIR_G) of 400 pmol/l would have a disposition index [defined as the product (SI × AIR_G)] of 800. Reduction in insulin sensitivity (for example, to 0.4 × 10−5 min−1 per pmol/l) would result in up-regulation of AIRg to 2,000 pmol/l, with the DI remaining constant at 800. Reduction in insulin sensitivity may be due to one of several factors, including pregnancy, increased adiposity, and puberty (556)

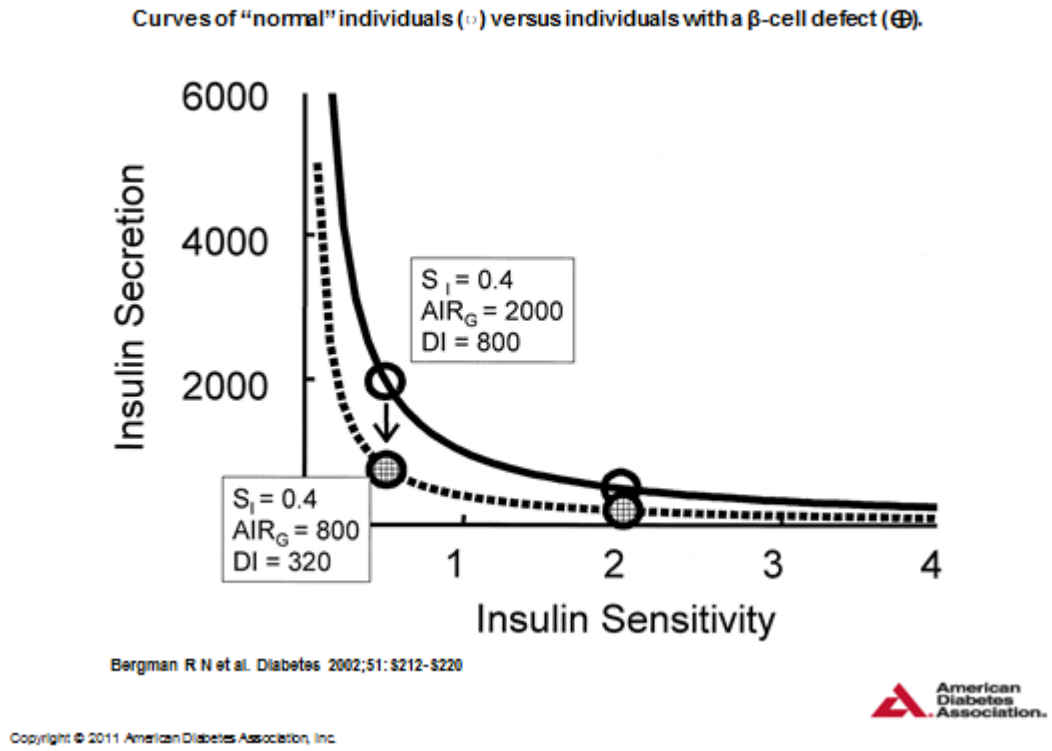


Figure 2-3 Curves of "normal" individuals (○) versus individuals with a β-cell defect (⊕).

Note that at elevated insulin sensitivity (SI ~2.0 × 10−5 min−1 per pmol/l), it would be difficult to detect differences in insulin response, as the AIRG is relatively low due to the minimal stress on the β-cells. When insulin resistance is present (SI = 0.4), there is a clearer differentiation between AIRG values, making it easier to differentiate β-cell function in individuals of similar reduced insulin resistance (556)

Chapter 3 Effect of lowering NEFA by overnight acipimox on insulin resistance in PCOS

3.1 Introduction

Women with PCOS suffer from hirsutism, oligo/amenorrhoea and sub-fertility related to hyperandrogenaemia. Previous studies showed that women with PCOS have several features of metabolic dysfunction including IR (12), obesity (54), hypertension (70), IGT (21), dyslipidaemia (286), metabolic syndrome (29, 66) and T2DM (18, 21, 24).

IR is thought to play a role in the pathogenesis of PCOS (12). Compensatory hyperinsulinaemia induces androgen secretion from insulin sensitive ovaries (557) and decreases hepatic SHBG production (558). The resultant hyperandrogenaemia contributes to features of PCOS such as hirsutism, anovulation and oligo/amenorrhoea by affecting the hypothalamic pituitary ovarian axis (559). Although obesity is not vital to the development of IR in PCOS (14), it can aggravate the metabolic dysfunction observed in PCOS. Obese women with PCOS are more insulin resistant (128), hyperandrogenic (557, 560), have higher plasma NEFA levels (128, 286, 560) and are more susceptible to progress to T2DM (19, 67) than lean women with PCOS. Reduction in weight improves IR and hyperandrogenism in PCOS (561).

The prevalence of PCOS in the general population is 8-9% but reaches to 11- 12% in women with BMI greater than 35 kg/m^2. Two thirds of women are obese at the time of PCOS diagnosis (54). The increasing incidence of obesity in PCOS parallels the obesity epidemic (54). Obesity per se is associated with the development of IR and dyslipidaemia (562). The mechanism by which obesity can lead to increased IR involves increased NEFA production and their effect on skeletal muscle (330, 408, 563). Increased availability of NEFA with limited mitochondrial beta oxidation could lead to accumulation of metabolites such as DAG, ceramides and intracellular LCFA acyl CoA. These could interfere with insulin action and reduce insulin-stimulated glucose uptake in skeletal muscle (409). Whilst this is well recognized, the magnitude of the effect of NEFA on IR varies between individuals and patient groups.

Chylomicrons are formed in the small intestine to transport dietary TG, cholesterol, and fat-soluble vitamins to the circulation (178, 564). These are hydrolysed by LPL in adipose tissue capillaries to deliver FFA that are mostly taken up by adipose tissue via a concentration gradient created by the activation of re-esterification of TG and by the inhibition of TG hydrolysis in the postprandial state (565-567). The remaining chylomicron remnants that contain cholesterol ester, fat- soluble vitamins, Apo B48 and Apo E are rapidly removed by LDL receptors and LDL receptor-related protein in the liver (182, 568). Apo B100 containing VLDL are synthesized in the liver and delivered to the circulation, and their rate of synthesis depends upon the availability of FFA (569). Chylomicrons and VLDL compete with each other for LPL hydrolysis in a common lipolytic pathway (178, 179). In the postprandial period, 80% of the increase in plasma TG level is due to a rise in chylomicrons and their remnants that contain a large amount of TG (180). In healthy subjects, 90% of the rise of cholesterol in the TRL fraction is the result of accumulation of large endogenous VLDL particles secondary to preferential lipolysis of chylomicrons (181). The postprandial increase in adipose tissue blood flow (570-572) and raised insulin level suppress adipose tissue HSL and up-regulate LPL (571, 573). This increases FFA influx from chylomicrons to adipose tissue in the fed state.

Physiological postprandial lipaemia is a transitory rise in TRLs lasting from 6 to 12 h after ingestion of a fatty meal. In normolipidaemic subjects, plasma TG typically increases 1.0 -2.0 mmol/L from a fasting level at 2–4 h after a meal (241). The postprandial rise of TG reflects an accumulation of TRLs in the circulation, intestinal derived Apo B48 containing chylomicrons and their remnants, and liver derived Apo B100 VLDL and VLDL remnants. Postprandial dyslipidaemia refers to an increase in the magnitude and duration of the postprandial rise in TRLs ad it has been described in detail in section 1.6.2.

Postprandial hypertriglyceridaemia is commonly associated with PCOS, T2DM and obesity (246, 285, 574). During the post-absorptive phase, adipose tissue HSL activity is maintained in obesity and insulin resistant states as in healthy subjects (571). During the postprandial phase, 75% of HSL activity is suppressed and LPL activity increased 1.5 fold in healthy subjects. However, HSL and LPL fail to respond to postprandial insulin in obese subjects (571, 575-578). The continued release of FFA enhances VLDL

synthesis and secretion from the liver (579, 580) which would compete with chylomicron clearance postprandially. In fact, IR is positively correlated with postprandial TG and NEFA levels (581). Contrary to this theory, a recent study suggested a down regulation of FFA release in obesity to maintain fasting FFA well within normal levels (321). The author suggested that adipose tissue from obese subjects had a defective storage function resulting in high, postprandial chylomicron-derived FFA that may well lead to ectopic fat deposition. Delayed, ineffective removal of chylomicron remnants by LDL receptors or LDL receptor-related protein in the liver (271) may also contribute to postprandial hypertriglyceridaemia. IR appears to affect hepatic clearance of lipids as in both, *in vitro* and *in vivo* animal studies (270), where insulin seems to enhance hepatic LDL receptor related protein (269). However, it is not clear whether the impairment in clearance of TRL is due to failure of insulin-stimulated LPL activity, or insulin-inhibited HSL activity (582, 583) that is a feature of IR, or whether it is simply due to competition for clearance with VLDL when TG clearance is rate limited.

Longitudinal studies have shown the importance of other lipid measurements such as HDL-c and TG in addition to LDL-c for the prediction of cardiovascular risk status (294, 584, 585). Fasting TG are preferred as they are reproducible but postprandial TG are normally present for many hours each day. Indeed, postprandial hypertriglyceridaemia is well recognised as an independent risk factor for CVD (311, 312, 586-589). Subjects with PCOS suffer from postprandial hypertriglyceridaemia that may contribute to and enhance cardiovascular risk.

3.1.1 Hypothesis

In the present study it was hypothesized that NEFA plays a significant role in the pathogenesis of IR in PCOS by interfering with insulin-stimulated glucose uptake in skeletal muscle. This effect would be prominent during the postprandial period when maximal insulin action is required.

It is also hypothesized that continued delivery of FFA from adipose tissue to the liver during the postprandial period would promote VLDL secretion thereby impeding chylomicron metabolism resulting in persistent postprandial hypertriglyceridaemia in women with PCOS.

This study examined these hypotheses using overnight acipimox treatment to produce a sustained reduction in plasma FFA concentration which would improve IR and postprandial hypertriglyceridaemia in response to a physiological mixed meal in women with PCOS.

3.2 Methods and materials

Details of study design, recruitment, protocols and procedures have been described in (section 2.1, 2.2.1, 2.3.1). This was a hypothesis generating, case control, interventional study. The ethical approval for the study was obtained from the Hull and East Riding ethics committee, and all participants gave their fully informed consent. The study was sponsored by the Research and Development Department, Hull and East Yorkshire hospitals NHS trust.

Ten women with PCOS and 10 age and BMI matched healthy volunteers completed the study. Participants underwent a 900 kcal mixed meal test on two occasions one week apart, the first at baseline, and the second after taking acipimox (one, 250 mg capsule at 20:00h, 23:00h and at 06:00h). Tripalmitin ([^{13}C]-labeled), was used to measure fat oxidation during meal tests. Participants avoided foods naturally enriched with [^{13}C] (e.g., maize products and cane sugar), strenuous exercise and alcohol for 2 days before the study. They were provided with a standard low fat evening meal before the study day to replicate between meal tests, after which, they drank only water until the study commenced.

Briefly, participants attended the research centre at 08:00h following an overnight 12 h fast on the study day. Height, weight, waist and hip circumference and resting blood pressure were measured and a specimen of expired air collected to measure exhaled [^{13}C] excretion. Fasting venous samples were taken via a cannula inserted into a forearm vein. Participants then consumed a standard 900 kcal mixed meal (45 g fat, 32 g protein, 92 g carbohydrates)(section 2.3.1). Thereafter, venous blood samples and specimens of exhaled air were collected at half-hourly intervals for the first 3 h and then hourly until study completion 6 h after label administration. Subjects were asked to collect four, further exhalation samples at home until 24 h after the labeled meal. The patients remained seated or supine for the duration of the study period. No additional foods or liquids, except for water, were permitted during the study. Blood

samples were centrifuged within 30 min of sampling at 1500 G for 15 min at 4° C to isolate plasma and serum that were stored at -80° C until analysis.

Biochemical analysis and statistical analysis were described in section 2.4, 2.5.

3.3 Results

3.3.1 Baseline characteristics

Baseline characteristics of women with PCOS and healthy controls are detailed in Table 3-1. Age and BMI were similar between control and PCOS groups. Women with PCOS had higher insulin, and HOMA_IR, androgen and TG levels, and lower HDL-c levels than controls. Fasting NEFA level and whole body fat oxidation measured by cumulative ^{13}C exhaled in breath were similar in both groups.

3.3.2 Postprandial lipid and glucose in response to a mixed meal

Following a physiological mixed meal, insulin secretion was persistently increased until 6 h post-meal in PCOS compared with controls. Postprandial NEFA levels were suppressed by a postprandial rise in insulin in both controls and PCOS. However, despite a two fold increase of postprandial insulin levels, PCOS women had significantly higher postprandial TG and a trend for a rise in plasma glucose compared to controls throughout the meal test.

Postprandial changes in lipid, glucose and insulin at baseline and after acipimox therapy are illustrated in Table 3-2 and Table 3-3.

Table 3-1 Baseline characteristics of participants

Parameters	Controls (n=10)	PCOS (n=10)	*p =
Age (year)	30.6± 6.9	30± 2.2	0.79
Body mass index (kg/m^2)	31.3± 5.7	32.9± 3.9	0.48
Waist (centimetre)	90.9± 10	102± 16	0.07
WHR	0.80± 0.05	0.87± 0.02	0.03
Testosterone (nmol/L)	1.12± 0.4	2.0± 1.3	0.05
Free androgen index	2.8± 0.95	6.6± 3.1	0.00
SHBG (nmol/L)	44.2± 17	31±12	0.08
Androstenedione (nmol/L)	7.3± 2.2	11± 2.6	<0.001
TC (mmol/L)	4.24± 0.29	4.43± 0.34	0.69
Triglycerides (mmol/L)	0.96±0.12	1.40± 0.14	0.03
HDL-c (mmol/L)	1.3± 0.11	1± 0.06	0.05
LDL-c (mmol/L)	2.55± 0.23	2.79± 0.27	0.51
Fasting NEFA (µmol/L)	421 (281, 580)	408 (320, 581)	0.65
Fasting glucose (mmol/L)	4.75± 0.19	5.32± 0.34	0.16
Fasting insulin (pmol/L)	55 (43, 72)	96 (62, 118)	0.03
HOMA-IR	1.62 (1.33, 2.19)	2.99 (2.18, 3.97)	0.01
HbA1c (mmol/mmoL)	32± 2.9	30± 8	0.46
Cumulative exhaled $^{13}CO_2$	19.8± 2.3	19.9± 1.97	0.55

Results are expressed as mean ± SD, or median (25th, 75th percentile). *p <0.05 is significant.

Table 3-2 Baseline postprandial lipid and glucose levels

Parameters	Controls (n=10)	PCOS (n=10)	*p =
TG AUC 2 h (mmol/L)	2.4± 0.9	3.38± 1.1	0.04
TG AUC 4 h (mmol/L)	5.19± 0.67	7.56± 0.90	0.05
TG AUC 6 h (mmol/L)	7.93± 1.06	11.3± 1.33	0.07
NEFA AUC 2 h (µmol/L)	408 (366, 435)	451 (285, 562)	0.33
NEFA AUC 4 h (µmol/L)	719 (654, 757)	813 (536, 911)	0.08
NEFA AUC 6 h (µmol/L)	1312 (1172, 1751)	1459 (1175, 1639)	0.94
PG AUC 2 h (mmol/L)	9.86± 2.0	11.3± 2.21	0.13
PG AUC 4 h (mmol/L)	19.2± 1.06	21.8± 1.2	0.12
PG AUC 6 h (mmol/L)	28.5± 1.3	31.1± 1.4	0.20
Insulin AUC 2 h (pmol/L)	651 (401, 1167)	1237 (810, 1690)	0.03
Insulin AUC 4 h (pmol/L)	161 (116, 293)	261 (196, 473)	0.04
Insulin AUC 6 h (pmol/L)	193 (130, 431)	300 (224, 564)	0.04

Results are expressed as mean ± SD, or median (25th, 75th percentile). *p < 0.05 is significant.

Table 3-3 Effect of acipimox on glucose and lipid levels

Parameters	Controls (n=10)			PCOS (n=10)		
	baseline	after acipimox	*p =	Baseline	after acipimox	*p =
Fasting TG (mmol/L)	0.96± 0.12	0.80± 0.14	0.05	1.40± 0.14	1.11± 0.12	0.01
Fasting NEFA (µmol/L)	421 (281, 580)	141 (82, 240)	0.01	408 (320, 581)	108 (73, 246)	0.01
Fasting PG (mmol/L)	4.75 (4.38, 5.2)	4.8 (4.25, 5)	0.37	5.0 (4.45, 6.28)	4.5 (4.1, 4.95)	0.03
HOMA-IR	1.62 (1.33, 2.19)	0.96 (0.67, 1.09)	0.03	2.99 (2.18, 3.97)	1.98 (1.39, 2.63)	0.02
24h Cumulative exhaled $^{13}CO_2$	19.8± 2.3	16± 2.3	0.048	19.9± 1.97	15.4± 0.75	0.05
Insulin AUC 2 h (pmol/L)	651 (401, 1167)	445 (325, 751)	0.01	1238 (810, 1691)	917 (644, 1070)	0.04
Insulin AUC 4 h (pmol/L)	161 (116, 293)	123 (75, 195)	0.005	261 (196, 473)	247 (152, 301)	0.05
Insulin AUC 6 h (pmol/L)	193 (130, 431)	144 (99, 239)	0.022	300 (224, 564)	291 (191, 344)	0.07
PG AUC 2 h (mmol/L)	9.94 (7.8, 10.84)	10.1 (8.3, 10.7)	0.72	11.3 (9.6, 12.7)	10.3 (8.93, 12.3)	0.26
PG AUC 4 h (mmol/L)	19.2± 1.06	19.2± 0.78	0.98	21.8± 1.2	20.1± 1.2	0.10
PG AUC 6 h (mmol/L)	28.5± 1.3	28.2± 0.94	0.745	31.1± 1.4	28.7± 1.4	0.06
TG AUC 2 h (mmol/L)	2.4± 0.9	2.2± 1.0	0.12	3.4± 1.1	2.7± 1.0	0.04
TG AUC 4 h (mmol/L)	5.2± 2.1	4.9± 2.5	0.52	7.6± 2.8	6.7± 1.8	0.34
TG AUC 6 h (mmol/L)	7.9± 3.4	7.7± 3.8	0.68	11.3± 4.2	10.6± 2.9	0.55
NEFA AUC 2 h (µmol/L)	408 (365, 435)	232 (186, 337)	0.01	452 (286, 562)	256 (163, 303)	0.01
NEFA AUC 4 h (µmol/L)	719 (653, 757)	589 (451, 773)	0.29	813 (536, 911)	582 (373, 898)	0.20
NEFA AUC 6 h (µmol/L)	1312 (1172, 1751)	2112 (850, 2476)	0.24	1459 (1175, 1639)	1552 (949, 2011)	0.51

Results are expressed as mean ± SD, or median (25th, 75th percentile). *p < 0.05 is significant.

3.3.3 Effect of overnight acipimox on postprandial glucose

All the participants have completed the study. Two subjects with PCOS and one control subject reported that they have had tolerable skin flushes within one to two hour after ingestion of acipimox tablets. Those symptoms resolved spontaneously after a few hours. Overnight acipimox lowered fasting NEFA effectively in both controls and PCOS (median NEFA μmol/L: controls baseline, 421 (281, 580); controls after acipimox, 141(82, 240), p<0.01; PCOS baseline, 408 (320, 581); PCOS after acipimox, 108 (73, 246), p=0.01). Overnight acipimox also effectively suppressed postprandial NEFA AUC levels 2 h in both controls and PCOS (median NEFA μmol/L: controls baseline, 408 (366, 435); after acipimox, 232 (186, 337), p<0.01; PCOS baseline, 452 (286, 563) PCOS after acipimox, 256 (163, 303), p=0.01).

The decreased availability of NEFA following treatment with acipimox led to a reduction of whole body fat oxidation in both groups. Exhaled $^{13}CO_2$, a marker of fat oxidation, fell from 19.5 (14.6, 23.9) to 17 (9.78, 20.3), p=0.05 in controls and from 21 (16, 24) vs.15.1 (13.9, 16.7), p=0.05 in PCOS.

Following overnight acipimox therapy, a fall in NEFA level was associated with a significant fall in fasting TG. Importantly, this was accompanied by a reduction in HOMA-IR in both controls and PCOS (median value: controls baseline, 1.62 (1.33, 2.19); controls after acipimox, 0.96 (0.67, 1.09), p=0.03; PCOS baseline, 2.99 (2.18, 3.97); PCOS after acipimox, 1.98 (1.39, 2.63), p=0.02). Post treatment HOMA-IR of PCOS became comparable to the baseline HOMA-IR of controls (Figure 3-1).

Furthermore, postprandial insulin fell significantly in both groups. Absolute change in NEFA AUC 2 h correlated with absolute change in insulin AUC 2 h (r=0.549, p=0.05). Despite the reduction of postprandial insulin, postprandial glucose was maintained, suggesting better insulin sensitivity at the target tissue with acipimox therapy. For summary of results, see in Table 3-3, Figure 3-2, Figure 3-4 and Figure 3-5.

3.3.4 Effect of overnight acipimox on lipid metabolism

Three doses of acipimox taken overnight led to a reduction of fasting NEFA and TG levels. Furthermore, the effective suppression of NEFA AUC 2 h post meal by acipimox was accompanied by a reduced TG AUC 2 h in PCOS (mean TG mmol/L ±

SEM: PCOS baseline, 3.4 ± 1.1; PCOS after acipimox , 2.7± 1.0, p=0.04). Participants took the last dose of acipimox at 06:00h and its effect had therefore declined a few hours after the meal. This was evidenced by a rebound rise in NEFA AUC at 4 h and 6 h post-meal. TG AUC levels at 4 h and 6 h were no longer significantly different after acipimox therapy. Similar findings were observed in healthy controls (Figure 3-2, Figure 3-3).

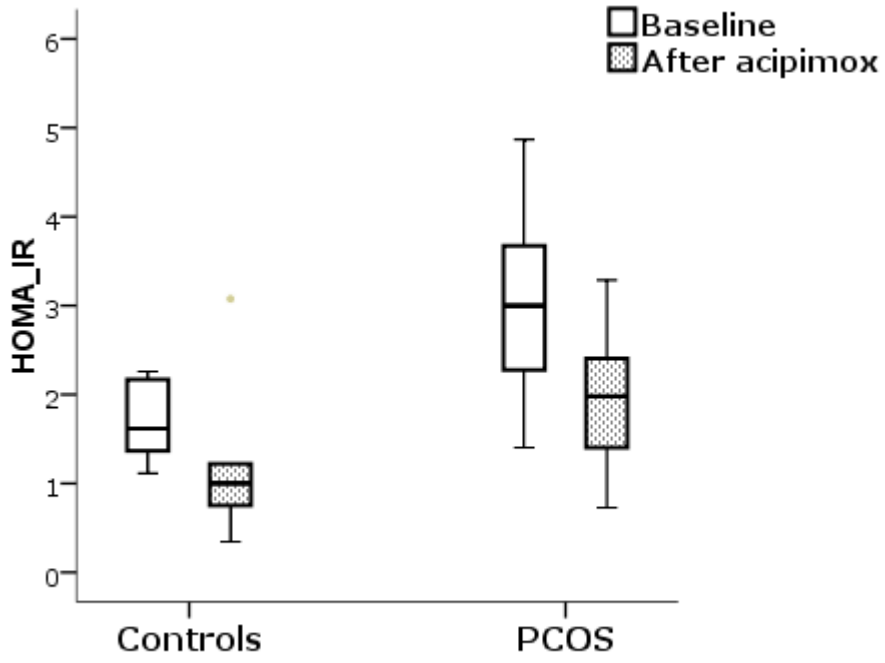


Figure 3-1Changes in HOMA-IR following overnight acipimox therapy
(The data are expressed as median ± interquartile range (box) and range (whisker).

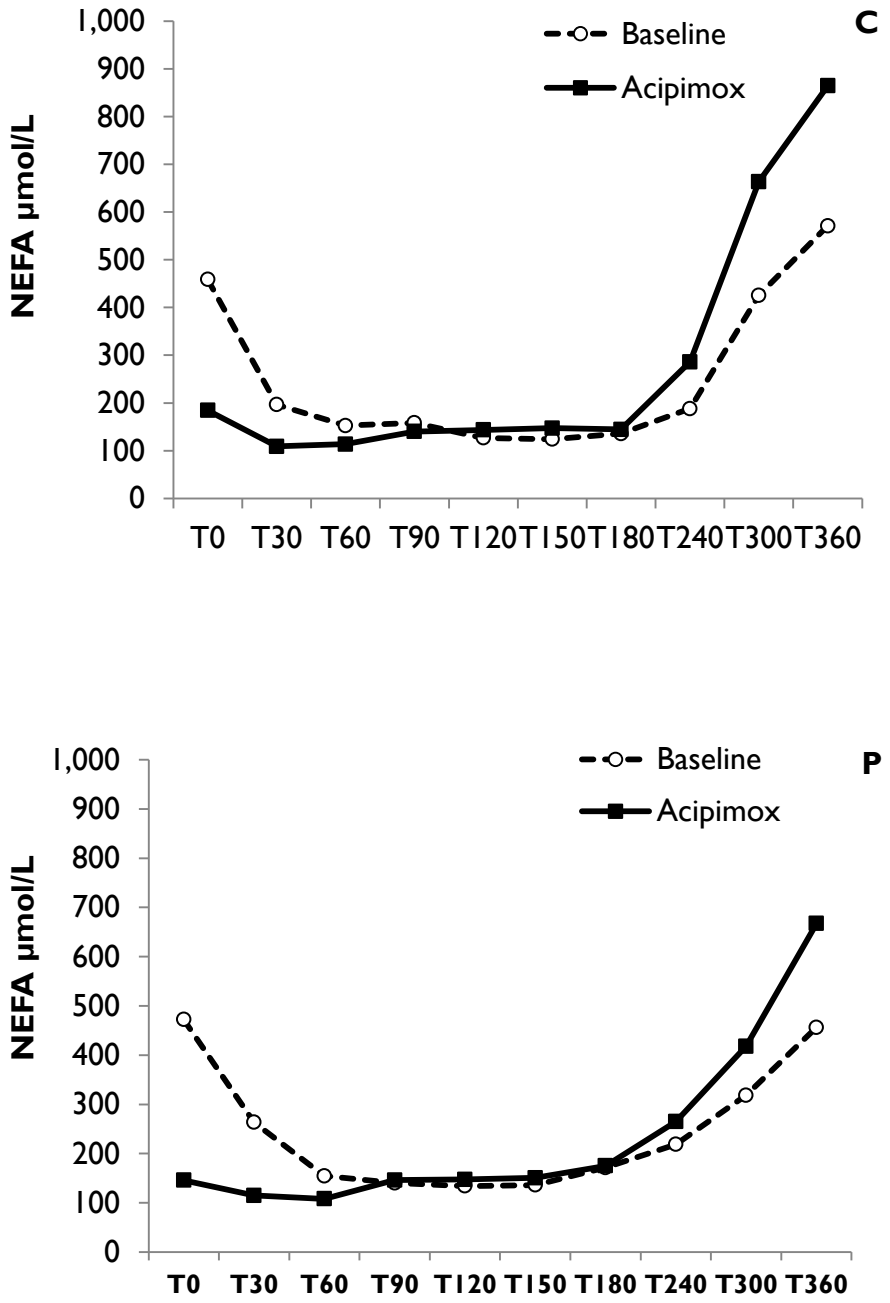


Figure 3-2 Postprandial NEFA levels at baseline and after acipimox therapy

Data are expressed as means. C= Controls, P=PCOS, T=time (minute)

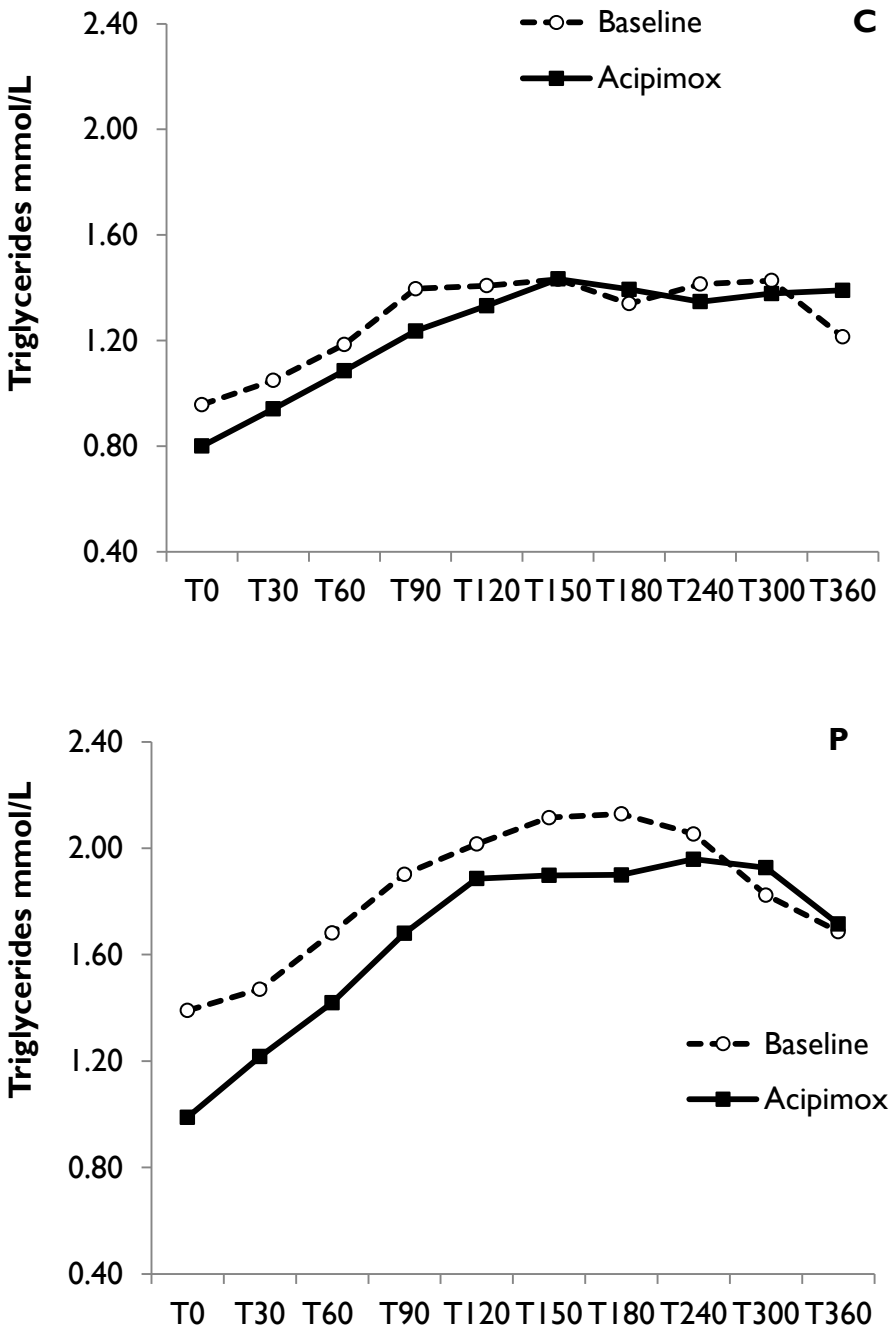


Figure 3-3 Postprandial triglyceride levels at baseline and after acipimox therapy

Data are expressed as means. C= Controls, P=PCOS, T=time (minute)

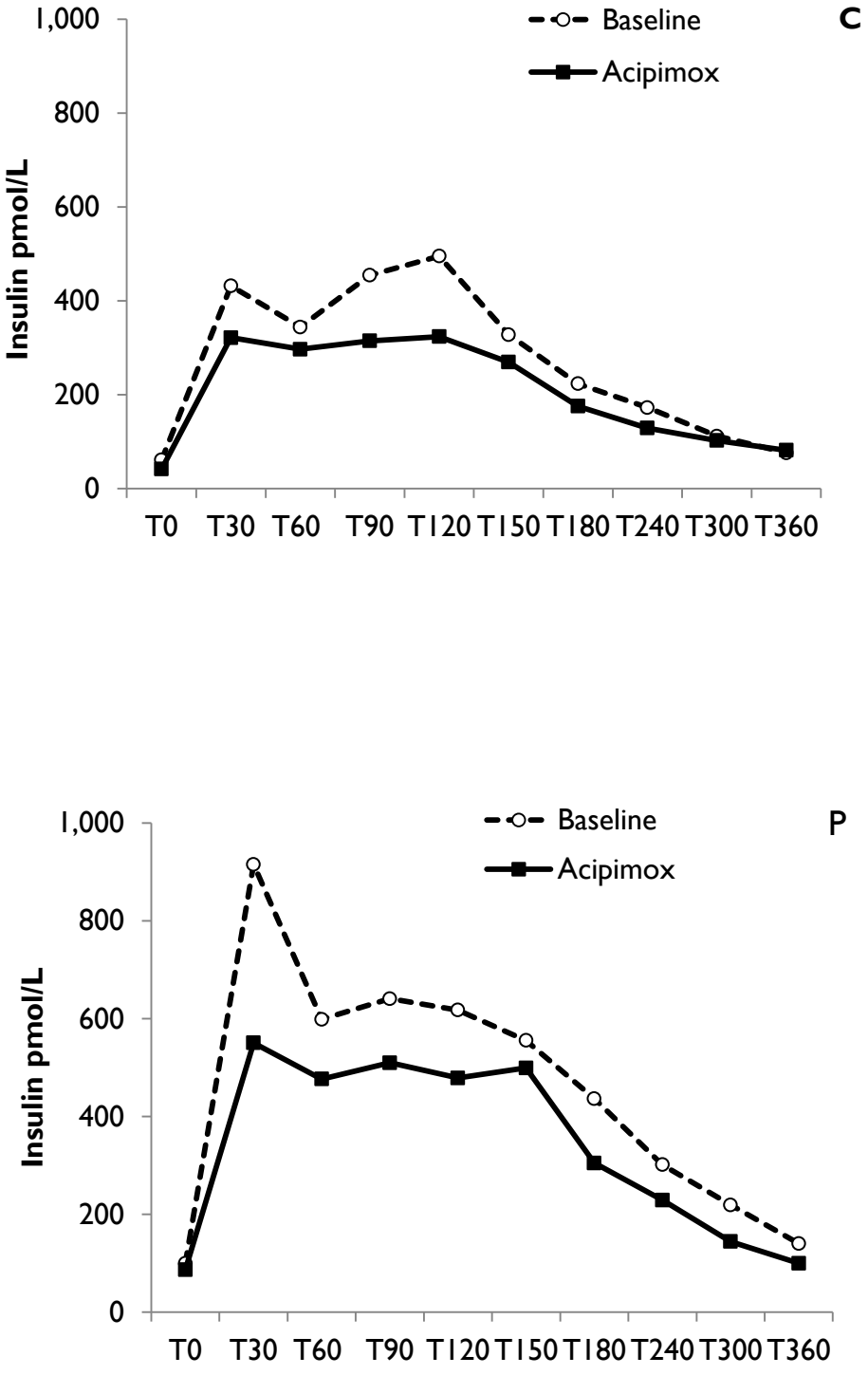


Figure 3-4 Postprandial insulin levels at baseline and after acipimox therapy

Data are expressed as means. C= Controls, P=PCOS, T=time (minute)

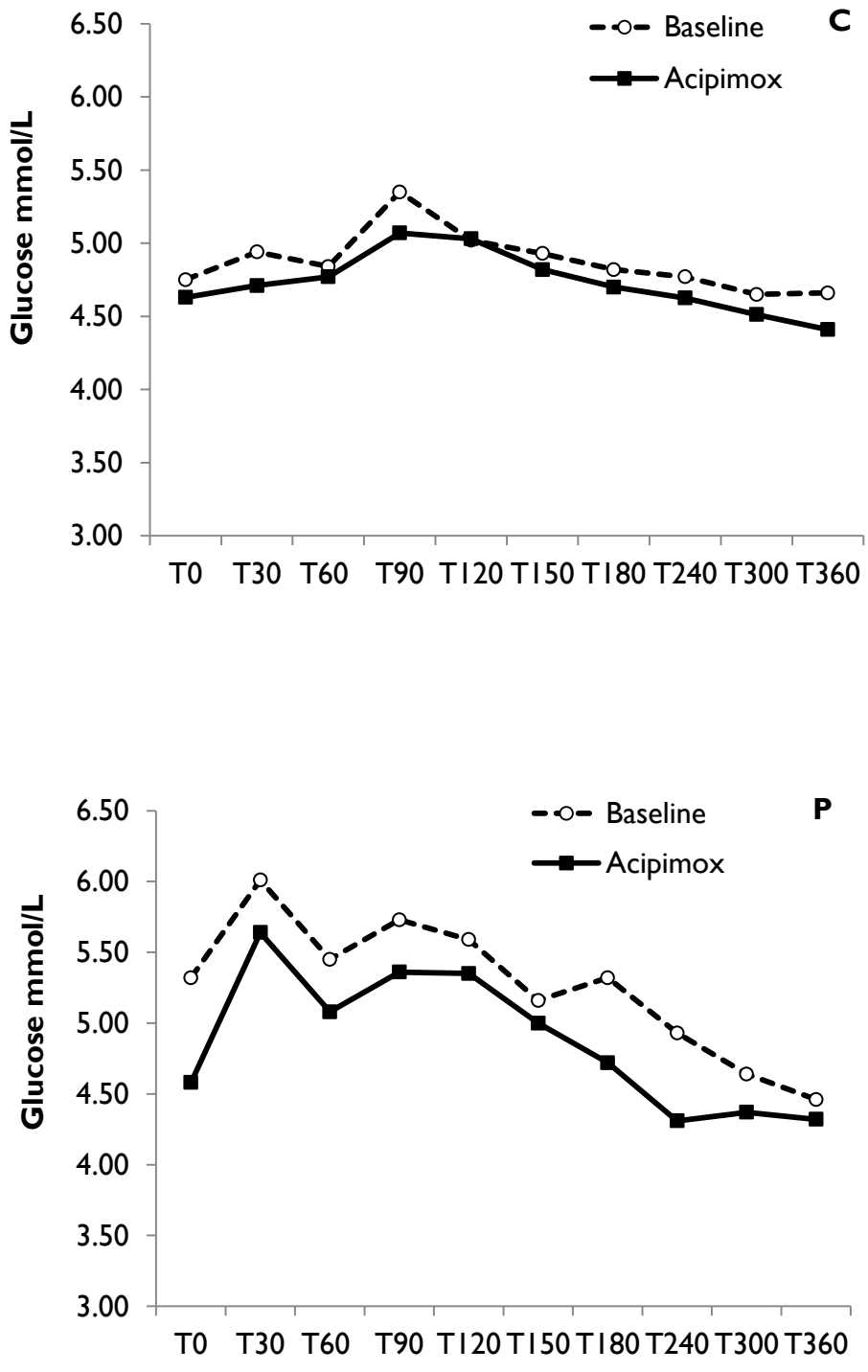


Figure 3-5 Postprandial glucose levels at baseline and after acipimox therapy

Data are expressed as means. C= Controls, P=PCOS, T=time (minute)

3.4 Discussion

In the present study, effective suppression of fasting NEFA by acipimox resulted in reduced IR in PCOS during fasting and in the postprandial period.

3.4.1 NEFA and insulin resistance

Baseline, fasting NEFA levels were not higher in subjects with PCOS although they had greater waist circumference and IR than healthy controls. This is in accord with findings in previous studies (590, 591). A recent review suggested that there is no consistent association of obesity with elevated fasting NEFA. In addition, NEFA delivery to non-fat tissues is increased even if plasma concentrations are only slightly raised in obesity (342).

Mobilization of NEFA from adipose tissue depends upon HSL activity which is highest during the post-absorptive state when the whole body, except the brain, relies on NEFA as an energy source, and at its lowest in the presence of high postprandial insulin. In insulin resistant states, adipose tissue HSL activity fails to respond to insulin postprandially although its activity is maintained during the post-absorptive state (571). This mechanism might explain why the ambient NEFA level was not raised in insulin resistant PCOS in the present study. Alternatively, the liver takes up excess NEFA and produces VLDL resulting in higher fasting TG in PCOS than controls and maintains a normal circulating NEFA level. According to studies by Mcquaid et al., there is a down regulation of NEFA release from adipose tissue in obese subjects to compensate expansion of adipose tissue mass which could explain why centrally obese women with PCOS had no increased fasting NEFA when compared with BMI matched but less centrally obese controls.

In 1963, Randle, Garland, Hales and Newsholme proposed the existence of a glucose fatty-acid cycle in which, excess lipid oxidation leads to inhibition of glucose oxidation in skeletal muscle (343). Both acute (< 24 h) and prolonged exposure (> 24 h) to intralipid, inducing elevated NEFA levels, lowered insulin sensitivity (365, 368) and subsequently reduced insulin-stimulated glucose oxidation in both healthy subjects (356-358) and T2DM (375). When NEFA levels were substantially increased following a 24 h fast, insulin sensitivity was reduced but that was corrected by 6 doses of

acipimox every 2 h in healthy subjects (386). In addition to a reduction of insulin-stimulated glucose uptake by skeletal muscle, elevated plasma NEFA augments hepatic gluconeogenesis and promotes hepatic glucose production suggesting hepatic IR (351, 355). The high NEFA level in patients with T2DM was highly related to the degree of fasting hyperglycaemia (331, 380, 381).

Reaven et al. documented that NEFA lowered by nicotinic acid, significantly improved fasting glucose through improved insulin action in diabetic rats (382). Similarly, NEFA lowered by acipimox, improved insulin-stimulated glucose oxidation as well as non-oxidative glucose disposal in patients with T2DM (383, 384), insulin treated poorly controlled T2DM (592), IGT (591) and healthy subjects with a strong family history of T2DM (593). A rebound rise of NEFA with long term acipimox use failed to improve IR in patients with T2DM (384, 594, 595). Therefore, it seems that NEFA levels are reciprocally and inversely related to insulin sensitivity in healthy and diabetic subjects.

In PCOS, a post receptor insulin signaling defect is detected in skin fibroblasts (143), skeletal muscle (142) and adipocytes (139). This defect is independent of obesity, metabolic derangement, body fat or sex hormone levels and therefore appears due to an inherited intrinsic abnormality in PCOS.

However, when skeletal muscle from PCOS subjects underwent repeated culture in a lipid free environment, a subsequent *in vitro* lipid challenge did not interfere with insulin-stimulated glucose transport despite the intrinsic insulin signalling defect *in vitro* (143). This suggested that IR in PCOS in vivo could be, in part, an acquired defect since IR in PCOS skeletal muscle decreased when the environmental factor (lipid) was removed from the *in vitro* environment. As mentioned earlier, NEFA plays a major role in IR in obesity and T2DM. Furthermore, increased FFA delivery and incomplete oxidation in skeletal muscle can lead to increased lipid metabolites which can interfere with insulin-mediated IRS-1 related PI3K activity and resultant glucose uptake via GLUT4 transporters (409). More information can be read in section 1.9.2. Therefore, the present study was conducted and determined the reversibility of IR in obese PCOS patients with an intrinsic insulin signalling defect by modulating NEFA levels.

When acipimox inhibited overnight NEFA production through suppression of HSL activity, the resultant fall in NEFA level was accompanied by a reduced IR in PCOS.

Interestingly, the post-treatment level of HOMA-IR in PCOS became similar to control baseline level suggesting complete reversal of IR with reduction of NEFA (Figure 3-1). Therefore, the present study confirms that IR in PCOS can be modified by improving NEFA levels.

In contrast, Ciampelli et al. found no effect of acipimox on insulin sensitivity despite effectively lowered NEFA in lean and obese PCOS, and controls (590). In the present study, a physiological mixed meal test was used and the relationship of NEFA and glucose metabolism was examined using the data gathered 2 h following a meal where maximal suppression of NEFA and the maximal response of insulin secretion to the meal. Our findings in PCOS subjects are consistent with findings in subjects with simple obesity and T2DM as mentioned above.

3.4.2 Effect of lowered NEFA on chylomicron clearance in control subjects

The first step in chylomicron clearance is dependent on an insulin-stimulated increase in LPL activity (582). Impaired chylomicron clearance i.e. a postprandial rise in TG, has been associated with visceral obesity (245), metabolic syndrome (596), polycystic ovary syndrome (285) and T2DM (597). This has been suggested to be due to IR as a failure of insulin action on HSL in adipose tissue (598), and on LPL at the luminal surface of the endothelium in adipose tissue (599), and on lipid oxidation. Insulin regulates both clearance and storage of TG from the circulation and the suppression of hydrolysis of stored NEFA in adipose tissue, promoting the storage of fat and metabolism of glucose in the postprandial phase. Unlike glucose homeostasis, there is no feedback loop for lipids with only a highly sensitive on or off regulation. The study examined whether impaired chylomicron clearance was a consequence of IR in PCOS or competitive inhibition by higher endogenous VLDL, competing for hydrolysis by LPL in adipose tissue with chylomicrons.

In the present study, the reduction of fasting NEFA by overnight acipimox inhibition of HSL activity (when most active- overnight fasting), was accompanied by a fall in fasting TG suggesting that the rate of VLDL synthesis in the liver was decreased. It was followed by improved chylomicron clearance in the postprandial phase in healthy subjects despite lower postprandial insulin levels suggesting that the improvement is

most likely due to reduced competition for hydrolysis in adipose tissue. Thus, TG clearance was maximally stimulated in the postprandial state, the rate of clearance being dependent on the total level. In healthy controls, higher fasting TG was associated with impaired chylomicron clearance. Considering whole body fat oxidation in the postprandial state was reduced following acipimox therapy, the absolute rate of chylomicron clearance was similar before and after acipimox. Thus, the improved postprandial clearance is due to reduced substrate and not faster hydrolysis and capture in adipose tissue or increased oxidation of NEFA. LPL clears chylomicrons in preference to VLDL in the postprandial period in healthy subjects (181). In the present study, a reduction in NEFA and VLDL (fasting TG) by acipimox further facilitated the clearance of chylomicrons by LPL as evidenced by lowered postprandial TG in the presence of lower postprandial insulin levels.

3.4.3 Effect of lowered NEFA on chylomicron clearance in PCOS

In the present study, women with PCOS had higher postprandial TG than their age and BMI matched controls in accord with previous reports of the association of postprandial hypertriglyceridaemia and insulin resistant states (245, 285, 596, 597). In healthy subjects, the rate of chylomicron clearance was partly dependent upon substrate competition for LPL rather than impaired activation of insulin.

Overnight acipimox therapy lowered NEFA by inhibiting HSL activity in PCOS. This was accompanied by a fall in fasting TG suggesting the rate of VLDL synthesis in the liver was decreased, similar to the control group. Comparison of postprandial NEFA between meal tests i.e. with or without acipimox therapy in PCOS, showed that it was suppressed to a similar extent by postprandial insulin even though postprandial insulin AUC was significantly lowered with acipimox therapy. In both tests, postprandial NEFA reached its nadir at 90 min. This finding suggested that postprandial HSL activity was suppressed by postprandial insulin in PCOS and controls with or without the effect of acipimox therapy. This effect may be due to baseline compensatory hyperinsulinaemia present in PCOS. Fat oxidation in controls and PCOS was similar at baseline and fell concomitantly after acipimox therapy suggesting that inhibitory action of insulin on lipolysis is well compensated in PCOS.

When fasting TG was suppressed by overnight acipimox and postprandial HSL activity blocked, there was a concomitant fall of postprandial TG in PCOS. Since postprandial HSL activity was successfully suppressed and fat oxidation decreased by acipimox, similar to controls, this suggests that substrate competition for LPL activity as the major influence in chylomicron clearance in PCOS. Continued synthesis of VLDL postprandially from NEFA supplied by spillover from LPL activity and delayed removal of chylomicron remnants by LDL receptors were considered as contributing factors to postprandial hypertriglyceridaemia. However, acipimox significantly improved postprandial TG with no effect on LPL activity or clearance of chylomicron remnants, suggesting substrate competition for LPL activity is a major contributing factor for chylomicron clearance in PCOS.

3.5 Summary

The present study has shown that effective lowering of NEFA and its decreased delivery to non-adipose tissue improves fasting and postprandial IR in PCOS despite its association with an intrinsic insulin signalling defect. This suggests that NEFA contributes significantly to IR in PCOS which affirms the necessity of environmental factors to impair insulin-mediated glucose transport in previous *in vitro* studies of skeletal muscle in PCOS. This finding also suggests the metabolic reversibility of IR in PCOS by modification of NEFA metabolism.

The present study showed that postprandial NEFA was equally suppressed by postprandial insulin levels in both PCOS and controls with or without acipimox therapy. This suggests that the inhibitory action of insulin on HSL activity is intact in PCOS at least in the presence of compensatory hyperinsulinaemia. A postprandial defect in lipid handling in patients with PCOS was found to be improved with acipimox lowered fasting VLDL-TG, in accord with observations in healthy controls. The concomitant fall in fasting TG and postprandial TG following acipimox therapy suggested that postprandial TG was due to competition for chylomicron clearance by endogenous VLDL rather than a manifestation of IR.

Chapter 4 Effect of rebound rise in NEFA during chronic use of nicotinic acid on insulin resistance and cardiovascular risk in PCOS

4.1 Introduction

PCOS is associated with multifaceted metabolic abnormalities such as obesity (283, 412, 600-602), metabolic syndrome (557), dyslipidaemia (286, 603) and insulin resistance (604). Women with PCOS are at risk of impaired glucose regulation (67) and subsequent cardiovascular disease (99). Typical dyslipidaemia found in PCOS is low HDL-c, preponderance of atherogenic small dense LDL-c (286, 603, 605), and fasting and postprandial hypertriglyceridaemia (285). Evidence of early arthrosclerosis such as impaired endothelial dysfunction (79, 606, 607), increased carotid intima-media thickness (89, 608, 609) and coronary artery calcium (90, 91) seem to be prevalent in PCOS.

In the insulin resistant state, insulin is unable to suppress completely, HSL activity in adipose tissue. This leads to an increased flux of NEFA to the liver resulting in increased synthesis and release of VLDL. As shown in section 3.4.2, 3.4.3, the excess VLDL competes for LPL and interferes with chylomicron clearance in the postprandial period. The delayed removal of chylomicrons and their remnants have been seen in diabetes (610), PCOS (285), renal failure (552) and familial combined dyslipidaemia. All of these conditions are well recognised to be associated with atherosclerosis and cardiovascular diseases such as hypertension and coronary artery disease.

Niacin raises HDL-c and reduces both LDL-c and TG, and on this basis, has been used to treat patients with hyperlipidaemia and CVD for over 50 years (611). Lowering TG and increasing HDL-c using fibrates has been shown to reduce cardiovascular events, albeit limited to patients with atherogenic metabolic dyslipidaemia (612) so it was hoped that niacin would show similar benefits in these patients especially since niacin has been shown to induce regression of common carotid intima-media thickness (516, 613) and decrease carotid atherosclerosis (614).

Obesity and the chronic elevation of NEFA are associated with insulin resistance in PCOS. IR plays a major role in the pathophysiology of hyperandrogenism, diabetes and CVD risk. Acute suppression of NEFA with acipimox improves IR in PCOS (section 3.3.3). Moderate intensity exercise that enhanced fatty acid oxidation improved IR and androgen profile in PCOS (section 5.3.4, 5.3.5). Whether this effect would be sustained with chronic suppression of NEFA is not known.

This study examined the prolonged effect of niacin on postprandial lipids and glucose metabolism, their relationships with NEFA and their impact on cardiovascular risk markers in obese women with PCOS using a mixed meal model. In addition, the effect of chronic suppression of NEFA on IR was investigated.

4.1 Materials and methods

4.1.1 Subjects and randomization

This was a double blind, randomized placebo controlled trial. A total of 37 PCOS patients were screened for the study and 34 of these, fulfilling the inclusion and exclusion criteria, were included in the study after obtaining their informed written consent. Participants were non-smokers with no concurrent illness, were not taking prescription or over-the-counter medication likely to affect insulin sensitivity or lipids including hormonal contraceptives for the preceding 3 months. None were breast-feeding or planning to conceive and all were using barrier contraception. They were advised not to change their lifestyle, including physical activity or dietary habits, during the study period. Details of recruitment, screening tests and study visits have been mentioned in section 2.1.5.2. They were randomly assigned to either niacin/laropiprant or placebo. Randomization was performed by a hospital pharmacist with 1:1 allocation in random blocks of 12. All investigators and patients were blinded to randomization and treatment assignment. The responsible pharmacist who did not see patients directly, held the randomization table and treatment assignment in a sealed container.

4.1.2 Procedures and protocols

Detailed procedures of visits, the EndoPAT test and mixed meal tests have been discussed in section 2.3.1, 2.3.2. In brief, after passing successful screening, subjects

underwent a baseline assessment, an EndoPAT test and a mixed meal test before the study medicine was prescribed.

On the study day, participants attended the research centre at 08:00h following an overnight 12 h fast. Weight, height, waist, hip and resting blood pressure were measured followed by an endothelial function assessment with EndoPAT 2000. A cannula was inserted in the antecubital vein and fasting blood samples were taken. Subjects consumed the study meal, a standard 900 kcal mixed meal (45 g fat, 32 g protein, 92 g carbohydrates: 9 g of those was refined sugar) consisting of breakfast cereal and milk, a cheese sandwich and a milkshake. Serial blood samples beginning 30 min after the meal were collected at half-hourly intervals for the first 3 h and then hourly for a further 3 h. This protocol was repeated at the end of the study.

Niacin/laropiprant (Nicotinic acid 1000 mg/ laropiprant 20 mg per tablet) or placebo began at one tablet per day for the first four weeks increasing to two tablets per day for 8 weeks. Participants were advised to take tablets whole, with food, at bedtime and were seen once in every 4 week to ensure their tolerability, safety and compliance with treatment. Compliance was checked by counting returned tablets.

4.1.3 Biochemical analysis

Measurement of total cholesterol, TG, HDL-c, LDL-c, NEFA, blood glucose, insulin, testosterone, SHBG, FAI and hsCRP were carried out according to the analytical assays mentioned in section 2.4.

4.1.4 Statistical analysis and calculations

The statistical analysis used is described in detail in section 2.5. The methods of calculation of total and incremental TG area under the response curve, and insulin, glucose and NEFA AUC and HOMA-IR are mentioned in section 2.6. The oral disposition index (ODI) was used as a measure of compensatory beta cell function in response to insulin resistance (555). The calculation of ODI, AIRg ($\Delta I0–30/\Delta G0–30$) and insulin sensitivity was described in section 2.6. Fasting beta cell function (HOMA-B %) was calculated using the HOMA calculator that uses the HOMA 2 model. The HOMA calculator is designed to estimate beta cell function (%B) and insulin sensitivity

(%S) for an individual from simultaneously measured fasting plasma glucose and fasting plasma insulin values (552).

4.1.5 Sample size determination

A sample size of 36 with 1:1 randomization was calculated to show a one standard deviation difference in the primary outcome measure, fasting HDL-c, between the two groups (80% power, 5% significance, two-tailed) with an assumption of 10% loss to follow-up. Although effect of tredaptive on NEFA was primarily aimed to study, there was no reference study for power calculation and therefore HDL-c, one of the cardiovascular stratified risk, was used for sample size calculation.

4.1.6 Pharmacovigilance and Safety Evaluation

After commencing stud medicine in visit 2, patients were instructed to return used and unused study drug bottles and packets in their next visit 3, 4 and 5. They were reviewed in visit 3 – 6 at the research centre and by telephone in visit 2A, 3A and 4A for their tolerance and safety measures. Participants were aware of the adverse effects of nicotinic acid/laropiprant listed in the patient information sheet and encouraged to report any adverse events. The contact numbers for research team at working hours as well as emergency numbers were given (Figure

Blood tests for full blood count, coagulation screen, urea, creatinine & electrolytes, bone profile, amylase, Liver function test, thyroid function test, lipid profile, creatinine kinase and uric acid were done at visit 1 as baseline data and visit 3, 4, 5 and 6 for safety monitoring of study medicine. All the adverse reactions were handled according to guidance of EU Directive 2001/20/EC.

Figure 4-1 A tredaptive study card for participants

4.2 Results

A total of 37 patients were screened for the study and 3 patients failed to meet the eligibility criteria. Thirteen of 17 (76%) participants in the niacin group and 12 of 17 (70%) patients in the placebo group completed the study. Two patients (12%) dropped out due to intolerance of hot flushes in niacin group. Two patients from the niacin group and four patients from the placebo group were withdrawn because of loss of follow up. One patient from the placebo group was withdrawn after she became pregnant despite using barrier contraception (Figure 4-2).

4.2.1 Baseline characteristics

At baseline, both groups were matched for age, BMI, WHR and degree of hyperandrogenaemia. The lipid profile, fasting NEFA, fasting plasma glucose and HbA1c and RHI were comparable between groups. HOMA-IR, 2 h postprandial glucose and hsCRP were not different between the groups (Table 4-1).

At the end of the study, there were no significant differences in BMI, WHR, SHBG or FAI and HbA1c before and after the intervention, or between either groups. There was a reduction in testosterone within the niacin group but this was not significant when compared the placebo group (Table 4-2).

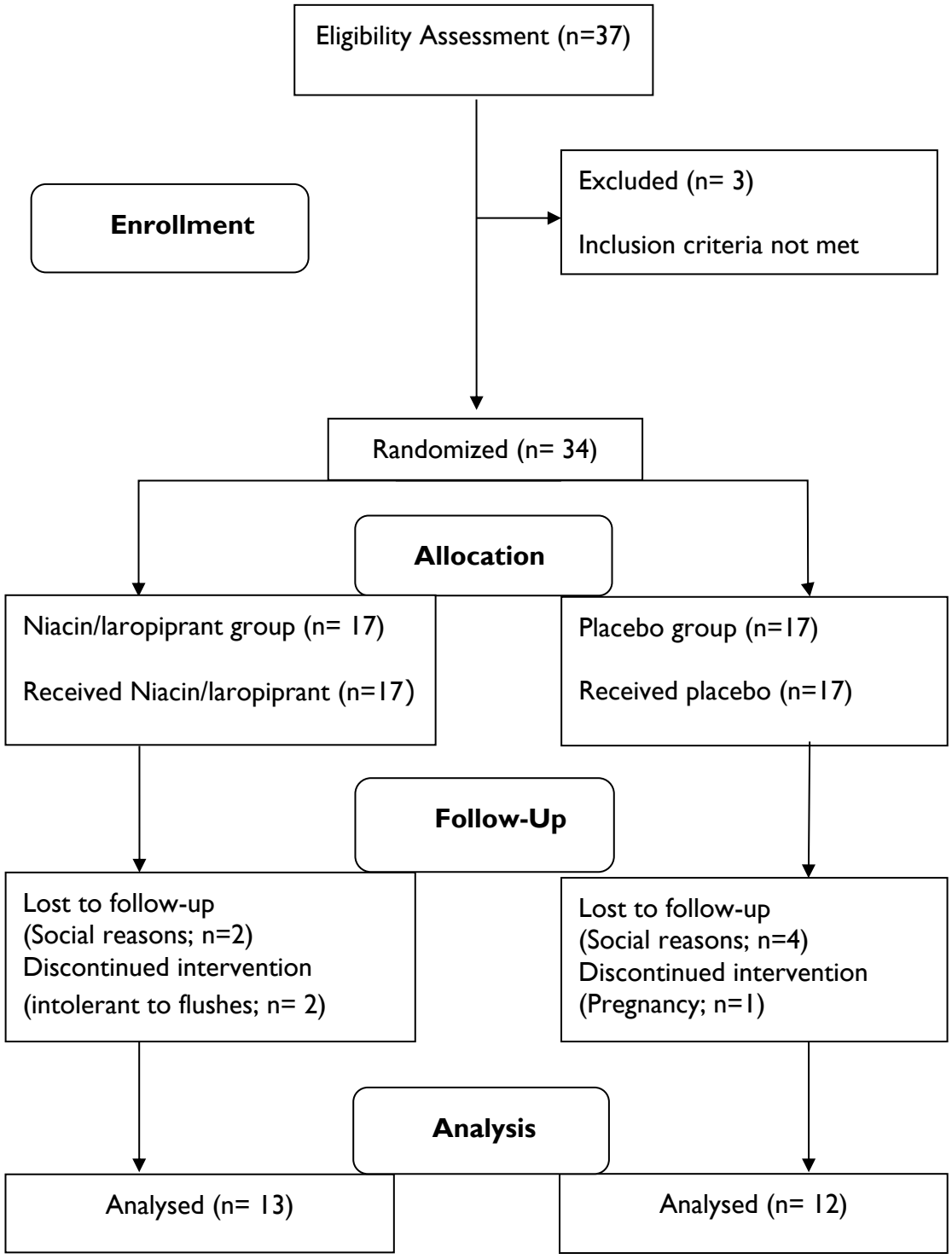


Figure 4-2 Clinical trial diagram

Table 4-1 Baseline characteristics of participants

Parameter	Niacin/Laropiprant (n=13)	Placebo (n=12)	*p =
Age (year)	31.0± 6.33	31.7± 6.51	0.80
BMI (kg/m^2)	35.8± 5.55	34.8± 5.03	0.65
Waist (cm)	108± 13.5	104± 13.5	0.55
WHR	0.88± 0.06	0.87± 0.08	0.62
SBP (mmHg)	125± 13.8	121± 12.5	0.53
DBP (mmHg)	80.2± 15.4	71.2± 6.55	0.07
Testosterone (nmol/L)	1.44± 0.58	1.41± 0.54	0.89
FAI	5.63± 2.40	5.93± 3.09	0.79
SHBG (nmol/L)	29.2± 12.5	27.6± 12.6	0.75
ALT (iu/L)	31.8± 11.3	31.5± 18.9	0.97
TC (mmol/L)	4.55± 1.17	4.65± 0.87	0.81
TG (mmol/L)	1.22± 0.62	1.29± 0.59	0.37
HDL-c (mmol/L)	1.20± 0.31	1.15± 0.25	0.66
LDL-c (mmol/L)	2.78± 0.93	2.84± 0.54	0.86
FPG (mmol/L)	5.14± 0.83	5.20± 0.42	0.82
PPG (mmol/L)	6.24± 2.27	7.16± 1.76	0.29
HbA1c (mmol/mmoL)	36.5± 3.64	34.1± 2.51	0.10
TSH (iu/L)	1.97± 1.38	1.25± 0.42	0.11
hsCRP (mg/mL)	6.14± 2.98	3.44± 2.19	0.10
Fasting NEFA (µmol/L)	458± 133	550± 138	0.24
HOMA- B%	92.4± 47	115.2± 33	0.17
HOMA- IR	2.2 (1.2,4.2)	3.3 (2.2,4,1)	0.22
Insulin (pmol/L)	51(37, 95)	96(59, 115)	0.22
ODI (mM^{-1})	10.2(3.1, 13.9)	4.08(2.41, 17.3)	0.69
RHI	1.97± 0.40	1.95± 0.50	0.50

Results are expressed as mean ± SD, or median (25th, 75th percentile).

*p < 0.05 is significant

Table 4-2 Effect of Niacin/laropiprant on cardiovascular risk

	Niacin/laropiprant(n=13)			Placebo (n=12)			Change %		
Parameters	Baseline	12 weeks	P *	Baseline	12 weeks	P *	Niacin	Placebo	P †
BMI (kg/m^2)	35.8± 5.55	35.9± 5.67	0.55	34.8± 5.03	34.6± 4.62	0.70	0.00	0.00	0.70
Waist (cm)	108± 13.5	106± 12.0	0.36	104± 13.5	103± 14	0.43	-0.02	-0.01	0.64
WHR	0.88± 0.06	0.86± 0.06	0.01	0.87± 0.08	0.85± 0.08	0.32	-0.16	-0.02	0.44
SBP (mmHg)	125± 13.8	127± 15.5	0.37	121± 12.5	118±11	0.24	0.02	-0.03	0.16
DBP (mmHg)	80.2± 15.4	80.9± 9.68	0.86	71.2± 6.55	76± 8	0.17	-0.07	0.07	0.24
hsCRP (mg/mL)	6.14± 3.98	5.11± 3.56	0.05	3.44± 2.19	3.58± 2.41	0.81	-0.09	0.11	0.30
RHI	1.97± 0.40	2.05± 0.58	0.34	1.95± 0.50	1.96± 0.47	0.76	0.12	0.03	0.47
Testosterone (nmol/L)	1.44± 0.58	1.24± 0.53	0.05	1.41± 0.54	1.40± 0.60	1.00	-0.07	0.08	0.13
FAI	5.63± 2.40	4.70± 2.49	0.48	5.93± 3.09	5.43± 2.6	0.17	-0.04	-0.05	0.96
SHBG (nmol/L)	29.2± 12.5	29.5± 15.5	0.89	25.6± 12.6	27± 9	0.92	0.02	0.04	0.70
TC (mmol/L)	4.55± 1.17	4.18± 1.01	0.01	4.65± 0.87	4.72± 0.83	0.55	-0.08	0.01	0.05
TG (mmol/L)	1.22± 0.62	0.93± 0.50	0.04	1.29± 0.59	1.37± 0.67	0.79	-0.21	0.04	0.05
HDL-c(mmol/L)	1.20± 0.31	1.33± 0.34	0.05	1.15± 0.25	1.14± 0.20	0.99	0.16	-0.01	0.03
LDL-c (mmol/L)	2.78± 0.93	2.36± 0.74	0.01	2.84± 0.54	2.89± 0.57	0.62	-0.10	0.02	0.05

Results are expressed as mean ± SD. *Significance from baseline within the groups, † Significant differences for the comparison between the groups

Table 4-3 Effect of Niacin/Laropiprant on postprandial lipids and glucose

	Niacin/laropiprant(n=13)			Placebo (n=12)			Change %		
Parameters	Baseline	12 weeks	P*	Baseline	12 weeks	P *	Niacin	Placebo	P †
Fasting PG(mmol/L)	5.54± 0.6	5.56± 0.61	0.81	5.31± 0.38	5.2± 0.5	0.23	0.01	0.03	0.45
Fasting Insulin (pmol/L)	51(37, 95)	113(39, 150)	0.01	96(59, 115)	95(73, 153)	0.33	0.6	0.02	0.04
HOMA- IR	2.2 (1.2,4.2)	3.8(1.3, 5.5)	0.02	3.3 (2.2,4,1)	3.2(1.8, 4.4)	0.48	0.54	-0.12	0.05
HbA1c (mmol/mmoL)	36.5± 3.6	37.2± 4.1	0.21	34.1 ± 2.5	35.1 ±2.7	0.24	0.02	0.03	0.65
Fasting NEFA (µmol/L)	414 (353, 583)	506(410, 656)	0.35	561(376, 653)	499 (387, 604)	0.58	0.23	0	0.58
TG AUC 6 h (mmol/L)	9.44± 2.9	7.74± 3.2	0.03	11.0± 5.0	11.4± 4.7	0.77	-0.17	0.14	0.06
NEFA AUC 6 h(µmol/L)	1567(1273, 1781)	1811(1304, 2021)	0.06	1547(1142, 2134)	1639(1406, 1813)	0.58	0.11	-0.01	0.35
2 h PG (mmol/L)	6.08± 1.1	6.72 ± 1.4	0.02	6.24 ± 0.9	6.56± 1.6	0.3	0.14	0.02	0.04
PG AUC 2 h (mmol/L)	13.1 ±2.9	14.0 ±2.8	0.05	12.9 ±2.0	13.0 ±2.8	0.89	0.08	0.01	0.05
Insulin AUC 2h (pmol/L)	814(717,1645	1140(587, 1946)	0.51	1102(844, 1908)	1042(785, 1282)	0.8	-0.14	-0.05	0.59
AIRg (pmol/moL)	424(211, 975)	257(122, 418)	0.04	320(244, 879)	564(334, 664)	0.96	-0.31	0.29	0.04
ODI (mM-1)	10.2(3.1, 13.9)	2.12(0.96, 4.7)	0.01	4.08(2.41, 17.3)	5.0(3.95, 8.73)	0.33	-0.61	0.06	0.04

Results are expressed as mean ± SD except NEFA, insulin, AIRg, ODI and HOMA-IR which are median values (25^{th}, 75^{th}centiles)

*Significance from baseline within the groups, † Significant differences for the comparison between the groups

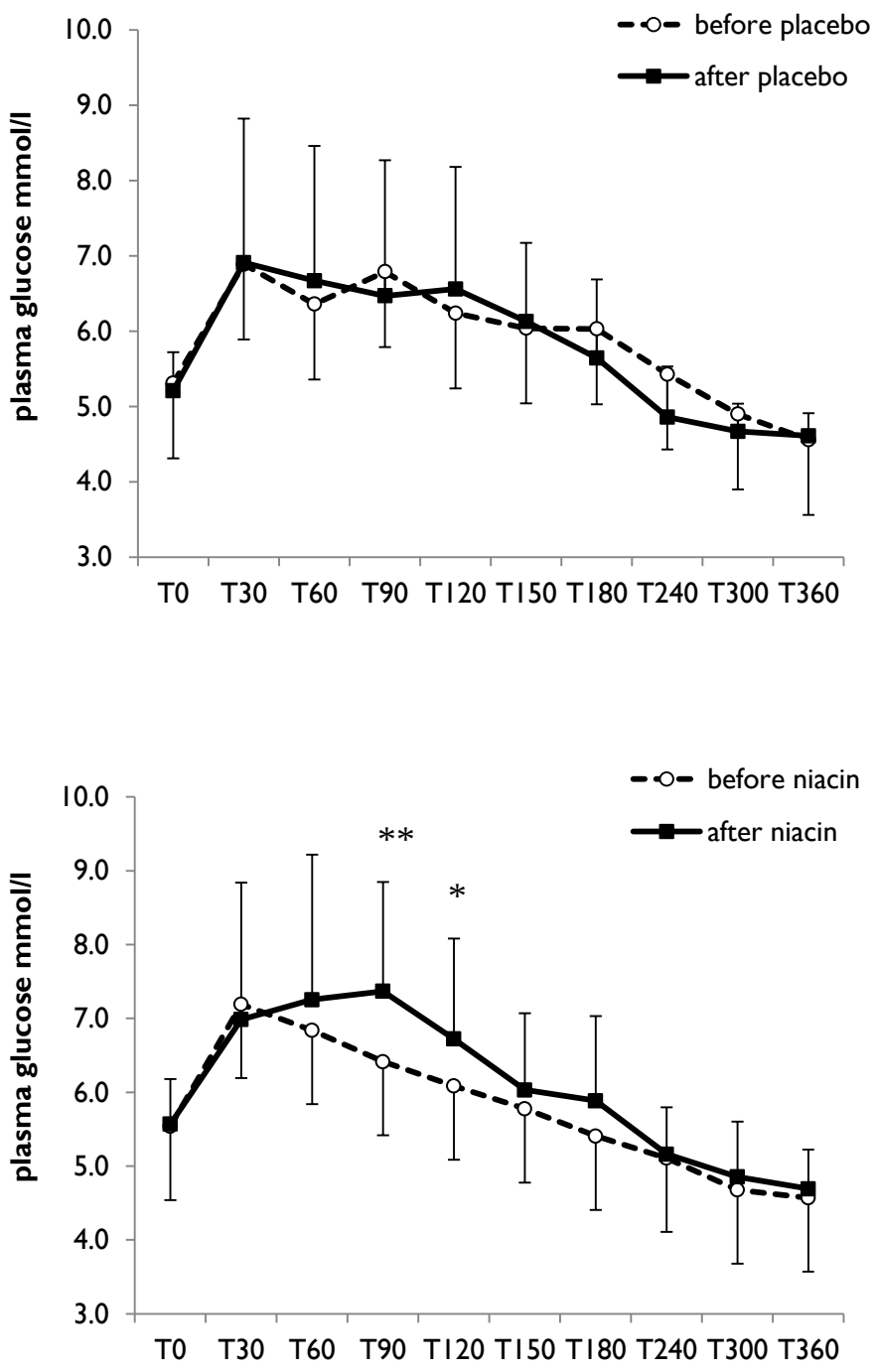


Figure 4-3 Postprandial glucose following a mixed meal test before and after intervention with placebo (top panel) or niacin (bottom panel)

Values were mean ± SD. $^{*}p < 0.05$; $^{**}p \leq 0.01$, T= time (minutes)

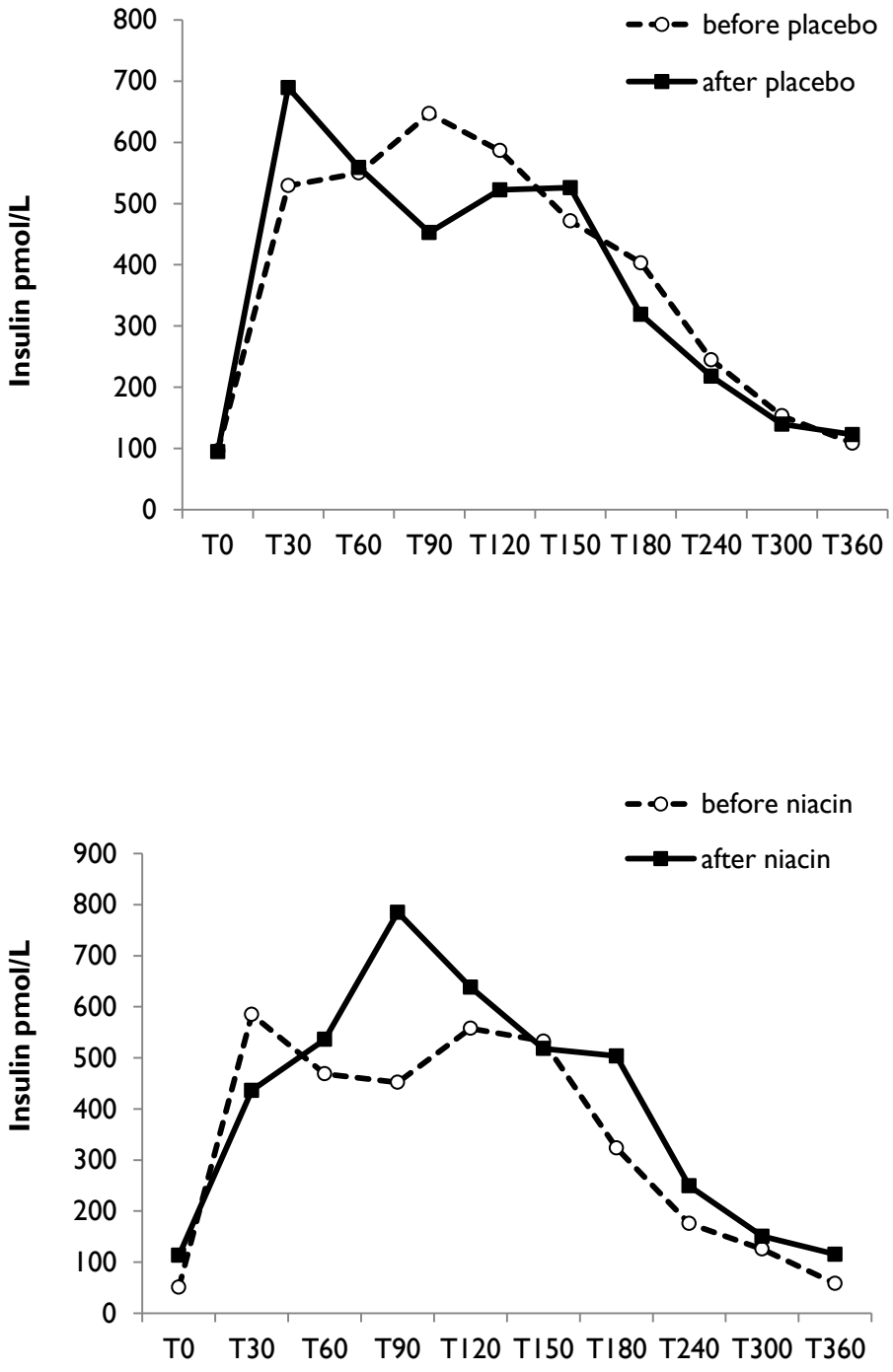


Figure 4-4 Postprandial insulin levels following a mixed meal test in PCOS women before and after intervention with placebo (top panel) or niacin (bottom panel)

Values were means. T= time (minutes)

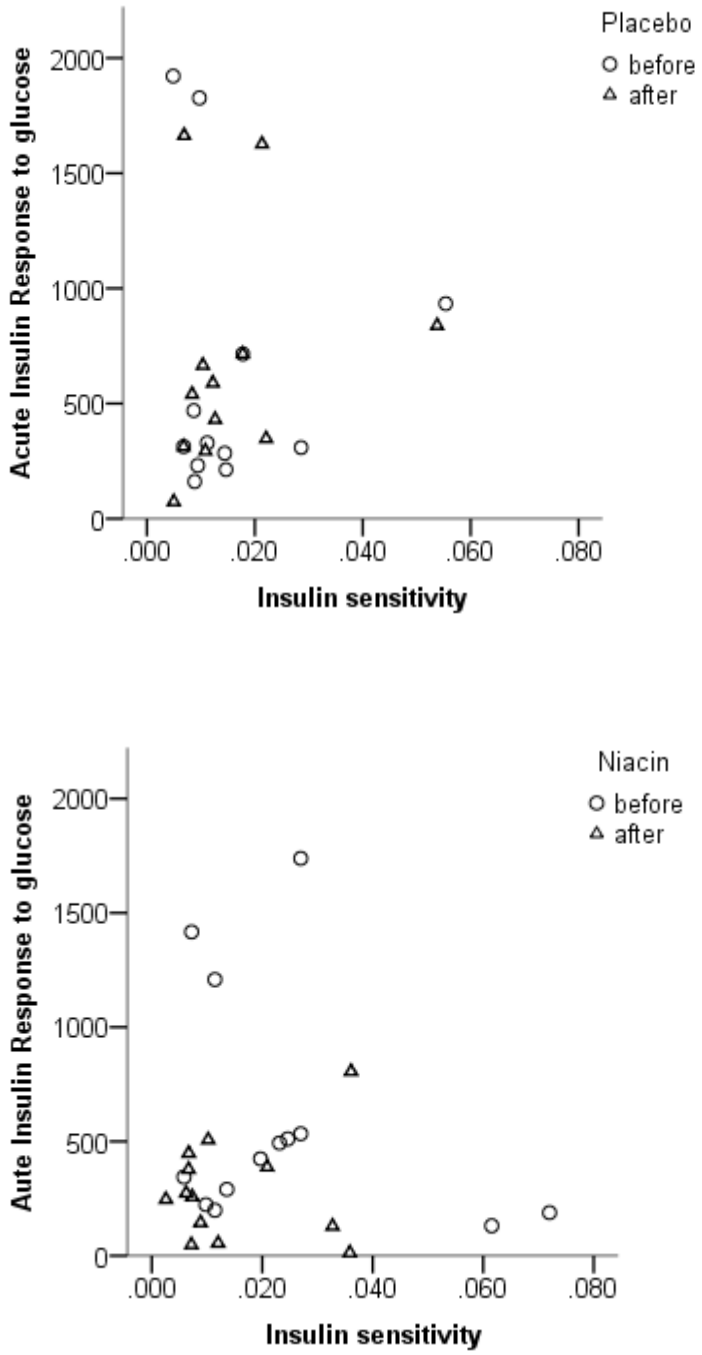


Figure 4-5 Relationship between insulin sensitivity ($pmol^{-}$) and acute insulin response to glucose (AIRg; pmol/mmoL) in PCOS women

With niacin treatment, insulin sensitivity was reduced which was not associated with compensated increase in AIRg resulting in reduction of disposition index (DI_o). No changes were seen with placebo treatment

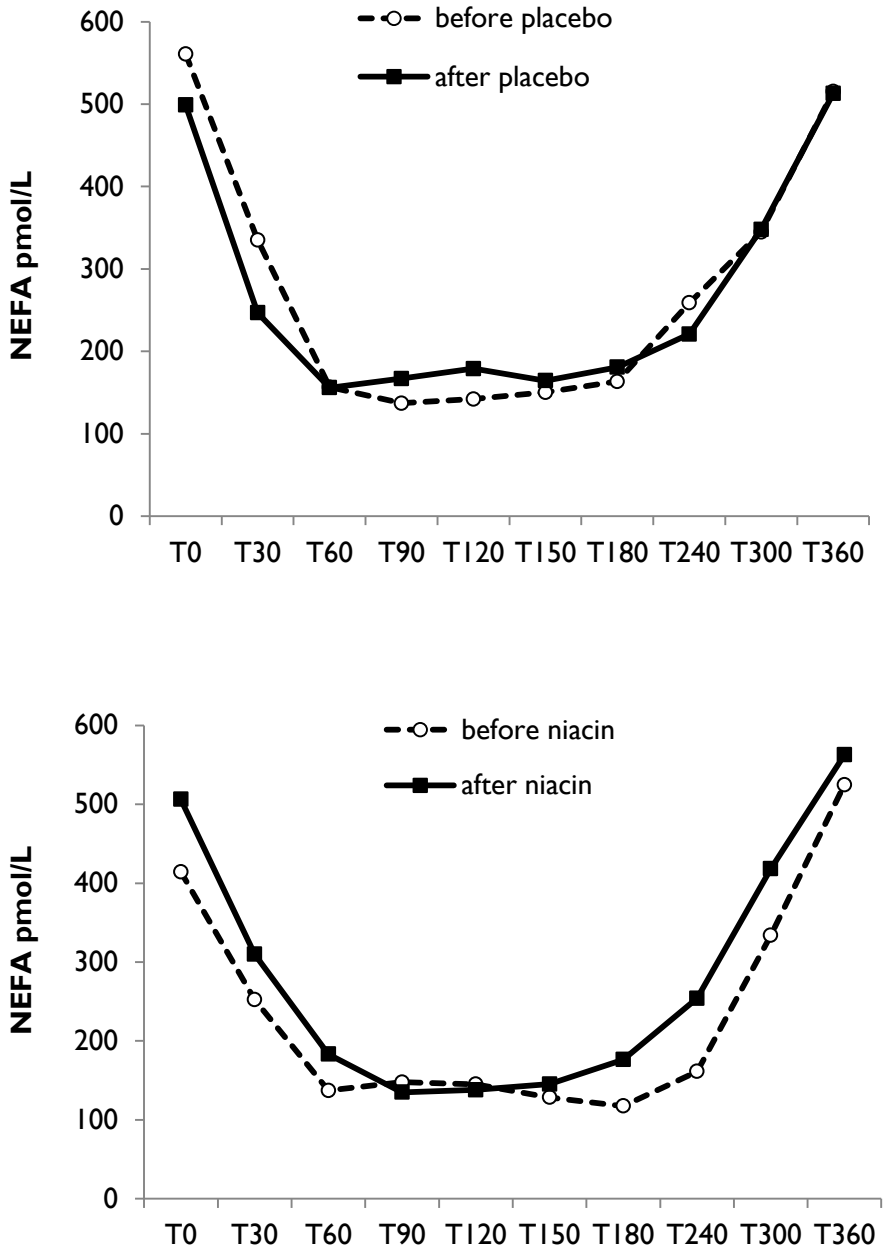


Figure 4-6 Postprandial NEFA levels following a mixed meal test in PCOS women before and after intervention with placebo (top panel) or niacin (bottom panel)

Values are means. T= time (minutes)

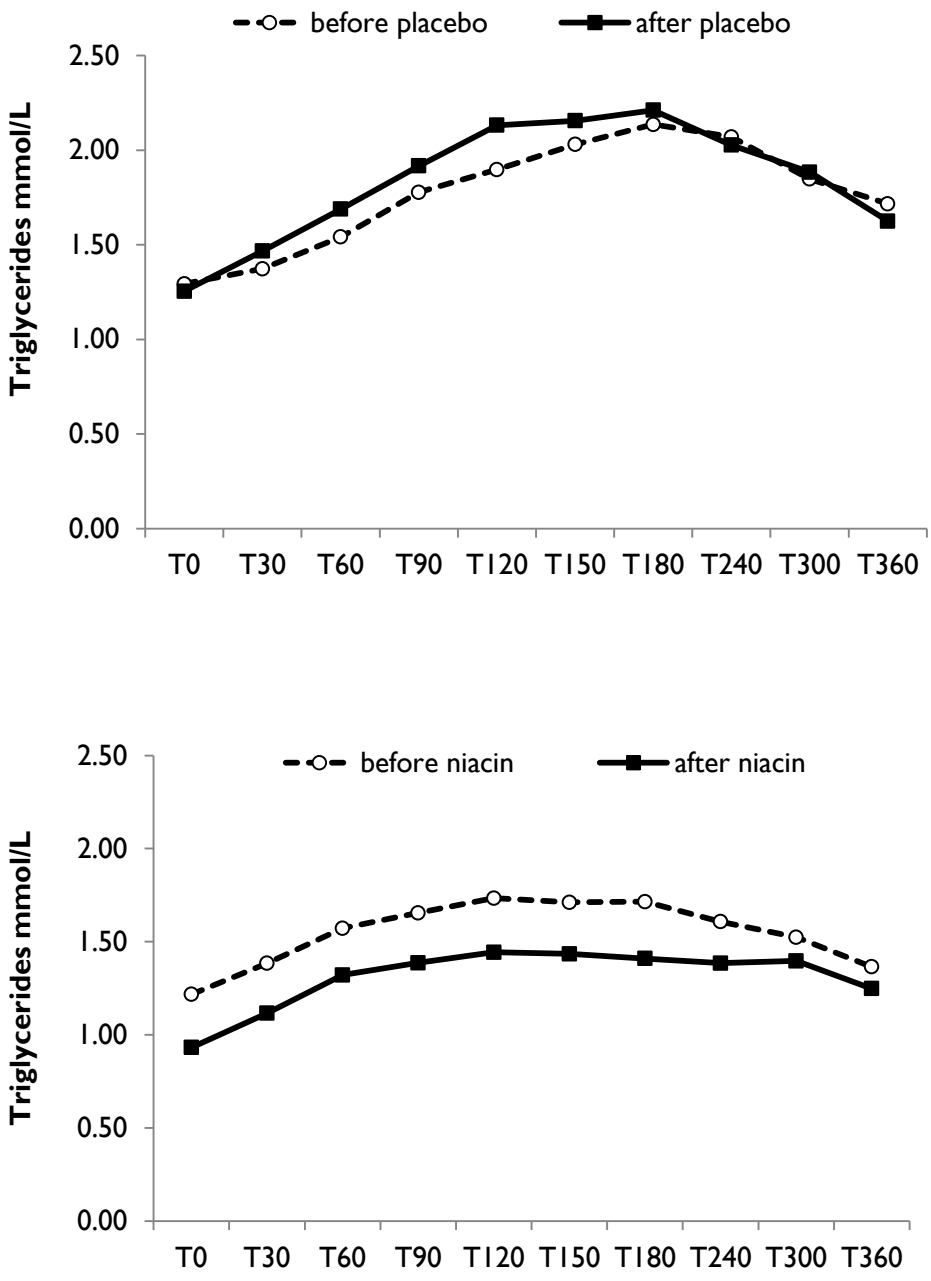


Figure 4-7 Postprandial triglyceride levels following a mixed meal test in PCOS women before and after intervention with placebo (top panel) or niacin (bottom panel)

Values were means. T= time (minutes).

4.2.2 Effect of niacin on fasting and postprandial lipids

At the end of the study, there was a significant difference (% change) between the niacin and placebo treated groups for fasting total cholesterol (-8% vs. 1%), TG (-21% vs. 4%), LDL-c (-13% vs. 3%) and HDL-c (17% vs. 1%) shown in Table 4-2. Although niacin decreased absolute postprandial TG AUC at the end of 6 h following a mixed meal (9.44± 2.9 vs. 7.74 ± 3.2 mmol/L; p = 0.03), it had no effect on the increment of postprandial TG AUC (2.69 ± 1.44 vs. 2.49 ± 1.14 mmol/L; p=0.64) above the fasting value. No change in postprandial TG was seen in the placebo group. By 12 weeks, fasting NEFA and NEFA AUC per 6 h were increased by 23% and 11% respectively in the niacin group and decreased by 2% and 1% respectively in the placebo group. However, the difference between the groups did not reach significance (Table 4-3, Table 4-3, Figure 4-5, and Figure 4-6).

4.2.3 Effect of niacin on cardiovascular risk

By 12 weeks, waist circumference and blood pressure remained the same after intervention in both groups. Although fasting lipids were improved, deterioration in insulin resistance was detected in the niacin group. No significant improvement in RHI was observed in either the niacin (1.97 ± 0.40 vs. 2.05 ± 0.58; p = 0.33) or placebo (1.95 ± 0.50 vs. 1.96 ± 0.47; p = 0.92) groups. There was a reduction in hsCRP within the niacin group but this was not significant when compared with the placebo group (Table 4-2).

4.2.4 Effect of niacin on fasting and postprandial glucose

After 12 weeks, HOMA-IR rose from baseline (2.2 (1.2, 4.2) vs. 3.8 (1.3, 5.5; p = 0.02) and was accompanied by a rise in fasting insulin levels (51 (37, 95) vs. 113 (39, 150) pmol/L; p = 0.01) but there was no change in fasting plasma glucose (5.54± 0.57 vs. 5.56 ± 0.61 mmol/L; p = 0.81) in the niacin group (Table 4-3). Similarly, there was no change in the placebo group. In contrast, after stimulation with a mixed meal, 2 h PG (6.08 ± 1.1 vs. 6.72 ± 1.4 mmol/L; p = 0.02) and postprandial plasma glucose AUC 2 h (13.1 ± 2.9 vs. 14.0 ± 2.8 mmol/L; p = 0.05) increased following niacin treatment. This was, at least partly, due to a decrease in AIRg (424 (211, 975) vs. 257 (122, 418) pmol/mmoL; p = 0.04). Consequently, the ODI was reduced (10.2 (3.1, 13.9) vs. 2.12

(0.96, 4.71) mM^{-1}; p = 0.01) in the niacin group. The differences were statistically significant between the niacin and placebo group (Table 4-3, Figure 4-3, Figure 4-4 and Figure 4-5).

4.2.5 Effect of niacin on cardiovascular risk

By 12 weeks, waist circumference and blood pressure remained the same after intervention in both groups. Although fasting lipids were improved, deterioration in insulin resistance was detected in the niacin group. No significant improvement in RHI was observed in either the niacin (1.97 ± 0.40 vs. 2.05 ± 0.58; p = 0.33) or placebo (1.95 ± 0.50 vs. 1.96 ± 0.47; p = 0.92) groups. There was a reduction in hsCRP within the niacin group but this was not significant when compared with the placebo group (Table 4-2).

4.3 Discussion

4.3.1 Tolerability

Niacin, a lipid-lowering agent, has been used to treat cardiovascular disease for over 50 years. Until recently, its use was limited because of cutaneous flushing, its principal adverse effect which is due to prostaglandin D_2 mediated vasodilatation from skin Langerhans cells (519). Laropiprant, a selective prostaglandin D2 receptor antagonist, is combined with niacin to reduce this effect. We have used tredaptive (slow release nicotinic acid/ laropiprant) in the present study where subjects seemed to tolerate it well since cutaneous flushing led to only 12% withdrawal from the study. A total of 4 patients in the active arm reported hot flushes and pins and needles which were resolved spontaneously. One patient has reported nausea which was self-limiting. One patient had a two fold increase in ALT in her visit3.

4.3.2 Intention to treat analysis concept and application

Intention to treat (ITT analysis) means inclusion of every subject who is randomized according to randomized treatment assignment. ITT analysis is usually described as "once randomized, always analyzed" (615). ITT analysis could be a potential solution for two major complications, i.e., noncompliance and missing outcomes found in randomized control trials (616). ITT is better regarded as a complete trial strategy for

design, conduct and analysis rather than as an approach to analysis alone (617, 618). The CONSORT statement for improving the quality of reports of RCTs states that number of participants in each group should be analyzed by "intention-to-treat" principle (619).

It avoids overoptimistic estimates of the efficacy of an intervention resulting from the removal of non-compliers by accepting that noncompliance and protocol deviations are likely to occur in actual clinical practice (620). On the other hand, including a patient who did not receive any treatment in the analysis, could dilute the actual effect of treatment and results in heterogeneity. Therefore a better application of the ITT approach is possible if complete outcome data are available for all randomized subjects. In this study, the end of trial meal tests was unable to perform in dropped out patients and therefore they were not included in the analyses.

4.3.3 Improvement in fasting lipids

Niacin lowered fasting LDL-c, TG, total cholesterol and increased HDL-c in the present study as observed in other studies (613, 621). Nicotinic receptors, G protein-coupled receptors GPR109A, are found in adipose tissue and immune cells. Niacin acting via these receptors inhibits the hydrolysis of TG in adipose tissue, reducing the release of NEFA into the circulation (507) and subsequently reducing the hepatic synthesis and release of VLDL and LDL-c (508).

In the present study, prolonged use of slow release niacin failed to lower fasting NEFA. This finding was consistent with previous studies. The niacin-decreased plasma NEFA is transient and followed by a rebound increase as shown in previous human and animal studies (511, 622). Vega et al. examined the effect of 2 g per day of extended release nicotinic acid on the NEFA level in patients with metabolic syndrome. There was a 30% reduction in NEFA from baseline at 4 h but 40-50% rise from baseline at 9 h after ingestion. Therefore, the extended release form of niacin failed to abolish the NEFA rebound (511) but the mechanism for this remains under investigation.

Thus, the effect of niacin on adipose tissue alone cannot explain the paradoxical improvement in lipids. Kashyap et al. reported that niacin enhances Apo B degradation and subsequently decreased TG synthesis in hepatoblastoma G2 cells (510). In

addition, niacin directly inhibits diacylglycerol acyltransferase 2 (DGAT2) resulting in decreased TG synthesis (509). Niacin also reduces the hepatic expression of Apo CIII and the peroxisome proliferator-activated receptor (PPAR) gamma coactivator-1beta (PGC-1beta), a transcriptional co- activator and subsequently, lowered TG (623). This might explain a reduction in fasting TG with no associated reduction in NEFA. The exact mechanism for the lowering of HDL-c by niacin is unclear. Niacin may increase HDL-c by decreasing hepatic expression and secretion of CETP (cholesterol ester transfer protein) (624), or by increasing expression of ABCA1 in primary hepatocytes, (625) and by inhibiting surface expression of ADP synthase beta chain, a receptor for HDL-c endocytosis (626). Therefore, the reduction of fasting TG with no apparent reduction in NEFA may be due to a direct effect of niacin on TG synthesis in the liver (509, 623).

4.3.4 Impact of niacin on postprandial lipids

Following a mixed meal, niacin did not influence the postprandial excursion of TG. Therefore, the improvement in total postprandial TG seen with niacin was mainly due to a decrease of fasting TG rather than an effect on any postprandial rise following a meal. This lack of change in postprandial hypertriglyceridaemia with niacin is likely to be related to the 23% increase in NEFA at the beginning of the meal and the overall 11% rise in the NEFA AUC, compared with no change in the placebo group. A similar finding was noted in a previous study in which men with either central obesity or hypertriglyceridaemia were treated with a course of niacin for 6 weeks but showed no effect on postprandial incremental TG (596). This suggested that the postprandial effect was largely related to a decrease in fasting TG. Hence, niacin has little or no effect on chylomicron metabolism or postprandial LPL activity. This explanation is supported by findings in an animal study in which niacin decreased Apo B100 concentration but had no effect on LPL mRNA expression in adipose tissue in obese insulin resistant dogs (627).

4.3.5 Postprandial TG as a CV risk

Over 30 years ago, Zilversmit postulated that atherogenesis may occur during the postprandial period due to a rise in plasma chylomicrons following a high fat meal (302). Two prospective population cohorts, the Women's Health Study (628) and

Copenhagen City Heart Study (629) reported that elevated non-fasting TG rather than fasting TG are associated with cardiovascular events independent of conventional cardiovascular risk factors. Furthermore, the incremental area under the response curve, rather than fasting TG or total TG AUC, most accurately described the TG response to an oral fat load in both healthy and T2DM subjects (630). T2DM patients with prior coronary events had higher, postprandial, incremental TG AUC than those without coronary artery disease and this suggested a rise in postprandial TG being at least a marker of high CV risk (631). The low RHI detected by EndoPAT was consistent with endothelial dysfunction and associated with late cardiovascular adverse outcome (632). In the ACCORD study, fenofibrate and simvastatin combined therapy, lowered PP TG as well as Apo B48 in patients with high fasting TG. This may be linked to the CV outcome improvement seen in that subgroup of patients (633). In the AIM-HIGH trial (Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides: Impact on Global Health Outcomes) involving 3400 patients treated with niacin for three years, niacin was shown to have no cardiovascular benefit despite a decrease in LDL-c and an increase in HDL-c (634). In the recent HPS2-THRIVE (Heart Protection Study 2-Treatment of HDL to Reduce the Incidence of Vascular Events) study, tredaptive plus statin therapy did not improve cardiovascular outcome compared to statin therapy alone. This was seen after a median of 3.9 years follow up in over 25,000 study patients at high risk of cardiovascular events. In accord with these studies, the present study has shown that niacin had no effect on either RHI or hsCRP, by both measures of CV risk. Hence, the failure of niacin to decrease the postprandial rise in TG AUC may have direct relevance for the clinical observation of its lack of cardiovascular protection.

4.3.6 Postprandial hyperglycaemia as a CV risk

With regard to the effect of niacin on glucose homeostasis, the present study showed that drug therapy increased insulin resistance as measured by HOMA-IR and this was accompanied by a rise in fasting insulin. Fasting plasma glucose was maintained at pre-treatment level, indicating that beta cells were able to compensate for a rise in insulin resistance in this unstimulated state. It would seem plausible that niacin increased insulin resistance relative to the rebound rise in NEFA, especially since acute and

chronic elevation of circulating NEFA is known to be associated with increased insulin resistance (373).

The information derived solely from the homeostasis model assessment (120) is limited as it does not determine the ability of beta cells to respond to rising glucose concentrations in the postprandial period. Although beta cell function and insulin sensitivity can be measured following an intravenous glucose infusion (635) or an oral glucose load, we used a mixed meal in preference since the presence of other nutrients such as proteins and fat make it a much more physiological challenge (553, 554). Using this, we found, by the end of niacin therapy, a significant reduction in AIR_g and a significant rise in both postprandial plasma glucose and postprandial plasma glucose AUC towards the end of 2 h. The ODI was assessed in more detail as it was able to give an indication of any appropriate beta cell response to change in insulin resistance. The significant fall in ODI following niacin suggests that beta cells did indeed fail to meet the larger insulin requirement demanded during the postprandial period. This means that the increased postprandial glucose and HbA1c found in a meta-analysis of niacin clinical trials could be as much due to the effect of the drug on beta cell function as it is on insulin resistance (636). Also of note is the observation that the ODI has been found previously to be predictive for the development of diabetes over 10 years (555), so it is, perhaps, not surprising that diabetes has been found more frequently in niacin treated patients. As mentioned above, even postprandial hyperglycaemia (short of frank diabetes) is regarded as an independent risk factor for atherosclerosis and CV events. In the Whitehall Study, the Paris Prospective Study, and the Helsinki Policemen Study, a 20-year follow-up of healthy, middle aged, working non-diabetic men showed that high postprandial, as well as fasting, glucose were risk factors for cardiovascular and all-cause mortality (637). The Gonzaga Diabetes Study reported that postprandial glucose was a better predictor for cardiovascular events than fasting glucose and HbA1c in T2DM (638). Postprandial hyperglycaemia impairs vascular endothelial dysfunction as seen by a reported reduction in FMD, an increase in lipid peroxidation and asymmetric dimethylarginine (ADMA) in healthy (639) as well as diabetic subjects (640). This niacin-induced postprandial rise in glucose may contribute to understanding why niacin therapy did not produce a cardiovascular benefit despite improvements in HDL-c and LDL-c (634).

4.3.7 Reasons for failed beta cell function

Failure of beta cell adaption in obese PCOS women following niacin therapy could be multifactorial. The capacity of the beta cell mass to increase in response to insulin resistance is essential to maintain normal glucose regulation. Although beta cell proliferation in adult human is very low, beta cell mass is dynamic and adaptable to changes in plasma glucose by change in number (hyperplasia) and individual volume of beta cells (hypertrophy) (641). Central obesity in this study group might have added to increased demand for beta cell function resulting in decompensation. This could be a genetic defect in beta cell mass as observed in a study in which subjects who were at risk of diabetes failed to increase insulin secretion in response to acute insulin resistance induced by intralipid infusion (373). However, incomplete beta cell adaptation was observed in healthy subjects without family history of diabetes following niacin-induced insulin resistance for a week (642). Failure of beta cell adaptation could be related to lipotoxicity of beta cells since there was a rebound rise in NEFA. Whilst transient or mild hyperglycaemia may be a favourable drive for beta cell growth, chronic hyperglycaemia appears to be harmful with loss of specific beta cell differentiation (643). Therefore, in the present study, beta cell compensation could be down regulated by chronic hyperglycaemia caused by insulin resistance due to chronic use of niacin. The effect of niacin or NEFA directly on pancreatic beta cells cannot be excluded.

4.3.8 Limitations of the study

The main limitation in the present study is the dropout rate of 23%. However, this was the same for both the placebo and active treatment groups and may help explain why many expected differences between groups only just managed or failed to reach statistical significance. Laropiprant was used in combination with slow release niacin to improve tolerability. Therefore, it was not possible to dissect the effect of laropiprant from that of niacin on outcome variables in the present study. However laropiprant is biologically impossible to exert any effect on lipid and glucose metabolism.

4.3.9 Summary

Unlike the acipimox study mentioned in section 3.3.3 and 3.3.4, Niacin/laropiprant, whose mode of action is via nicotinic receptors in adipose tissue, failed to suppress fasting and postprandial NEFA in the present study. Furthermore, niacin/laropiprant did not improve postprandial TG excursions in response to a mixed meal. The drug did, however, significantly increase postprandial glucose through an increase in IR and a detrimental effect on beta cell function. Both metabolic dysfunctions seem related to rebound rise in fasting and postprandial NEFA. Together, these findings are likely to offset many of the potentially beneficial effects of niacin/laropiprant on fasting lipids and so may help explain the observed clinical ineffectiveness of niacin on cardiovascular outcomes.

In addition, women with PCOS compensated adequately to the increased IR related to prolonged use of niacin by increased beta cell function at fasting. However, the beta cells adaptation appeared to fail to respond adequately when the insulin requirement is particularly high, such as during the postprandial period. These findings add to the understanding of the effect of the rebound rise in NEFA on IR in PCOS and its impact on CV risk modification.

Chapter 5 Effect of moderate intensity exercise on lipid induced insulin resistance and cardiovascular risk in PCOS

5.1 Introduction

Insulin resistance (IR) is an essential component in the development of T2DM, PCOS and metabolic syndrome. Chronically elevated plasma NEFA levels are seen in all of these conditions (136, 286, 353, 644-646) and postulated to play a major role in the pathophysiology of IR.

NEFA levels are positively correlated with age, BMI, IR and Waist-Hip Ratio and negatively correlated with rate of glucose disposal i.e. insulin sensitivity of skeletal muscle in a study using euglycaemic hyperinsulinaemic clamps in healthy individuals. The association between NEFA levels and IR was independent of obesity (239).

Adipose tissue regulates whole body metabolism. It acts not only as an energy store but also as an endocrine organ producing hormones and as a regulating organ to balance fat storage and mobilization. Visceral fat has a higher lipolysis rate for the release of FFA in response to catecholamines, and less sensitivity to the anti-lipolytic action of insulin compared to peripheral fat (647, 648). Moreover, the ability of insulin to suppress lipolysis in adipose tissue is reduced in IR states such as obesity and T2DM (649). Hence, the larger the amount of visceral fat then the greater FFA release into the circulation. In contrast, McQuaid et al. reported that FFA release from adipose tissue is down regulated in obesity (321). At the same time, the author found adipose tissue uptake of chylomicron derived FFA was severely impaired in obesity which might lead to ectopic fat accumulation in non-adipose tissue. Other studies also suggested that a decreased mobilization of FFA from adipose tissue leads to decreased entrapment of FFA derived from lipolysis of chylomicrons resulting in spill over into the circulation (319, 320). Therefore, obese people may experience high circulating NEFA levels either due to increased delivery from adipose tissue or failure to remove diet-derived NEFA in the postprandial period which is the major part of the day.

Increased availability (414, 650), and reduced rate of oxidation of FFA lead to the accumulation of intramyocellular lipid metabolites such as LCFA Acyl Co A, DAG and ceramides. These are thought to interfere with insulin signalling with impairment of

insulin-mediated glucose transport and/ or phosphorylation resulting in skeletal muscle IR. As skeletal muscle constitutes 40% of body mass and uses 80% of the glucose load, its impaired glucose utilisation leads to compensatory hyperinsulinaemia and systemic IR. Whilst this is well recognized, the magnitude of the effect of FFA on IR varies between individuals and patient groups (343). PCOS status is well recognized to have an intrinsic post receptor insulin signalling defect in skeletal muscle (143) which may augment the detrimental effect of FFA on insulin sensitivity. Obesity is associated with the development of IR and dyslipidaemia (562). Although IR in PCOS is independent of obesity, the latter can potentiate the adverse effect of PCOS on IR (14).

The majority of women with PCOS are obese. Although the prevalence of PCOS does not linearly increase with increasing obesity in the population, increasingly more PCOS women are overweight at diagnosis according to a longitudinal study (54). Both lean and obese PCOS women, especially those with hyperandrogenism and menstrual irregularities, have hyperinsulinaemia and IR. However, obese PCOS women are more insulin resistant (128), hyperandrogenic (557, 560), with higher plasma NEFA and metabolic dyslipidaemia (128, 286, 560). They have increased progression to T2DM (19, 67) and a worse cardiovascular risk profile than lean PCOS women (99). IR in PCOS correlates well with hyperandrogenism, oligomenorrhoea and subfertility. It has been shown that acute elevation of FFA, induced by intravenous lipid and heparin infusions, impairs insulin sensitivity and augments circulating androgen level in healthy volunteers (651).

It has been recognised that lean PCOS women have more visceral fat compared to lean controls (283). More centrally obese PCOS women are associated with higher fasting insulin levels (652), elevated NEFA levels and subsequent skeletal muscle IR (143). The mechanism by which obesity can lead to increased IR is via increased FFA production and its effect on skeletal muscle (330, 408, 563). Therefore, elevated NEFA could be the underlying driver for PCOS but the effect is complex and seems to be related to fat distribution and the metabolic competence of skeletal muscle i.e. its ability to completely metabolise stored IMTG. In a state of metabolic incompetence, incomplete metabolism of stored IMTG could lead to accumulation of intermediate metabolites that interfere with glucose uptake.

It is not uncommon to observe an improvement in insulin sensitivity, dyslipidaemia, hirsutism, restoration of menstrual periods and fertility with weight loss in PCOS in the clinic and during research studies (561, 653-657). Weight reduction improved the androgen profile and IR in women with upper body obesity (648).

Moderate intensity exercise uses FFA as fuel for skeletal muscle energy production. It increases the blood flow through the adipose tissue and enhances lipolysis and delivery of FFA for use in skeletal muscle. It improves FA oxidation and reduces the accumulation of intramyocellular lipid metabolites with subsequent improvement of IR (430).

Hypothesis:

Women with PCOS might have metabolic incompetence that could lead to an inability to cope with a chronic elevation of NEFA resulting in skeletal muscle IR and subsequent hyperandrogenism. It may be reversible when lipid metabolism is modulated by endurance exercise.

The aims of this study were:

- To establish if PCOS patients are 'metabolically incompetent' and, if so, are they less able to cope with an acute elevation of NEFA than metabolically competent controls. This was investigated by raising NEFA by intralipid infusion and comparing its effect in PCOS patients and controls
- To determine the effect of endurance exercise upon lipid-induced IR in PCOS patients
- To identify the effect of exercise upon the androgen profile and cardiovascular risk in PCOS patients

5.2 Materials and methods

Details of recruitment, screening tests, study visits (section 2.1.5.3, 2.1.2, 2.1.3, 2.1.4, 2.1.5.3), exercise program (section 2.2.4) and clamp procedures (section 2.3.4) have been described in the previous chapter.

5.2.1 Study subjects

A total of thirteen PCOS patients and twelve female healthy volunteers who have fulfilled the inclusion and exclusion criteria were included in the study after obtaining their fully informed written consent.

5.2.2 Protocols

All study measurements including anthropometry, fasting blood tests, and the hyperinsulinaemic normoglycaemic clamp were performed at the beginning of the study. A body volume scanner was used for anthropometric measurements. All participants underwent two day procedures of infusions of either normal saline or 20% intralipid with unfractionated heparin for 5 h with a measurement of insulin sensitivity i.e. rate of glucose disposal by a hyperinsulinaemic euglycaemic clamp in the last 2 h of infusions. Following this, subjects attended the sports laboratory for the measurement of VO_2 max. Subsequently, all subjects underwent an 8 week program of moderate intensity exercise. All of the procedures detailed above were repeated at the end of the exercise program (Figure 5-1).

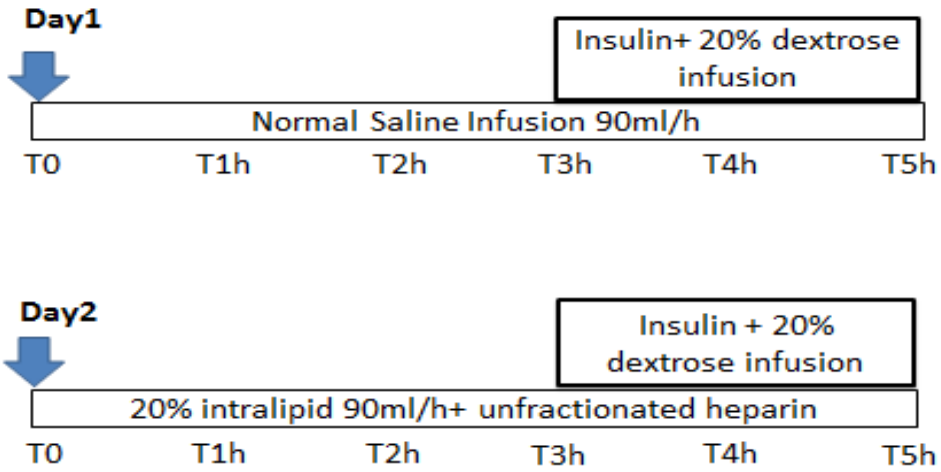


Figure 5-1 Procedure protocols (T=time)

5.2.3 Measurement of insulin sensitivity

The hyperinsulinaemic euglycaemic clamp procedure is described in detail in section 2.3.4. Briefly, following a 12 h overnight fast, participants underwent a hyperinsulinaemic euglycaemic clamp that measured insulin sensitivity whilst receiving infusion of either normal saline on the first occasion followed by intralipid infusion on the second occasion within a week interval. An 18 gauge intravenous cannula was inserted into an ante-cubital vein to administer test infusions and a retrograde cannula was inserted into the dorsal hand vein on the contralateral hand. This hand was heated (60°C) to arterialize venous blood for the measurement of blood glucose. On the lipid infusion day, subjects received one more cannula at the contralateral antecubital vein as lipid emulsion cannot be infused in the same cannula with other infusate. After fasted blood samples were taken, either normal saline 1.5 mL/min or 20% intralipid 1.5 mL/min along with unfractionated heparin sodium 0.3 unit/kg/min was infused for 5 h. At 180 min, a 2 h hyperinsulinaemic euglycaemic clamp was commenced by infusing soluble insulin (Humulin S, Eli Lilly and Co., Indianapolis, IN) at a rate of 80 mU/m^2 surface area/min for the first 20 min followed by a constant rate of 40 mU/m^2 surface area/minute for 100 min. Plasma glucose was clamped 5.0 mmol/L with a variable rate of 20% dextrose infusion. The dextrose infusion rate was adjusted relative to arterialized blood glucose measurements undertaken every 5 min. Blood samples were taken at baseline and every hour for 5 h. These samples were centrifuged at 1500 G for 15 min at 4°C within 15 min of sampling and plasma and serum stored at -80°C until analysis.

5.2.4 Exercise intervention

In a case control study design, all participants underwent an incremental treadmill test until exhaustion was reached at the beginning and at the end of the study (658) to determine their VO_2 max. All participants completed a structured exercise program for an hour, three times per week for 8 weeks in a laboratory setting at the Department of Sport, Health and Exercise Science, University of Hull. All supervised exercise sessions were performed on a motorized treadmill and achieved a target heart rate equivalent to 60% of their baseline VO_2 max. Their moderate exercise intensity was recalculated at the end of 4 weeks by repeating the VO_2 max test to account any improvement in fitness during the first half of the program.

5.2.5 Biochemical analysis

Measurement of total cholesterol, triglycerides (TG), HDL-c, LDL-c, NEFA, blood glucose, insulin, testosterone, SHBG, FAI and hsCRP were carried out according to the analytical assay methods described in (section 2.4).

5.2.6 Statistical analysis

Statistical analysis is described in detail (section 2.5, 2.6).

5.3 Results

A total of 14 PCOS patients and 13 healthy volunteers who met the eligibility criteria entered the study. Two PCOS and three healthy subjects were withdrawn because they were unable to comply with required sessions of the exercise program. In total, 10 of 12 PCOS and 8 out of 10 controls have completed all that was required by the end of the study procedures. Two subjects from each group were unable to complete the study clamp procedures owing to time commitments and social reasons.

5.3.1 Baseline characteristics of participants

Baseline characteristics of subjects are summarised in Table 5.1. Age and BMI were comparable between groups. Subjects with PCOS had larger WHR (mean WHR ± SEM: PCOS, 0.85 ± 0.02; controls, 0.78 ± 0.02; p=0.02), higher FAI (mean FAI ± SEM: PCOS, 6.18 ± 0.82; controls, 1.57 ± 0.3; p=<0.001), lower SHBG (mean SHBG nmol/L ± SEM: PCOS, 26 ± 6.1; controls 74 ± 9.4; p<0.001), and lower HDL-c (mean HDL-c mmol/L ± SEM: PCOS, 1.16 ± 0.07; controls, 1.6 ± 0.15; p=0.01) and higher IR (mean HOMA-IR ± SEM: PCOS, 2.63 ± 0.53; controls, 1.18 ± 0.22; p=0.04) than controls. Fasting TG was higher in PCOS than controls but not significantly (mean TG mmol/L ± SEM: PCOS, 1.33 ± 0.24; controls, 0.87 ± 0.06; p=0.12). However, fasting NEFA was similar between controls and PCOS. Healthy subjects were physically fitter than PCOS at baseline (mean VO_2 max ml/kg/min ± SEM: controls, 36.6 ± 2.4; PCOS, 27.9 ± 1.20; p<0.01).

5.3.2 Blood glucose levels during clamps

In the present study, blood glucose levels of 5.0 mmol/L were aimed to achieve by administering 20% dextrose whilst infusing a fixed dose of insulin during the hyperinsulinaemic euglycaemic clamp. The rate of administration of 20% dextrose was adjusted by the arterialized blood glucose levels measured every 5 min during the clamp. A total of 4 clamps were carried out during the saline and lipid trials before and after exercise. The mean glucose levels achieved during these clamps were the lowest 4.97 mmol/L and highest 5.15 mmol/L, the coefficients of variation of blood glucose were 7.9% and 11.6% respectively in both groups (Figure 5-2, Figure 5-3).

Table 5-1 Baseline characteristics of participants

Parameters	Controls (N=8)	PCOS (N=10)	p =
Age (year)	24.9 ±2.4	28.0 ± 2.3	0.36
BMI (kg/m^2)	25.7 ± 2.0	29.0 ± 1.5	0.20
Waist (cm)	81.2 ± 5.4	94.4 ± 4.6	0.08
WHR	0.78 ± 0.02	0.85 ± 0.02	0.02
SBP (mmHg)	115 ± 3.4	117 ± 2.6	0.75
DBP (mmHg)	74 ± 3.4	76 ±2.5	0.49
Testosterone (nmol/L)	1.03 ± 0.13	1.32 ± 0.19	0.24
FAI	1.57 ± 0.36	6.18 ± 0.82	<0.001
SHBG (nmol/L)	74 ± 9.4	26 ± 6.1	<0.001
ALT (iu/L)	17.3 ±2.2	25.5 ± 5.3	0.20
TC (mmol/L)	4.85 ± 0.21	4.15 ± 0.21	0.04
TG (mmol/L)	0.87 ± 0.06	1.33 ± 0.24	0.12
HDL-c (mmol/L)	1.6 ± 0.15	1.16 ± 0.07	0.01
LDL-c (mmol/L)	2.75 ± 0.21	2.33 ± 0.17	0.14
FPG (mmol/L)	4.8 ± 0.19	4.9 ± 0.17	0.88
HbA1c (mmol/mmoL)	33 ±2.3	34 ±0.89	0.60
TSH (iu/L)	2.03 ±0.32	1.7 ±0.18	0.34
HOMA-IR	1.1 (0.67, 1.57)	2.14 (1.14, 4.19)	0.02
NEFA (µmol/L)	472 (392, 657)	553 (313,673)	0.86
hsCRP (mg/mL)	1.92 ±1.04	2.9 ±0.47	0.47
VO_2 max (ml/kg/min)	36.6 ±2.4	27.9 ±1.20	<0.001

P value < 0.05 is significance of difference

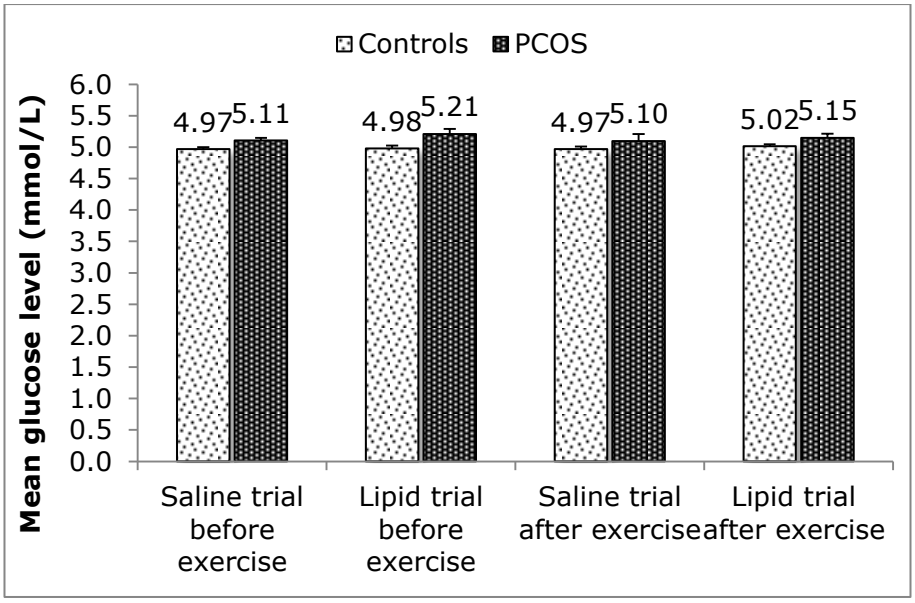


Figure 5-2 Mean blood glucose levels achieved during hyperinsulinaemic euglycaemic clamps (Data are expressed as mean± SEM)

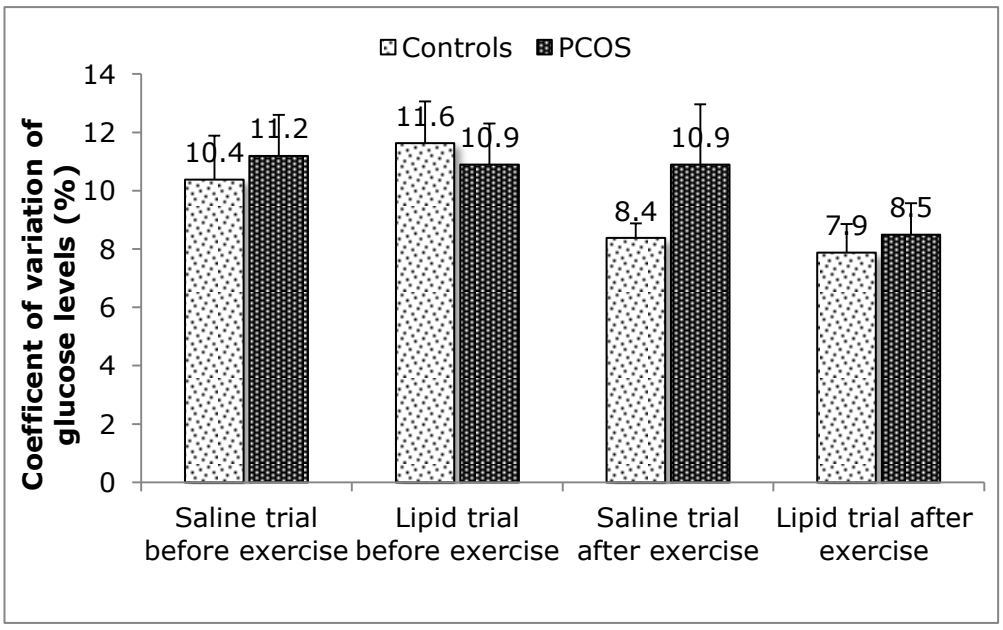


Figure 5-3 Coefficients of variation for blood glucose levels during hyperinsulinaemic euglycaemic clamp (Data are expressed as mean± SEM)

5.3.3 Effect of intralipid on glucose homeostasis

During saline infusion, NEFA AUC 3 h (NEFA μmol/L: PCOS, 1881 (1499, 2061); controls, 1978 (1350, 2201); p = 0.66) and glucose AUC at 3 h (mean glucose mmol/L ± SEM: PCOS, 14.1 ± 0.27; controls, 14.2 ± 0.32; p = 0.85) were not different between PCOS and controls. However, TG AUC 3 h (mean TG mmol/L ± SEM: PCOS, 3.75 ± 0.64; controls, 2.40 ± 0.17; p = <0.01) and insulin AUC 3 h (insulin pmol/L: PCOS, 161 (111,250); controls, 73 (48, 156); p<0.01) were higher in PCOS compared to those of controls. Lipid infusion led to a 3.5- 4 fold rise in NEFA AUC and TG AUC in both groups. This was accompanied by a rise in plasma glucose AUC 3 h in PCOS from 14.1± 0.27 to 14.9± 0.39 mmol/L, p=0.08 and in controls from 14.2± 0.32 to 15.1± 0.20 mmol/L, p=0.04.The insulin AUC 3 h was unchanged with lipid infusion in PCOS (insulin pmol/L: saline, 161(111,250); intralipid 170 (69, 344); p=0.89), and controls (insulin pmol/L: saline, 73 (48, 156); intralipid, 68 (48, 107); p=0.52). This suggested that pancreatic beta cells did not compensate for lipid-induced IR resulting in high glucose levels in both groups. Data are shown in Table 5-2.

During the saline infusion, insulin sensitivity was significantly lower in PCOS than controls (rate of glucose disposal mg/kg/min: PCOS, 3.14 (2.64, 3.63); controls, 6.04 (4.33, 6.48); p=0.01). Lipid infusion lowered insulin sensitivity further in PCOS (rate of glucose disposal mg/kg/min: saline, 3.14 (2.6, 3.6); intralipid, 0.93 (0.55, 1.65); p=0.01) and controls (rate of glucose disposal mg/kg/min: saline 6.04 (4.33, 6.48); intralipid 2.74 (1.81, 4.17); p=0.005). The rate of glucose disposal fell by 67% in PCOS and 45% in controls with lipid infusions (Figure 5-5).

5.3.4 Effect of exercise on insulin resistance and cardiovascular risk

The endurance exercise for 8 week improved cardiovascular fitness in both PCOS patients and controls (mean VO_2 max ml/kg/min± SEM: PCOS before 8 week exercise, 27.9 ± 1.20; PCOS after 8 week exercise, 30.4 ± 1.4; p=0.05) and (mean VO_2 max ml/kg/min± SEM: controls before 8 week exercise, 36.6 ± 2.4; controls after 8 week exercise, 39.6 ± 2.03 ml/kg/min; p=0.03). This was accompanied by a significant reduction in HOMA-IR in PCOS (HOMA-IR: PCOS before 8 week exercise, 2.14 (1.1, 4.2); after 8 week exercise, 0.98 (0.7, 1.7); p=0.03) although not significantly in controls (HOMA-IR: controls before 8 week exercise 1.1(0.67, 1.6); after 8 week exercise, 0.65

(0.5, 1.2); p=0.16). The post exercise level of HOMA-IR of PCOS was reduced to that of pre-exercise level in controls. The reduction in IR was independent of weight reduction as there was no significant weight loss in either group. Waist circumference and diastolic blood pressure fell in PCOS. Total cholesterol, LDL-c and HDL-c and NEFA were unchanged but TG fell in both groups. . HsCRP fell in both groups but not significantly. Data are summarised in Table 5-3.

Table 5-2 Effect of intralipid infusion on insulin sensitivity and lipid parameters

Parameters	Controls (n=8)			PCOS (n=10)		
	Normal Saline	Intralipid	*P =	Normal Saline	Intralipid	*P =
glucose disposal mg/kg/min	6.04 (4.33,6.48)	2.74 (1.81, 4.17)	0.005	3.14 (2.64, 3.62)	0.93 (0.55, 1.65)	0.01
NEFA AUC 3 h (µmol/L)	1978 (1350, 2201)	6227 (5366, 7994)	0.005	1881 (1499, 2061)	6655 (4672, 8460)	0.01
TG AUC 3 h (mmol/L)	2.40 ± 0.17	10.3 ± 1.3	0.005	3.75 ± 0.64	12.98 ± 1.65	0.01
Glucose AUC 3 h (mmol/L)	14.23 ± 0.32	15.12 ± 0.20	0.06	14.14 ± 0.27	14.86 ± 0.39	0.08
Insulin AUC 3 h (pmol/L)	73 (48, 156)	68 (48, 107)	0.52	161 (111, 250)	170 (69, 344)	0.89
NEFA AUC 5 h (µmol/L)	2377 (2019, 2581)	12960 (8448, 14418)	0.005	2403 (1632, 2738)	12041 (8871, 13320)	0.01
TG AUC 5 h (mmol/L)	3.5± 0.52	21.55± 1.82	0.005	5.76±0.73	26.05± 1.1	0.01
Glucose AUC 5 h (mmol/L)	23.68± 0.76	25.53±0.78	0.22	24.41± 0.58	25.62±0.94	0.16
Insulin AUC 5 h (pmol/L)	809 (622, 1116)	570 (403, 748)	0.07	513 (399, 603)	411 (395, 535)	0.16

Skewed variables are shown as median (25th, 75th percentile). Normally distributed variables are shown as mean ± SEM

*p value<0.05 is significance of difference

Table 5-3 Demographic and biochemical changes with exercise

Parameters	Controls (n = 8)			PCOS (n = 10)		
	Exercise			Exercise		
	Before	After	*p =	Before	After	*p =
BMI (kg/m^2)	25.7 ± 2.0	25.3 ± 1.9	0.10	29.0 ± 1.5	28.8 ± 1.6	0.49
Waist (cm)	81.2 ± 5.4	80.1 ± 5.3	0.13	94.4 ± 4.6	89.4 ± 4.2	0.05
SBP (mmHg)	115 ± 3.4	109 ± 4.5	0.05	117 ± 2.6	111 ± 3.8	0.13
DBP (mmHg)	74 ± 3.4	72 ± 3.3	0.89	76 ± 2.5	67 ± 2.9	0.05
Testosterone (nmol/L)	1.03 ± 0.13	1.2 ± 0.16	0.11	1.3 ± 0.19	0.82 ± 0.12	<0.001
Free androgen index	1.6 ± 0.36	1.7 ± 0.37	0.10	6.9 ± 0.82	2.9 ± 0.54	<0.001
SHBG (nmol/L)	74 ± 9.4	74 ± 8.7	0.93	26 ± 6.1	43 ± 10.3	0.05
FPG (mmol/L)	4.8 ± 0.19	4.59 ± 0.15	0.01	4.9 ± 0.17	4.68 ± 0.1	0.31
TC (mmol/L)	4.85 ± 0.21	4.66 ± 0.29	0.33	4.15 ± 0.21	4.03 ± 0.27	0.50
TG (mmol/L)	0.87 ± 0.06	0.70 ± 0.09	0.14	1.33 ± 0.24	1.05 ± 0.14	0.05
HDL-c (mmol/L)	1.6 ± 0.15	1.6 ± 0.12	0.88	1.16 ± 0.07	1.17 ± 0.08	0.89
LDL-c (mmol/L)	2.75 ± 0.21	2.7 ± 0.27	0.87	2.33 ± 0.17	2.12 ± 0.16	0.22
HOMA-IR	1.1(0.67, 1.6)	0.65(0.5, 1.2)	0.16	2.14(1.1, 4.2)	0.98 (0.7, 1.7)	0.03
NEFA (µmol/L)	553 (313,673)	513 (396, 593)	0.86	472 (392, 657)	448 (323,551)	0.51
hsCRP (mg/mL)	1.92 ± 1.04	0.81 ± 0.24	0.22	2.9 ± 0.47	2.6 ± 0.69	0.34
VO_2 max (ml/kg/min)	36.6 ± 2.4	39.6 ± 2.03	0.03	27.9 ±1.20	30.4 ±1.4	0.05

Normally distributed variables are shown as mean ± SEM. Skewed variables are shown as median (25th, 75th percentile).

*p value<0.05 is significance of difference

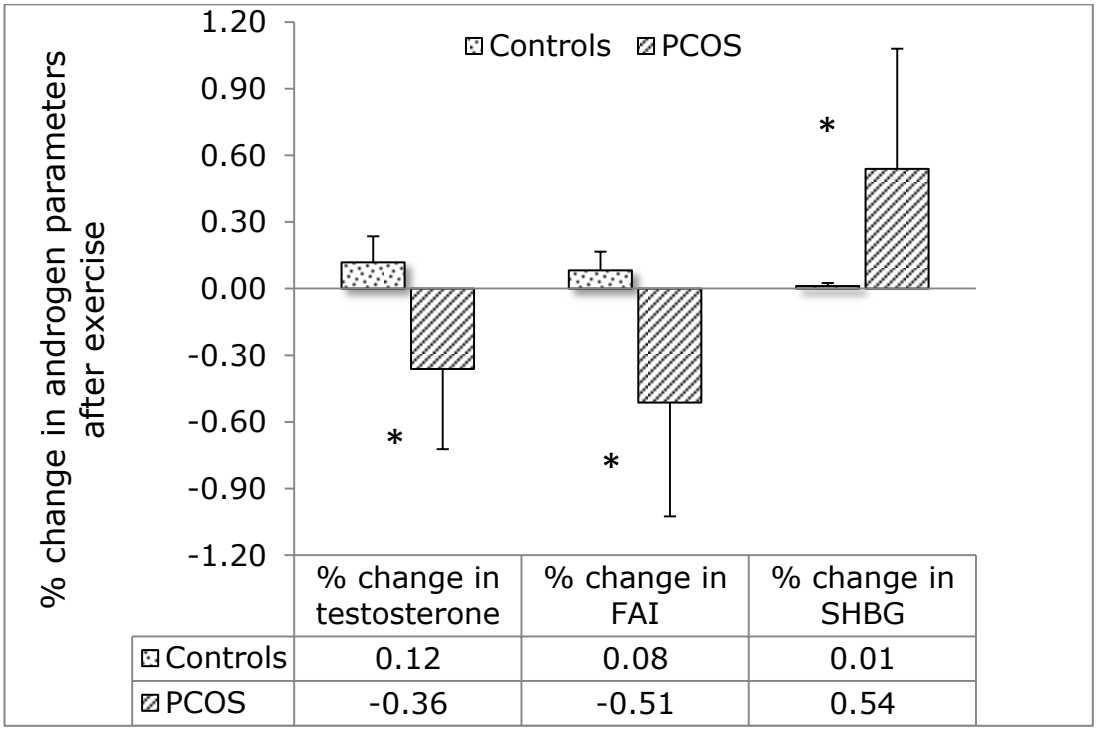
Table 5-4 Effect of exercise on intralipid induced insulin resistance

		Controls (n = 8)			PCOS (n = 10)					
		Exercise			Exercise			Change %		
Parameters	Infusion	Before	After	*p =	Before	After	p =	Controls	PCOS	*p =
glucose disposal mg/kg/min	Saline	6.04 (4.33,6.5)	6.71 (4.84, 7.7)	0.09	3.14 (2.64, 3.6)	3.33 (2.92, 5.6)	0.05	0.1	0.25	0.41
	Intralipid	2.74 (1.8, 4.2)	3.14 (2.28, 4.5)	0.05	0.93 (0.55, 1.7)	1.53 (0.93, 2.3)	0.03	0.2	0.57	0.03
NEFA AUC 3 h (µmol/L)	saline	1978 (1350,2201)	1499(1329,1631)	0.09	1881(1499, 2061)	1365(1208, 1751)	0.09	-0.3	-0.15	0.70
	intralipid	6227(5366, 7994)	5751(4358,7487	0.78	6655(4672, 8460)	4832 (4512,6775)	0.11	-0.0	-0.18	0.86
TG AUC 3 h (mmol/L)	Saline	2.40 ±0.17	2.26 ±0.47	0.78	3.75 ± 0.64	2.84 ± 0.48	0.05	-0.1	-0.21	0.08
	Intralipid	10.3 ±1.3	8.77 ±0.86	0.11	12.98 ±1.65	11.3 ±1.5	0.10	-0.1	-0.13	0.24
Glucose AUC 3 h (mmol/L)	Saline	14.23 ±0.32	13.79 ±0.22	0.08	14.14 ± 0.27	14.06 ± 0.54	0.85	-0.01	-0.01	0.56
	Intralipid	15.12 ± 0.20	14.37 ± 0.31	0.09	14.86 ± 0.39	14.64 ± 0.38	0.66	-0.1	-0.01	0.61
Insulin AUC 3 h (pmol/L)	saline	73 (48, 156)	103 (59, 156)	0.58	161 (111,250)	157 (89, 218)	0.37	0.22	-0.12	0.04
	intralipid	68 (48, 107)	91 (43, 178)	0.4	170 (69, 344)	99 (76, 289)	0.06	0.08	-0.43	0.03

Skewed variables are shown as median (25th, 75th percentile). Normally distributed variables are shown as mean ± SEM.
*p value<0.05 is significance of difference. Change % = relative change before and after exercise

5.3.5 Effect of exercise on hyperandrogenaemia

Following exercise, testosterone levels fell (Testosterone nmol/L mean ± SEM: PCOS pre-exercise, 1.3 ± 0.19; PCOS post-exercise, 0.82 ± 0.12; p<0.001) and SHBG rose (SHBG nmol/L ± SEM: PCOS pre-exercise, 26 ± 6.1; post-exercise, 43 ± 10.3; p=0.05) resulting in a fall in FAI (FAI mean ± SEM: PCOS pre-exercise, 6.9 ± 0.82; post-exercise, 2.9 ± 0.54; p=<0.001). In other words, endurance exercise reduced total testosterone by 36% in PCOS patients reaching equivalent control levels of baseline testosterone and SHBG increased by 54% and lowered FAI by 51%. SHBG, although improved, remained significantly lower than normal, thus a degree of hyperandrogenaemia persisted after exercise. The androgen profiles in controls were unchanged following the exercise programme. Data are summarised in Table 5-3, Figure 5-4.



	% change in testosterone	% change in FAI	% change in SHBG
Controls	0.12	0.08	0.01
PCOS	-0.36	-0.51	0.54

Figure 5-4 Changes in androgen profiles following moderate intensity exercise

Values are expressed as mean± SD.

5.3.6 Effect of exercise on lipid induced insulin resistance

After 8 weeks of moderate intensity exercise, there were concomitant rises in insulin sensitivity, significant in PCOS but not in controls (median glucose disposal mg/kg/min: PCOS pre-exercise, 3.14 (2.64, 3.63); post-exercise 3.33 (2.92, 5.6) mg/kg/min, p=0.05; controls pre-exercise, 6.04 (4.33, 6.48); post-exercise, 6.71 (4.84, 7.7); p=0.09) during the hyperinsulinaemic euglycaemic clamp with the saline infusion. Similar findings were observed during the lipid infusion (median glucose disposal mg/kg/min: PCOS pre-exercise 0.93 (0.55, 1.65); post-exercise, 1.53 (0.93, 2.34); p=0.03; controls pre-exercise 2.74 (1.8, 4.17) post-exercise, 3.14 (2.28, 4.51); p=0.05). Data are summarised in Table 5-4. The improvement in insulin sensitivity was far greater in PCOS than controls. For example, exercise increased the rate of glucose disposal by 10% and 25% in controls and PCOS during saline infusions and by 20% and 57% respectively following lipid infusions. Before the exercise program, the lipid challenge reduced insulin sensitivity by 67% in PCOS and 45% in controls. In contrast, at the end of the exercise program, the degree of lipid induced reduction in insulin sensitivity appeared comparable between PCOS and controls: i.e. lipid infusion reduced the rate of glucose disposal by 55% in Figure 5.5.

Despite a significant improvement of insulin sensitivity during both saline and lipid infusion, insulin sensitivity remained significantly lower in PCOS than controls towards the end of the exercise.

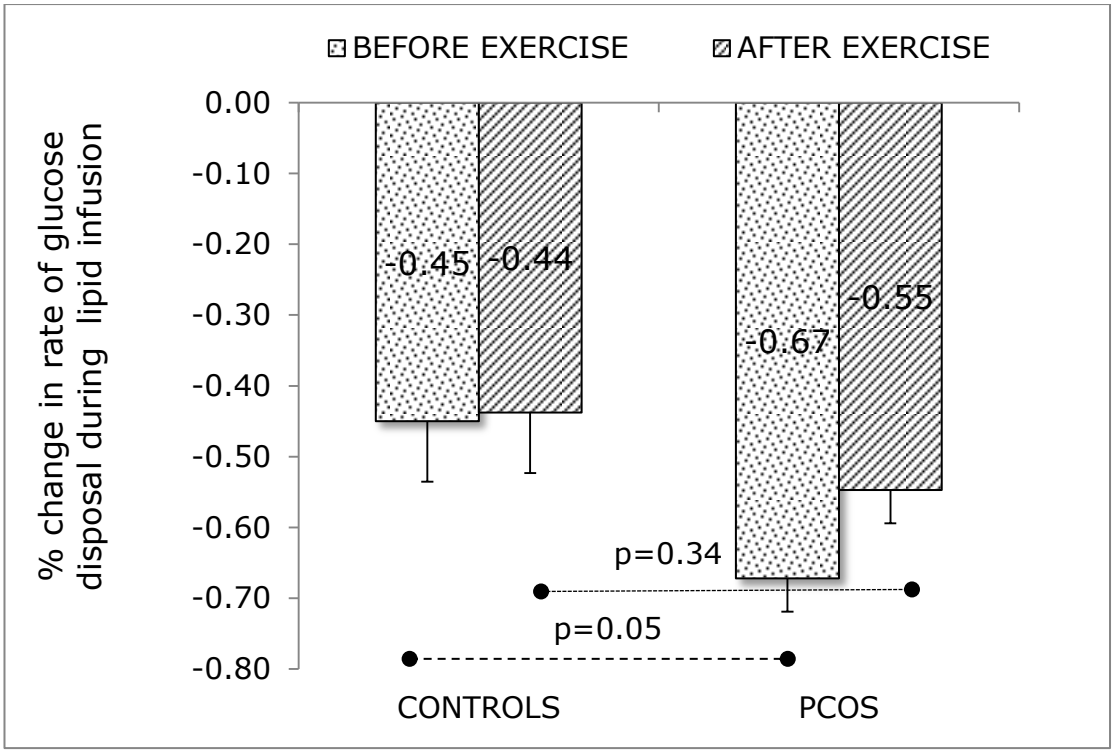


Figure 5-5 Effect of intralipid on the rate of glucose disposal before and after exercise (Data are expressed as mean± SEM)

5.3.7 Effect of exercise on lipid metabolism

Exercise reduced TG AUC 3 h in PCOS (mean TG mmol/L ± SEM: PCOS pre-exercise, 3.75 ± 0.64; post-exercise, 2.84 ± 0.48; p=0.05) during the saline infusion and during lipid infusions (mean TG AUC mmol/L ± SEM: PCOS pre-exercise, 12.98 ± 1.7; PCOS post-exercise, 11.3 ± 1.5; p=0.10). There was a concomitant fall in NEFA AUC 3 h during saline (median NEFA µmol/L: PCOS pre-exercise, 1881 (1499, 2061); post-exercise, 1365 (1208, 1751); p=0.09) and lipid infusion (median NEFA µmol/L: PCOS pre-exercise, 6655 (4672, 8460); post-exercise, 4832 (4512, 6775); p=0.11) although these changes were not significant. Data are summarised in Table 5-4.

5.4 Discussion

5.4.1 Effect of exercise on NEFA levels

Fasting NEFA levels before exercise were not different between groups despite women with PCOS being slightly more overweight than controls. This could be due to down regulation of NEFA release from adipose tissue (321) or hormone HSL activity being unaffected by IR during the post-absorptive period (571). Following exercise, there was a trend for reduced fasting NEFA as well as NEFA AUC during lipid infusion suggesting effective removal of circulating NEFA. TG levels fell with exercise thus it is unlikely to be an increased uptake by the liver to synthesize VLDL. Therefore the fall in NEFA levels after exercise may well represent increase uptake of NEFA by skeletal muscle in PCOS.

5.4.2 Effect of exercise on insulin resistance

In the present study, moderate intensity exercise with weight maintenance resulted in a reduction of IR in the post absorptive state. Previous studies have shown the benefit of exercise and weight loss in PCOS with a reduction of IR (659, 660). In those studies, reduced IR and improved androgenaemia appeared mostly dependent on weight loss. It is, therefore, difficult to tease out whether exercise alone may be beneficial. Exercise training preferentially reduces abdominal fat independently of weight loss (661). Moderate intensity exercise in adolescents (426) and adults (662) improves insulin sensitivity in conjunction with favourable alterations in lipid partitioning and an enhanced lipid oxidative capacity within muscle. In the present study, the exercise program appeared to enhance NEFA uptake and oxidation by skeletal muscle as shown by lowered NEFA AUC levels during either saline or intralipid infusion and subsequent improvement of IR after exercise.

5.4.3 Effect of exercise on hyperandrogenaemia

The present study also shows that a reduction of IR is accompanied by a significant increase in SHBG, and a decrease in total testosterone with a consequent fall in FAI in women with PCOS. The improved insulin sensitivity and reduced insulin levels probably contributed to a decrease in testosterone and a rise in SHBG since hyperinsulinaemia enhances androgen production (663). Both the suppression of

insulin secretion by diazoxide (664) and improved insulin sensitivity by thiazolidinediones (665) have been shown to decrease circulating androgen levels. This action of insulin appeared to be restricted to insulin resistant states since lowering of insulin levels in controls showed no effect on androgen profiles (666). Insulin can directly decrease hepatic SHBG production (558). Therefore, enhanced insulin sensitivity through exercise without weight changes probably explains the improvement in total testosterone (37%), SHBG (30%) and FAI (48%) levels in PCOS but not in controls seen in the present study. Velazquez et al. reported that an 8 week course of metformin therapy in 26 women with PCOS resulted in a modest reduction in waist hip ratio (2.5% from baseline) and weight (1.5%) accompanied by a 44% decrease in total testosterone, 33% in SHBG, 49% in FAI and 7.5% in mean systolic blood pressure (667). This suggests that the magnitude of the effect of exercise in our study was comparable with that of metformin on the metabolic and androgenic parameters in PCOS.

The few studies undertaken to examine specific effects of exercise training in PCOS on reproductive outcomes have shown improvements in menstrual cyclicity and/or ovulation in 50% of PCOS women. For example, Aubuchon et al. showed, in their uncontrolled small study, that six women (46%) became pregnant during the 14 week exercise study or within 3 months of completion (668). Similarly, aerobic exercise assisted in the restoration of normal menstrual cycles with improvements in body weight, waist circumference and IR in PCOS women (669, 670). In the present study, changes in menstrual cycle, fertility and hirsutism score were not examined because of the relatively short study period.

In PCOS, exercise, when compared to diet alone provided more favourable effects on body composition with greater reduction in fat mass, preservation of fat-free mass and a reduction in waist circumference with weight loss in most (659, 668, 669) but not all studies (671). There was no change in body composition after aerobic exercise and combined aerobic and resistance exercise (659). Palomba et al. compared 24 weeks of aerobic exercise (30 min cycling, 3 times per week) with a hypocaloric, high-protein diet (670). Although both interventions reduced body weight, waist circumference, fasting insulin, IR and improved the reproductive hormone profile, exercise produced greater improvements than dieting in waist circumference, SHBG, FAI and insulin levels

despite greater weight loss in the dieting subjects. This suggests that exercise training may offer greater benefits for improving IR and reproductive hormones compared with diet induced weight loss alone. However, Thomson et al. did not find any benefit of adding aerobic exercise to a hypocaloric diet on IR or hormonal profile (659). In the present study, moderate intensity exercise alone improved both metabolic and hormonal profiles in accordance with the Palomba et al. study (670) and this also achieved reduced waist circumference and improved physical fitness, but no weight loss.

5.4.4 Effect of exercise on cardiovascular risk in PCOS

In the present study, moderate intensity exercise improved physical fitness and cardiometabolic risk factors such as IR, blood pressure and fasting TG similarly in both PCOS and control subjects whilst body weight remained constant. HsCRP was also improved by exercise in both groups but not to a significant level. As discussed in section 1.9.6, there is a powerful, positive relationship between sedentary lifestyle/low cardiorespiratory fitness and poor health outcomes such as T2DM, CVD mortality, and all-cause mortality (436-438). Several studies have already proved that physical activity reduces CVD and all-cause mortality by a least one third in the general population (439). In fact, both duration and intensity of exercise influence the reduction all-cause mortality (440, 441), CVD mortality (441, 442) and the risk of CAD (443, 444). Increased physical activity as a part of lifestyle modification improves not only cardiovascular risk but also glucose and insulin homeostasis and subsequent risk of diabetes (218, 446). Based on our findings, women with PCOS who are at risk of diabetes should, at least, exercise with moderate intensity i.e. brisk walking, 180 min per week to improve androgen and metabolic profiles and decrease cardiovascular risk.

Moderate intensity exercise is defined as activity with an energy expenditure of 3-6 metabolic equivalents (MET) and is generally comparable to a brisk walk on level ground at 3-4 miles per hour that noticeably accelerates the heart rate (672, 673). It is recommended that all adults should undertake moderate intensity aerobic (endurance) physical activity for a minimum of 150 min per week to promote and maintain their health (672). This moderate intensity exercise is known to increase fat oxidation greater than low or high intensity exercise (322). The exercise programme, followed

in the present study, was designed to meet this requirement and was standardized as exercise training to achieve a heart rate equivalent to 60% of baseline VO_2 max (674) by a brisk walking on a treadmill with a heart rate monitor under supervision.

Moderate intensity exercise alone decreased fat mass, increased physical fitness, enhanced insulin sensitivity and basal fat oxidation in older people with IGT who were at high risk of developing diabetes and CVD (675). In a longitudinal study of the Chinese population, intervention with exercise alone, led to a significant decrease in the incidence of diabetes over a 6 year period among those with IGT (676). In PCOS, a combination of diet and exercise with resultant weight loss improved endothelial function and androgen profiles (677). A hypo-calorific diet with or without aerobic exercise in PCOS women, decreased blood pressure, TG, total cholesterol, LDL-c, testosterone and insulin level with weight reduction (659). However, the study failed to prove the beneficial effect of the addition of aerobic or combined aerobic-resistance exercise to the hypo-calorific diet. A 16 week exercise program improved cardiopulmonary fitness which was followed by an improvement in FMD of the brachial artery, independent of changes in fatness or IR (678). Similar to our findings, a 12 week supervised and intensified exercise with a target of VO_2 max 90-100%, improved cardiovascular risk markers such as physical fitness, IR and reduction of visceral fat and TG without a significant weight loss in women with PCOS (679). In the present study, comparable cardiovascular risk reduction was achieved by a lesser intensity-supervised exercise that is achievable and equivalent to brisk walking.

The most common perceived barriers to physical activity and healthy eating encountered by young women, particularly those with children, were high cost, lack of motivation, time and social support (430). This may lead to the development of a perception of optimum lifestyle changes as unachievable and unrealistic (680). Our findings of the effectiveness of moderate intensity exercise on cardiometabolic and androgen profiles provide a valuable asset to women with PCOS. Moderate intensity exercise i.e. simple brisk walking 3 h per week is a simple, achievable and reliable prescription with a predictable outcome and no cost involved. In addition, this strategy, together with the provision of a supportive environment, would fit within the context of a young woman's daily routine to enable physical activity participation and to overcome the perceived barrier.

5.4.5 Effect of acute rise in NEFA on IR in PCOS

It is well recognised that women with PCOS are more insulin resistant than their weight and age matched controls (15). Chronic elevation of NEFA plays a significant role in the pathogenesis of IR in PCOS (286). However, their tolerance to an acute rise in NEFA has not been studied in comparison with non-PCOS women. In the present study, unfractionated heparin was given together with intralipid to enhance the action of LPL for the hydrolysis of infused TG. An acute rise in NEFA due to lipid and heparin infusion decreased the rate of glucose disposal from their baseline levels by 67% in PCOS and 45% in controls. This suggests PCOS women are metabolically incompetent to tolerate acutely raised NEFA levels as control subjects can do.

An intrinsic insulin signalling defect could be one of the reasons for metabolic incompetence in PCOS. However, intralipid has had no effect on proximal signalling, namely basal or insulin-stimulated IRS-1 tyrosine phosphorylation, tyrosine-associated PI3K activity, or IRS-1-associated PI3K activity (681). Therefore, it is more likely that acutely elevated NEFA levels act synergistically rather than enhancing the underlying insulin signalling defect in skeletal muscle glucose transport in PCOS. Elevation of the plasma NEFA level impairs pancreatic beta-cell function and reduces glucose stimulated insulin secretions in obese non-diabetic subjects (374, 378, 379). In the present study, there was no compensatory increase in insulin levels in response to lipid-induced IR following a 3 h intralipid infusion. Therefore, the lipotoxicity of pancreatic beta cells could provide another explanation for lipid-induced IR in PCOS.

An inherited insulin signalling defect in skeletal muscle in PCOS has been detailed in section 1.3.7.1. Briefly, PCOS has profound peripheral IR due to a reduction in IRS-1-associated PI3K activity in skeletal muscle (140). Obesity and IR enhance mobilization of NEFA from adipose tissue particularly in the postprandial period due to decreased effective inhibition of insulin on HSL (571). It has been recognised that elevated NEFA interferes with skeletal muscle glucose transport via increased production of intermediate lipid metabolite (409). This is compatible with findings in the present study which has shown that elevated NEFA enhances IR more severely in PCOS than controls. This hypothesis is reinforced by the findings of a failure to decrease skeletal muscle insulin-mediated glucose uptake in lipid free culture media (144). The novel finding in this study was that women with PCOS were significantly less tolerant to an

acute NEFA rise than controls, probably due to their intrinsic insulin signalling defect. This finding highlights the important role of environmental factors in the pathogenesis of a decrease in insulin-mediated glucose transport in PCOS. This may also suggest that acute weight gain in PCOS would contribute to a more significant adverse effect on IR when compared to controls.

5.4.6 Effect of exercise on fasting and lipid induced IR in PCOS

Lifestyle changes in dietary fat intake, physical activity and weight loss may improve metabolic flexibility in skeletal muscle, and thereby contribute to improve IR (682). This study examined the effect of exercise on metabolic competency without dietary modification and with no intention to modify body weight in PCOS. Exercise was shown to significantly lower HOMA-IR and improved the rate of glucose disposal during clamps in both PCOS and controls. Exercise also improved the rate of glucose disposal during lipid infusion in both groups. An acute rise in NEFA reduced the rate of glucose disposal by 67% before exercise and improved to 55% from baseline levels during saline infusion after exercise in PCOS. In controls, lipid infusion reduced the rate of glucose disposal by about 45% after exercise a value similar to that before exercise. After exercise, PCOS women became more tolerant to acute fat load but still did not reach to the level observed in controls. This improvement was achieved with no significant change in weight but with increased physical fitness. The rate of glucose disposal in PCOS, in a lipid trial, remained lower than that of controls suggesting that short term exercise has no significant impact to completely reverse chronically elevated IR. However, exercise in PCOS reversed metabolic incompetence related to an acute rise in NEFA but failed to achieve complete reversal of IR related to chronic metabolic incompetence.

In a recent study, 8 healthy subjects underwent 1 h of one legged knee extensor exercise followed by 7 h of saline or intralipid infusion. During the last 2 h, a hyperinsulinaemic euglycaemic clamp was performed. Intralipid reduced the glucose uptake of the leg which was alleviated by prior one-legged knee extensor exercise. In addition, prior exercise normalised insulin-stimulated glucose uptake in the lipid trial to the level observed in the resting, control leg in the saline trial (683). This improvement was related to the fact that exercise increased insulin which stimulated both glucose uptake and glycogen synthase activity. However, prior exercise did not up-regulate

proximal signalling events such as insulin-induced receptor tyrosine kinase activity, serine phosphorylation of Akt, or serine phosphorylation of glycogen synthase kinase-3 (GSK-3) in human skeletal muscle in that study (684).

In the present study, the exercise program improved fasting IR as well lipid-induced IR in controls. Unlike other studies, the exercise program failed to reverse lipid induced IR completely back to the level observed during the saline infusion. This might be related to the time gap between exercise and the lipid infusion trial. In other words, the effect of prior exercise on insulin- stimulated glucose uptake during the lipid trial is not a sustained effect. This finding may highlight the importance of the time gap between meal and exercise in promoting insulin-mediated glucose uptake during the postprandial period.

Bruce et al. reported the effect of 8 weeks of moderate intensity exercise on insulin sensitivity and IMTG content in subjects with T2DM. After exercise, insulin sensitivity was improved by 30% to the same extent in both groups with training but IR was not completely reversed in diabetic patients (428). Similar to the diabetes patients, exercise improved IR at baseline and at the lipid challenge in PCOS but it failed to reverse either to the level observed in controls at baseline or during the lipid trial in the present study. This might be related to an inherited proximal insulin signaling defect in PCOS. However, neither intralipid nor exercise has impacted on the proximal pathway of insulin signaling i.e. the defect observed in PCOS. It suggests that endurance exercise attenuated IR induced by lipids but was unable to overcome the underlying IR of PCOS. Alternatively, the failure of complete reversal of IR by exercise in PCOS may be due to the time gap between the acute rise in NEFA and exercise activity which has been noted in the control group in the present study. Therefore, exercise should be recommended prior to meals to produce its maximum effect on lipid metabolism during the postprandial period.

Failure of moderate intensity exercise to reverse lipid-induced IR could be due to multiple factors in PCOS. It could be related to metabolic incompetency of skeletal muscle influenced by FA availability, uptake and oxidation. Randle and colleagues thought that increased FFA availability would lead to an increased FFA oxidation and then interfere with glucose uptake. Subsequent studies proved that accumulation of lipids and lipid metabolites (DAG, ceramides and LCFA Acyl CoA) interferes with

insulin signalling and glucose uptake (420, 451). Detail information has been described in **s**ection 1.7.4**.**

Increased availability of fatty acids i.e. elevated plasma NEFA levels may lead to increase FFA uptake and cause IR in skeletal muscle. For example, chronically elevated NEFA levels were associated with insulin resistant states such as obesity, T2DM and PCOS. Suppression of NEFA release by acipimox that inhibits HSL activity in adipose tissue improves IR in PCOS as previously described in section 3.3.3. Moreover, a rebound rise in NEFA with chronic use of slow release nicotinic acid deteriorated fasting as well as postprandial IR that was shown in the tredaptive PCOS study, described in section 4.2.4. Infusion of intralipid decreased the rate of glucose disposal. Therefore, the increased NEFA availability clearly contributes to IR in PCOS. Similar results were found in T2DM, IGT relatives and healthy subjects in whom low grade intralipid infusion reduced insulin sensitivity and increased whole body fat oxidation and decreased whole body glucose oxidation (377). In the present study, there was a trend of decreased plasma NEFA AUC during saline as well as lipid trials in both groups with exercise. However, the level of NEFA AUC in the lipid trial was still 4 times higher than the saline trial. This persistently significant rise in NEFA may well contribute to the incomplete reversal of insulin sensitivity after exercise.

Impairment of FA oxidation/ utilization may also contribute to the aetiology of skeletal muscle IR. Following a 60 min moderate intensity exercise, there was no difference in total energy expenditure or in total carbohydrate and fat oxidation among subjects with T2DM, with OGT and healthy controls. However the plasma FFA oxidation was lower but triglyceride-derived FA oxidation was higher in subjects with IGT and T2DM compared with obese subjects at rest and tended to be lowered during exercise (452, 685).

Muscle FA metabolism is impaired in obesity and IR, reflected in reduced rates of FA oxidation and accumulation of lipids. Bruce et al. investigated the effect of 8 weeks of moderate intensity endurance exercise on the rate of mitochondrial, FA oxidation and lipid content in muscle of obese subjects and their relation to glucose tolerance. Endurance exercise improved glucose tolerance with an increase in mitochondrial FA oxidation. It also decreased the DAG and ceramide content of skeletal muscle but had no effect on muscle TG content (430). The acipimox study has shown that whole

body FA oxidation during the postprandial period in PCOS was not different from controls in section 3.3.4.

Therefore, normal mitochondrial oxidative phosphorylation is required to maintain the balance of FA uptake and oxidation to protect excessive accumulation of IMTG and their metabolites. In women with PCOS, the impaired insulin-stimulated total, oxidative and non-oxidative glucose disposal are associated with a consistent down regulation of OXPHOS (mitochondrial oxidative phosphorylation) gene expression in skeletal muscle that coupled with reduced levels of peroxisome proliferator-activated receptor gamma co-activator alpha and cannot be ascribed to obesity and diabetes (429). This defect could also be plausible potential explanation for the failure of exercise to completely reverse post-absorptive HOMA-IR and lipid-induced IR in PCOS in this study.

5.5 Summary

In the present study, women with PCOS were more insulin resistant, more androgenic than controls at baseline. Lipid infusion induced more severe IR in PCOS than in controls. This highlights the importance of the effect of an acute fat load and/ or acute weight gain on IR in PCOS. Supervised, moderate intensity exercise improved physical fitness, androgen profile and cardiovascular risk markers such as post-absorptive IR (HOMA-IR), fasting TG and systolic blood pressure in PCOS without a significant weight reduction. Moderate intensity exercise improved lipid-induced plasma TG AUC 3 h and NEFA AUC 3 h during lipid infusion. This was accompanied by an increase in the rate of glucose disposal during saline and lipid infusion in both groups. PCOS women had improved tolerance to acutely raised plasma NEFA level since the relative change in lipid-induced reduction in insulin sensitivity was parallel with the change observed in controls after exercise. However, women with PCOS failed to completely reverse their IR in the saline and lipid trials to levels observed in controls, suggesting the presence of chronic metabolic incompetence in relation to insulin signalling defect in the presence of chronically elevated NEFA. However, incomplete reversal in IR may well be due to the time gap between exercise and lipid challenge and this may highlight the beneficial effect of exercise just prior to acute fat load i.e. postprandial period.

Chapter 6 Effect of acute hypertriglyceridaemia on insulin resistance and platelet activation in PCOS

6.1 Introduction

Insulin resistance (IR) and dyslipidaemia commonly occur in obese women with PCOS (278, 686). Consequently, IGT and metabolic syndrome, recognized as the predictors of T2DM and premature CVD mortality (687, 688), are prevalent in PCOS (24, 689). IR is known to be an independent CV risk in the general population (690) as well as in patients with T2DM (691). IR is associated with a dyslipidaemia that usually involves hypertriglyceridaemia, which may, itself, independently increases the risk of development of CVD in both adult men and women (290, 291). Dyslipidaemia plays a major role in the development of atherothrombosis, the pathology that underlies CVD. A 1 mmol/L increase in TG was associated with a relative risk increase of 14% in men and 37% in women for incident CVD (292). TG, even at levels as low as 2.25 mmol/L were associated with increased CVD mortality in a 14 year follow up study (293). Lowering TG using fibrates in patients with either isolated hypertriglyceridaemia or atherogenic metabolic dyslipidaemia significantly reduces CVD events (692, 693). However, the nature of the link between hypertriglyceridaemia and CVD is poorly defined. The positive association of high TG and low plasma HDL-c with CVD is well documented. Plasma concentrations of HDL-c and TG are inversely related (694, 695). In fact, increased plasma TG levels have been suggested as a major independent cause of decreased HDL-c levels because of the substantial loss of cholesteryl ester from HDL particles as they become enriched with TG (696, 697). The association of low HDL-c with high TG has been reported in obesity, obesity related metabolic disorders (698-700) and many heritable lipoprotein disorders (701, 702). Several clinical trials have shown that elevating HDL-c and lowering TG, in addition to reducing LDL-c, improved the risk of CVD (703-705). However, there has been difficulty in separating high TG and low HDL-c as individual risk factors since they are interrelated and occur conjointly in many metabolic disorders. Hypertriglyceridaemia may play an indirect role in the causation of atherosclerosis (295, 296) as a central component of the "atherogenic dyslipidaemia triad" together with reduced levels of HDL-c (297) and a preponderance of small,

dense LDL-c (299). It is also possible that hypertriglyceridaemia has a direct effect on the cells involved in atherogenesis, although evidence for this is less well developed.

Excessive platelet activation induced by activation mediators such as ADP, thrombin and thromboxane A_2 is normally tightly controlled by both endothelial and platelet derived antagonists such as PGI_2 and NO (457, 458). Vascular endothelial cells, under basal conditions and in response to various vasoactive agents, synthesize and release PGI_2. PGI_2 stimulates adenylate cyclase and increases intracellular cAMP dependent, protein kinase activation (463, 706). The protein kinases reduce intracellular Ca^{2+} flux which suppresses glycoprotein IIb/IIIa expression. This change is required for binding integrin to fibrinogen, thereby decreasing the number and affinity of fibrinogen binding sites on the platelet's surface (463). PGI_2, the most powerful inhibitor of platelet activation, seems to play a pivotal role in atherothrombosis (707).

Platelets, once activated, enhance their further recruitment and aggregation, release chemokines, cytokines and immunomodulatory ligands, contribute to endothelial activation, recruit neutrophils, facilitate inflammatory responses, exert immunomodulatory activity (708) and complement the initiation of the coagulation cascade (464). Platelet activation thus appears to be a common thread that linking inflammation, thrombosis and atherogenesis (464). Pathological increase in platelet activation are associated with an increased risk of atherothrombosis and subsequent cardiovascular events (457). Effective platelet function at all times is critical since, in addition to their classical role in acute thrombosis, platelets are involved in the initiation of atheroma, modulation of inflammatory responses and contribute to endothelial dysfunction (454). Platelets can adhere to intact, activated endothelium in the absence of exposed extracellular matrix proteins (709). These adherent platelets may play a critical role in atherogenesis by the secretion of the chemokines CCL5 (RANTES), CXCL4 (platelet factor 4) and interleukin-1 (710).

In intact endothelium, platelet activation, induced by either shear stress (711) or endothelial dysfunction due to chronic exposure to risk factors such as smoking, facilitates the progression of atherosclerosis (468). In the context of endothelial erosion and atherosclerotic plaque rupture, the exposure of vascular matrix components to the blood stream triggers extensive platelet adhesion and activation eventually leading to aggregation and thrombus formation (464). The final pathway for

platelet aggregation is regulated by activation of the platelet GPIIb/IIIa receptor, the most abundant protein on the platelet surface, and its binding to fibrinogen from plasma or platelet origin (470).

The effect of hypertriglyceridaemia on platelet function was not consistently reported in previous studies. Patients with familial hypertriglyceridaemia have a lower threshold for aggregation in response to ADP and collagen compared to normal controls (475). In another study, no difference was found in platelet reactivity between controls and hypertriglyceridaemic subjects (476). In vitro, VLDL increased platelet aggregation while HDL-c gave the opposite effect (712). In animal studies, a high fat, high calorie diet induced hyperlipidaemia, insulin resistance and obesity and was associated with increased reactivity of platelets to ADP and collagen (713).

Insulin resistant states such as type 2 diabetes are associated with increased platelet activation (495). PCOS patients are insulin resistant and associated with increased CV risk (714). Plasma platelet derived microparticles are elevated in overweight/obese women with PCOS compared with BMI-matched controls (715). Women with PCOS had significantly higher platelet aggregation in response to ADP than controls (83). However, platelet response to inhibitory mediators such as PGI_2 and the effect of hypertriglyceridaemia on platelet activity has not been studied previously in PCOS.

The mechanisms underlying platelet hyperactivity in insulin resistant states are ill defined, and hypertriglyceridaemia may play an important but undefined role. Therefore, in the present study we sought to determine the influence of an intralipid induced, acute hypertriglyceridaemia and insulin resistant state upon multiple aspects of platelet function in patients with PCOS and their age and BMI matched healthy women.

6.2 Methods and materials

6.2.1 Study subjects

A total of thirteen PCOS patients and twelve female healthy volunteers who fulfilled the inclusion and exclusion criteria were included in the study. Fully informed, written consent was obtained from all subjects (figure 6-1). Details of recruitment, screening tests and study visits have been mentioned in section 2.1, 2.1.5.3. This study was done

in the visit 2 of the exercise study and therefore the study population was the same as the exercise study which has been described in chapter 5.

6.2.2 Protocols

All participants underwent two day procedures of either normal saline or 20% intralipid with unfractionated heparin infusion for 5 h with a measurement of insulin sensitivity; i.e. rate of glucose disposal by a hyperinsulinaemic euglycaemic clamp in the last 2 h of infusions. Detailed procedures were described in section 4, 5.2.3. A schematic diagram of procedural protocols can be seen in Figure 6-2.

For the platelet study, blood samples were taken at 2 h and at 5 h of either saline or intralipid infusions to compare the effect of intralipid and the effect of hyperinsulinaemia on platelet activation. After 2 h (before the insulin clamp was initiated), of saline or intralipid infusion, blood was collected in 3.8% tri-sodium citrate with the initial 5 mL discarded to avoid artificial platelet activation as per protocol described in section 2.3.5.3. A second blood sample, for the platelet function test, was taken at 5 h after the saline or intralipid infusion i.e. at the end of the insulin clamp.

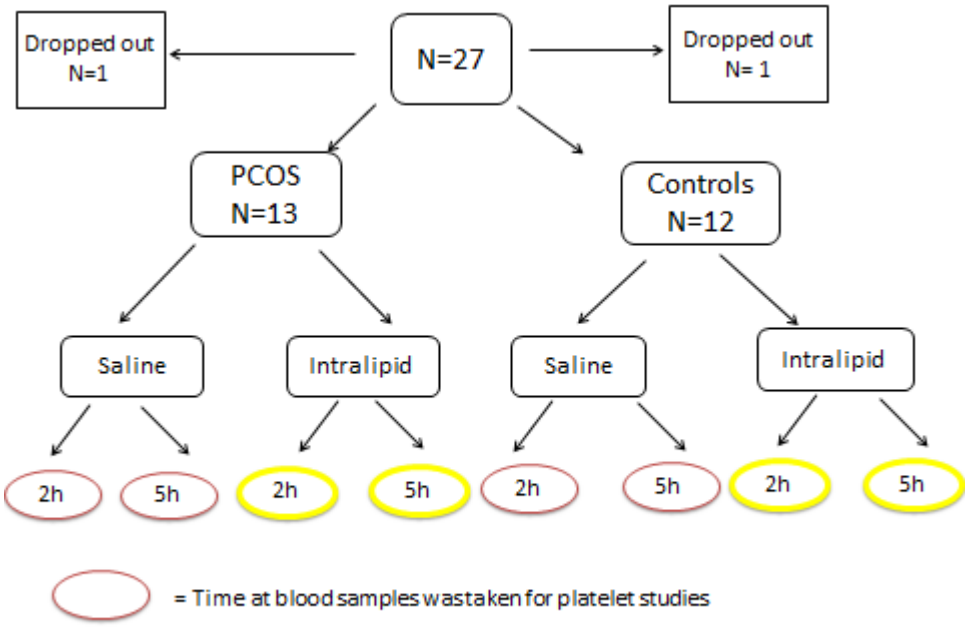


Figure 6-1 Recruitment and study design

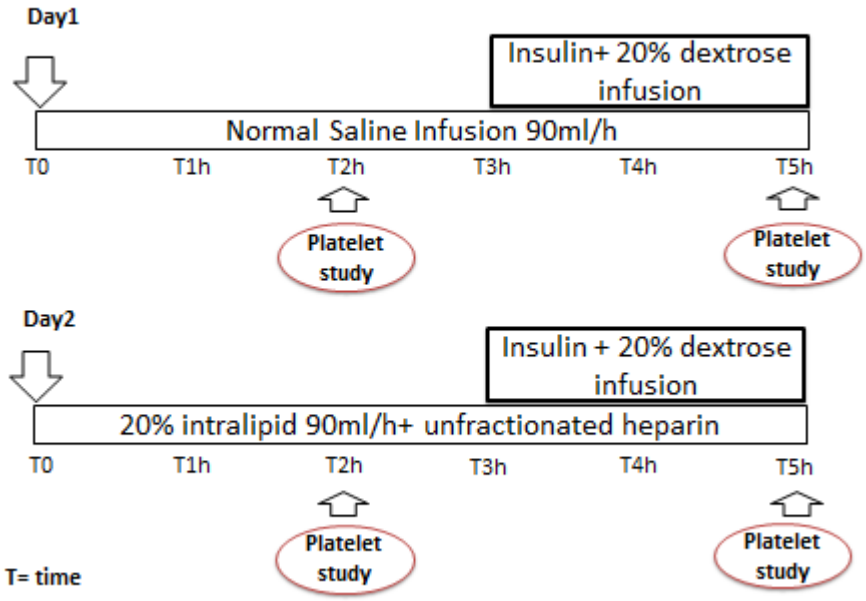


Figure 6-2 Procedural protocols

6.2.3 Biochemical analysis

Measurement of total cholesterol, TG, HDL-c, LDL-c, NEFA, blood glucose, insulin, testosterone, SHBG, FAI and hsCRP were carried out according to analytical assays described above in section 2.4.

6.2.4 Statistical analysis

Statistical analysis is described in detail in section 2.5.

6.2.5 Flow cytometric analysis of platelet activation

Details of the preparation and analysis of platelet assay tubes have been described in section 2.3.5.5 (Figure 6-3, Figure 6-4). Platelet immunostaining was performed in a manner designed to minimise sample manipulation and artefactual *ex vivo* activation. Platelet activity was assessed at basal state and in response to three graded concentrations of ADP (0.01, 1, 10 μM). For the assessment of platelet inhibition, platelets were primed with either three concentrations of PGI_2 (0.001, 0.01, 0.1 μM)

or 8-CPT-6-Phe-cAMP (50, 100, 200 μM) before stimulation with 1 μM ADP. A total of 10,000 events were recorded and more than 95% of cells in the gated study population were platelets. The presence of fibrinogen binding receptors and P selectin receptors on the activated platelet surface was recognised by appropriate antibodies. Data are presented as means of duplicate samples of the percentage of platelets activated.

Figure 6-3 A FACS Aria flow cytometer

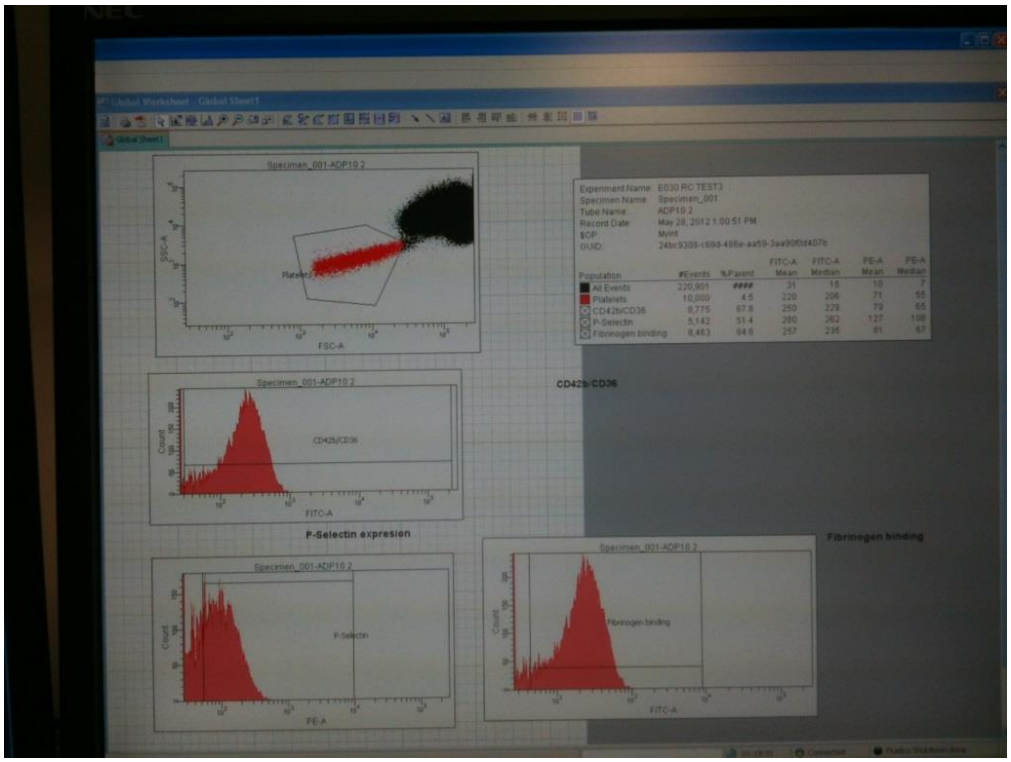


Figure 6-4 Gating platelet population, and detecting fluorescent activities of activated platelets

6.3 Results

Baseline characteristics of PCOS and controls are shown in Table 6-1. In the present study, PCOS women were more overweight, centrally obese and hyperandrogenic than controls.

6.3.1 Biochemical changes during intralipid infusion

Biochemical changes with lipid infusion were compared to those with saline infusion Table 6-2. Lipid infusion increased plasma TG and NEFA levels in both groups. This was accompanied by significantly decreased insulin sensitivity: rate of glucose disposal (3.15 (2.94, 3.85) vs. 1.06 (0.72, 1.43) mg/kg/min; $p<0.001$) and (5.25 (3.30, 6.48) vs. 2.60 (0.88, 3.88) mg/kg/min; $p<0.001$) in PCOS and controls, respectively. In both groups, there was no significant difference in plasma glucose between samples taken at 2 h of saline and intralipid infusion, or 5 h, where glucose concentration was clamped at 5mmol/L by design.

6.3.2 Platelet activation

The binding of fibrinogen to the platelet surface is an early marker of platelet activation, and P selectin is only expressed on activated platelets. Both these criteria were examined to evaluate platelet activity in whole blood samples taken before and after hyperinsulinaemic euglycaemic clamp during either saline or intralipid infusion.
Incubation of whole blood with ADP led to a concentration-dependent increase in platelet activation in all samples in both groups. Addition of either PGI_2 or 8-CPT-6-Phe-cAMP resulted in concentration-dependent decrease in ADP-induced, platelet activation. Detailed results are given in Table 6-3, Table 6-5.

Table 6-1 Baseline characteristics of participants

Parameters	Controls (n=12)	PCOS (p=13)	*P=
Age (year)	24.1±5.8	28.0±6.3	0.13
BMI (kg/m^2)	25.5±5.0	29.7±6.0	0.07
Waist (cm)	80.9±2.5	96.5±17	0.02
WHR	0.79±0.07	0.86±0.02	0.04
SBP (mmHg)	116±9	118±11	0.56
DBP (mmHg)	74±9	75±8	0.73
Testosterone (nmol/L)	1.03±0.34	1.51±0.77	0.05
FAI	1.6±1.0	6.6±3.2	<0.001
SHBG (nmol/L)	75±30	28±19	<0.001
ALT (iu/L)	17±5	27±13	0.01
TC (mmol/L)	4.7±0.79	4.1±0.63	0.14
TG (mmol/L)	0.96±0.32	1.12±0.51	0.35
HDL-c (mmol/L)	1.58±0.48	1.2±0.37	0.06
LDL-c (mmol/L)	2.31±0.49	2.68±0.58	0.1
FPG (mmol/L)	4.69±0.51	4.74±0.45	0.08
PPG (mmol/L)	4.78±1.8	5.7±1.7	0.21
HOMA-IR	1.33±0.69	2.50±1.5	0.03
NEFA (µmol/L)	551 (330, 658)	441 (345, 544)	0.23

Results are expressed as means ± SD except NEFA, Insulin and HOMA-IR that were expressed as medians (25th, 75th centiles). *p value < 0.05 is significance of difference.

Table 6-2 Biochemical changes following intralipid or saline infusion

Parameters	Controls (n=12)			PCOS (n=13)		
	Saline	Intralipid	*P=	Saline	Intralipid	*P=
glucose disposal mg*kg*min	5.25 (3.30, 6.48)	2.6 (0.88, 3.9)	<0.001	3.15 (2.94, 3.9)	1.06 (0.72, 1.43)	<0.001
Triglycerides AUC 2 h (mmol/L)	1.67± 0.37	5.98± 1.8	<0.001	2.21± 1.37	7.23± 2.83	<0.001
Triglycerides AUC 5 h (mmol/L)	3.78± 0.86	22.5± 7.8	<0.001	5.29± 2.99	23.9± 11.4	<0.001
NEFA AUC 2 h (mmol/L)	1.13 (0.86, 1.36)	3.2 (2.29, 4.0)	0.01	1.1 (0.81, 12.4)	3.30 (2.71, 4.80)	0.01
NEFA AUC 5 h (mmol/L)	2.24 (1.83, 2.86)	12. (8.5, 13.5)	<0.001	2.26 (1.8, 2.55)	12.4 (9.25, 1.44)	<0.001

Skewed variables are shown as median (25th, 75th percentile). Normally distributed variables are shown as mean ± SEM

*p value<0.05 is significance of difference

Table 6-3 Effect of 2 h intralipid infusion on platelet expression of fibrinogen binding receptors

Percentage of Platelets expressed fibrinogen binding						
	Controls (n=12)			PCOS (n=13)		
agents that added	2-h saline	2-h intralipid	*P=	2-h saline	2-h intralipid	*P=
Basal	2.30 (1.93, 2.55)	2.50 (2.34, 2.89)	0.07	2.20 (2.10, 2.78)	2.35 (2.20, 2.65)	0.41
+0.1μM ADP	4.30 (3.06, 8.10)	8.30 (4.44, 12.8)	0.01	5.30 (3.00, 6.58)	8.70 (4.95, 14.0)	0.01
+1 μM ADP	57.9 (46.7, 68.0)	78.7 (67.9, 82.3)	0.01	51.4 (44.4, 61.8)	71.8 (58.7, 81.0)	0.01
+10 μM ADP	78.5 (73.9, 84.2)	88.9 (82.8, 91.8)	0.14	76.7 (72.1, 83.5)	85.0 (78.6, 90.4)	0.05
+0.01 μM PGI_2+1μM ADP	11.6 (8.44, 26.0)	67.6 (39.5, 83.8)	<0.001	8.40 (4.90, 13.0)	34.9 (17.1, 50.9)	<0.001
+0.05 μM PGI_2+1μM ADP	2.50 (1.90, 3.42)	20.9 (4.45, 36.3)	<0.001	2.45 (2.08, 2.95)	3.30 (2.75, 6.48)	0.01
+0.1 μM PGI_2+1μM ADP	2.30 (1.88, 3.38)	5.00 (3.30, 15.3)	0.01	2.20 (1.98, 2.75)	2.80 (2.38, 4.73)	0.04
+50 μM 8CPT+1μMADP	46.4 (26.7, 58.6)	68.5 (58.6, 72.8)	<0.001	35.5 (29.0, 48.5)	50.2 (43.4, 67.0)	<0.001
+100 μM 8CPT+1μMADP	31.6 (19.1, 44.8)	40.9 (31.5, 52.6)	<0.001	28.0 (16.0, 30.7)	35.0 (23.8, 44.1)	0.05
+200 μM 8CPT+1μMADP	16.0 (10.3, 21.6)	14.2 (11.2, 26.8)	0.01	10.7 (7.55, 13.9)	11.1 (6.38, 16.0)	0.35

Results are expressed as medians (25th and 75th centiles), *p value < 0.05 is significance of difference. 8CPT=8-CPT-6-Phe-cAMP

Table 6-4 Effect of 5 h intralipid infusion on platelet expression of fibrinogen binding receptors

Percentage of Platelets expressed fibrinogen binding						
	Controls (n=12)			PCOS (n=13)		
agents that added	5-h saline	5-h intralipid	*P=	5-h saline	5-h intralipid	*P=
Basal	2.35 (1.56, 2.60)	2.53 (2.08, 2.96)	0.46	2.45 (1.98, 268)	2.35 (2.18, 2.80)	0.16
+0.1 μM ADP	3.95 (3.20, 6.79)	6.50 (3.88, 13.9)	0.02	3.90 (3.15, 6.58)	7.30 (5.78. 11.8)	<0.001
+1 μM ADP	45.8 (38.2, 62.4)	62.8 (51.8, 73.3)	0.01	53.3 (46.3, 64.6)	66.5 (56.3, 74.3)	0.02
+10 μM ADP	76.7 (59.5, 84.6)	86.7 (75.1, 88.4)	0.01	78.2 (69.4, 84.2)	81.0 (74.7, 89.0)	0.05
+0.01 μM PGI_2+1 μM ADP	8.18 (6.19, 34.0)	40.9 (23.8, 60.9)	<0.001	12.0 (9.00, 16.2)	31.8 (21.4, 45.4)	<0.001
+0.05 μM PGI_2+1 μM ADP	2.08 (1.48, 4.76)	6.78 (3.06, 26.8)	<0.001	2.85 (2.48, 3.35)	4.10 (2.50, 6.10)	0.01
+0.1 μM PGI_2+1 μM ADP	2.43 (1.91, 3.68)	3.30 (2.44, 7.34)	<0.001	2.40 (2.18, 2.63)	3.05 (2.25, 3.73)	0.01
+50 μM 8CPT+1 μMADP	42.0 (24.2, 50.7)	54.0 (46.3, 67.1)	0.01	32.6 (27.3, 46.8)	55.2 (48.5, 59.5)	<0.001
+100 μM 8CPT+1 μMADP	25.9 (16.6, 37.5)	38.2 (29.6, 49.5)	0.04	17.8 (16.3, 28,8)	34.3 (31.2, 49.1)	<0.001
+200 μM 8CPT+1 μMADP	12.9 (6.08, 18.6)	16.7 (10.8, 31.0)	0.04	9.90 (7.90, 11.5)	12.5 (7.95, 17.1)	0.02

Results are expressed as medians (25th and 75th centiles), *p value < 0.05 is significance of difference. 8CPT=8-CPT-6-Phe-cAMP

Table 6-5 Effect of 2 h intralipid infusion on platelet expression of P selectin receptors

Percentage of Platelets expressed P selectin						
	Controls (n=12)			PCOS (n=13)		
agents that added	2-h saline	2-h intralipid	*P=	2-h saline	2-h intralipid	*P=
Basal	1.98 (1.03,2.90)	1.90 (1.65, 2.61)	0.75	1.85 (1.28, 4.18)	1.70 (2.50, 2.18)	0.2
+0.1μM ADP	2.80 (1.76, 5.21)	4.55 (2.35, 7.99)	0.07	3.80 (1.88, 5.78)	4.00 (2.84, 7.05)	0.13
+1 μM ADP	30.5 (25.2, 47.2)	54.1 (31.5, 63.4)	0.02	36.2 (23.9, 47.4)	44.9 (35.5, 54.6)	0.02
+10 μM ADP	53.8 (45.1, 69.7)	68.1 (45.5, 75.8)	0.18	61.6 (42.8, 67.5)	59.6 (50.2, 70.5)	0.75
+0.01 μM PGI_2+1 μM ADP	13.4 (8.35, 20.9)	49.6 (26.4, 65.8)	<0.001	10.6 (8.23, 13.1)	28.3 (13.6, 37.1)	<0.001
+0.05 μM PGI_2+1 μM ADP	5.58 (3.24, 7.71)	15.6 (3.15, 23.6)	0.03	6.40 (4.00, 8.35)	5.95 (4.00, 11.2)	0.38
+0.1 μM PGI_2+1 μM ADP	3.83 (3.45, 6.99)	5.60 (3.15, 10.3)	0.48	6.35 (3.83, 7.60)	4.28 (3.48, 7.10)	0.7
+50 μM 8CPT+1 μMADP	30.6 (16.5, 41.0)	45.8 (27.5, 55.9)	0.01	24.4 (15.3, 36.7)	27.9 (20.0, 48.5)	0.05
+100 μM 8CPT+1 μMADP	22.3 (13.3, 31.4)	27.9 (18.5, 35.8)	0.05	28.9 (7.65, 28.7)	23.1 (12.5, 27.1)	0.15
+200 μM 8CPT+1 μMADP	9.08 (4.76, 13.2)	8.65 (7.40, 22.2)	0.03	8.85 (2.85, 10.2)	7.45 (3.60, 11.9)	0.65

Results are expressed as medians (25th and 75th centiles), *p value < 0.05 is significance of difference. 8CPT=8-CPT-6-Phe-cAMP

Table 6-6 Effect of 5 h intralipid infusion on platelet expression of P selectin receptors

	Percentage of Platelets expressed P selectin					
	Controls (n=12)			PCOS (n=13)		
agents that added	5-h saline	5-h intralipid	*P=	5-h saline	5-h intralipid	*P=
Basal	2.35 (1.66, 3.01)	2.48 (1.95, 3.29)	0.31	3.40 (1.58, 4.15)	2.40 (1.78, 3.40)	0.53
+0.1 μM ADP	2.93 (2.68, 3.70)	5.82 (3.30, 7.45)	<0.001	4.30 (3.18, 5.45)	5.55 (4.28, 7.78)	0.05
+1 μM ADP	31.1 (21.7, 47.9)	41.3 (28.0, 46.8)	0.05	30.3 (27.1, 44.8)	44.4 (37.4, 60.1)	0.01
+10 μM ADP	55.2 (40.8, 70.0)	57.3 (48.4, 66.4)	0.51	55.7 (47.3, 61.3)	62.2 (53.5, 74.7)	0.05
+0.01 μM PGI_2+1 μM ADP	10.0 (6.88, 29.3)	30.3 (12.5, 46.0)	0.02	13.0 (10.8, 14.2)	22.3 (18.7, 40.0)	0.01
+0.05 μM PGI_2+1 μM ADP	5.88 (3.11, 7.36)	8.70 (3.65, 22.5)	0.05	5.95 (4.28, 7.53)	7.70 (5.10, 12.7)	0.12
+0.1 μM PGI_2+1 μM ADP	5.00 (3.13, 6.70)	6.98 (3.76, 13.6)	0.03	5.90)3.63, 6.40)	6.40 (4.80, 7.70)	0.12
+50 μM 8CPT+1 μMADP	25.3 (18.2, 41.1)	35.4 (27.9, 41.0)	0.06	22.7 (18.4, 34.8)	36.7 (29.2, 45.0)	<0.001
+100 μM 8CPT+1 μMADP	17.3 (11.8, 27.3)	24.6 (18.6, 33.7)	0.03	14.3 (11.8, 20.2)	22.4 (19.2, 33.8)	<0.001
+200 μM 8CPT+1 μMADP	7.68 (5.60. 10.2)	11.6 (6.73, 19.7)	0.02	6.70 (5.43, 10.7)	9.95 (5.73, 16.1)	0.04

Results are expressed as medians (25th and 75th centiles), *p value < 0.05 is significance of difference, 8CPT=8-CPT-6-Phe-cAMP

Table 6-7 Effect of exogenous insulin on platelet expression of fibrinogen binding receptors

	Percentage of Platelets expressed fibrinogen binding					
	Controls (n=12)			PCOS (n=13)		
agents that added	2-h intralipid	5-h intralipid	*P=	2-h intralipid	5-h intralipid	*P=
Basal	2.50 (2.34, 2.89)	2.53 (2.08, 2.96)	0.98	2.35 (2.20, 2.65)	2.35 (2.18, 2.80)	0.92
+0.1 μM ADP	8.30 (4.44, 12.8)	6.50 (3.88, 13.9)	0.25	8.70 (4.95, 14.0)	7.30 (5.78. 11.8)	0.22
+1 μM ADP	78.7 (67.9, 82.3)	62.8 (51.8, 73.3)	0.02	71.8 (58.7, 81.0)	66.5 (56.3, 74.3)	0.17
+10 μM ADP	88.9 (82.8, 91.8)	86.7 (75.1, 88.4)	0.18	85.0 (78.6, 90.4)	81.0 (74.7, 89.0)	0.25
+0.01 μM PGI_2+1 μM ADP	67.6 (39.5, 83.8)	40.9 (23.8, 60.9)	0.01	34.9 (17.1, 50.9)	31.8 (21.4, 45.4)	0.38
+0.05 μM PGI_2+1 μM ADP	20.9 (4.45, 36.3)	6.78 (3.06, 26.8)	0.05	3.30 (2.75, 6.48)	4.10 (2.50, 6.10)	0.4
+0.1 μM PGI_2+1 μM ADP	5.00 (3.30, 15.3)	3.30 (2.44, 7.34)	0.08	2.80 (2.38, 4.73)	3.05 (2.25, 3.73)	0.33
+50 μM 8CPT+1 μMADP	68.5 (58.6, 72.8)	54.0 (46.3, 67.1)	0.02	50.2 (43.4, 67.0)	55.2 (48.5, 59.5)	0.75
+100 μM 8CPT+1 μMADP	40.9 (31.5, 52.6)	38.2 (29.6, 49.5)	0.16	35.0 (23.8, 44.1)	34.3 (31.2, 49.1)	0.14
+200 μM 8CPT+1 μMADP	14.2 (11.2, 26.8)	16.7 (10.8, 31.0)	0.13	11.1 (6.38, 16.0)	12.5 (7.95, 17.1)	0.51

Results are expressed as medians (25th and 75th centiles), *p value<0.05 is significance of difference, 8CPT=8-CPT-6-Phe-cAMP

Table 6-8 Effect of exogenous insulin on platelet expression of P selectin receptors

Percentage of Platelets expressed P selectin						
	Controls (n=12)			PCOS (n=13)		
agents that added	2-h intralipid	5-h intralipid	*P=	2-h intralipid	5-h intralipid	*P=
Basal	1.90 (1.65, 2.61)	2.48 (1.95, 3.29)	0.11	1.70 (2.50, 2.18)	2.40 (1.78, 3.40)	0.13
+0.1 μM ADP	4.55 (2.35, 7.99)	5.82 (3.30, 7.45)	0.64	4.00 (2.84, 7.05)	5.55 (4.28, 7.78)	0.15
+1 μM ADP	54.1 (31.5, 63.4)	41.3 (28.0, 46.8)	0.04	44.9 (35.5, 54.6)	44.4 (37.4, 60.1)	0.97
+10 μM ADP	68.1 (45.5, 75.8)	57.3 (48.4, 66.4)	0.31	59.6 (50.2, 70.5)	62.2 (53.5, 74.7)	0.81
+0.01 μM PGI_2+1 μM ADP	49.6 (26.4, 65.8)	30.3 (12.5, 46.0)	0.01	28.3 (13.6, 37.1)	22.3 (18.7, 40.0)	0.7
+0.05 μM PGI_2+1 μM ADP	15.6 (3.15, 23.6)	8.70 (3.65, 22.5)	0.31	5.95 (4.00, 11.2)	7.70 (5.10, 12.7)	0.51
+0.1 μM PGI_2+1 μM ADP	5.60 (3.15, 10.3)	6.98 (3.76, 13.6)	0.59	4.28 (3.48, 7.10)	6.40 (4.80, 7.70)	0.22
+50 μM 8CPT+1 μMADP	45.8 (27.5, 55.9)	35.4 (27.9, 41.0)	0.02	27.9 (20.0, 48.5)	36.7 (29.2, 45.0)	0.13
+100 μM 8CPT+1 μMADP	27.9 (18.5, 35.8)	24.6 (18.6, 33.7)	0.06	23.1 (12.5, 27.1)	22.4 (19.2, 33.8)	0.35
+200 μM 8CPT+1 μMADP	8.65 (7.40, 32.2)	11.6 (6.73, 19.7)	0.18	7.45 (3.60, 11.9)	9.95 (5.73, 16.1)	0.15

Results are expressed as medians (25th and 75th centiles), *p value <0.05 is significance of difference, 8CPT=8-CPT-6-Phe-cAMP

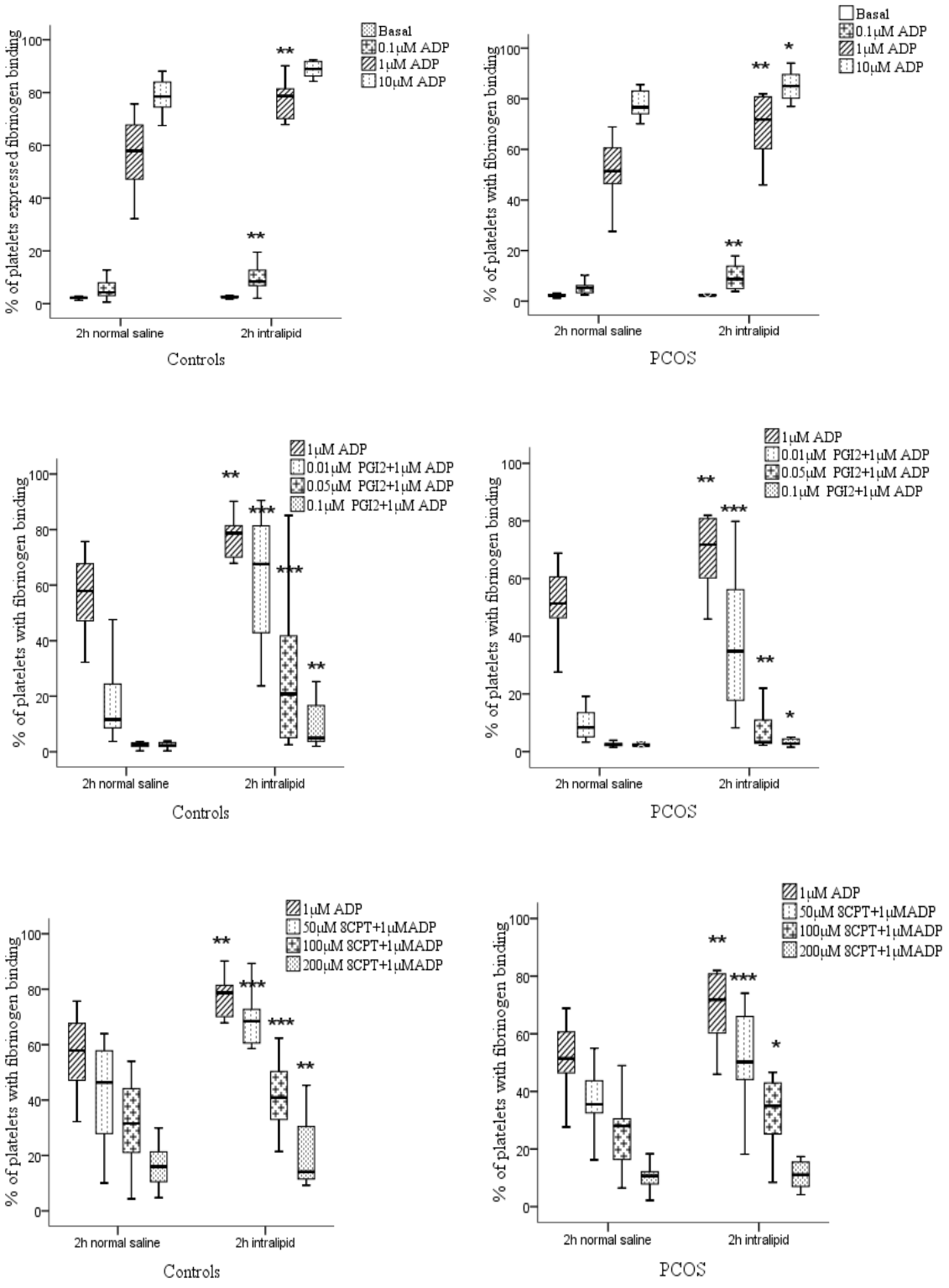


Figure 6-5 Platelet expression of fibrinogen binding receptors with 2 h of either saline or intralipid infusion

Platelet response to ADP, ADP+PGI_2, ADP + 8-CPT-6-Phe-cAMP, in controls (left panels) and PCOS patients (right panels). The data are expressed as percentage positive cells for fluorescence and represent median ± interquartile range (box) and range (whisker). Asterisk (*p <0.05, ** p<0.01, *** p<0.001) compared effect of saline to intralipid within the groups

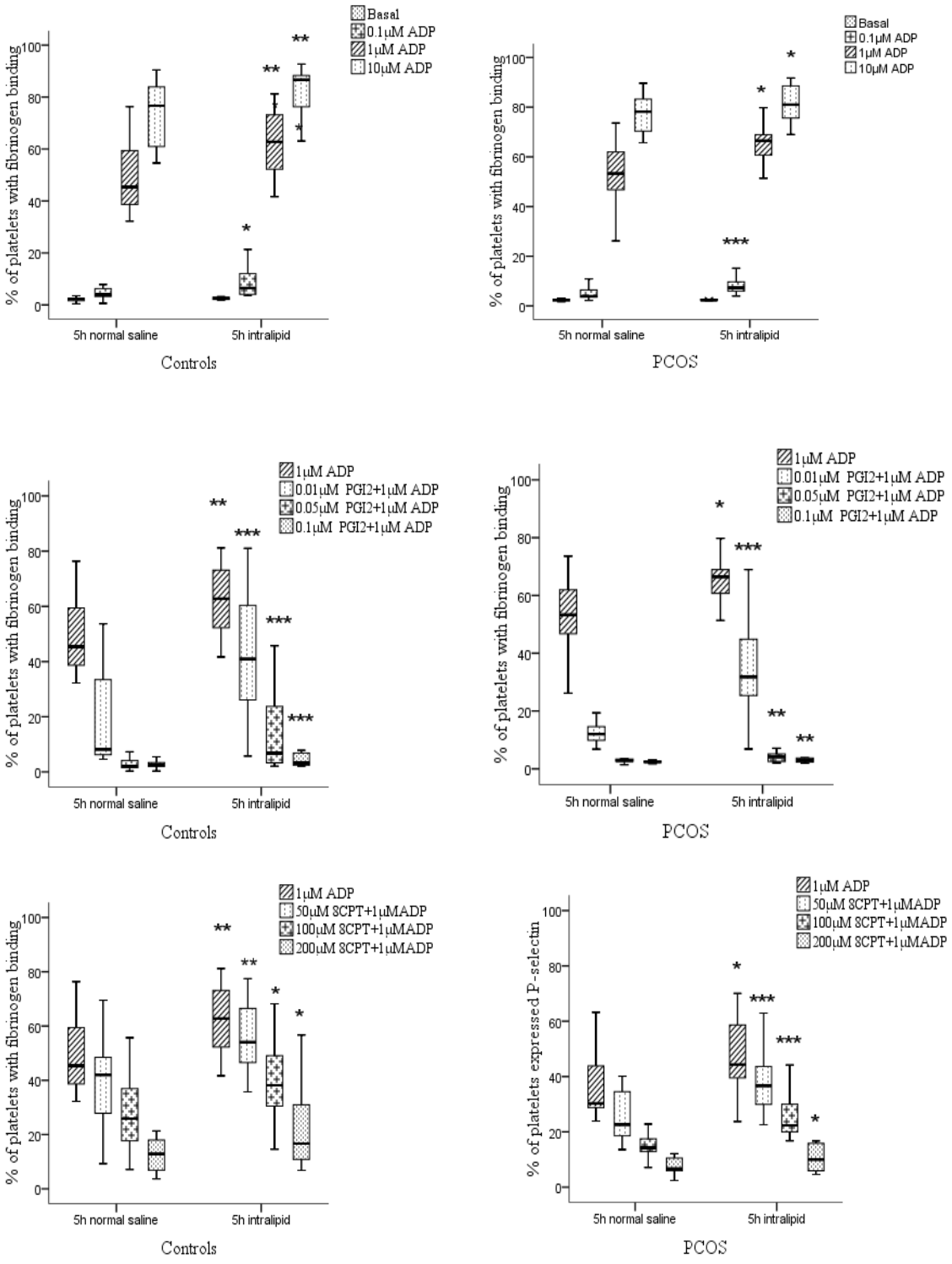


Figure 6-6 Platelet expression of fibrinogen binding receptors with hyperinsulinaemic clamp and either saline or intralipid infusion

Platelet response to ADP, ADP+PGI_2, ADP + 8-CPT-6-Phe-cAMP, in controls (left panels) and PCOS patients (right panels). The data are expressed as percentage positive cells for fluorescence and represent median ± interquartile range (box) and range (whisker). Asterisk (*p <0.05, ** p<0.01, *** p<0.001) compared effect of saline to intralipid within the groups.

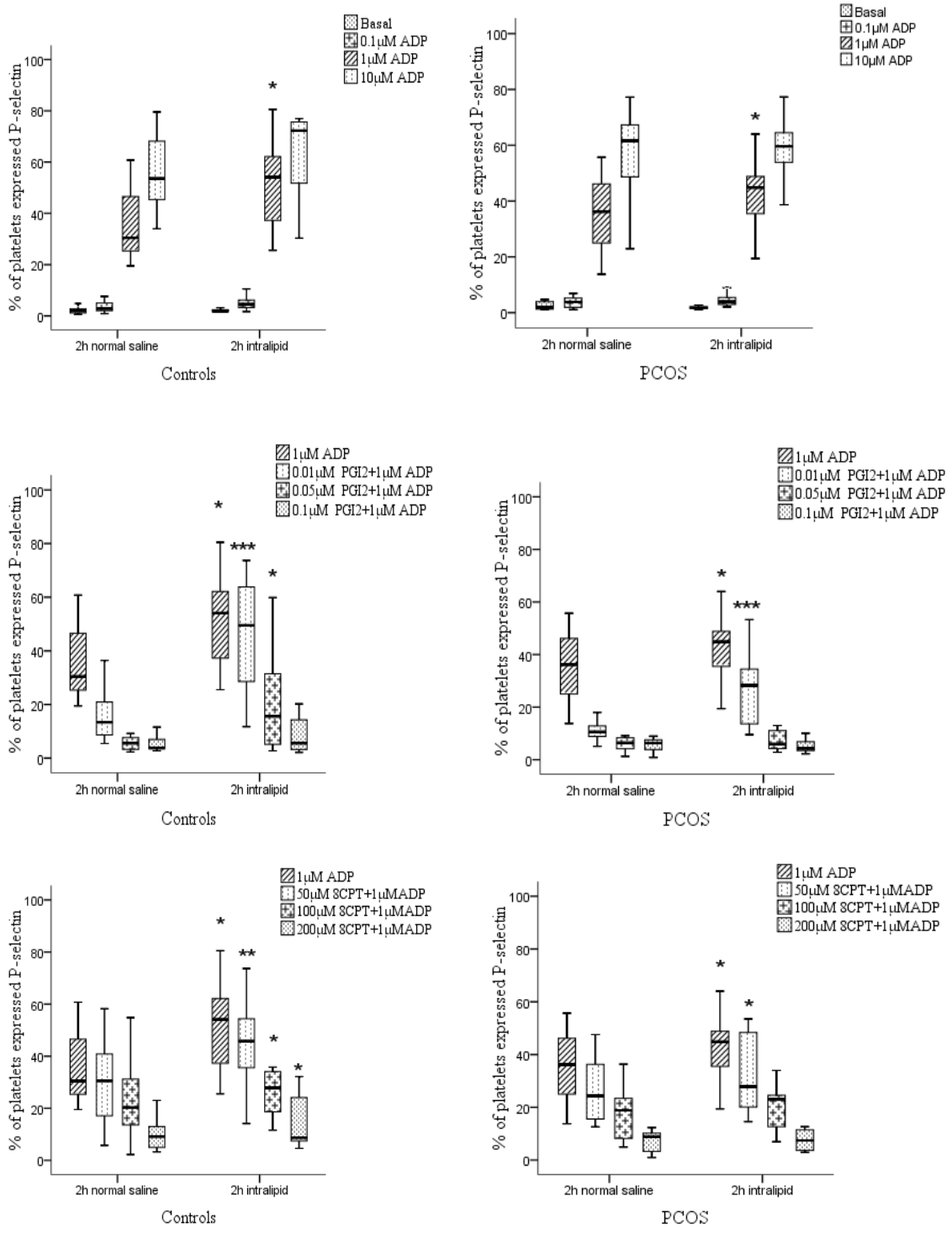


Figure 6-7 Platelet expression of P selectin receptors with 2 h either saline or intralipid infusion

Platelet response to ADP, ADP+PGI_2, ADP + 8-CPT-6-Phe-cAMP in controls (left panels) and PCOS patients (right panels), The data are expressed as percentage positive cells for fluorescence and represent median ± interquartile range (box) and range (whisker). Asterisk (*p <0.05, ** p<0.01, *** p<0.001) compared effect of saline to intralipid within the groups.

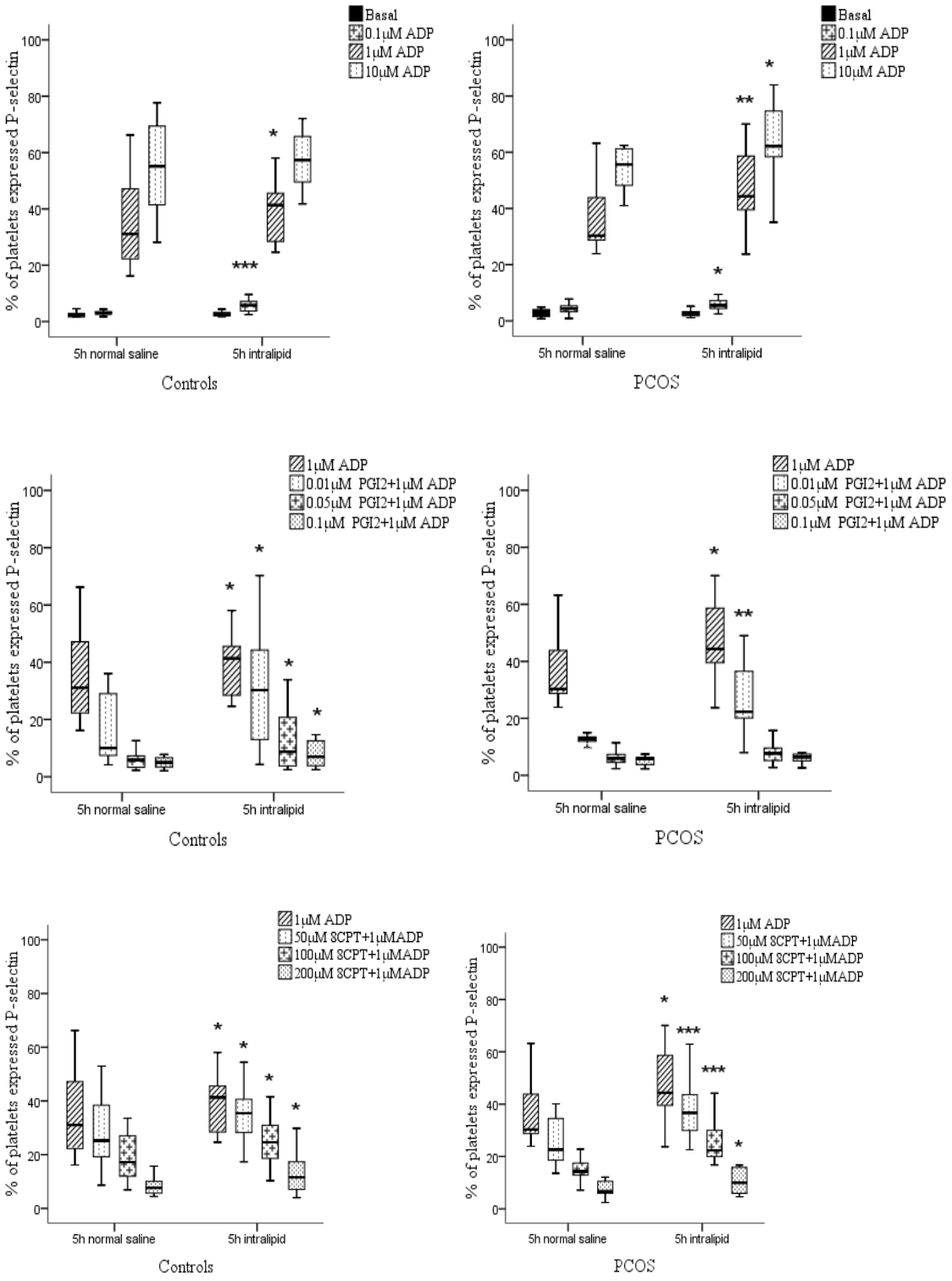


Figure 6-8 Platelet expression of P selectin receptors during either saline or intralipid infusion with hyperinsulinaemic euglycaemic clamp

Platelet response to ADP, ADP+PGI_2, ADP + 8-CPT-6-Phe-cAMP, in controls (left panels) and PCOS patients (right panels). The data are expressed as percentage positive cells for fluorescence and represent median ± interquartile range (box) and range (whisker). Asterisk (*p <0.05, ** p<0.01, *** p<0.001) compared effect of saline to intralipid within the groups.

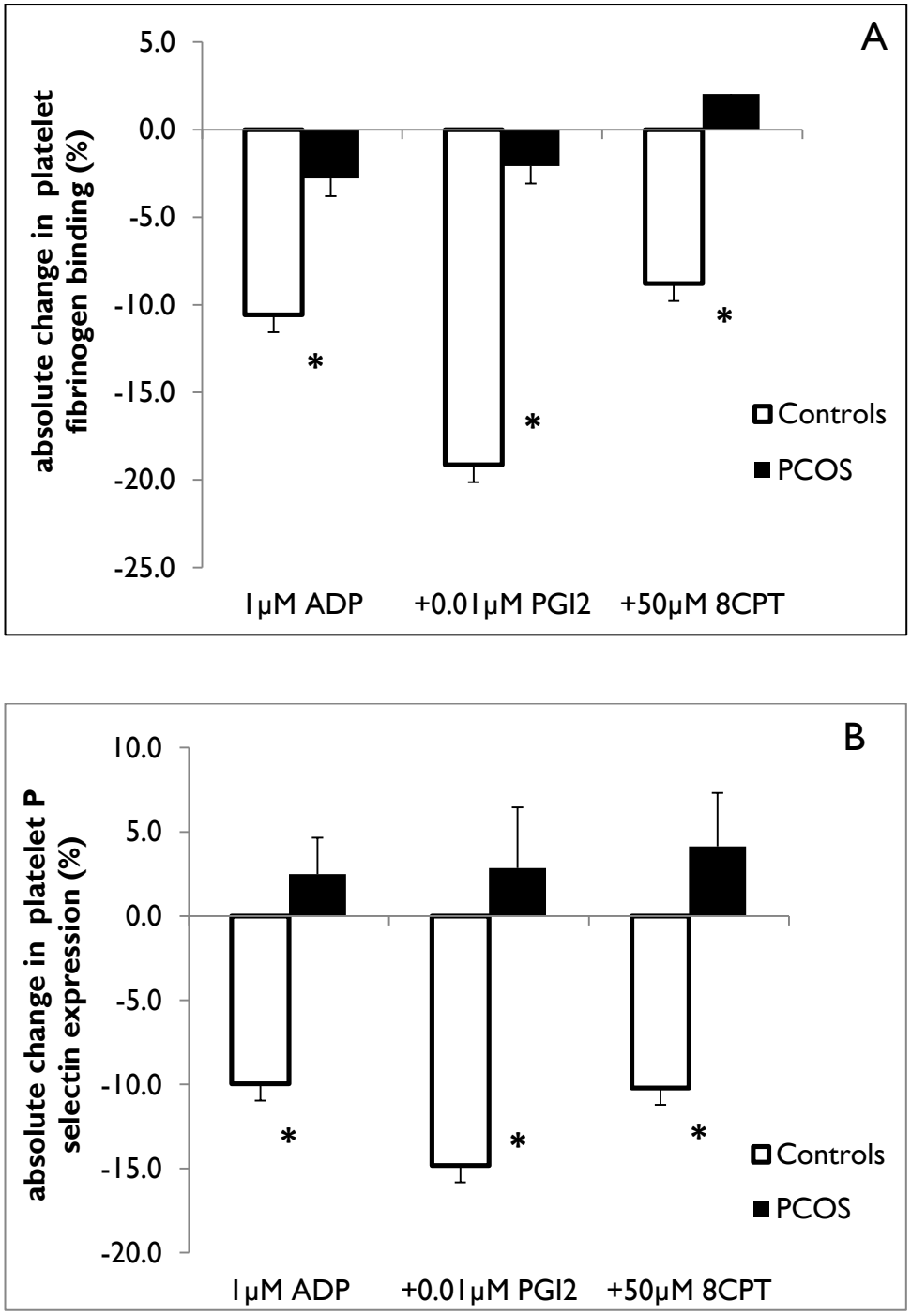


Figure 6-9 Comparison of effect of insulin on platelet fibrinogen binding receptor (A) and P selectin (B) expression during intralipid infusion

(Absolute change is calculated by deduction of % platelet activation before and after hyperinsulinaemic clamp, data shown as mean ± SEM and compared by independent sample t test between the groups; *$p < 0.05$)

6.3.2.1 Platelet activation in physiological milieu

During the physiological saline infusion, platelet fibrinogen binding and P selectin expression was at baseline, in response to ADP, ADP with PGI_2, or ADP with 8-CPT-6-Phe-cAMP and comparable between PCOS and controls (Table 6-3, Table 6-5).

6.3.2.2 Platelet activation during intralipid infusion

The lipid infusion had no effect on basal levels of fibrinogen binding in either group. Platelets were stimulated with ADP to examine if lipid infusion sensitised platelets to activation. The propensity for platelet activation was increased in both groups following lipid infusion (Table 6-3, Figure 6-5). For example, in the control group, ADP (1 μM) increased fibrinogen binding to 57.9% (46.7, 68) after saline infusion for 2 h, but this was 78.7% (67.9, 82.3), p=0.01) after lipid infusion for 2 h. Similarly, in PCOS, fibrinogen binding in response to ADP was increased from 51.4% (44.4, 61.8) to 71.8% (58.7, 81; p=0.01) after lipid infusion. A similar pattern was obtained using P selectin as an alternative marker of activation, ADP-induced P-selectin expression increased from 30.5% (25, 47) to 54.1% (31.5, 63.4) in the control group and from 36.2 (23.9, 47.4) to 44.9 (36, 55; $p<0.05$) in PCOS by lipid infusion (Table 6-5, Figure 6-7). These data suggested that platelet activation was increased by lipid infusion as shown by increased fibrinogen binding and P selectin expression in response to ADP.

To examine if this was due to increased sensitivity to agonists or diminished responsiveness to endogenous inhibitors, the influence of PGI_2 on platelet function was investigated. The effect of PGI_2 (0.01 - 0.1 μM) on ADP (1 μM) stimulated fibrinogen binding was assessed. PGI_2 caused a concentration-dependent inhibition of fibrinogen binding following 2 h of saline infusion in both groups. However, the ability of PGI_2 to inhibit platelet fibrinogen binding was diminished after infusion of intralipid for 2 h. To ensure that this effect was not only an unselective effect of PGI_2, the experiments were repeated using the cell permeable cAMP analogue, 8-CPT-6-Phe-cAMP, as a direct activator of PKA. The cAMP analogue, 8-CPT-6-Phe-cAMP (50 μM), was able to inhibit ADP-induced fibrinogen binding in both control and PCOS groups (Table 6-3, Figure 6-5).

However, the inhibitory effects were less potent after infusion of intralipid rather than saline. Consistent with these data, this study found that the ability of both PGI_2 and 8-CPT-6-Phe-cAMP to inhibit P-selectin expression was attenuated after lipid infusion (Table 6-5, Figure 6-7).

The intralipid infusion induced an increase in platelet response to ADP but a decrease in platelet sensitivity to PGI_2 and 8-CPT-6-Phe-cAMP 2 h after the commencement of infusion. The effect was maintained towards the end of the infusion at 5 h (Table 6-5).

6.3.2.3 Effect of insulin infusion on platelet activation

The hyperinsulinaemic euglycaemic clamp was applied for the last 2 h of both saline and intralipid infusion to assess the effect of supra-physiological levels of insulin on platelet activity whilst maintaining plasma glucose levels at 5 mmol/L in both controls and PCOS.

In controls, infusing a supra-physiological dose of insulin did not significantly alter platelet activation/inhibition during the physiological saline infusion. It is likely that platelet homeostasis is maximally optimized in the physiological milieu. However, the insulin infusion decreased platelet fibrinogen binding in response to 1 μM ADP from 78.8% (67.9, 82.3) to 62.8% (51.8, 73.3; p=0.02), in response to 0.01 μM PGI_2+1 μM ADP from 67.6% (39.5, 83.8) to 40.9% (23.8, 60.9; p=0.01) and to 50 μM 8-CPT-6-Phe-cAMP +1 μM ADP from 68.5% (58.6, 72.8) to 54.0% (46.3, 67.1; p=0.02) during the lipid infusion (figure 6-6, figure 6-8).

In contrast to controls, a similar amount of insulin during intralipid infusion made no improvement to platelet fibrinogen binding 71.8% (58.7, 81) to 68.5% (56.3, 74.3; p=0.17) or P selectin expression 44.9% (35.5, 54.6) to 44.4% (37.4, 60.1; p=0.97) in response to ADP in PCOS (figure 6-6, figure 6-8). Similarly, no improvement was found in platelet response to PGI_2 and 8-CPT-6-Phe-cAMP (Table 6-4). When comparing absolute reductions in platelet activation in response to ADP alone or with either PGI_2 or 8-CPT-6-Phe-cAMP after infusion of supra-physiological doses of insulin, there was significantly lower platelet activation in the controls than the PCOS group (Figure 6-9). Therefore, PCOS platelets appeared resistant to insulin and remained in the increased and activated state throughout the intralipid infusion.

6.4 Discussion

In the present study intralipid infusion induced a significant reduction in the rate of glucose disposal i.e. whole body glucose utilization following an acute rise in TG and NEFA levels in both PCOS and controls. This was in accord with other studies in which intralipid was well recognized to induce insulin resistance by reduced insulin-stimulated glucose oxidation, an increase of insulin inhibited lipid oxidation (349) and hepatic glucose production (357).

6.4.1 Effect of acute hypertriglyceridaemia on platelet activation

The infusion of intralipid had no effect on baseline unstimulated platelet fibrinogen binding or P selectin expression in either group, suggesting that increased plasma lipid per se did not activate platelets *in vivo*. Indeed, De Man et al. reported that there were no differences in baseline platelet expression of P-selectin and fibrinogen binding in patients with primary hypertriglyceridaemia when compared to age and sex matched controls (476). Therefore, both acute and chronic rise of TG do not appear to induce platelet activation in the resting state.

In contrast to these baseline findings, we have found that platelets have a greater propensity for activation when challenged with ADP and infused with intralipid. This is accompanied by a reduced sensitivity to the key physiological regulator of platelets, $PGI_{2.}$ Interestingly, these changes in platelet function were observed in both PCOS and control subjects. This could suggest that acute lipaemia primes platelets for activation by a two-pronged mechanism; firstly, platelets are more readily activated when confronted by activating stimuli, and secondly, platelets have reduced sensitivity to inhibitory stimuli. In combination, these two mechanisms could exaggerate platelet responsiveness once activated, contributing to the pro-coagulant phenotype in dyslipidaemia. The mechanism by which acute hypertriglyceridaemia influences sensitivity to ADP and PGI_2, or whether the effects represent separate mechanisms is unclear. However, this study proves for the first time that there may be an unreported dimension to this platelet hyperactivity, that is, increased sensitivity to agonists is accompanied by a hyposensitivity to cAMP-mediated inhibition.

This platelet increased responsiveness to ADP and decreased responsiveness to PGI_2 may well lead to atherothrombosis in the presence of endothelial dysfunction since platelet activity can be switched on or triggered by high or low shear stress even in the absence of discontinuity in endothelial surface (464, 716) or endothelial denudation (464).

The effect of either hypertriglyceridaemia or atherogenic dyslipidaemia on platelet function was not consistent in previous studies. Patients with familial hypertriglyceridaemia have a lower threshold for aggregation in response to ADP and collagen compared to normal controls (475). Another study reported no difference in platelet reactivity between controls and hypertriglyceridaemic subjects (476). Some studies provide indirect evidence that high TG might affect platelet function and subsequent CV risk (717). Induction of hyperlipidaemia, IR and obesity in Ossabaw miniature swine using a high fat, high calorie diet was associated with increased reactivity of platelets to ADP and collagen (713). In other animal experiments, intralipid infusion enhanced ADP-stimulated platelet aggregation, and induced early atherosclerosis in the aorta of laboratory rats (718, 719). A rise of TG by intralipid infusion also impaired fibrinolysis supported by an increased level of endothelial derived plasminogen activation inhibitor (717).

A postprandial rise in TG appears to decrease endothelial function as evidenced by a reduction in flow mediated dilatation (720), increased plasma endothelial derived micro-particles levels (721) and increased endothelial activation markers sICAM-1, soluble E-selectin and vWF in healthy subjects and diabetics (722, 723). This increased expression of endothelial surface makers and the down regulation of NO and PGI_2 due to endothelial dysfunction promote platelet activation and result in the progression of atherosclerosis in denuded endothelium (464). Lowering TG (by omega3 acid ethyl esters) in patients with coronary artery disease has been shown to decrease platelet reactivity to ADP (724). Lowering TG by fenofibrate in patients with combined dyslipidaemia, decreased sCD40L (soluble CD40 ligand), and improved endothelium-dependent flow mediated vasodilatation (725). Therefore, the presence of high TG in the circulation could act as a driver of atherosclerosis and atherothrombosis though platelet activation in PCOS and controls.

6.4.2 Effect of PCOS status on platelet activation

Dereli et al. found that PCOS women showed increased platelet aggregation in response to ADP compared to controls (83). In the present study, such difference in platelet activation during physiological saline infusion between PCOS and controls were not seen. As an explanation for this discrepancy the effect of small sample size was considered but a trend of difference between the groups was not detected. Therefore, it is more likely to be due to compensation in chronic hyperinsulinaemia in PCOS maintaining platelet homeostasis at the baseline physiological state.

The present study showed for the first time that the infusion of insulin during a hyperlipidaemic state is able to rectify changes in platelet reactivity. However, this protective effect of insulin was only observed in control subjects, demonstrating that PCOS platelets showed insulin resistance in the presence of intralipid. In controls, insulin attenuated platelet responsiveness to ADP and enhanced the response to PGI_2, but a similar amount of insulin made no improvement in PCOS and resulted in prolonged lipid induced platelet activation.

IR is recognised as an independent CV risk in both the general population (690) and patients with T2DM (691). Previous observations report that insulin may inhibit platelet activation (487), enhance platelet activation (726) or have a differential effect depending upon concentration (488). Insulin decreased platelet sensitivity to agonists and enhanced platelet sensitivity to PGI_2 in healthy subjects (727). In IR states, such as T2DM, platelets are less sensitive to PGI_2 (728) *in vivo* and more responsive to ADP in *in vitro* studies than those in insulin sensitive subjects. The findings in the present study suggest platelet insulin resistance in PCOS at hyperlipidaemic milieu.

Whilst reduced platelet sensitivity to PGI_2 in PCOS was demonstrated in the present study, platelet inhibition through direct activation of PKA using the cell permeable activator, 8-CPT-6-Phe-cAMP, was also shown to be compromised in PCOS compared to controls. The mechanisms underlying compromised PKA activity in PCOS are unclear and require further investigation but suggest that IR associated with acute lipidaemia in the context of PCOS, suppresses activity of the whole cAMP signalling pathway. In addition, the diminished platelet response to PGI_2 is may be due to a direct effect of lipid on the platelet rather than high lipid interference with PGI_2

receptors on the platelet surface. Insulin resistance in PCOS appeared to deprive the apparent cardioprotective effects of insulin on platelet activation during acute lipaemia that has been seen in controls.

6.5 Study limitation

Majority of women with PCOS were obese (54). In the present study, women with PCOS were slightly more overweight than controls. However, this chronic obesity did not appear to be related to increased platelet activation in this PCOS group as there was no difference in platelet activation at basal state i.e. during saline infusion between PCOS and control groups.

The strength of the study lies in the use of flowcytometric analysis with whole blood for platelet function assessment. While all ex-vivo studies of platelet function are prone to artefactual platelet activation, whole blood flowcytometric analysis has the advantage that it examines individual platelets directly with a high degree of sensitivity in their native environment and with minimal sample manipulation or consequent artefactual platelet activation (547, 729).

6.6 Summary

In summary, the findings in the present study suggested that acute metabolic derangement with hypertriglyceridaemia and acute insulin resistance induced by lipid infusion, enhanced and prolonged platelet activation in both PCOS and controls. In contrast to controls, women with PCOS failed to improve lipid induced platelet activation by insulin infusion. Thus, PCOS platelets were more resistant to insulin as shown by prolonged and enhanced activation despite supra-physiological doses of insulin compared to controls. Therefore, hypertriglyceridaemia and worsening insulin resistance in PCOS may well increase cardiothrombotic risk by increased platelet activation.

Chapter 7 Summary and future direction

7.1 Summary

PCOS is one of the commonest endocrine disorders of reproductive women. Insulin resistance is thought to play a pathogenic role in PCOS, particularly in women who are obese (12). Increasing incidence of obesity in PCOS parallels with obesity epidemic (54), with the latter itself associated with the development of IR and dyslipidaemia (562). Obesity could be associated with increased delivery of FFA to non-adipose tissue either due to failure of suppression of HSL in postprandial period by insulin (330, 408, 563) or decreased trapping of NEFA released from hydrolyzed chylomicrons by adipose tissue. The increased uptake of fatty acids with decreased fatty acids oxidation could lead to accumulation of intramyocellular lipid metabolites such as DAG, LCFA acyl CoA and ceramides. Those can interfere with insulin signalling and glucose uptake in skeletal muscle (409). Whilst this is well recognized, the magnitude of the effect of NEFA on IR varies between individuals and patient groups (343). In addition, muscles from PCOS women showed insulin signalling defect in *in vitro* studies (730). Women with PCOS are at risk of metabolic dyslipidaemia and type 2 diabetes. Long term studies of cardiovascular risk in women with PCOS are conflicting with some showing increased carotid intima-media thickness predicting enhanced cardiovascular risk (86), but others showing no long term detriment (99, 731).

In this research work, acute lowering of NEFA by overnight acipimox improved fasting and postprandial IR suggesting a significant contribution of NEFA to IR in PCOS. It also affirms the necessity of high NEFA to impair insulin-mediated glucose transport in skeletal muscle of PCOS found in previous *in vitro* studies (143). This may well be translated as acute weight gain could impose worsening of IR in PCOS. Reduction of fasting NEFA was accompanied by a decrease in hepatic TG synthesis evidenced by reduction in fasting TG and reduction in whole body lipid oxidation. Reduction in fasting TG was associated with improved postprandial hypertriglyceridaemia suggesting postprandial hypertriglyceridaemia in PCOS is probably due to competition of endogenous VLDL to chylomicrons for LPL hydrolysis. The importance of NEFA in pathogenesis of IR in PCOS was further confirmed by the niacin study. Niacin/laropiprant, which mode of action is to suppress HSL activity via nicotinic

receptors in adipose tissue, failed to suppress fasting and postprandial NEFA. As a result, the drug did significantly increase postprandial glucose through an increase in IR and had a detrimental effect on beta cell function which was evidenced by a reduction in oral disposition index. This proposes that the beta cells adaptation failed to respond adequately when the insulin requirement is particularly high, such as during the postprandial period. Furthermore, niacin/laropiprant did not improve postprandial TG excursions in response to a mixed meal.

Both metabolic dysfunctions seem related to rebound rise in fasting and postprandial NEFA. Despite the reduction in fasting TG and HDL-c with niacin therapy, there was no improvement in RHI and HsCRP. These findings add to the understanding of the effect of the rebound rise in NEFA on IR in PCOS and its impact on CV risk modification.

To understand the effect of acute fat load on IR in PCOS, the rate of glucose disposal during hyperinsulinaemic euglycaemic clamp was measured whist giving intralipid infusion. Acutely induced hypertriglyceridaemia resulted in an exaggerated fall in rate of glucose disposal in PCOS compared to controls. It suggests women with PCOS are more metabolically susceptible to acute fat load than controls. After 8 week course of moderate intensity exercise, women with PCOS showed significant improvement in baseline HOMA-IR and lipid induced IR. The effect of acute fat load on the rate of glucose disposal measured with clamps became comparable between PCOS and controls. It is a promising finding that the detrimental effect of acute fat load such as postprandial state or acute weight gain could be attenuated by regular moderate intensity exercise in PCOS. In addition, moderate intensity exercise improved hyperandrogenaemia in PCOS.

Increased platelet responsiveness to ADP and decreased responsiveness to PGI_2 could lead to atherothrombosis in the presence of plaque rupture and promote acceleration of atherosclerosis in the presence of endothelial dysfunction. This research work found that acute hypertriglyceridaemia increased platelet fibrinogen binding and p selectin expression in response to ADP which was not corrected by PGI_2 resulting in sustained platelet activation. This effect was attenuated by insulin infusion in controls but not in PCOS. The findings in the present study suggested that acute metabolic

derangement with hypertriglyceridaemia and acute IR induced by lipid infusion, enhanced and prolonged platelet activation in PCOS.

7.2 Future direction

The studies described in this thesis pose as well as answer many questions related to effect of NEFA and endurance exercise on insulin resistance in PCOS. This research work clearly illustrates that lowering of NEFA level improves IR and a rise in NEFA level worsens IR in PCOS. Compared to controls, women with PCOS are more susceptible to acute fat load resulting in increased skeletal muscle IR and this can be improved by moderate intensity exercise.

Further a large scale study of endurance exercise with primary outcomes of improvement in lipid induced IR and hyperandrogenaemia and fertility in PCOS would be of use for clinical implications. Subsequent studies of changes in intramyocellular lipid and insulin signalling pathways in skeletal muscle with exercise would add on understanding the mechanism how exercise improves lipid induced IR. Endurance exercise improved fasting HOMA-IR but was not able to reverse completely lipid induced IR. Timing of the exercise seems to have impact on lipid induced IR. Therefore it would be of use if a study can further elicit the effect of prior exercise on lipid induced IR in PCOS. Constant lipid infusion has had significant impact on skeletal muscle IR. Like glucose, the TG level varies from time to time throughout the day. Therefore it would be worthwhile to study the effect of fluctuation in lipid levels on oxidative stress makers, platelet function and beta cell function.

In this research work, postprandial insulin levels were able to suppress NEFA postprandially in insulin resistant PCOS similar to healthy controls. Therefore it poses a question of how postprandial hypertriglyceridaemia develops in PCOS with no evidence of excessive NEFA delivery postprandially. Like hepatic and skeletal muscle IR, the adipose tissue IR i.e. effect of IR on HSL and LPL activities which governs the postprandial lipid and glucose metabolism would be interesting to be studied especially functionality of adipose tissue plays a significant role in energy metabolism and subsequent lipid and glucose metabolism.

The finding from platelet study poses several interesting hypotheses that may be translatable to several conditions and disease states. Additional experiments will be needed to determine the mechanistic basis underlying the major findings such as dissecting whether increased platelet activation was due to lipaemia itself or PCOS platelet abnormalities. Therefore, by testing effect of lipaemic plasma on platelets taken from healthy subjects would satisfy that hypertriglyceridaemia induced the exaggerated platelet activation. Further studies on the platelet activating and inhibiting pathways affected by hypertriglyceridaemia would add on understanding of how hypertriglyceridaemia implicates in CV risk. It would be worthwhile to study the effect of combined hyperlipidaemia and hyperglycaemia, which we would see in clinical emergencies in patients with diabetes, on platelet activation.

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