The Effect of Metformin, Flutamide and Dietary Nicotinic Acid on Dyslipidemia and Cardiometabolic Risk in the JCR:LA-cp Rodent Model of PCOS

by

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Abstract

Polycystic ovary syndrome (PCOS) is a highly prevalent metabolic-endocrine disorder affecting up to 18% of adolescent and adult females in their reproductive years. PCOS is associated with an increased risk of cardiovascular disease (CVD) and type 2 diabetes. Atherogenic dyslipidemia occurs in 70% of PCOS patients, however at present the independent role of testosterone and insulin in modulating lipid metabolism in PCOS is not understood. Currently, there is no single therapy that is safe and effective to improve plasma lipids and cardiometabolic risk in PCOS. The primary aim of this thesis was to determine the effect of the insulin sensitizer, metformin, and the androgen receptor blocker, flutamide, and a lipid-lowering vitamin, nicotinic acid, on insulin and glucose, lipid and apoB-lipoprotein metabolism in the JCR:LA-*cp* rodent model of PCOS. The results showed metformin improved fasting plasma insulin and HOMA-IR, and attenuated intestinal chylomicron-cholesterol secretion and this was concomitant with changes in insulin signaling and lipogenic gene expression. Flutamide reduced fasting plasma TG, apolipoprotein (apo) B48 and apoB100, as well as intestinal TG secretion, but there was no effect on mRNA expression of lipogenic genes. Nicotinic acid treatment alone improved postprandial plasma insulin concentrations and tended to decrease intestinal chylomicron-apoB48 secretion. Addition of metformin reduced fasting plasma insulin, glucose and HOMA-IR, insulin and glucose response to a 'meal tolerance test' and tended to lower fasting plasma TG and apoB48 concentrations. Collectively, the results from these studies demonstrate: i) the androgen receptor and testosterone mediate regulation of lipid and lipoprotein metabolism in the liver and intestine and this is independent of insulin, ii) nicotinic acid has beneficial effects

on insulin-glucose metabolism and may have the potential to target dyslipidemia in PCOS conditions, as demonstrated in the JCR:LA-*cp* rodent model of PCOS.

Preface

This thesis is an original work by Maria Kupreeva. Valuable contributions from Metabolic and Cardiovascular Diseases Laboratory staff and trainees were made to facilitate the realization of the research project, of which this thesis was a part. Animal feeding, body weight measurements and blood collection procedures were performed by Mrs. Sharon Sokolik. Mesenteric lymph cannulation procedures were completed by Mrs. Sandra Kelly. Gene mRNA expression experiments and training were facilitated by Dr. Faye Borthwick. The research project received research ethics approval from the University of Alberta Research Ethics Board, Project Name “The Characterization of Metabolism, Vascular, Intestinal and Ovarian Function in the JCR-LA-cp Rodent Model of Chronic Disease”, No. AUP00000197, 12/01/2014.

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3β-HSD	3β-hydroxysteroid dehydrogenase
17β-HSD	17β-hydrosteroid dehydrogenase
A	androstenedione
ACAT	acyl-CoA:cholesterol acyltransferase
ACC	acetyl-CoA cardoxylase
ACTH	adrenocorticotropic hormone
AEPCOSS	Androgen Excess and PCOS Society
AES	Androgen Excess Society
AKT	protein kinase B
AMH	anti-mullerian hormone
AMHR2	anti-mullerian hormone receptor type II
AMPK	AMP-activated protein kinase
AR	androgen receptor
Apo	apolipoprotein
ATGL	adipose triglyceride lipase
AUC	area under the curve
BMP15	bone morphogenic protein 15
CA	corpus albicans
CE	cholesteryl ester
CETP	cholesterol ester transfer protein
CL	corpus luteum
CM	chylomicron
CMr	chylomicron remnant
CRH	corticotropin-releasing hormone
CVD	cardiovascular disease
DGAT	diacylglycerol acyltransferase
DHEAS	dehydroepiandrosterone sulfate
DHT	dihydrotestosterone
E2	17β-estradiol
ER	estrogen receptor
ERK	extracellular-regulated kinase
FAI	free androgen index
FAS	fatty acid synthase
FC	free cholesterol
FFA	free fatty acids
FSH	follicle-stimulating hormone
FT	free testosterone
GDF9	growth differentiation factor 9
GnRH	gonadotropin-releasing hormone
GPR	G protein-coupled receptor
GR	glucocorticoid receptor
GSK3β	glycogen synthase kinase 3 beta

HDL	high-density lipoprotein
HL	hepatic lipase
HMGR	3-hydroxy-3-methyl-glutaryl-CoA reductase
HSL	hormone-sensitive lipase
iAUC	incremental area under the curve
IDL	intermediate-density lipoprotein
IDOL	inducible degrader of the low-density lipoprotein receptor
IGF1	insulin-like growth factor 1
IR	insulin resistance
IRS1	insulin receptor substrate
JNK	c-Jun N-terminal kinase
NIH	National Institute of Health
LCAT	lecithin-cholesterol acyltransferase
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LH	luteinizing hormone
LPL	lipoprotein lipase
LXR	liver X receptor
MAPK	mitogen-activated protein kinase
MetS	metabolic syndrome
MGL	monoglyceride lipase
MTP	microsomal triglyceride transfer protein
MTT	meal tolerance test
NPY	neuropeptide Y
OC	oral contraceptives
OGTT	oral glucose tolerance test
P4	progesterone
P450scc	P450 side chain cleavage
PCOS	polycystic ovary syndrome
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PPARα	peroxisome proliferator-activated receptor alpha
PPARγ	peroxisome proliferator-activated receptor gamma
PTEN	phosphatase and tensin homolog
PTPN1	tyrosine-protein phosphatase non-receptor type 1
RCT	reverse-cholesterol transport
SCAP	sterol regulatory element-binding protein cleavage activating protein
SHBG	serum hormone binding globulin
SR-B1	scavenger receptor B1
SREBP	sterol regulatory element-binding protein
StAR	steroidogenic acute regulatory
T2D	type 2 diabetes
T	testosterone

TC	total cholesterol
TG	triglyceride
TR	thyroid hormone
TRL	triglyceride-rich lipoproteins
VLDL	very-low density lipoprotein
VTV	very-low density lipoprotein transport vesicles

1. Literature Review

1.1 The Problem: Cardiometabolic Risk in Adolescents and Women with PCOS

Polycystic ovary syndrome (PCOS) is a highly prevalent metabolic-endocrine disorder in 5-18% of adolescent and adult females of reproductive age. PCOS women are affected by hyperandrogenism and often have insulin resistance. Metabolic syndrome and atherogenic dyslipidemia are commonly seen in PCOS and predispose these women to higher risk for early development of cardiovascular disease (CVD) and Type 2 Diabetes (T2D). Despite the prevalence of PCOS, the pathophysiology and mechanisms associated with dyslipidemia are poorly understood. In particular, the role of hyperandrogenemia and insulin resistance in the development of PCOS-associated dyslipidemia are not well studied. To prevent and improve CVD risk, a number of lifestyle and therapeutic approaches have been developed in the field, however no one intervention treats both PCOS symptomology and cardiometabolic risk, in particular dyslipidemia. Insulin sensitizing agents are commonly used to reduce insulin resistance in PCOS and the limited lipid-lowering therapy options available inconsistently target dyslipidemia in young women with PCOS. Collectively, current interventions do not consistently improve cardiometabolic risk and atherogenic dyslipidemia and cannot be used in all patients affected by PCOS. Therefore, there is a need to understand the physiological mechanisms involved in cardiometabolic risk, in particular dyslipidemia in PCOS, and how insulin resistance and hyperandrogenemia contribute to atherogenic dyslipidemia. In addition, finding a safe and effective treatment to target PCOS-associated dyslipidemia and to reduce subclinical CVD risk is a key area of PCOS research highlighted by the Androgen Excess and PCOS Society (AEPCOSS) (Azziz *et al* 2009). The focus of this literature review is to provide background on the normal physiology of female reproductive-endocrine function, and lipid and lipid metabolism, the pathophysiological roles of hyperandrogenemia and insulin resistance in reproductive-endocrine dysfunction and cardiometabolic risk, in particular dyslipidemia in PCOS, and current treatment options to reduce cardiometabolic risk in PCOS.

1.2 Characteristic Features of PCOS

PCOS is a metabolic-endocrine disorder, which is heterogenous in nature, with patients presenting with varying phenotypes of the condition. Menstrual cycle irregularity is one of the most common features in PCOS with an estimated 32% percent of cycles being ovulatory (Azziz *et al* 2001, Laven *et al* 2002). It has been shown that up to 95% of women with oligo-ovulation or anovulation have PCOS (Kumarapeli *et al* 2008). Hirsutism affects 40-90% of PCOS patients and is considered to be a reliable marker of hyperandrogenemia (Kopera *et al* 2010). Dihydrotestosterone (DHT) is a product of testosterone (T) conversion by 5-alpha reductase enzyme in hair follicles leading to a male hair pattern growth (Lowenstein 2006). Despite being a good marker of hyperandrogenemia, plasma androgen levels need to be tested due to a subjectivity of the hirsutism clinical assessment (Lujan *et al* 2008). Obesity has been found to worsen reproductive and metabolic outcomes in PCOS and is prevalent in 14-74% of PCOS patients depending on a population studied (Lim *et al* 2012). Insulin resistance, dyslipidemia, hyperandrogenism, irregular menstrual cycles and infertility are known to be exacerbated in patients with higher body weights (Lim *et al* 2012).

1.3 Diagnostic Criteria and Prevalence

Stein and Levental first described PCOS as a disorder associated with enlarged follicles, hirsutism and chronic anovulation (Stein and Levental 1935). In later years, hyperandrogenemia and ovarian morphology assessed by ultrasonography led to improvements in diagnostic capacity (Raj *et al* 1978, Adams *et al* 1986). In 1990, National Institutes of Health (NIH) proposed diagnostic criteria to classify PCOS. The presence of clinical or biochemical hyperandrogenemia and chronic oligo- or anovulation were the two features for the characterization (Zawadski and Dunaif 1992). However, there was an apparent need for a broader classification due to a rising awareness of the clinical expression of the disorder, which comprises a broad spectrum of symptoms of ovarian dysfunction. In 2003, the Rotterdam consensus on diagnostic criteria recommended the criteria to include the presence of polycystic ovaries, which are defined as containing ≥ 12 follicles measuring 2-9 mm and/or ovary volume of > 10 cm^3 to the list of existing features. Hence, two out of three features needed to be

present for a PCOS diagnosis (Dewailly and Tarlatzis 2004). The most recent diagnostic criteria were proposed in 2006 by AEPCOSS (Azziz *et al* 2009), which requires two factors to be present – signs of clinical or biochemical hyperandrogenemia, and ovarian dysfunction (Table 1-1). Other etiologies of androgen excess and related disorders need to be excluded prior to PCOS diagnosis (Lujan *et al* 2008, Azziz *et al* 2009). These comprise thyroid dysfunction, hyperlactinemia, congenital adrenal hyperplasia, Cushing syndrome, idiopathic hirsutism, androgen-secreting neoplasms and other syndromes associated with severe insulin resistance and hyperandrogenism (Azziz *et al* 2009).

Table 1-1. Diagnostic criteria of polycystic ovary syndrome.

National Institutes of Health (1990)

Patient demonstrates two of the following:

1. Hirsutism and/or hyperandrogenemia
2. Oligo- or anovulation

Rotterdam Guidelines (2003)

Patent demonstrates two out of three of the following:

1. Oligo- or anovulation
2. Hirsutism and/or hyperandrogenemia
3. Polycystic ovaries

Androgen Excess and PCOS Society (2006)

Patient demonstrates two of the following:

1. Hirsutism and/or hyperandrogenemia
2. Oligo- or anovulation and/or polycystic ovaries

In addition to the criteria listed above, the AEPCOSS recognized insulin resistance, dyslipidemia and obesity as metabolic disorders associated with the syndrome, and that these should be tested at the time of PCOS diagnosis, though they are not used as a part of PCOS definition (Azziz *et al* 2009). In addition, four phenotype categories emerged to further classify PCOS based on the refinement of diagnostic criteria (Table 1-2), but these still do not include other metabolic abnormalities associated with PCOS (Dewailly and Tarlatzis 2004, Panidis *et al* 2011, Zawadski and Dunaif 1992).

Table 1-2. Definitions of the PCOS phenotypes based on Rotterdam criteria (2003).

PCOS Phenotype	Hyper-androgenemia	Oligo-/ Anovulation	Polycystic Ovaries
I – severe	✓	✓	✓
II – anovulatory, hyperandrogenic	✓	✓	✗
III – ovulatory	✗	✓	✓
IV – mild	✓	✗	✓

Phenotypes I & II were included in the NIH (National Institutes of Health) 1990 criteria

Despite PCOS being a common endocrine disorder in women of reproductive age, prevalence estimates are highly variable. Prevalence of PCOS cannot be precisely estimated as there are three different criteria sets used for PCOS diagnosis and there is a lack of larger cohort studies for prevalence assessment and most of the prevalence study samples are small and are from specific populations (March *et al* 2010). A study by Yildiz and colleagues assessed PCOS prevalence in cross-section of Turkish women using NIH, Rotterdam and AES criteria. The prevalence was determined to be 6.1%, 19.9% and 15.3% respectively between ages 18 and 25 yrs (Yildiz *et al* 2012). Later a cohort of 728 South Australian women (27-34 yrs) was conducted and the results have shown the prevalence of 8.7%, 17.8% and 12.0% using NIH, Rotterdam and AES criteria respectively (March *et al* 2010). In addition, the prevalence rates of PCOS may vary between ethnic groups (Azziz *et al* 2004). Despite the variance in prevalence statistics, PCOS is the most common endocrine abnormality in females of reproductive age yet the etiology of PCOS and associated cardiometabolic anomalies are poorly understood (Carmina *et al* 2006).

1.4 Etiology and Pathophysiology of PCOS

The exact etiology of PCOS remains unclear due to the heterogeneity of the clinical and biochemical features of the syndrome. A broad range of phenotypic variations (Table 1-2) and endocrine-metabolic abnormalities suggest that multiple factors contribute to the pathophysiology of the disorder. There is evidence that both genetic and environmental factors contribute to PCOS development (Diamanti-Kandarakis *et al* 2006). To date, there is no single gene identified that causes PCOS, however multiple studies have been conducted to underpin candidate genes. Those genes found to play a role in folliculogenesis, androgen synthesis, insulin signaling pathways, adiponectin secretion, and fat and obesity associated gene (Kosova

and Urbanek 2012, Simoni *et al* 2008, Urbanek 2007). In addition, it has been proposed that obesity, insulin resistance and T2D affect male and female first-degree relatives of women with PCOS at a higher frequency (Legro *et al* 2002, Ehrmann *et al* 2005). However, the exact inheritance pattern is still not clear due to the heterogeneity of phenotypic variations associated with PCOS (Kosova and Urbanek 2012). Environmental factors are also implicated in the development of PCOS. These include prenatal exposure to excess androgens (Abbott *et al* 2008, Li and Huang 2006), poor dietary pattern and sedentary lifestyle (Moran *et al* 2009), as well as exposure to certain environmental toxins (Diamanti-Kandarakis *et al* 2009). Overall, the etiology of PCOS appears to be polygenic and multifactorial (Diamanti-Kandarakis *et al* 2006). These potential genetic predispositions and environmental exposures are thought to mediate the pathogenesis of PCOS, which have been proposed to predominately relate to androgen excess and insulin resistance (Norman *et al* 2007). To understand the pathogensis of PCOS, in particular ovarian dysfunction and cardiometabolic risk, normal reproductive-endocrine and lipid metabolism will be briefly outlined.

1.4.1 Reproductive and endocrine physiology

The human menstrual cycle is represented by two phases, follicular and luteal, and these are regulated by the hypothalamic-pituitary-ovarian axis (Fig 1-1). The hypothalamus secretes gonadotropin-releasing hormone (GnRH), which stimulates the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary (Keye *et al* 1973, Tsutsumi and Webster 2009). FSH secretion is favoured by slow GnRH pulse frequency with low amplitude and LH secretion is favoured by rapid pulse frequency with high amplitude (Tsutsumi and Webster 2009). These two gonadopropins act on the ovaries to regulate the ovarian cycle and follicle development. The ovarian follicle is composed of two major cell types – theca and granulosa cells (Figure 1-2). During the follicular phase of the cycle, FSH secretion is increased initially (Figure 1-3), stimulating follicle recruitment. Granulosa cells secrete estradiol (E2) under FSH stimulation. E2 is made form androstenedione (A) and testosterone (T) that is secreted form theca cells under LH stimulation (Figure 1-4). In the late follicular phase, the increasing estradiol in circulation produces a positive feedback on GnRH, LH and FSH secretion from the

hypothalamus and anterior pituitary. This positive feedback is necessary for the mid-cycle LH and FSH surge, which precedes ovulation (Carr *et al* 2006) (Figure 1-3). The luteal phase is initiated after ovulation and is characterized by the formation of the corpus luteum (CL) from theca and granulosa cells following ovulation of the oocyte. Under LH stimulation, P450 side chain cleavage (P450scc) enzyme activity is increased, converting cholesterol to pregnenolone, and decreased 17α-hydrolase and 17,20-lyase enzyme activity, shifts steroid synthesis pathways to progesterone (P4) production. Therefore, during the luteal phase CL predominantely produces P4 and a smaller amount of E2. At this stage, these two hormones provide negative feedback to the hypothalamus and anterior pituitary, lowering FSH and LH secretion. CL also produces inhibin, which helps to reduce FSH secretion and prevents a new oocyte maturation cycle. If no implantation occurs the CL degenerates to a corpus albicans, which no longer produces P4 and E2, and indicates the end of the luteal phase and transition into the menstrual phase (Carr *et al* 2006).

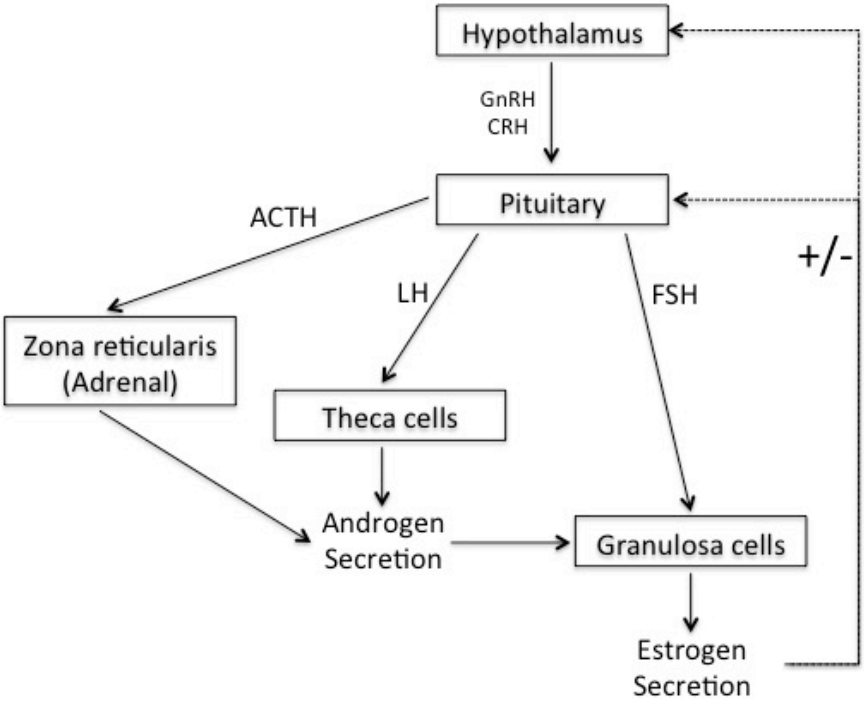


Figure 1-1. Regulation of hormone secretion by the hypothalamic-pituitary-ovarian and hypothalamic-pituitary-adrenal axis. During early to mid-follicular phase, low levels of estradiol provide negative feedback to hypothalamic release of gonadotropin releasing hormone (GnRH), and pituitary release of luteinizing hormone (LH) and follicle stimulation hormone (FSH). During the late follicular phase, high estradiol concentration provide positive feedback to GnRH, LH and FSH release. ACTH stimulates production of androgens from the adrenal gland, which do not provide feedback regulation to hypothalamus or pituitary. ACTH, adrenocorticotropic hormone; CRH, corticotropin-releasing hormone. (Adapted from Visser *et al* 2010 and Walker *et al* 2010).

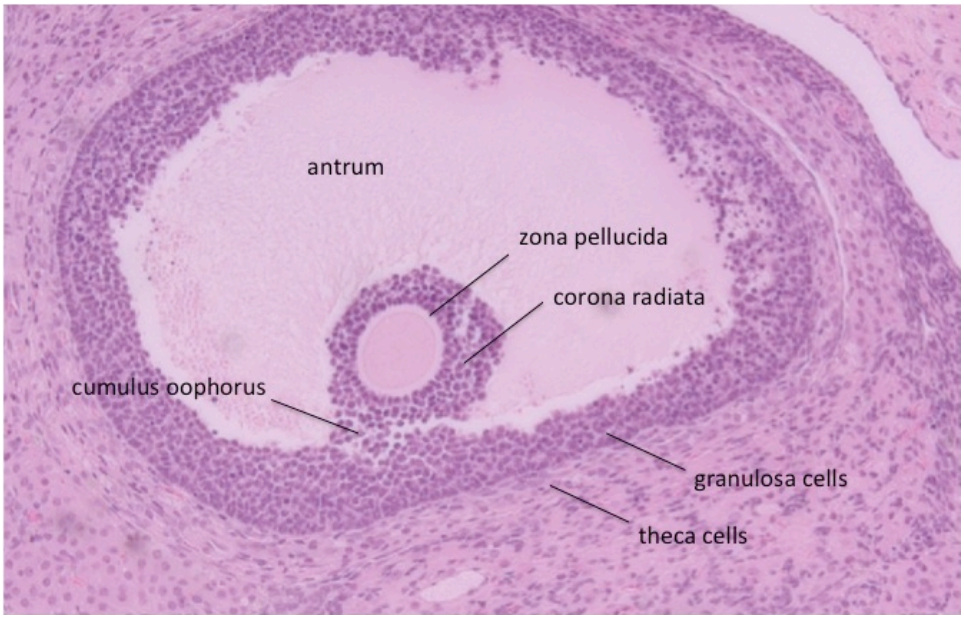


Figure 1-2. Structure of tertiary follicle in ovary.

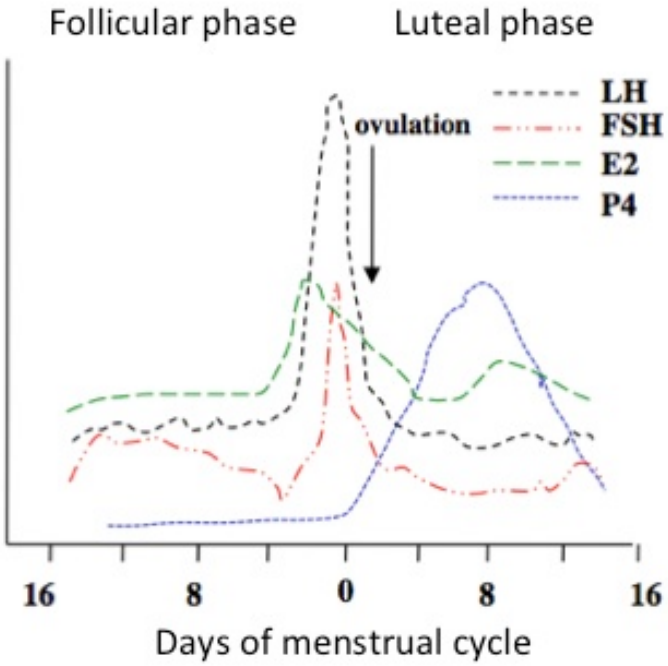


Figure 1-3. Hormone profile during follicular and luteal phases in a normal menstrual cycle. E2, estradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone; P4, progesterone. (Adapted form Neill *et al* 2006).

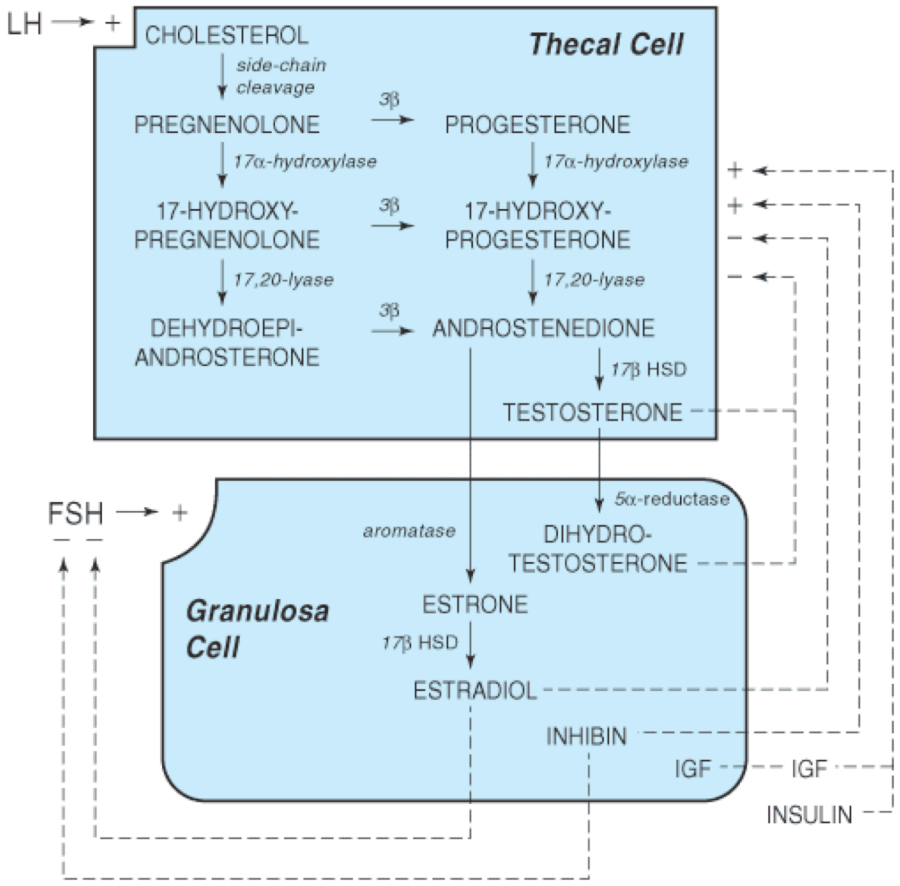


Figure 1-4. Theca and granulosa cell androgen and estrogen biosynthesis in response to luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH stimulates theca cells to express cholesterol side-chain cleavage cytochrome P450, 17α-hydroxylase, 17,20-lyase, and 3β-hydroxysteroid dehydrogenase (3β), therefore testosterone and androstenedione are synthesized from cholesterol in these cells. Androstenedione and testosterone diffuse across the basal lamina into granulosa cells, which contain P450 aromatase and 17β-hydrosteroid dehydrogenase (17β-HSD) enzymes under FSH stimulation and convert androstenedione to estradiol. Testosterone is converted into dihydrotestosterone (DHT) by 5α-reductase enzyme in granulosa cells. Testosterone, DHT and estradiol provide negative feedback to theca cells, while insulin-like growth factor (IGF) 1 provides positive feedback. Insulin enhances LH stimulation of theca cell androgen production. Inhibin, produced in granulosa cells, promotes androgen synthesis in theca cells. Inhibin and estradiol also reduce FSH stimulation of granulosa cells. (Adapted from Gardner *et al* 2007).

1.4.2 Hyperandrogenemia and dysregulation of androgen metabolism in PCOS

Hyperandrogenemia remains the principle feature of PCOS affecting 70-80% of women with PCOS, and hyperinsulinemia has been proposed to be involved in the pathogenesis of

hyperandrogenism, albeit there are other metabolic pathways that may be dysfunctional leading to PCOS (Baptiste *et al* 2010) (Table 1-3). A dysfunction of the hypothalamic-pituitary-ovarian-adrenal axis, and alterations in LH secretion have been proposed to be a major cause of androgen excess in PCOS (Barontini *et al* 2001, Doi *et al* 2005) (Figure 1-1). It has been shown that women with PCOS have a prevailed rapid-frequency GnRH secretion from hypothalamus, thus favoring LH synthesis and secretion from pituitary gland (Doi *et al* 2005). Polycystic ovaries tend to have a thickened theca cell layer, which secrete excessive androgens in response to LH stimulation (Norman *et al* 2007). Excess LH leads to a rise in circulating levels of T and A via direct upregulation of steroidogenic acute regulatory (StAR) protein, P450 scc, 17α-hydroxylase, 17,20-lyase, and 3β-hydroxysteroid dehydrogenase (3β-HSD) enzymes in theca cells (Jakimiuk *et al* 2001, Zhang *et al* 2000) (Figure 1-5), and is exacerbated by lowered circulating serum hormone binding globulin (SHBG) (Norman *et al* 2007) leading to an increased circulating levels of free T. Studies on cultured theca cells isolated from polycystic ovaries were found to produce significantly greater amount of T in response to normal LH stimulation compared to cells from unaffected women, suggesting the PCOS-ovary is more sensitive to LH stimulation of T synthesis (Wickenheisser *et al* 2006, Wood *et al* 2003). It has been shown that theca cells from PCO tend to have a greater number of LH receptors (Jakimiuk *et al* 2001). Interestingly, the excess T and A secreted from ovaries does not appear to induce negative feedback inhibition on LH secretion from the pituitary. Similarly, DHEA and androstenedione produced by the adrenal do not appear to work in the negative feedback pathway to reduce ACTH production in women (Baptiste *et al* 2010) (Figure 1-1), therefore increased circulating androgen concentration does not reduce ACTH stimulation of andrenal androgen excess. In addition, some of the key enzymes in the ovary and adrenal gland that are involved in androgen production have been found to be upregulated in PCOS (Figure 1-5). These enzymes include 17α-hydrolase and 17,20-lyase, which are the rate-limiting enzymes of the androgen production pathway, 3β-HSD type II, 17β-hydrosteroid dehydrogenase type V and the P450 side chain cleavage enzyme. In addition, activity of the Cyp19 (P450 aromatase) enzyme, which converts T to E2 and A to estrone, has been shown to be reduced in PCOS ovary (Figure 1-5), which leads to an increase in A and T production, and a decrease in conversion of A to estrone,

and T to E2 (Baptiste *et al* 2010, Norman *et al* 2007, Wood *et al* 2003). How these enzymes in androgen metabolism are dysregulated or become dysregulated in PCOS remains unclear, however a genetic predisposition and insulin resistance may play a role in the altered expression or function of these enzymes (Baptiste et al 2010) (Table 1-3, Figure 1-5).

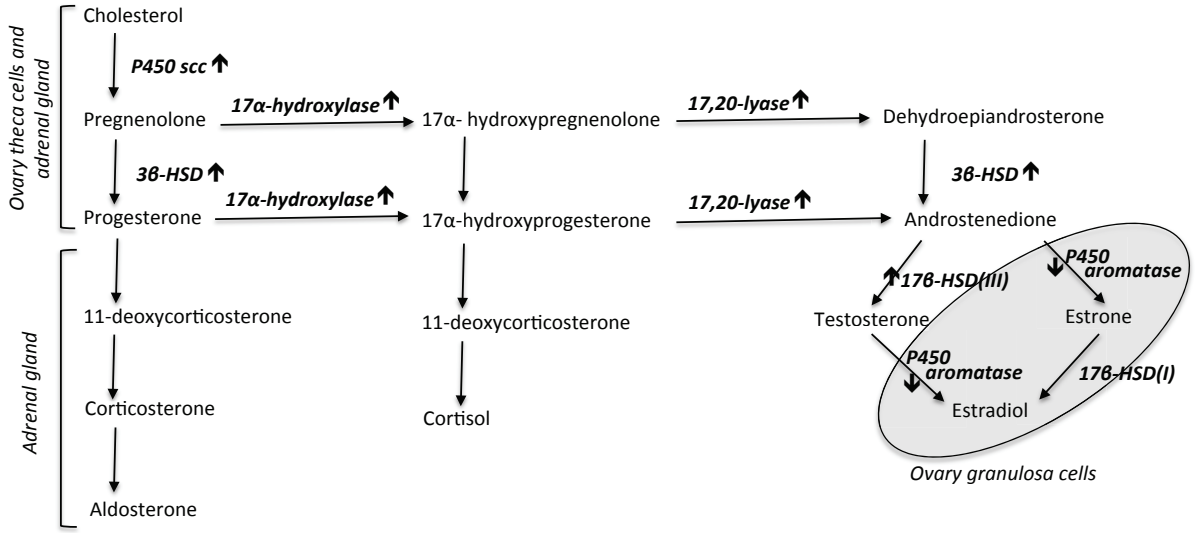


Figure 1-5. Steroidogenesis occurs in ovaries and adrenal gland in females. Two types of cells in the ovary are involved in the process of androgen secretion and conversion – theca and granulosa cells. In PCOS, some of the key enzymes of androgen production are upregulated leading to an increases androgen production. These are cholesterol side-chain cleavage cytochrome P450 (P450 scc), 17α-hydroxylase, 17,20-lyase, 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydrosteroid dehydrogenase (17β-HSD). Conversion of testosterone to estradiol is reduced in granulosa cells due to downregulation of P450 aromatase enzyme. (Adapted from Payne *et al* 2004, Baptiste *et al* 2010).

Table 1-3. Potential pathological pathways and mechanisms in the etiology of PCOS.

Genetic factors:
Familial clustering
Alterations in genes involved in folliculogenesis, androgen synthesis, insulin signaling, adiponectin secterion and fat and obesity associated gene
Environmental factors:
Prenatal exposure to androgens
Diet and lifestyle
Environmental chemicals and toxins
Abnormal GnRH action and LH secretion:
Altered amplitude and frequency of GnRH pulse
Altered amplitude and frequency of LH pulse
Altered FSH/LH ratio
Hyperandrogenemia:
Increased sensitivity of theca cells to LH
Increased adrenal androgen production
Decreased conversion of testosterone to estradiol in granulosa cells
Decreased SHBG production in liver
Hyperinsulinemia:
Amplifies androgen production in ovaries and adrenal gland
Reduces SHBG production in liver

GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone

1.4.3 Androgens and ovarian follicular development

Androgen oversecretion, impaired folliculogenesis and ovulation are contributors to ovarian dysfunction in PCOS. There is an apparent failure of a dominant follicle to develop and maturation of fluid-filled antral follicles, and follicles tend to be smaller in size. These effects are likely due to higher serum LH:FSH ratio reducing the stimulatory effect of FSH on follicular development (Norman *et al* 2007). Women with PCOS have an abnormal LH secretion pattern and the LH surge magnitude is altered, with a higher LH response to GnRH leading to increased circulating LH levels and an increased LH:FSH ratio (Dale *et al* 1992, van Hooff *et al* 2000). This further mediates increased androgen synthesis in theca cells (Baptiste *et al* 2010, Franks *et al* 2008). If cytochrome P450 aromatase activity is decreased, testosterone and androstenedione are not readily converted to estradiol and estrone (Norman *et al* 2007) (Figure 1-5). There is not only an increase in the number of small antral follicles, but also the number of primary and

secondary follicles in ovaries of PCOS women compared to women with a normal ovulatory cycle (Webber *et al* 2003). If androgens are not converted to estradiol (as shown in Figure 1-4, 1-5), there is failure of initiation of follicular development and increased granulosa cell proliferation surrounding the oocyte (Forsdike *et al* 2007, Steckler *et al* 2007). It has been shown that only large dominant follicles respond to LH in the normal ovary (Franks *et al* 2008). However, in PCOS, granulosa cells of smaller follicles are responsive to LH under conditions of hyperinsulinemia and excess LH secretion, leading to an arrest in follicular growth (Franks *et al* 2008). It has also been proposed that genes in the transforming growth factor-beta superfamily are involved in normal follicular development, and these genes may be altered in PCOS leading to arrested oocyte maturation (Sproul *et al* 2010). These genes include growth differentiation factor 9 (GDF9) (early follicle growth promoter), bone morphogenic protein 15 (BMP15) (oocyte maturation and follicle development), anti-mullerian hormone (AMH) (early follicle growth inhibitor), and anti-mullerian hormone receptor type II (AMHR2). It has been shown that mRNA expression of follicle growth promoters, DGF9 and BMP15, is decreased in PCOS ovaries, whereas the inhibitors of growth, AMH and AMHR2, have increased expression in follicular fluid (or antrum) in PCOS (Sproul *et al* 2010).

1.4.4 Hyperinsulinemia and dysregulation of androgen metabolism and follicular development

It is well known that most women with PCOS are more likely to be insulin resistant and have compensatory hyperinsulinemia, which has been proposed to promote androgen synthesis and the pathogenesis of PCOS (Azziz *et al* 2009, Baptiste *et al* 2010, Ehrmann *et al* 1995). Hyperinsulinemia has been proposed to act through multiple mechanisms leading to excess androgen production (Table 1-3). It has been shown that cultured theca cells produce more androgens in response to LH stimulation and insulin stimulation (Baillargeon *et al* 2005, Franks *et al* 1999). Insulin also potentiates ACTH-stimulated steroidogenesis in the andrenal gland (Arslanian *et al* 2002, Moghetti *et al* 1996). Serum concentration of 17α-hydroxypregnenolone, 17α-hydroxyprogesterone and DHEA, but not cortisol, were significantly increased with ACTH stimulation in hyperinsulinemic women with PCOS. In addition, adrenal 17,20-lyase activity was also increased. Insulin has been shown to act through the insulin receptor in human theca cells

to induce testosterone production (Nestler *et al* 1998). Insulin has been shown to directly stimulate P450 side chain cleavage enzyme and 3β-HSD enzyme expression and activity in theca cells, which would increase production of pregnenolone and progesterone, which are the precursors for A and T. In addition, in theca cells insulin inhances LH stimulation of androgen biosynthesis via interacting with the signaling pathway downstream of cAMP, through PI3K/AKT interaction (Carvalho *et al* 2002). In has been shown to increase StAR protein biosynthesis, which directly increases steroidogenic pathway activity in follicles (Diamanti-Kandarakis and Papavassiliou 2006). Insulin-like growth factor 1 (IGF1) is critical for ovarian follicle development. lGF1 binds to IGF1 and insulin receptors, which elicits PI3K/AKT and mitogen-activated protein kinase (MAPK) transduction pathways activation. This leads to protein phosphorylation of downstream targets, stimulating granulosa cell proliferation and differentiation (Baillargeon *et al* 2006, Baptiste *et al* 2010, Dib *et al* 1998, Homburg *et al* 1992, Nestler *et al* 1998). Insulin has been shown to stimulate phosphatase and tensin homolog (PTEN) expression in granulosa cells, which negatively regulates the PI3K/AKT pathway activation (Figure 1-6), preventing phosphorylation of AKT downstream targets and leading to decreased follicular growth and maturation (Iwase *et al* 2009).

In addition, it has been demonstrated that high plasma insulin levels inhibit hepatic synthesis of SHBG, increasing the concentrations of free T (Jayagopal *et al* 2003). Numerous studies have demonstrated that improvements in insulin resistance can reduce plasma total and free T levels and this is associated with improvements in ovulatory function (Ehrmann *et al* 1997, Biallargeon *et al* 2003). Overall, there is a putative link between hyperinsulinemia and androgen excess although the exact mechanisms remain to be elucidated. It has also been suggested that the reverse relationship exists where androgens dysregulate insulin metabolism, as anti-androgen therapies such as spironolactone, cyproterone acetate, flutamide and GnRH agonists, have been shown to improve insulin sensitivity in some studies, however the mechanisms are unknown (Arslanian *et al* 2002, Dahlgren *et al* 1998, la Marca *et al* 1999, Moghetti *et al* 1996).

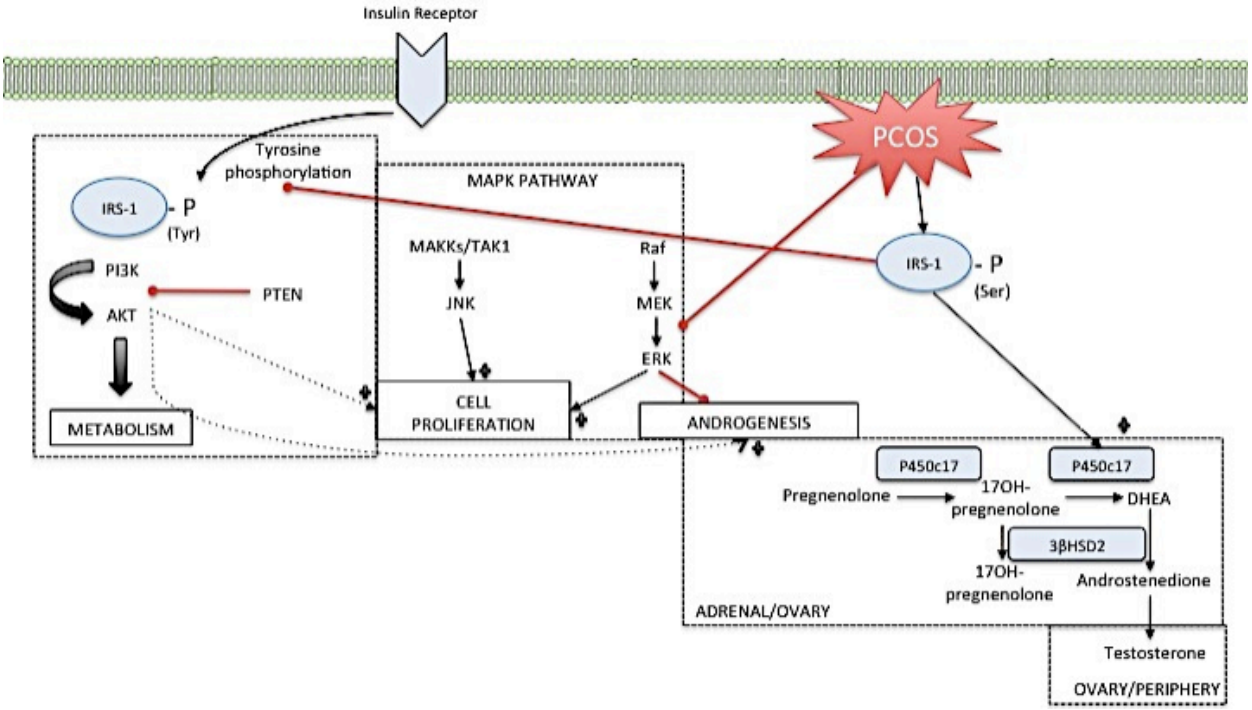


Figure 1-6. Proposed cellular mechanisms involved in insulin-stimulated androgen biosynthesis, PCOS-associated defects and increased androgen production in insulin-sensitive and androgen-secreting cells. Insulin binds to insulin receptor resulting in tyrosine phosphorylation of the receptor and insulin receptor substrate 1 (IRS1). IRS1 activates phosphoinositide-3-kinase (PI3K) and protein kinase B (AKT), which mediate insulin-glucose metabolism in insulin-responsive cells. Serine phosphorylation of IRS1 prevents PI3K activation, which inhibits insulin-signaling cascade. Serine-phosphorylated IRS1 increases P450c17 enzyme activity and decreases MEK/ERK activity, which leads to increase in androgen biosynthesis. (Adapted form Baptiste *et al* 2010).

1.5 Cardiometabolic and CVD Risk in PCOS

Women with PCOS demonstrate multiple risk factors for cardiovascular disease (CVD). These include the metabolic syndrome, abdominal obesity, dyslipidemia, insulin resistance and hypertension (Dokras 2008, Teede *et al* 2006). Although evidence for cardiovascular events in women affected by PCOS during their reproductive years is limited, multiple studies suggest that PCOS women are predisposed to the early development of atherosclerosis (Wild *et al* 2010). Indeed, adolescents and women with PCOS have early subclinical signs of CVD including increased carotid artery intima media thickness, endothelial dysfunction, ventricular hypertrophy and left ventricular dysfunction (Jovanovic *et al* 2010, Karoli *et al* 2012, Meyer *et al* 2012, Orio *et al* 2004). In longer-term health outcome studies, such as the Nurses Health Study,

menstrual dysfunction was shown to be associated with higher incidence of end stage CVD events and risk factors for CVD (Solomon *et al* 2002, Pierpoint *et al* 1998).

The prevalence of the metabolic syndrome (MetS) in PCOS ranges from 20% to 45% depending on the population studied and is two fold higher compared to the general population (Apridonidze *et al* 2005, Carmina *et al* 2006, Ehrmann *et al* 2005). MetS comprises a major risk factor for CVD risk and is linked to atherogenic dyslipidemia, chronic inflammation, oxidative stress and endothelial dysfunction (Wild *et al* 2010). The prevalence of obesity in PCOS is two-fold higher compared to the general female population, affecting more than half of women with PCOS, and obesity is associated with an exacerbation of IR and atherogenic dyslipidemia in PCOS (Martinez-Bermejo *et al* 2007, Gambineri *et al* 2002). Normal lipid and lipoprotein metabolism will be reviewed followed by the association with IR and hyperandrogenemia.

1.5.1 Lipid and lipoprotein metabolism

1.5.1.1 Lipoprotein classification and function

Transport of lipids in plasma accurs via the formation of lipoproteins, which are classified by their densities upon ultracentrifugal separation and represent major lipoprotein classes. These include high-density lipoprotein (HDL), low-density lipoprotein (LDL), intermediate-density lipoprotein (IDL), very-low density lipoprotein (VLDL), chylomicrons (CM) and LDL- and CM-remnants. Lipoproteins share a common structure and consist mainly of a hydrophobic core (TG and cholesteryl esters) surrounded by amphiphatic molecules (phospholipids, cholesterol and apolipoproteins) (Nilsson-Ehle *et al* 1980). Apolipoproteins (apo) play a role in regulation of plasma lipid and lipoprotein metabolism and help to solubilize the lipids in the core. Briefly, apoB100 is required for assembly of VLDL in the liver and is present in associated lipoproteins following lypolysis, IDL and LDL. ApoB48 is required for chylomicrom assembly in the intestine, and apoA is an integral component of HDL (Ginsberg *et al* 2005). There are other types of apolipoproteins associated with lipoproteins that have distinct metabolic functions and these are listed in Table 1-4.

Table 1-4. Classification and metabolic function of major apolipoproteins and association with lipoprotein classes. Adapted from Ginsberg *et al* 2005.

Apolipoprotein	Lipoprotein	Origin	Metabolic function
ApoA(I-IV)	HDL, CM	Liver, intestine	Structural activation of HDL, LCAT activation Modulation of TG metabolism through LPL Apolipopotein transfer between HDL and CM
ApoAIV	HDL	Liver	LPL activation, modulation of TG metabolism
ApoB48	CM	Intestine	CM assembly and secretion from the intestine
ApoB100	VLDL, IDL, LDL	Liver	VLDL assembly and secretion form the liver
ApoC(I-III)	CM, VLDL, IDL, LDL	Liver, Intestine	May inhibit hepatic LDL and CM uptake via LDL-R (I,III), LPL activation (II), LPL inhibition (III)
ApoE	CM, VLDL, IDL, LDL	Liver, Intestine	LDLR-apoB100-E receptor binding

CM, chylomicron; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein metabolism; LCAT, lecithin-cholesterol acyltransferase; LDL, low-density lipoprotein; LDLR, LDL receptor; LPL, lipoprotein lipase; VLDL, very low-density lipoprotein

1.5.1.2 Lipid and lipoprotein pathways of metabolism

Lipid and lipoprotein metabolism and homeostasis is maintained by multiple key regulators, which include hormones (e.g. insulin, leptin), nuclear receptors (e.g. peroxisome proliferator-activated receptors (PPAR), liver X receptor (LXR)) and other regulatory proteins (e.g. sterol regulatory element-binding protein (SREBP)). The liver and intestine are the major sites of lipid and lipoprotein synthesis and metabolism, and adipose, muscle and other tissues play a role in metabolism of lipids delivered to these tissues. Lipid transport and metabolism pathway is summarized in Figure 1-7.

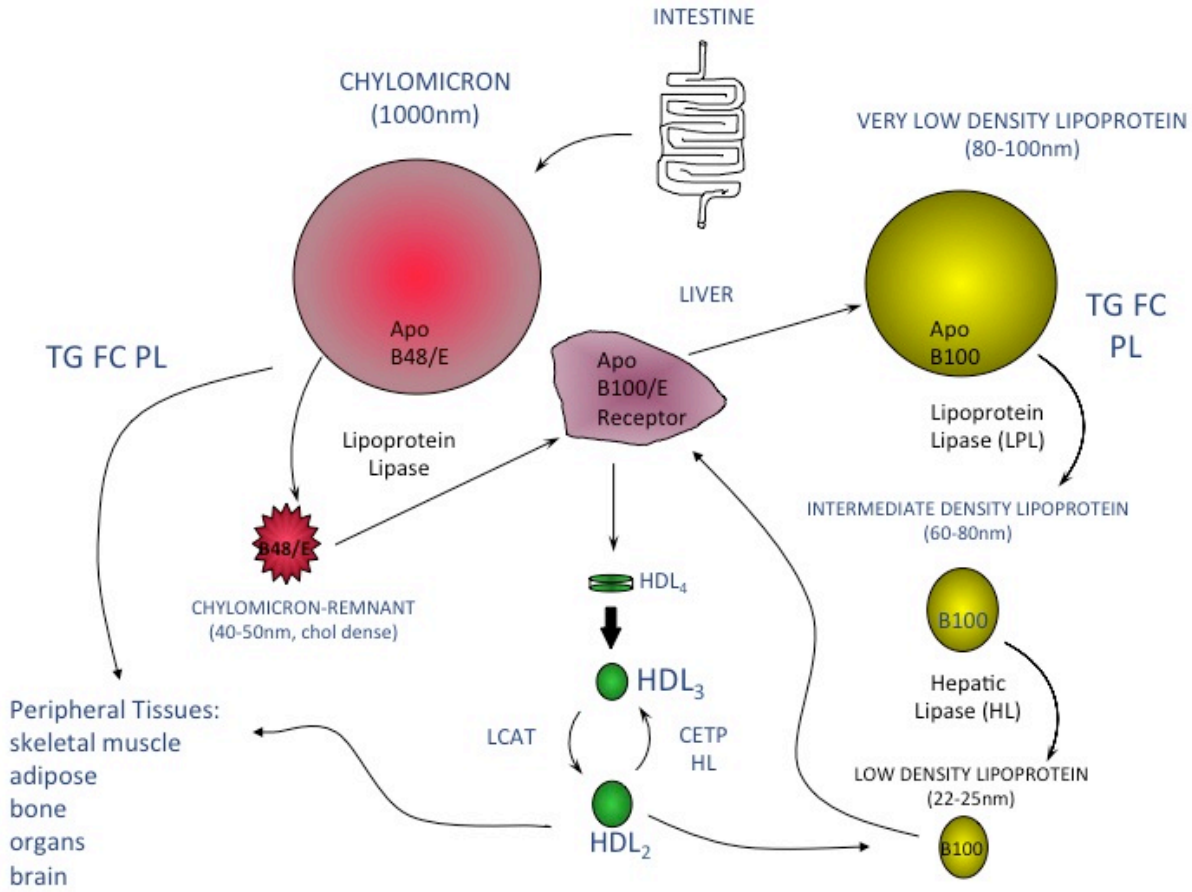


Figure 1-7. Lipid transport and metabolism pathway. CETP, cholesterol ester transfer protein; FC, free cholesterol; HDL, high-density lipoprotein; LCAT, lecithin-cholesterol acyltransferase; TG, triglycerides.

1.5.1.2.1 Chylomicron metabolism

Chylomicrons are continually synthesized in the enterocyte and following a meal the particles enlarge to incorporate absorbed dieatary lipids (Figure 1-7). Dietary TG and cholesterol are digested and absorbed by the small intestine. In the enterocytes, dietary monoglycerides and FFA are primarily re-esterified into TG. Newly synthesized TG and esterified cholesterol, are incorporated into apoB48-containing chylomicrons and secreted into the lymphatic system, and enter the circulation via superior vena cava (Ginsberg *et al* 2005). When CMs are in the circulation, endothelial lipoprotein lipase (LPL) interacts with apoCII, facilitates the hydrolysis of TG to FFAs and monoglycerides, delivering these substrates to tissues. In adipocytes, FFAs are re-esterified and stored as TG. Chylomicron remnants (CMr), which are formed through this lypolytic process, contain less TG and are enriched in cholesteryl esters (CE). CMr are removed

from the circulation predominately by the liver via LDL receptor-related protein (LRP), LDLR-apoB100-E receptor and hepatic lipase (HL) interaction (Cooper 1997, Ginsberg *et al* 2005) (Figure 1-7).

1.5.1.2.2 VLDL metabolism

VLDLs are synthesized and secreted by the liver and this process occurs similarly to CM assembly in the intestine. VLDL assembly relies on TG availability and requires microsomal triglyceride transfer protein (MTP), which transfers lipid to synthesized apoB100, forming a lipid-poor primordial particle in endoplasmic reticulum (Shelness and Sellers 2001). If lipidation of nascent apoB is insufficient due to low TG availability, apoB undergoes degradation (Tiwari and Siddiqi 2012). A mature VLDL particle is formed upon apoB100-containing precursor particle fusion with a lipid droplet. FFA influx form the adipose tissue, CM remnants and *de novo* synthesized TG comprise the TG pool available for VLDL assembly. Diacylglycerol acyltransferase (DGAT) and acyl-CoA:cholesterol acyltransferase (ACAT) enzymes facilitate production of TG and CE and the formation of the cytosolic lipid droplets. Newly synthesized VLDL particles are then transported in VLDL transport vesicles (VTV) into Golgi, where they undergo further maturation, packadged into post-Golgi vesicles and then secreted into blood (Tiwari and Siddiqi 2012). Once in plasma, VLDL particles are hydrolyzed by LPL forming IDL and LDL. Small LDL remnants are then removed form the circulation by the liver via LDLR (Ginsberg *et al* 2005).

During the fasted state, adipose tissue lipolysis is a major fuel source providing free fatty acids and glycerol. Adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoglyceride lipase (MGL) are the major lipases facilitating FFA release form the adipose tissue. During short-term fasting both ATGL and HSL activity is increased, facilitating the release of FFA into the plasma (Viscarra and Ortiz 2013). FFA are taken up by tissues for use as energy and a small fraction is delivered to the liver. FFA may be re-esterified into TG and incorporated into newly synthesized VLDL (Jensen *et al* 2001, Viscarra and Ortiz 2013).

1.5.1.2.3 HDL metabolism

HDL particles are involved in reverse-cholesterol transport (RTC), which promotes efflux of excess cholesterol from the extrahepatic tissues. Nascent phospholipid-rich and cholesterol-poor HDL particles are synthesized by the liver and intestine. In plasma, they acquire phospholipids and cholesterol via cellular efflux and LPL-mediated lipolysis of triglyceride-rich lipoproteins (TRL) (Lewis and Rader 2005). There are multiple apolipoproteins that are associated with HDL (Table 1-4). Some of these exhibit enzymatic activity and facilitate maturation of the particle and enable RCT (Nofer *et al* 2002). Lecithin-cholesterol acyltransferase (LCAT) facilitates HDL maturation via extracellular cholesterol uptake and CE synthesis. Cholesteryl ester transfer protein (CETP) promotes redistribution of CE and TG between HDL and TRL in the circulation, depleting CE and enriching TG in the HDL particle (Lewis and Rader 2005). Scavenger receptor (SR)-BI facilitates removal of lipids from HDL particles and has high affinity for CE and free cholesterol (Ginsberg *et al* 2005, Lewis and Rader 2005).

1.5.2 Insulin resistance in PCOS

Insulin resistance affects approximately 30% of lean and 70% of overweight women with PCOS (Randeva *et al* 2012), and obesity exacerbates insulin insensitivity or glucose intolerance in PCOS (Dunaif 1999). Impaired insulin-glucose metabolism is observed in PCOS under fasting and non-fasting conditions, and incidence of T2D in women with PCOS is 5 to 10-fold higher compared to women without PCOS (Elting *et al* 2001, Ovalle *et al* 2002). It has been suggested that lean PCOS subjects may exhibit normal fasting insulin and glucose levels, but have glucose intolerance following an oral glucose tolerance test (Legro *et al* 1999, Salley *et al* 2007). It has been suggested that the severity of the PCOS phenotype (Table 1-2) is related directly to the presence of IR (Panidis *et al* 2011). Women who exhibit oligo-/anovulation and/or have polycystic ovaries (phenotypes I & II) are more likely to have hyperinsulinemia and have an exacerbated or elevated insulin response to a glucose tolerance test independent of their body weight (Panidis *et al* 2011). A study in adolescent girls with PCOS who exhibited an increased glucose response to an oral glucose tolerance test (OGTT), were shown to exhibit increased

hepatic glucose production using isotopic glucose infusion method, decreased insulin secretion and decreased glucose clearance under hyperinsulinemic–euglycemic clamp conditions (Arslanian *et al* 2001). Other studies have shown that insulin resistant women with PCOS tend to have inappropriate beta-cell compensation in response to glucose infusions or a meal tolerance test. They also tend to have a reduced postprandial incremental insulin response to a glucose challenge is suggestive of inadequate beta-cell function and insulin secretion (Dunaif *et al* 1996, Ehrmann *et al* 1995, Panidis *et al* 2012). Reduced peripheral insulin sensitivity has also been suggested in PCOS, as indicated by a decrease in GLUT-1 and GLUT-4 expression in cultured muscle cells and adipocytes isolated from women with PCOS (Rosenbaum *et al* 1993, Corbould *et al* 2005). As a result, women with PCOS may have decreased sensitivity and/or responsiveness to insulin. It has been reported that insulin receptor post-binding defects impairing insulin action may be present in PCOS. Excessive serine phosphorylation of insulin receptor down-stream signaling proteins such as insulin receptor substrate (IRS) 1, and decreased PI3K/AKT activity has been reported in cultured adipocytes and muscle cell biopsies isolated from PCOS women (Baptiste *et al* 2010, Venkatesan *et al* 2001). Cultured skeletal muscle cells and myotubes from obese women with PCOS have exhibited signaling defects with increased serine rather than tyrosine phosphorylation of IRS1 and 2 in response to insulin leading to a decrease in insulin-mediated cellular glucose uptake (Corbould *et al* 2005) (Figure 1-6). In addition, it has been demonstrated in primary rat myotubes that low and high-dose testosterone exposure induces IRS-1 phosphorylation at $Ser^{636/639}$ residues, and decreases PI3K activity, therefore impairing insulin signaling in the muscle (Allermand *et al* 2005, 2009).

The prevalence of hypertension is nearly 3-fold higher in PCOS women compared to the general female population and has been associated with insulin resistance (Elting *et al* 2001). Hyperinsulinemia may directly exacerbate blood pressure by enhancing sodium retention in the kidneys via increased sympathetic nervous system activity (Reaven *et al* 1996, Sowers *et al* 1994), and suppression of nitric oxide secretion through PI3-kinase and Akt phosphorylation pathways (Zeng *et al* 2000). It has been shown that insulin resistance is associated with endothelial dysfunction, increased vascular stiffness and early atherosclerosis risk in both lean

and obese PCOS women (Christopher *et al* 2002, Tarkun *et al* 2004). IR is a major factor in increasing predisposition to transition to T2D (Norman *et al* 2001), and increases cardiovascular disease risk in women with PCOS (Sam *et al* 2003, Bickerton *et al* 2005).

1.5.3 Hyperandrogenemia and endothelial and cardiac dysfunction

Hyperandrogenemia affects up to 80% of women diagnosed with PCOS (Shroff *et al* 2007), and the direct and long term effects of hyperandrogenemia on cardiovascular disease risk in PCOS is limited. Ferrer and colleagues have shown that supraphysiological doses of androgens impair endothelium-dependent and independent dilatation of aortic rings in rabbits (Ferrer *et al* 1994). PCOS-prone JCR:LA-*cp* rats with hyperandrogenemia and insulin resistance have impaired vascular function and develop atherosclerotic disease later in life (Dolphin *et al* 1990, O'Brien *et al* 2000). It has been shown that high doses of testosterone (200-800 mg/mo) in women who are undergoing transsexual transformation are associated with increased arterial diameter and decreased vascular reactivity to nitroglycerine stimulation (McCredie *et al* 1998).

It is well known that generally females are thought to be protected from early CVD development due to estrogen's protective actions on endothelium-dependent (assessed via endothelium ability to release nitric oxide and cause vasodilation in response) and independent (in response to nitroglycerine, NO donor, infusion) responses of systemic arteries without atherogenic risk factors (Sader *et al* 2002). Administration of exogenous estrogens in males and post-menopausal females has been associated with beneficial effects on endothelial function, such as arterial reactivity and flow-mediated dilation, and this effect remains significant after adjusting for plasma lipid concentrations (Gerhard *et al* 1998, McCrohon *et al* 1996, 1997). However, it is important to note that androgens can be converted intracellularly to both, DHT and estradiol, which may result in androgenic and estrogenic actions (Sader *et al* 2002). However, more research is needed to assess the mechanisms and effect of hyperandrogenemia on atherogenic blood lipid profile and cardiovascular function and CVD risk in PCOS.

1.5.4 Dyslipidemia in PCOS

Dyslipidemia appears to be a consistent cardiometabolic risk factor in PCOS, affecting more than 70% of overweight-obese and 30% of lean patients (Rocha *et al* 2011). BMI is significantly associated with an atherogenic lipid profile, including increased total cholesterol (TC), TG and LDL-C, and decreased HDL-C levels (Rocha *et al* 2011). A recent meta-analysis by Wild and colleagues showed women with PCOS have increased fasting plasma TG, total cholesterol and non-HDL-C levels regardless of their body mass index, putting them at a higher risk for cardiovascular disease (Wild *et al* 2011). Other studies report the association of insulin resistance, hyperandrogenemia and altered HDL serum profile, with decreased HDL_{2a} and increased HDL_{3c}, subclasses as well as increased plasma TG levels (Legro *et al* 1999). It has ben shown that PCOS women tend to have higher concentrations of small dense LDL-C (class III and IV) particles, which are considered to be readily taken up by the arterial wall and of higher atherogenicity compared to larger LDL-C particles (Berneis *et al* 2007).

Non-fasting or postprandial hyperlipidemia, and increased apoB-cholesterol remnant lipoproteins (chylomicron remnants and LDL) are now implicated in increased CVD risk and end-stage coronary heart disease events (Karpe *et al* 1995, Frieberg *et al* 2008, Varbo *et al* 2013). Impaired postprandial clearance of intestinal chylomicron (apoB48)-lipoproteins has been recognized as an independent risk factor for atherogenesis (Sharrett *et al* 2001, Tomkin and Owens 2012, Vine *et al* 2008). It has been shown that women with PCOS have an impaired TG and apoB clearance from plasma following a high fat meal (Bahceci *et al* 2007, Velazquez *et al* 2000) (Table 1-5). Following a high fat meal women with PCOS have elevated postprandial TG and total cholesterol response compared with weight and age-matched controls, with increased area under the curve $(AUC)_{TG}$, AUC_{TChol}, $AUC_{VLDL\text{-}C}$ and AUC_{ApoB} (Bahceci *et al* 2007). Delayed clearance of TRLs (VLDL and CM), is associated with increased circulating concentrations of cholesterol-dense apoB48 chylomicron remnants and apoB100-LDL, and the consequent increased uptake of these remnants by the arterial wall (Alipour *et al* 2008, Mangat *et al* 2007, Su *et al* 2009, Vine *et al* 2008). However, apoB48-CMr have been implicated to have greater atherogenicity due to increased propensity to deliver two fold more cholesterol to the

subendothelium, and these particles have impaired efflux from the arterial wall compared to apoB100-LDL particles (Mamo *et al* 1998, Mangat *et al* 2007, Proctor *et al* 2004). The pathogenesis of dyslipidemia in PCOS remains unclear and there is potential involvement of many risk factors related to obesity, insulin resistance and hyperandrogenemia (Diamanti-Kandarakis *et al* 2007, Wild *et al* 2011, 2012).

Table 1-5. Studies reporting the incidence and type of dyslipidemia in PCOS.

Reference	# PCOS subjects	Hyper-androgenemia	Insulin/glucose levels	Fasting lipids	Non-fasting lipids
Rocha et al 2011	124	↑TT	↑Ins, ↑HOMA-IR	↔TG, ↔TC, ↔LDL-C, ↓HDL-C	-
Wild et al 1985	29	↑TT, ↑FT, ↑DHEAS, ↓SHBG	-	↑TG, ↔TC, ↔LDL-C, ↓HDL-C	-
Legro et al 1999	16	↑TT	↔Ins	↔TG, ↔TC, ↔LDL-C, ↔HDL-C (↓HDL_{2a}, ↑HDL_{3c}), ↓apoAI, ↔apoB	-
Von Eckardstein et al 1996	26	↑TT	-	↑TG, ↑TC, ↑LDL-C, ↓HDL-C, ↔apoAI, ↔apoB	-
Legro et al 2001	195	↑TT, ↑FT	↑Ins[1]	↑TG[1], ↑TC, ↑LDL-C, ↓HDL-C[1]	-
Carmina et al 2005	290	↑TT, ↑FT	↑Ins	↑TG, ↑TC, ↑LDL-C, ↓HDL-C	-
Berneis et al 2007	30	-	↑Ins	↑TG, ↔TC, ↔LDL-C, ↓HDL-C	-
Valkenburg et al 2008	557	↑TT, ↓SHBG	↑Ins	↑TG, ↑TC, ↑LDL-C, ↓HDL-C, ↓apoAI, ↔apoB	-
Roa Barrios et al 2009	62	-	↑HOMA-IR, ↑Glc	↑TG, ↑LDL-C, ↔HDL-C	-
Macut et al 2001	29	-	↑Ins, ↑HOMA-IR	↑TG, ↑TC, ↑LDL-C, ↔HDL-C, ↑apoB	-
Velazquez et al 2000	18	↑FT	↑Ins, ↑AUC_{Ins}	↑TG, ↑TC[1], ↓HDL-C	↑AUC_{TG}, ↔AUC_{HDL-C}
Bahceci et al 2007	20	↔FT, ↑DHEAS, ↓SHBG	↑Ins, ↑HOMA-IR, ↔AUC_{Ins}, ↔AUC_{glc}	↑TG, ↔TC, ↑VLDL-C, ↑apoB	↑AUC_{TG}, ↑AUC_{TChol}, ↑AUC_{VLDL-C}, ↑AUC_{ApoB}, ↔AUC_{LDL-C}, ↔AUC_{HDL-C}, ↔AUC_{ApoA1}

[1] Improvement in these parameters occurred in obese patients only. A, androstenedione; DHEAS, dehydroepiandrosterone; E2, 17β-estradiol; FT, free testosterone; SHBG, sex hormone-binding globulin; TT, total testosterone; AUC, area under the curve; TG, triglycerides, TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol

1.5.4.1 Insulin resistance and dyslipidemia in PCOS

It has been shown that PCOS women with IR have an atherogenic plasma lipid profile as opposed to their insulin-sensitive counterparts. They tend to have higher plasma TG, total cholesterol and lower HDL-C levels (Kalra *et al* 2006, Slowinska-Srezednicka *et al* 1991, Wild *et al* 2010, 2011, 2012). This association remains significant after correcting for body weight, and non-obese women with PCOS and IR tend to have higher plasma lipid levels compared to healthy normal weight women (Talbott *et al* 2001). In the insulin resistant state, dyslipidemia is driven by multiple mechanisms (Figure 1-8). In normal physiology, insulin acts to inhibit ATGL and HSL activity in adipocytes, however in insulin resistance this effect is diminished leading to increased lipolysis and free fatty acid (FFA) release form adipose tissue (Kershaw *et al* 2006, Yu *et al* 2005, Zechner *et al* 2005). Increased substrate FFAs become available for hepatic TG synthesis leading to increased VLDL production (Ginsberg *et al* 2005) (Figure 1-8).

Intestinal and hepatic *de novo* lipogenesis is upregulated in IR leading to increased secretion of both chylomicrons and VLDL (Duez *et al* 2006, Veilleux *et al* 2012, Vine *et al* 2007). Insulin activates liver X receptor (LXR)-dependent fatty acid synthesis occurring through activation of sterol response element-binding protein (SREBP)-1c and increase in fatty acid synthase (FAS) expression (Horton *et al* 2002, Kabashima *et al* 2003, Gavroliva *et al* 2003). Increased SREBP-1c and FAS activation drive FFA and TG synthesis, increasing substrate availability for TRL assembly and secretion in the liver and intestine (Adeli and Lewis 2008, Lewis *et al* 2004, Shojaee-Moradie *et al* 2013, Xiao *et al* 2011). MTP was shown to be upregulated in the hepatocytes and enterocytes in insulin resistance, therefore contributing to increased biosynthesis of these lipoproteins (Adeli and Lewis 2008, Federico *et al* 2006). It has been shown that TRL particles, enriched in TG and depleted in CE, are prevalent in insulin resistant individuals (Austin *et al* 1990, Krauss *et al* 2004). LPL activity is also reduced in IR leading to reduced and prolonged lipolysis of VLDL and CM particles in plasma (Panarotto *et al* 2002), however to date there are no studies that have examined LPL, AGTL or HSL activity in women with PCOS.

Overall, increased lipoprotein particles in plasma contributes to an increase and prolonged residency of in remnant particles in the circulation, CMr and LDL, increasing the risk for arterial wall uptake in the insulin resistant state (Alipour *et al* 2008, Mangat *et al* 2007, Proctor *et al* 2002). Federico and colleagues have shown that diet-induced insulin insensitivity in hamsters resulted in overproduction of apoB48 and SREBP-1c activation under fasting and non-fasting conditions, which was proposed to be a result of upregulation of the ERK pathway (Federico *et al* 2006). It has recently been demonstrated that insulin stimulates apoB degradation in the liver acting through phosphatidylinositide-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK)/extracellular-regulated kinase (ERK) pathway activation, and therefore acts to inhibit or decrease VLDL secretion, and this apoB-degradation is impaired in insulin resistance (Allister *et al* 2008, Sparks *et al* 2013). Although these mechanisms of insulin resistance contributing to dyslipidemia are promising and could explain PCOS-associated insulin resistance and its role in dyslipidemia, there has been a limited number of studies exploring these mechanisms in PCOS (Lo *et al* 2006, Wild *et al* 2011). It has been shown that certain apoE and apoC protein alleles are linked to increased plasma lipids and apoB-lipoproteins in PCOS (Cetinkalp *et al* 2009, Huang *et al* 2010) due to decreased LPL activation and slower lipoprotein removal form plasma via LDLR (Table 1-4).

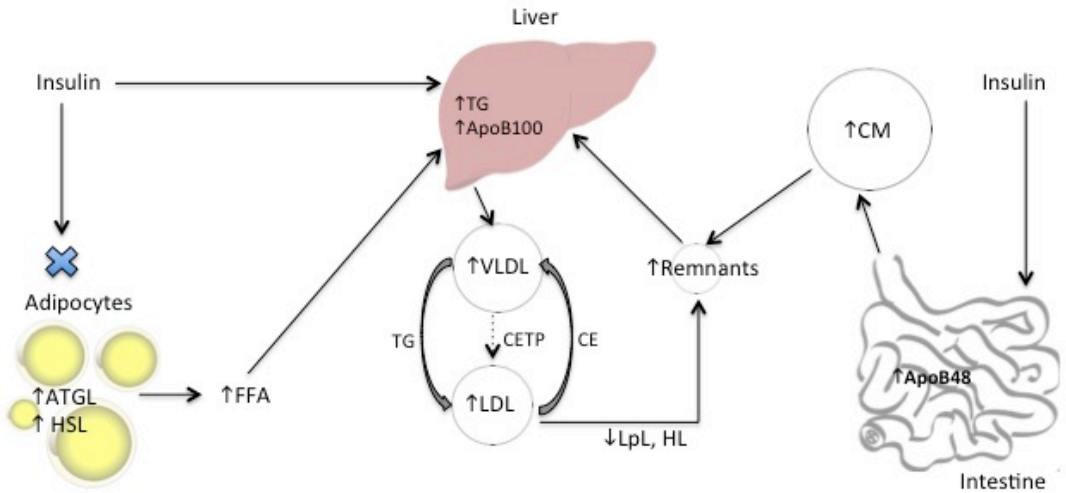


Figure 1-8. Lipoprotein metabolism in insulin resistance. ATGL, adipocyte triglyceride lipase; CE, cholesterol esters; CETP, cholesterol ester transfer protein; CM, chylomicron; FC, free cholesterol; FFA, free fatty acids; HDL, high-density lipoprotein; HL, hepatic lipase; HSL, hormone sensitive lipase; LPL, lipoprotein lipase; LCAT, lecithin-cholesterol acyltransferase; LDL, low-density lipoprotein; TG, triglycerides; VLDL, very low-density lipoprotein.

1.5.4.2 Hyperandrogenemia and dyslipidemia in PCOS

The prevalence of dyslipidemia is higher in women who have hyperandrogenemia compared to those with normal androgen concentrations in PCOS (Carmina *et al* 2005, Wiltgen *et al* 2010), and atherogenic changes in lipid profile are more pronounced in PCOS women with higher serum androgen concentrations (Valkenburg *et al* 2008). It has been shown that hyperandrogenemia in post-menopausal women is associated with a higher atherogenic index of plasma (AIP), calculated based on plasma TG and HDL-C concentration, and this association remains significant after adjusting for BMI and insulin resistance (Lambrinoudaki *et al* 2006). AIP has been associated with higher risk for coronary artery disease and significant carotid atherosclerosis in post-menopausal women (Mudali *et al* 2005). Evidence from studies in rats, sheep and rhesus monkeys suggest that androgen over-exposure during fetal development and early in life leads to impaired insulin-glucose and MetS in adulthood featuring elevated insulin and glucose (Abbott *et al* 1998, Alexanderson *et al* 2007, Demissie *et al* 2008, King *et al* 2007). It has been shown that fetal androgen overexposure may contribute to visceral adiposity and insulin resistance later in life, which could lead to the development of dyslipidemia (Abbott *et al* 2008). Central adiposity is proposed to cause increased release of FFA from adipocytes and inflammatory cytokines, which then may induce both reproductive and metabolic disturbances in PCOS (Mai *et al* 2006, Mlinar *et al* 2007, Kelly *et al* 2001). Androgens are proposed to stimulate lipolysis in adipocytes and release of FFA by increasing the number of beta-adrenergic receptors on the surface (De Pergola *et al* 2000). The androgen receptor (AR) mRNA and protein expression is more abundant in visceral compared to subcutaneous adipose tissue in humans (Rodriguez-Cuenca *et al* 2005), suggesting that visceral adiposity may contribute to increased FFA release under conditions of hyperandrogenemia in PCOS.

Evidence from cancer cell lines suggests androgens via AR receptor interaction with key regulatory proteins such as SREBP1c, SREBP2, LXR, SREBP cleavage activation protein (SCAP) and fatty acid synthase (FAS) may upregulate lipid synthesis in the intestine and liver of hyperandrogenemic PCOS subjects (Figure 1-9). However, exploration of the interactions between AR and lipogenic genes has been limited to cell culture studies in cancer cell lines

(Heemers *et al* 2006, Krycer *et al* 2011). Although clinical studies have found a strong correlation between high FT, FAI, and high plasma TG and low HDL-C levels (Carmina *et al* 2005, Fruzzetti *et al* 2009, Valkenburg *et al* 2008), the molecular mechanisms of how hyperandrogenemia and AR activation may contribute to dyslipidemia in PCOS has not been investigated in PCOS.

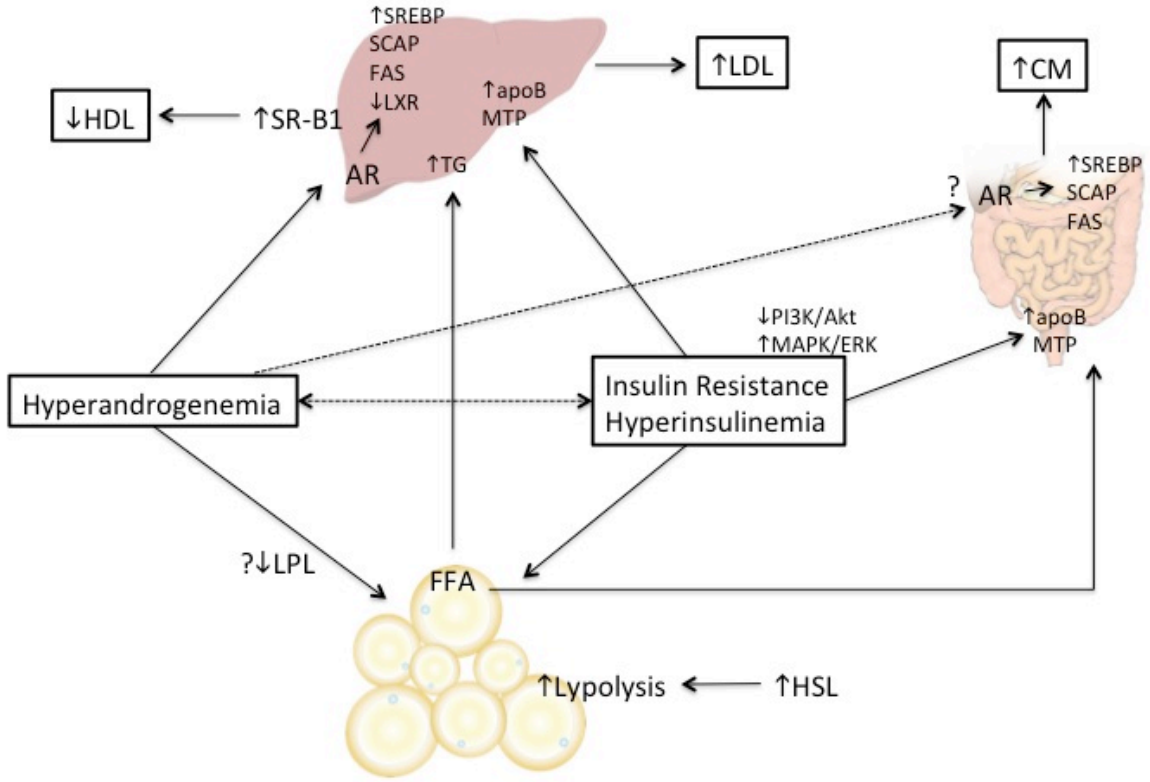


Figure 1-9. Possible mechanisms of dyslipidemia in PCOS. Insulin resistance and hyperandrogenemia induce lipolysis in adipocytes leading to increased free fatty acid (FFA) release into plasma, increasing substrate availability for triglyceride (TG) synthesis in the liver and intestine for triglyceride-rich lipoprotein assembly. Hyperandrogenemia upregulates lipogenic genes in liver and intestine through androgen receptor (AR) activation, and this may increase very low-density lipopretein (VLDL) and chylomicron (CM) synthesis. Insulin resistance is shown to upregulate apoB and MTP protein expression in liver and intestine. Abbreviations: Akt, protein kinase B; ERK, extracellular signal-regulated kinase; FAS, fatty acid synthase; HDL, high-density lipoprotein; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; LXR, liver X receptor; MAPK, mitogen activated protein kinase; MTP, microsomal triglyceride transfer protein; PI3K, phosphoinositide 3-kinase; SCAP, SREBP cleavage activating protein; SR-B1, scavenger receptor b1; SREBP, sterol regulatory element-binding protein. (Adapted from Diamanti-Kandarakis *et al* 2007).

1.6 Therapeutic approaches in PCOS

Various therapeutic approaches have been used to treat metabolic and reproductive aberrations associated with PCOS. PCOS is a highly heterogeneous disorder and finding a suitable treatment option is difficult because many factors play a role in the development of the syndrome (Norman *et al* 2002, Moran *et al* 2006). Various approaches such as lifestyle and dietary modifications, insulin sensitizers and lipid-lowering agents have been used to manage endocrine-reproductive aberrations and lower CVD risk in women with PCOS.

1.6.1 Diet and lifestyle modification

Lifestyle modifications are recommended as the first line therapy for all overweight and obese women diagnosed with PCOS (Wild 2012). Overweight status worsens underlying metabolic disturbances, therefore improvement in body weight is deemed beneficial in attenuating endocrine and metabolic abnormalities in PCOS (Moran *et al* 2011). Weight loss achieved through dietary and lifestyle modifications has been shown to improve insulin resistance and reproductive indices such as ovulation and fertility (Moran *et al* 2011), however moderate to severe caloric restriction and vigorous exercise regimes are difficult to sustain long-term (Norman *et al* 2002). Recent data demonstrates that concurrent use of dietary and exercise approaches are more beneficial to endocrine-reproductive profile in PCOS (Genazzani *et al* 2014, Haqq *et al* 2014). Interventions with caloric restriction (by 500-1000 kcal) and regular exercise (walking, cycling, weigh resistance exercises) for a period of 3-12 months demonstrated significant improvements in FSH, SHBG, total T and free androgen index (FAI) (Haqq *et al* 2014). However, approximately 30-60% of PCOS patients do not respond or have no improvements in cardiometabolic risk with dietary or exercise interventions regardless of weight loss, and it has been suggested that PCOS patients are inherently resistant to shifts or improvements in metabolism (Pasquali *et al* 2010).

In terms of lipid metabolism, a recent Cochrane review reported no evidence that lifestyle modification is effective at attenuating lipid abnormalities in PCOS (Moran *et al* 2011, Marsh *et al* 2010, Stamets *et al* 2004). Some dietary interventions, such as hypocaloric diets and diets

with modified macronutrient distribution, reduced fasting plasma lipids such as TG, total cholesterol, LDL-C and HDL-C (Moran *et al* 2003, Thomson *et al* 2008), although these effects are not maintained during the weight maintenance phase (Moran *et al* 2003). Even though lifestyle modification has been shown to improve a few parameters of metabolic abnormalities in PCOS, there is some evidence that other therapeutic approaches need to be applied because not all patients benefit from the lifestyle intervention. Weight loss therapies, inclusive of diet and exercise, were shown to be more beneficial in targeting plasma lipids in conjunction with pharmaceutical interventions such as insulin sensitizers, anti-androgens and oral contraceptives (Gambineri *et al* 2006, Hoeger *et al* 2004, 2008, Tang *et al* 2005). It has been shown that PCOS prevalence among regularly exercising lean individuals is approximately 9%, and are in need for alternative treatments (Khademi *et al* 2010).

1.6.2 Insulin sensitizer use in PCOS

Insulin resistance plays a critical role in the development of PCOS and is present in both lean and obese women affected by the syndrome. IR has been associated with an increased risk of T2D, hypertension, dyslipidemia, endothelial dysfunction and early cardiovascular disease (Sharma *et al* 2006). In both adolescent and adult women, Insulin sensitizing drugs are aimed to improve PCOS-associated insulin resistance, restore normal ovulatory cycles and fertility (Geller *et al* 2011, Sharma *et al* 2006). There are two major categories of the insulin-sensitizing agents – biguanides (metformin) and thiazolidinediones.

1.6.2.1 Thiazolidinediones

This class of insulin-sensitizing medications, thiazolidinediones, ameliorates insulin resistance through agonizing peroxisome proliferator-activated receptor gamma (PPARγ) therefore increasing insulin sensitivity in adipocytes and restoring adipocyte glucose uptake (Geller *et al* 2011). Pioglitazone and rosiglitazone have been shown to decrease fasting and postprandial insulin concentrations, and improve hirsutism, menstrual cyclicity, serum FT and SHBG (Ortega-Gonzalez *et al* 2005, Tarkun *et al* 2005). However, these treatments seem to have an adverse effect on body weight and waist circumference, and do not improve atherogenic dyslipidemia

(Tarkun *et al* 2005). Despite proven efficacy in improving hyperinsulinemia and reproductive indices, most of the thiazolidinediones were removed from the market due to hepatotoxicity and adverse cardiovascular health outcomes such as increased risk of congestive heart failure (Lago *et al* 2007, Geller *et al* 2011). Pioglitazone is currently considered safer for use compared to other thiazolidinediones (Tolman 2011), although it is not advisable to use in adolescents or young women of reproductive age in PCOS (Geller *et al* 2011).

1.6.2.2 Metformin

Metformin, a biguanide, has been used for decades to treat insulin resistance and compensatory hyperinsulinemia. Clinically, metformin is commonly used to lower fasting glucose and insulin concentrations, as well as insulin and glucose response to an oral glucose tolerance test in women with PCOS (Lord *et al* 2003, Tang *et al* 2012). Metformin acts through multiple mechanisms to ameliorate insulin resistance. It is proposed to decrease intestinal glucose absorption, suppress gluconeogenesis and hepatic glucose secretion, and to improve insulin sensitivity in peripheral tissues (Bargiota *et al* 2012). Metformin has been shown to lower de-novo glucose production by up to 30% (Stumvoll *et al* 1995). Metformin is proposed to inhibit mitochondial respiratory-chain complex 1 resulting in increased AMP-to-ATP ratio (Viollet *et al* 2012). Increased binding of AMP to gamma subunit of AMP-activated protein kinase (AMPK) results in the enzyme activation, and AMPK serves as a major regulator of the energy metabolism as it restores intracellular ATP levels by switching off catabolic pathways such as gluconeogenesis and lipogenesis (Palomba *et al* 2009). Activated AMPK upregulates small heterodimer partner (SHP), which inhibits cAMP response element binding protein (CREB), therefore down-regulating gluconeogenic enzyme transcription (Kim *et al* 2008). In addition, it has been shown that AMPK leads to insulin receptor and downstream insulin receptor substrate 1 and 2 activation (Cheng *et al* 2006, Chopra *et al* 2012). Cell culture studies in adipocytes and skeletal muscle cells demonstrate an upregulatory effect of metformin on glucose transporter membrane translocation (Matthaei *et al* 1992, Sarabia *et al* 1992). Thus, metformin increases glucose uptake and oxidation in skeletal muscles, the major glucose user in the body, therefore improving whole-body insulin sensitivity (Bailey and Turner 1996). A

recent meta-analysis of 38 clinical trials reported beneficial effect of metformin on reproductive-endocrine indices such as ovulation, menstrual cyclicity and clinical pregnancy rate in PCOS (Tang *et al* 2012). As mentioned in preceding sections, hyperinsulinemia may up-regulate androgen production in the ovaries and adrenal gland in PCOS. Therefore, increasing insulin sensitivity with metformin treatment and reducing circulating insulin levels may ameliorate PCOS-associated hyperandrogenemia. Clinically, metformin has been shown to lower serum total T, but did not improve SHBG concentration. In some studies, metformin improved one or more of FAI, total T and SHBG (Aghahosseini *et al* 2010, Ehrmann *et al* 1997, Sahin *et al* 2007, Velazquez *et al* 1994), but failed to do so in others (Harborne *et al* 2005, Moghetti *et al* 2000, Singh *et al* 2010) (Table 1-6).

Metformin has been shown to have no effect or to inconsistently reduce fasting plasma TG, total cholesterol, LDL-C and HDL-C in a few studies, as outlined in Table 1-6 (Tang *et al* 2012). Generally, the study cohort in reports to date are variable due to age of patients, overweight status, the treatment regimen and diagnostic criteria used, therefore the results have been mixed. Some studies report that overweight women with PCOS respond better to the metformin treatment (Aleyasin *et al* 2011, Banaszewska *et al* 2006) but other studies report no difference (Maciel *et al* 2004). Overall, if there is an improvement in plasma total testosterone and/or SHBG concentrations and insulin-glucose parameters, there appears to be a greater improvement in plasma lipids, supporting the involvement of insulin resistance and hyperandrogenemia in potentiating dyslipidemia in PCOS.

Metformin's action to improve lipoprotein metabolism has been proposed to be through induction of hepatic free fatty acid oxidation, thus decreasing free fatty acid availability for TG synthesis, at least in diet-induced hypertriglyceridemic rats. This process most likely occurs through AMPK activation and the induction of fatty acid oxidation pathways (Tessari *et al* 2008). Metformin has also been shown to decrease exocytosis of apoB-lipoprotein particles through AMPK activation as shown by hepatic human tumor cell line studies (Puljak *et al* 2008). Hepatocyte cell culture and diabetic rat studies have shown that metformin suppresses acetyl-CoA carboxylase activity (ACC) leading to decrease in fatty acid synthesis and thus TG synthesis

(Cleasby *et al* 2004, Zang *et al* 2004). In addition, metformin has been shown to down-regulate SREBP-1c gene expression via AMPK phosphorylation at Ser^{372} residue (Li *et al* 2011, Zhou *et al* 2001), therefore reducing *de novo* lipidogenesis in the liver, lowering plasma TG, TC and LDL-C as well as reducing atherosclerotic leasion areas in LDLR knock-out mice (Li *et al* 2011).

Table 1-6. Summary of studies on the effect of metformin on endocrine profile, insulin-glucose metabolism and plasma lipids in PCOS.

Study	Participants and treatment	Endocrine profile	Insulin and glucose	Plasma lipids
Velazquez et al, 1994	N=29 1000 mg (8 wks)	↓LH, TT, FT, DHEAS, FAI ↑FSH	↔AUC_{Glc}, ↓AUC_{Ins}	↔TG, TC, HDL-C, LDL-C, apoB
Moghetti et al, 2000	N=23 1000 mg (6 mo)	↓FT, ↔LH, FSH, A, DHEAS, SHBG	↔Glc, Ins, AUC_{Glc}, AUC_{Ins}	↔TG, TC, LDL-C, ↑HDL-C
Ng et al, 2001	N=35 1000 mg (3 mo)	↔LH, FSH, A, DHEAS, SHBG	↔Glc, Ins, AUC_{Glc}	↔TG, TC, HDL-C, LDL-C ↓wt
Fleming et al, 2002	N=39 1700 mg (14 wks)	↔TT, ↑E2	↔Glc, Ins, AUC_{Glc}	↔TG, TC, LDL-C, VLDL-C, ↑HDL-C
Chou et al, 2003	N=30 500 mg (3 mo)	↓TT, ↔LH, FSH, SHBG	↔Ins, HOMA-IR	↔TG, HDL-C, LDL-C ↓TC
Maciel et al, 2004	N=29 1000 mg (6 mo)	↓TT, FT, A, DHEA ↔LH, FSH	↔Glc, AUC_{Glc} ↓Ins, AUC_{Ins}	↔TG, TC, HDL-C, LDL-C
Harborne et al, 2005	N=83 1500 mg (8 mo) 2550 mg (8 mo)	↓A, ↔TT, SHBG, FIA	↔Glc, ↓Ins (2550 mg), ↔HOMA-IR	↔TG, ↓TC, LDL-C ↑HDL-C (2550 mg)
Lord et al, 2006	N=40 1000 mg (3 mo)	↔TT, LH, FSH, A, DHEAS, FAI, SHBG	↔Glc, Ins, HOMA-IR	↔TG, HDL-C, ↓TC, LDL-C
Aghahosseini et al, 2010	N=69 2500 mg	↓FT, ↑SHBG, ↔TT, A, DHEAS	↓Ins	↔TG, TC, HDL-C, ↓LDL-C
Karimzadeh et al, 2010	N=90 1500 mg (3-6 mo)	↔TT, ↑SHBG	↔Glc, AUC_{Glc}	↔HDL-C, LDL-C
Otta et al, 2010	N=30 1500 mg (4 mo)	↓TT, ↔LH, FSH, A, DHEAS	↓Ins, HOMA-IR ↔AUC_{Glc}, AUC_{Ins}	↔TG, TC, HDL-C, LDL-C
Romualdi et al, 2010	N=15 1000 mg (6 mo)	↓TT, ↔LH, FSH, A, DHEAS, FAI, SHBG	↔ AUC_{Glc}, AUC_{Ins}	↔TG, TC, HDL-C, LDL-C, FFA
Singh et al, 2010	N=44 500 mg (3 mo)	↔TT	↔Glc, ↓Ins, ↔AUC_{Glc}	↔TG, TC, VLDL ↓LDL-C, ↑HDL-C
Kocer et al, 2014	N=15 1700 mg (6 mo)	↓FT, DHEAS ↑SHBG, ↔TT, E2, LH, FSH, A	↓HOMA-IR	↓TG, TC, LDL-C ↑HDL-C ↔ApoB

A, androstenedione; DHEAS, dehydroepiandrosterone; E2, 17β-estradiol; FAI, free androgen index; FSH, follicle-stimulating hormone; FT, free testosterone; LH, luteinizing hormone; SHBG, sex hormone-binding globulin; TT, total testosterone; AUC, area under the curve; TG, triglycerides, TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol

1.6.3 Lipid-lowering medication use in PCOS

Atherogenic dyslipidemia is a major risk factor for CVD development, and it affects a large proportion of women with PCOS. Insulin-sensitizing drugs may alleviate disturbances in insulin-glucose metabolism and may improve serum testosterone and SHBG concentrations, but as shown above these medications do not consistently improve dyslipidemia in women with PCOS (Tang *et al* 2012). Statins, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, are used in dyslipidemic women with PCOS as cholesterol-lowering and anti-inflammatory therapies (Kodaman and Duleba 2008). In PCOS, statins have been shown to alleviate plasma total cholesterol, TG and LDL-C concentrations (Duleba 2012, Raja-Khan *et al* 2011), but adversely affect insulin sensitivity (Banaszewska *et al* 2007, Raja-Khan *et al* 2011). Furthermore statins are contraindicated for use in pregnancy or in women of reproductive age due to potential teratogenic effects (Godfrey *et al* 2012, Kazmin *et al* 2007). In addition, clinical studies with statins have reported adverse effects on insulin sensitivity, which can counterweight potential improvements in lipid metabolism (Banaszewska *et al* 2007, Raja-Khan *et al* 2011).

Oral contraceptives (OC) and anti-androgens have been used to improve cyclicity and androgen metabolism in PCOS. Oral contraceptive therapies may reduce LH secretion, adrenal and ovarian androgen production and increase hepatic SHBG production (Bargiota *et al* 2012). Despite a positive effect of OCs on endocrine profile in PCOS, potential negative effects on insulin-glucose and lipid metabolism have emerged. OCs has been shown to cause an impaired response to oral glucose tolerance tests and decrease insulin sensitivity in women with PCOS (Nader and Diamanti-Kandarakis 2007). A Cochrane review has concluded a significant effect of OC therapy on improving insulin-glucose metabolism, but OCs increase fasting plasma TG levels (Costello *et al* 2007). OC formulations, such as drospirenone and those containing more androgenic progestins, a low amount or no estrogens, have been linked to hypertriglyceridemia (Godsland *et al* 1990, Nader and Diamanti-Kandarakis 2007). Mastorakos and colleagues have shown that one-year desogestrel and ethinyl estradiol OC treatment negatively affects total cholesterol and LDL-C, whereas a combination of cyproterone acetate and ethinyl estradiol

tended to increase fasting plasma triglycerides in adolescent girls with PCOS (Mastorakos *et al* 2002). It has been shown that use of low-dose second- and third-generation OC formulations, containing progestins, drospirenone and cyproterone acetate may increases risk of cardiac and vascular arterial events such as myocardial infarctions and ischemic stroke in PCOS patients that are already at higher CVD risk (Baillargeon *et al* 2005, Carmina 2013). Therefore, if OC's are chosen as a course of treatment, careful selection of the type of oral contraceptive and monitoring the effects on plasma lipids, insulin-glucose metabolism and possibly long term CVD risk needs to occur.

1.6.3.1 Anti-androgens

Various anti-androgen therapies are used to lower plasma androgens, but they have different effects on lipid metabolism in PCOS (Bargiota *et al* 2012). Spironolactone, cyproterone acetate and flutamide are currently the most used anti-androgens in PCOS. The primary mechanism of action of these agents is to antagonize or inhibit the AR or to decrease androgen production and action in tissues (Bargiota *et al* 2012).

Spironolactone, a steroidal antimineralocorticoid, antagonizes androgen action by competitive inhibition of the androgen and mineralocorticoid receptor and reduces testosterone biosynthesis via inhibition of 5α-reductase enzyme (Bargiota *et al* 2012, Studen *et al* 2011). Spironolactone has been shown to reduce serum testosterone and DHEAS, and to reduce hirsutism scores in women with PCOS (Nakhjavani *et al* 2009). Spironolactone has been shown to adversely increase plasma LDL and decrease HDL cholesterol in PCOS (Nakhjavani *et al* 2009), and to increase plasma TG levels when used in combination with ethinyl estradiol/cyproterone acetate (Karakurt *et al* 2008). However, other studies have reported no effects of spironolactone treatment on blood lipid profile (Zulian *et al* 2008). Cyproterone acetate is also an effective antiandrogen, which has been shown to restore ovulation and reduce hirsutism in women with PCOS (Bargiota *et al* 2012). It has been shown to reduce plasma free and total testosterone, and increase SHBG concentration (Batukan *et al* 2007, Cagnacci *et al* 2003, Diamanti-Kandarakis *et al* 2003). Despite improvement in PCOS-associated

hyperandrogenemia, cyproterone acetate does not improve insulin-glucose and lipid metabolism (Armstrong *et al* 2000, Falsetti *et al* 2000).

Flutamide, a non-steroidal AR inhibitor with high selectivity, has been shown to reduce clinical and biochemical manifestations of hyperandrogenemia in PCOS (Bargiota *et al* 2012). Flutamide binds to the AR preventing binding to androgen response elements on target genes (Lu *et al* 1999). Clinically, flutamide has a neutral effect on insulin-glucose metabolism in PCOS (Diamanti-Kandarakis *et al* 1995) and does not improve insulin sensitivity (Table 1-7). However, flutamide has been shown to decrease fasting plasma TG, TC and LDL-C cholesterol, and to increase HDL-C in women with PCOS independent of their body weight (See Table 1-7) (Diamanti-Kandarakis *et al* 1998, Gambineri *et al* 2004, Ibanez et al 2000). However, the effect of flutamide on non-fasting lipids and apoB-lipoprotein metabolism is not well studied. The mechanism of androgen action on lipid metabolism in PCOS is poorly understood. Studies in prostate cancer cell lines have shown that androgen mediated AR activation can directly stimulate mRNA SREBP expression and activity, as well as SREBP cleavage activating protein (SCAP) leading to an upregulation of lipid synthesis (Heemers *et al* 2006). However the mechanisms of action of the AR antiagonist flutamide and effects on lipid and lipoprotein metabolism associated with hyperandrogenemia in PCOS remain unclear. Furthermore, flutamide is also contraintervened in young women and women of reproductive age planning to conceive due to potential teratogenicity (Kassim *et al* 1997, Ohbuchi *et al* 2009)

Table 1-7. Summary of changes in endocrine profile, insulin-glucose metabolism and dyslipidemia in PCOS with flutamide treatment.

Study	Participants and treatment	Endocrine profile	Insulin and glucose	Plasma lipids
Diamanti-Kandarakis et al, 1998	N=17 500 mg (12 wks)	↓A, DHEAS, SHBG ↔TT, FT, E2	↔Glc, Ins, AUC_{Glc}, AUC_{Ins}	↓TG, TC, LDL-C ↔HDL-C
Gambineri et al, 2004	N=40 500 mg (6 mo)	↓FT, TT, DHEAS, A ↔SHBG	↔Glc, Ins, AUC_{Glc}, AUC_{Ins}	↓TC, LDL-C ↔TG, HDL-C
Gambineri et al, 2006	N=80 500 mg (12 mo)	↓A, DHEAS ↔TT, FAI, SHBG	↔Glc, AUC_{Ins} ↓Ins, AUC_{Glc}	↓LDL-C ↔TG, HDL-C
Ibanez et al, 2000	N=18 250 mg (18 mo)	↓TT, FAI, DHEAS, A ↑SHBG ↔LH, FSH	↔Glc, Ins	↓TG, TC, LDL-C ↔HDL-C
Bahceci et al, 1999	N=30 750 mg (6 mo)	↓TT, FT, LH ↔SHBG, FSH	↔Glc, Ins	↓TC, LDL-C ↔TG, ↑HDL-C

A, androstenedione; DHEAS, dehydroepiandrosterone; E2, 17β-estradiol; FAI, free androgen index; FSH, follicle-stimulating hormone; FT, free testosterone; LH, luteinizing hormone; SHBG, sex hormone-binding globulin; TT, total testosterone; AUC, area under the curve; TG, triglycerides, TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol

1.6.3.2 Nicotinic acid

Nicotinic acid is a well-established lipid-lowering agent, which has been used for more than 50 years to treat atherogenic dyslipidemia and lower CVD risk (Altschul *et al* 1955). Another isoform of niacin, nicotinamide, does not exhibit the same lipid-lowering capacity (Bogan *et al* 2008, Carlson 1963), but has been shown to be effective in modulating pro-inflammatory cytokines (Yu *et al* 2007). Nicotinic acid has been associated with neutral or negative effects on insulin-glucose metabolism (Aye *et al* 2014, Goldberg *et al* 2000, Grundy *et al* 2002). Although other studies have shown a beneficial effect of nicotinic acid on adipocyte insulin sensitivity (Linke *et al* 2009, Plaisance *et al* 2009, Westphal *et al* 2008). Nicotinic acid therapy has been shown to reduce incidence of stroke and myocardial infarction as well as alleviate coronary artery revascularization in individuals with metabolic syndrome (Duggal *et al* 2010, Lavigne *et al* 2013). In addition, patients with coronary heart disease, T2D and MetS seem to benefit from niacin treatment due to reduction in carotid media thickness and restored endothelial function (Holzhauser *et al* 2011, Meyers *et al* 2004, Thoenes *et al* 2007, Wu *et al* 2010).

Despite niacin's use to target dyslipidemia for over half century, the mechanism of action of niacin is still under investigation. The hypolipidemic effects of niacin have been attributed to inhibition of the hepatic DGAT2 enzyme, to decrease TG synthesis and increase apoB degradation, therefore lowering lipidogenesis and apoB-lipoprotein secretion as shown by cell culture and animal studies (Ganji *et al* 2004, Jin *et al* 1999, Le Bloch *et al* 2010) (Figure 1-10). Furthermore, nicotinic acid has been shown to inhibit lipolysis in adipose tissue through activation of GPR109A receptor and to lower expression of hormone sensitive lipase, carnitine palmitoyl transferase 2 and fatty acid synthase (Karpe *et al* 2004, Linke *et al* 2009). This leads to decreased FFA release form adipose tissue and a limited pool of FFA for TG synthesis and VLDL assembly in the liver. Nicotinic acid also favorably affects HDL-C metabolism. It decreases hepatic apoAI catabolism without affecting apoAI *de novo* synthesis and increases HDL particle half-life (Jin *et al* 1997). In addition, nicotinic acid has been shown to decrease β-chain adenosine triphosphate synthase expression in hepatocytes, therefore reducing HDL particle uptake, but not SR-BI-mediated cholesterol ester uptake form plasma (Acton *et al* 1996,

Kamanna and Kashyap 2008). Furthermore hepatocyte cell culture studies have shown that niacin decreases Lp(A-I) particle uptake, which are deemed to be more efficient in cholesterol ester delivery than Lp(A-I+A-II) particles, therefore, niacin treatment increases Lp(A-I) particle retention in plasma and may favorably affect RCT (Sakai *et al* 2001). In addition to alleviating fasting dyslipidemia, nicotinic acid is effective at reducing postprandial TG response following high-fat meal consumption (Aye *et al* 2014, King *et al* 1994, Plaisance *et al* 2008). The use of nicotinic acid to treat dyslipidemia in PCOS has received limited investigation. To date, there is only one study published that assessed the effect of nicotinic acid treatment in women with PCOS. A 12-week therapy lowered fasting plasma TG, LDL-C, TC and reduced postprandial TG response to a 45%-fat meal (Aye *et al* 2014). Given niacin's potential to target fasting and postprandial lipemia, it becomes a promising potential treatment option for women with PCOS.

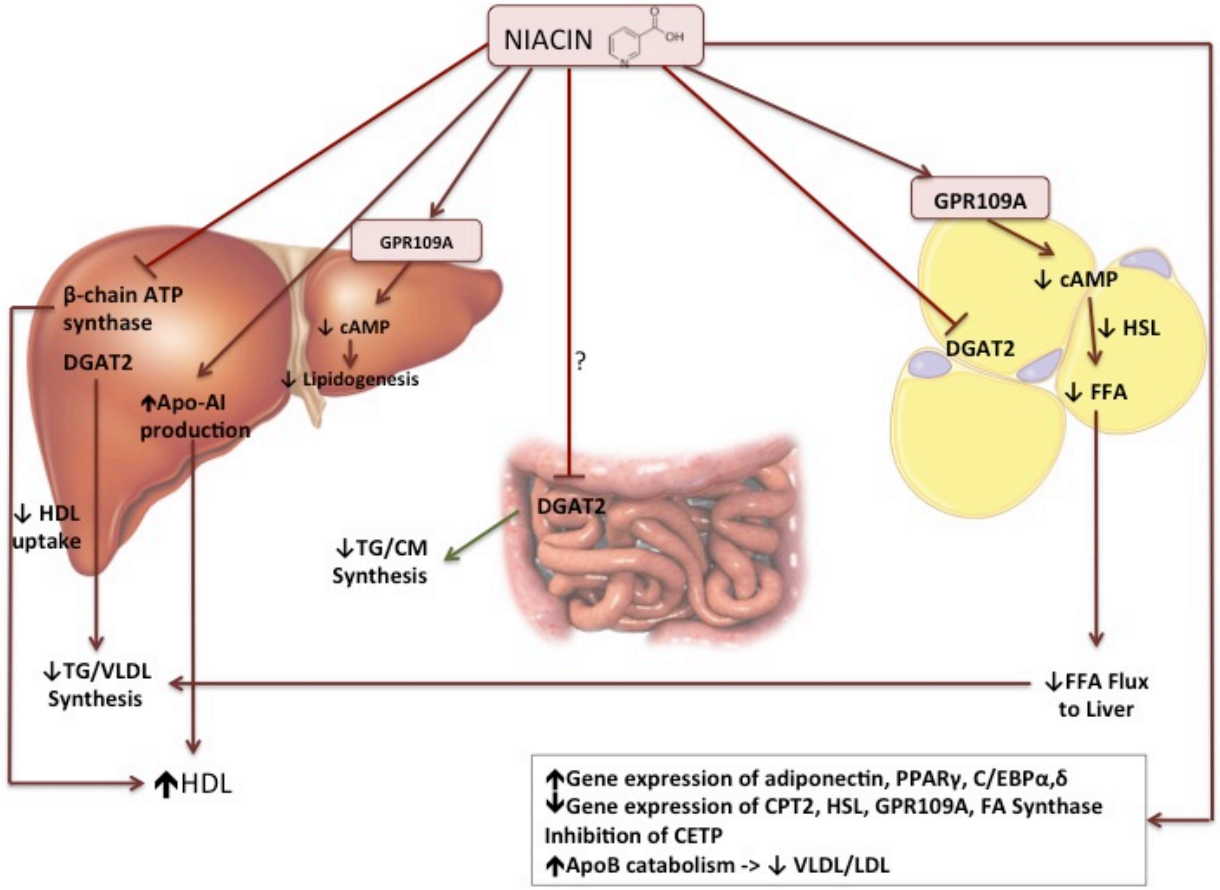


Figure 1-10. Proposed mechanisms of action of nicotinic acid in dyslipidemia. Nicotinic acid acts through its own receptor, G protein-coupled receptor (GPR) 109A in liver and adipose tissue and decreases cAMP levels, therefore decreasing free fatty acid (FFA) release form adipose tissue, limiting substrate availability for triglyceride (TG) synthesis and very low-density lipopretein (VLDL) assembly in hepatocytes. Nicotinic acid directly inhibits diglyceride acyltransferase (DGAT2) in the liver, adipose tissue and intestine. It has been shown to increase apoAI production and inhibit beta-chain ATP synthase, thus increasing plasma high-density lipoprotein (HDL) concentration. (Adapted from Kei and Elisaf 2012).

2. Study Rationale

2.1 Rationale

2.1.1 PCOS incidence and cardiometabolic risk

Polycystic ovary syndrome (PCOS) is recognized as the most common endocrine disorder in females causing anovulation and is often linked to several comorbidities (Norman *et al* 2007, Azziz *et al* 2009). There is heterogeneity in the clinical symptoms of PCOS including the reproductive-endocrine aberrations and cardiometabolic risk factors such as dyslipidemia and insulin resistance, which renders this disease a challenge to manage and treat. The metabolic syndrome and atherogenic dyslipidemia are common in PCOS, predisposing women to higher risk for the early development of type 2 diabetes (T2D) and cardiovascular disease (CVD). Dyslipidemia is the most common cardiometabolic abnormality in PCOS, affecting 70% of obese and 30% of lean individuals (Rocha *et al* 2011). These women tend to have higher fasting plasma TG, total cholesterol and LDL-C, and lower HDL-C compared to their healthy counterparts (Wild *et al* 2011). Non-fasting dyslipidemia associated with triglyceride rich apoB-lipoproteins and accumulation of apoB-remnant lipoproteins are recognized as independent risk factors for CVD (Varbo *et al* 2013, Tomkin and Owens 2012, Vine *et al* 2008). Furthermore, non-fasting dyslipidemia has been observed in adolescents and women with PCOS (Bahceci *et al* 2007, Velazguez *et al* 2000).

2.1.2 Etiological causes of PCOS and dyslipidemia in PCOS

The etiological causes of PCOS remain unclear, although underlying environmental and genetic factors contribute to the development of the syndrome and cardiometabolic risk (Diamanti-Kandarakis *et al* 2006a). The major pathophysiological factors are proposed to be hyperandrogenemia and insulin resistance (Diamanti-Kandarakis *et al* 2006b, 2007). Hyperandrogenemia affects the majority (80%) of women with PCOS and results from increased luteinizing hormone secretion, adrenal and ovarian testosterone secretion (Shroff *et al* 2007). Increased plasma testosterone concentrations in women have been shown to be an independent risk factor for decreased endothelial function, coronary artery disease and carotid

atherosclerosis (Mudali *et al* 2005, Sader *et al* 2002). Testosterone is positively correlated with an exacerbated plasma lipid profile in PCOS (Carmina *et al* 2005, Wiltgen *et al* 2010). Furthermore, testosterone-induced PCOS animal models develop dyslipidemia, in particular hypertriglyceridemia (Manneras *et al* 2009). In both in-vitro cell models and *ex vivo* studies in cancer tissues, androgen receptor (AR) activation by testosterone and dihydrotestosterone (DHT) has been shown to upregulate lipogenic genes (Krycer *et al* 2011, Heemers *et al* 2006, Swinnen *et al* 1996). However, the role of the AR in lipid metabolism, in particular in mediating dyslipidemic pathways in PCOS remains unknown. There have also been no studies to date assessing the regulation or expression of the AR and lipogenic genes in PCOS. To add to the complexity, it has been shown that insulin resistance is associated with atherogenic dyslipidemia in PCOS (Kalra *et al* 2006, Talbott *et al* 2001). Hyperinsulinemia has been shown to increase free fatty acid release from adipocytes, supplying substrates for increased TG synthesis in the liver and intestine (Ginsberg *et al* 2005). Various proteins such as SREBP1c, FAS, apoB and MTP are commonly overexpressed in insulin resistance, and these may be linked to MAPK/ERK pathway upregulation (Federico *et al* 2006, Horton *et al* 2002, Kabashima *et al* 2003, Gavroliva *et al* 2003). However, no studies to date have assessed the direct role of insulin and the expression of genes involved in insulin and lipogenic pathways in PCOS. Overall, the pathogenesis of dyslipidemia in PCOS remains unclear and there is potential involvement of many risk factors, in particular insulin resistance and hyperandrogenemia (Diamanti-Kandarakis *et al* 2007, Wild *et al* 2010, 2012). This forms the premise of the primary objective of this thesis to determine the physiological and mechanistic contribution of hyperandrogenemia and hyperinsulinemia to atherogenic dyslipidemia in PCOS.

2.1.3 Current therapeutic approaches to manage cardiometabolic risk and dyslipidemia in PCOS

To manage the metabolic and reproductive aberrations associated with PCOS, various therapeutic approaches are used. Lifestyle and dietary approaches are a first-line therapy and have been shown to improve body weight, hyperinsulinemia and fasting plasma lipids. However, these interventions are not effective in all patients and appear dependent on the PCOS phenotype (Moran *et al* 2011). In addition, PCOS patients are considered resistant to

these diet and lifestyle-induced shifts in metabolism and it is difficult to sustain these interventions long-term (Moran *et al* 2003, 2011, Pasquali *et al* 2010). Insulin sensitizers, such as metformin, and androgen receptor antagonists, such as flutamide, have been used to target hyperinsulinemia and hyperandrogenemia, respectively. Metformin has been shown to improve reproductive-endocrine indices, and plasma insulin and glucose levels, but it is not a consistently effective lipid-lowering agent in patients with PCOS (Tang *et al* 2012). Clinically, flutamide, an androgen receptor blocker, has been shown to improve hyperandrogenemia and fasting atherogenic dyslipidemia in PCOS, supporting the link between hyperandrogenemia and lipidogenesis in the syndrome (Bargiota *et al* 2012). However, there are no studies to date that have assessed the relationship between androgen receptor expression and activation, and lipogenic gene expression in the hyperandrogenemic and hyperinuslinemic conditions of PCOS. Using metformin and flutamide to modulate hyperinsulinemia and hyperandrogenemia, respectively, may give insight into the contribution of these factors to atherogenic dyslipidemia and the possible mechanisms involved in PCOS. In the specific objectives 1 and 2, metformin will be used to modulate hyperinsulinemia and AR function, respectively to determine the role of these pathways in insulin-glucose and lipid metabolism in PCOS.

Lipid-lowering drugs, such as statins, and androgen-receptor blockers such as flutamide, are not recommended as lipid lowering treatments in young women of reproductive age due to potential teratogenic and hepatotoxic effects (Godfrey *et al* 2012, Ohbuchi *et al* 2009). Therefore a safe dietary or nutrient approach to target atherogenic dyslipidemia in PCOS has been proposed. Nicotinic acid, a B vitamin, has been used clinically to treat dyslipidemia and improve cardiovascular function in patients with T2D, metabolic syndrome and established CVD (Canner *et al* 2006, Grundy *et al* 2002, Holzhauser *et al* 2011, Meyers *et al* 2004). However, to date there is only one study that has investigated the plasma lipid lowering effects of nicotinic acid in women with PCOS (Aye *et al* 2014). Nicotinic acid has been associated with neutral to negative effects on insulin-glucose metabolism, which may offset the potential of niacin to lower plasma lipids. However, slow release formulas have been shown to have no adverse effects on insulin-sensitivity (Goldberg *et al* 2000, Grundy *et al* 2002). Currently, the effects of niacin on fasting and non-fasting intestinal-hepatic lipid and lipoproteins, and insulin-glucose

metabolism have not been explored in PCOS. Given metformin is routinely used to target hyperinsulinemia in PCOS, metformin in combination with niacin, and niacin alone will be used in specific objective 4 to determine the effects on insulin-glucose and lipid metabolism in a rodent model of PCOS.

2.2 Aims, Objectives and Hypotheses

2.2.1 Primary aims

The primary aim of this study was to understand the physiological and mechanistic contribution of hyperandrogenemia and hyperinsulinemia to cardiometabolic risk, in particular atherogenic dyslipidemia, and to investigate the effect of dietary niacin to improve atherogenic dyslipidemia in the JCR:LA-*cp* rodent model of PCOS. The secondary aim of this study was to explore the effects of modulating androgens and insulin, and dietary niacin on reproductive-endocrine effects in the JCR:LA-*cp* rodent model of PCOS.

2.2.2 Primary hypotheses

The primary hypothesis of this study was that treatment with the insulin sensitizer, metformin, the androgen receptor antagonist, flutamide, and nicotinic acid would independently improve atherogenic dyslipidemia, and these compounds in combination with metformin would improve insulin resistance to modulate cardiometabolic risk in the JCR:LA-*cp* rodent model of PCOS. The secondary hypothesis was that treatment with metformin, flutamide and dietary nicotinic acid would improve reproductive-endocrine outcomes in the JCR:LA-*cp* rodent model of PCOS.

2.2.3 Specific objectives

The specific objectives were aimed to test the above hypotheses and determine the effect of metformin, flutamide and nicotinic acid on lipid and apoB-lipoprotein metabolism, insulin-glucose metabolism and reproductive-endocrine indices:

Specific objective 1: To assess the effect of metformin, flutamide and their combination on plasma lipid and apoB48- and apoB100-lipoprotein metabolism and insulin resistance in the JCR:LA-*cp* rodent model of PCOS (as shown in Chapter 3) by:

i) Assessing the fasting plasma lipids and apoB-lipoproteins, including triglycerides (TG), total cholesterol (TC), free fatty acids (FFA), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) and apoB48- and apoB100-lipoproteins.

ii) Assessing the postprandial or non-fasting plasma lipid and apoB-lipoprotein response, including TG, TC, and apoB48 and apoB100-lipoproteins, following a high-fat meal challenge test.

iii) Determining the intestinal lymphatic secretion of TG, TC and apoB48 in the fasting and non-fasting states.

iv) Assessing the fasting and non-fasting insulin-glucose metabolism using a meal tolerance test (MTT).

Specific hypothesis 1: Metformin and flutamide independently improve fasting and non-fasting plasma lipid and apoB48- and apoB100-lipoprotein metabolism, and metformin-flutamide combination additively improve lipid and apoB-lipoprotein metabolism and insulin resistance indices in the JCR:LA-cp rodent model of PCOS.

Specific objective 2: To assess the effect of metformin, flutamide and their combination on androgen, lipogenic and inulin-glucose gene expression in the liver and intestine in the JCR:LA-*cp* rodent model of PCOS (as shown in Chapter 3) by:

i) Determining the hepatic and intestinal mRNA expression of the nuclear androgen receptors AR, ERα, and ERβ.

ii) Determining the hepatic and intestinal mRNA expression of lipogenic genes including LXRα, PPARα, SREBP1, SREBP2, LDLR, HMGR, apoB, MTP, ACC, FAS, SCAP, DGAT1, and DGAT2.

iii) Determining the hepatic and intestinal mRNA expression of insulin signaling genes including insulin receptor (IR), MAPK1, AKT2, PTPN1, JNK and GSK3β.

Specific hypothesis 2: Metformin and flutamide independently, and in combination regulate the expression of androgen and estrogen receptors, and lipogenic and insulin signalling genes in the JCR:LA-cp rodent model of PCOS.

Specific objective 3: To determine the effect of metformin, flutamide and their combination on the reproductive-endocrine indices in the JCR:LA-*cp* rodent model of PCOS (as shown in Chapter 3) by:

i) Assessing plasma concentrations of free (FT) and total testosterone (TT), estradiol (E2), sex hormone binding globulin (SHBG) and free androgen index (FAI) as biomarkers of androgen-estrogen metabolism.
ii) Assessing ovarian follicular morphology.

Specific hypothesis 3: Metformin, flutamide and metformin-flutamide combination treatments improve reproductive-endocrine indices in the JCR:LA-cp rodent model of PCOS.

Specific objective 4: To assess the effect of dietary nicotinic acid (slow release formula) and its combination with metformin on plasma lipid and apoB48- and apoB100-lipoprotein metabolism and insulin resistance and in the JCR:LA-*cp* rodent model of PCOS (as shown in Chapter 4) by:

i) Assessing the fasting lipids and apoB-lipoproteins, including TG, TC, FFA, HDL-C, LDL-C and apoB48- and apoB100-lipoproteins.
ii) Assessing the postprandial or non-fasting lipid and apoB-lipoprotein response, including TG and TC, following a high-fat meal challenge test.
iii) Determining the intestinal lymphatic secretion of TG, TC and apoB48 in the fasting and non-fasting states.
iv) Assessing the fasting or non-fasting insulin-glucose metabolism by an MTT.

Specific hypothesis 4: Nicotinic acid alone and nicotinic acid-metformin combination treatments improve lipid and apoB-lipoprotein metabolism, and both treatments have a neutral effect on insulin resistance indices in the JCR:LA-cp rodent model of PCOS.

Specific objective 5: To determine the effect of nicotinic acid and its combination with metformin on the reproductive-endocrine indices in the JCR:LA-*cp* rodent model of PCOS (as shown in Chapter 4) by:

i) Assessing plasma concentrations of FT, TT, E2, SHBG and FAI as biomarkers of androgen-estrogen metabolism
ii) Measuring estrus cyclicity by assessing daily vaginal cytology.
iii) Assessing ovarian follicular morphology.

Specific hypothesis 5: Dietary nicotinic acid alone and nicotinic acid-metformin combination treatment improve reproductive-endocrine indices in the JCR:LA-cp rodent model of PCOS.

2.3 Expected outcomes

Based on the specific objective 1, metformin, flutamide and metformin-flutamide combination treatments are expected to independently improve fasting plasma TG, TC, FFA, HDL-C, LDL-C, apoB48 and apoB100, and non-fasting TG, TC, apoB48 and apoB100 following a high-fat meal challenge test. The treatments are also expected to beneficially modulate intestinal secretion of TG, TC and apoB48. Insulin and glucose concentrations are anticipated to be lowered by metformin and in combination with flutamide in the fasting and non-fasting states. As stated in the specific objective 2, androgen and estrogen receptors, lipidogenic and insulin-signaling gene expression in the liver and intestine are anticipated to be modulated by flutamide and metformin treatments.

Metformin, flutamide and metformin-flutamide combination are expected to lower plasma concentratins of FT, TT and E2, and increase SHBG. In addition, these treatments are expected to improve ovarian follicular morphology as described in specific objective 3.

Based on specific objective 4, nicotinic acid alone and in combination with metformin is expected to lower fasting plasma TG, TC, FFA, HDL-C, LDL-C, apoB48 and apoB100, and non-

fasting TG and TC in response to a high-fat meal challenge test. The treatments are also expected to beneficially modulate intestinal secretion of TG, TC and apoB48. Nicotinic acid and nicotinic acid-metformin combination treatments are anticipated to have a neutral effect on insulin and glucose concentrations in the fasting state and in response to a meal tolerance test.

As proposed in specific objective 5, nicotinic acid independently and in combination with metformin is expected to modulate plasma concentrations of FT, TT and E2, and increase SHBG. In addition, these treatments are expected to improve ovarian follicular morphology and estrous cyclicity.

3. Investigation of the effect and mechanism of action of metformin and flutamide to improve atherogenic blood lipid profile, insulin-glucose metabolism and reproductive-endocrine indices in the JCR:LA-*cp* rodent model of polycystic ovary syndrome.

3.1 Introduction

Polycystic Ovary Syndrome (PCOS) is a highly prevalent metabolic-endocrine disorder affecting 6-18% of women in their reproductive years (Carmina *et* al 2006, March *et al* 2010, Moran *et al* 2013). Currently the etiological causes of the disorder are not fully understood, however genetic and environmental factors are likely to contribute to the onset of hyperandrogenemia and insulin resistance, which are implicated in the development of PCOS (Franks *et al* 2012, Diamanti-Kandarakis *et al* 2006, 2008, Baptiste *et al* 2010). It has been estimated that up to 85% of women exhibit clinical or biochemical hyperandrogenemia (Moghetti *et* al 2013, Panidis *et al* 2013), and 70% of lean and 95% of overweight women diagnosed with PCOS have impaired glucose tolerance and elevated plasma insulin concentrations in the fasting and non-fasting state (Sirmans *et al* 2012, Yildir *et al* 2013). Atherogenic dyslipidemia is common in PCOS, occurring in 70% of individuals, and is defined as elevated fasting plasma triglycerides (TG), apoB-lipoproteins, lower high-density lipoprotein cholesterol (HDL-C) (Jovanovic *et al* 2010, Silva Dantas *et al* 2013, Velazquez *et al* 2000), and elevated excursions in non-fasting triglycerides and apoB-lipoproteins (Bahceci *et al* 2007, Sidhwani *et al* 2011, Velazquez *et al* 2000, Wild 2012). The most frequent aberrations in lipid metabolism observed in PCOS also include increased fasting plasma total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and total apoB, and postprandial or non-fasting lipemia have been reported in PCOS (Sidhwani *et al* 2011, Wild 2012). Atherogenic dyslipidemia predisposes young women to the early development of atherosclerosis and cardiovascular disease (Barcellos *et al* 2013, Silva Dantas *et al* 2013). Women and adolescents with PCOS have been shown to have early signs of cardiovascular disease including increased carotid artery media thickness, endothelial dysfunction, ventricular hypertrophy and left ventricular dysfunction (Jovanovic *et al* 2010, Karoli *et al* 2012, Meyer *et al* 2012, Orio *et al* 2004). Insulin resistance and hyperandrogenemia have been proposed to act independently and/or synergistically in the development of

atherogenic dyslipidemia and cardiovascular disease risk in PCOS (Cussons *et al* 2007, Alexander *et al* 2009, Polotsky *et al* 2012).

The mechanisms involved in the dysregulation of lipid metabolism in PCOS are proposed to relate to androgen and insulin-mediated effects on lipogenic pathways, though the mechanisms remain unclear. Furthermore, the pathophysiology of dyslipidemia may vary depending on the metabolic phenotype of an individual with PCOS (Wild 2012, Diamanti-Kandarakis *et al* 2007, Cussons *et al* 2007). Testosterone induction of PCOS leads to unchanged or increased plasma TG and a reduction in HDL-C in animal models, including rodents, sheep and rhesus monkeys (Abbott *et al* 1998, 2005, King *et al* 2007 Koecking *et al* 2005, Manneras *et al* 2007). Clinically, hyperandrogenemia has been associated with a more aberrant lipid profile in PCOS and this appears to be independent of body weight (Valkenburg *et al* 2008, Sidhwani *et al* 2011). In PCOS patients, hyperandrogenemia is correlated with higher concentrations of plasma TG, lower HDL-C levels and small dense LDL particles (Carmina *et al* 2005, Dejager *et al* 2002). Indeed flutamide, the androgen receptor (AR) antagonist, has been shown in several clinical studies to lower fasting plasma lipids (TG, total cholesterol and LDL-C), as well as an increase in HDL-C, and these observations were found independent of body weight (Diamanti-Kandarakis *et al* 1998, Gambineri *et al* 2004, Ibanez *et al*, 2000). The effects of flutamide on non-fasting plasma lipids and apoB-lipoproteins, and the mechanisms associated with decreasing lipidogenesis are currently unknown. Flutamide is known to inhibit testosterone binding to the AR resulting in a reduction of androgen-induced augmentation of the AR, and consequent modulation of AR's regulatory actions (Lu *et al* 1999). The AR competes with other nuclear receptors such as LXR and PPARγ for coactivators (Ting *et al* 1999). The AR may antagonize the function of these nuclear receptors through competiting for coactivators, but not by direct binding to these nuclear receptors (Krycer *et al* 2011). The AR, at least in prostrate cancer cell lines, can directly stimulate maturation of the sterol regulatory element-binding protein (SREBP) precursor and SREBP activation, as well as increase SREBP cleavage activating protein (SCAP) expression leading to an upregulation in lipidogenesis (Heemers *et al* 2006).

Recent genetic studies have explored AR polymorphisms and the association with the onset of PCOS. It has been reported that the presence of shorter and longer $(CAG)_n$ repeats in the AR gene sequence is a risk factor for PCOS development (Lin *et al* 2013), and positively correlates with higher plasma testosterone levels in PCOS patients (Zhang *et al* 2013). In addition, AR activity could be affected by ligand availability, as flutamide has been shown to reduce 5α-reductase activity, which may lead to a decrease in synthesis of 5α-dihydrotestosterone (5α-DHT) from testosterone, as shown in female rodent liver (Graef *et al* 1987). It has been recently demonstrated that intervention with flutamide for a period of three years improves menstrual cyclicity and spontaneous ovulation rate in PCOS patients (Paradisi *et al* 2013), however there is a limited number of studies that have explored the mechanisms of flutamide action in the ovary. Other anti-androgens such as cyproterone acetate and spironolactone have been used to improve hirsuitism and increase ovulation rate, though these do not necessarily improve dyslipidemia in PCOS (Bargiota *et al* 2012).

It is well established that insulin resistance is associated with increased lipogenesis and overproduction of hepatic (apo-B100-VLDL) and intestinal (apo-B48-chylomicron) TG rich lipoproteins and increased circulating concentrations of remnant particles (Duez *et al* 2008, Garg 1996, Eckel *et al* 1984, Phillips *et al* 2002, Vine *et al* 2007, Yki-Jarvinen *et al* 1984). Furthermore, recent evidence from the Copenhagen City Heart Study and ACCORD lipid study shows that increased fasting and non-fasting concentrations of apoB-remnant lipoproteins are positively associated with end stage CVD events (Chapman *et al* 2011, Twickler *et al* 2005, Varbo *et al* 2011, 2013, Fruchart *et al* 2008, 2010). Sidhwani and colleagues have shown that women with PCOS, who have elevated free testosterone and insulin levels tend to have increased concentrations of smaller remnant LDL and HDL particles, and higher plasma TG concentration, independent of body weight compared to healthy controls (Sidhwani *et al* 2011). It has been shown that postprandial hypertriglyceridemia in women with PCOS is positively associated with insulin resistance, independent of body weight (Bahceci *et al* 2007). In insulin resistance, lower lipoprotein lipase (LPL) activity may reduce the rate of lipolysis of TG rich lipoproteins (TRL's: VLDL and CM) leading to prolonged circulation of TRL's and remnant

lipoproteins (Panarotto *et al* 2002). PCOS patients have been proposed to have impaired clearance of apoB-lipoprotein remnants due to altered apo-A1, -C-I/II/III and -E expression, which hinders lipoprotein lipolysis and uptake, therefore reducing the clearance of apoB-remnant lipoproteins from circulation (Huang *et al* 2010, Wild *et al* 1992). Insulin sensitizing agents have been used to improve insulin resistance in PCOS and this may include beneficial effects on lipid and apoB-lipoprotein metabolism (Bargiota *et al* 2012, Diamanti-Kandarakis *et al* 2010). Thiazolinediones, PPARγ agonists are known to improve the insulin sensitivity of peripheral tissues, including adipose and skeletal muscle via promoting lipid storage and inhibiting free fatty acid release from adipocytes. This may reduce circulating FFAs in plasma and limit substrate availability for TG synthesis in the liver (Bargiota *et al* 2012, Evans *et al* 2004, Pasquali *et al* 2006). However these agents do not consistently improve plasma lipids in PCOS (Bargiota *et al* 2012). Metformin is a standard therapy to treat IR in PCOS (Bargiota *et al* 2012). Metformin is proposed to decrease intestinal glucose absorption, suppress gluconeogenesis and hepatic glucose secretion, and to improve insulin sensitivity in peripheral tissues (Bargiota *et al* 2012). There is some evidence that metformin may reduce plasma TG concentrations by suppressing de novo lipogenesis in the liver through downregulation of key lipogenic genes such as SREBP1, acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) and 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) (Zhou *et al* 2001). Metformin may also increase mitochondrial fatty acid oxidation in the insulin resistant state as shown by studies in isolated primary rat hepatocytes from high-fat fed Wistar rats (Cleasby *et al* 2004, Zhou *et al* 2001). Although some studies show beneficial effects of metformin to reduce plasma lipids, the evidence in PCOS remains limited (Bargiota *et al* 2012). In addition, evidence suggests that metformin beneficially improves the frequency of spontaneous ovulation, menstrual cyclicity and pregnancy rates in women with PCOS (Fleming *et al* 2002, Heard *et al* 2002, Ng *at al* 2001). In human granulosa cells metformin has been shown to inhibit FSH action and reduce number of FSH receptors. This may be beneficial in the PCOS ovary as they show an increased number of FSH receptors leading to hyper-responsiveness and premature luteinization, therefore metformin therapy may be an effective anovulation treatment in PCOS (Rice *et al* 2013). Metformin therapy in women with PCOS also appears to reduce both adrenal and ovarian

steroidogenesis, reducing levels of testosterone, E2, DHEAS and free androgen index (FAI) (Kolodziejejczyk *et al* 2000, Nestler *et al* 1996, 1997, Velazquez *et al* 1994). However, these findings are not consistent (Ehrmann *et al* 1997, Marca *et al* 1999, Vrbikova *et al* 2001) and it is unclear if the reduction in androgens is associated with improvements in plasma lipid metabolism in these studies.

Metformin-flutamide combination therapy in PCOS has shown improvements in fasting plasma total cholesterol and LDL-C in obese IR women with PCOS treated for 6 mths, but no change in total plasma triglycerides was observed (Gambineri *et al* 2004). A longer-term intervention of 12 mths in conjunction with a hypocaloric diet showed similar results (Gambineri *et al* 2009). Although, the diet alone improved insulin resistance, plasma lipid profile was improved only in conjunction with metformin and flutamide therapy. In young normal weight insulin resistant PCOS women, a low dose metformin, flutamide, pioglitazone and oral contraceptive combination treatment for 30 mths was shown to improve hyperandrogenemia, HDL-C and carotid intima media thickness, however fasting plasma TG concentrations were significantly increased (Vinaixa *et al* 2011). Despite the use of metformin, flutamide and combination treatments to improve insulin resistance with varying effects on blood lipids, the mechanisms of how metformin and flutamide regulate plasma lipid and apoB-lipoprotein metabolism in PCOS remains unclear. The aim of this study was to investigate the effects of metformin and flutamide, alone and in combination, on plasma lipid and apoB-lipoprotien metabolism, insulin-glucose metabolism and endocrine-reproductive indices in an established spontaneous PCOS-prone rodent model: the JCR:LA-*cp* rodent.

3.2 Methods

3.2.1 Animal model and study design

Female JCR:LA-*cp* rats, n=60, were raised in the established breeding colony at the University of Alberta. The JCR:LA-cp rodent is an established model for the spontaneous development of PCOS and the MetS and this is due to a polygenic recessive corpulent (*cp*) defect in the leptin receptor that on homozygous genotyping presents with the PCOS phenotype (Vine et al 2009, 2012). PCOS-prone rats, n=48 were homozygous for the recessive *cp* gene (*cp/cp*) and lean control animals, n=12, were either homozygous normal (+/+) or heterozygous (+/?). Rats were weaned at 21 days of age and housed in the 12/12-hour reversed light cycle to allow establishment of a normal diurnal cycle. At 10 weeks of age, PCOS-prone rats were randomly assigned to one of the three treatment groups or a control group. PCOS-prone control animals (n=12) were fed a standard chow diet, the treatment groups of PCOS-prone rats were fed a diet supplemented with 1) metformin (300 mg/kg body weight, n=12), 2) flutamide (10 mg/kg body weight, n=12) or 3) metformin-flutamide combination (n=12). Metformin dose was established based on a previous pilot study in the female genonotype (Wang *et al* 2012), and as per Food and Drug Administration estimation. The dose 300 mg/kg is equivalent to approximately 48 mg/kg of dose equivalent for patients (US Food and Drug Admonistration, 2005) and this is comparable to clinical doses used in PCOS patients (Adhahosseini *et al* 2010, Harborne *et al* 2005). Flutamide doses of 5-25 mg/kg have been used in animal studies to restore cyclycity and endocrine profile without causing toxicity, therefore a dose of 10mg/kg was chosen in this study (Kim et al 2002, Clark et al 2003). Clinically, doses of 250-750 mg/d have been shown to be effective in lowering plasma testosterone and to improve dyslipidemia in women with PCOS (Chapter 1, Table 1-7). Lean control and PCOS-prone rats were fed ad libitum their randomly assigned diet. The chow for all animal groups was prepared using standard dry chow powder (5001, PMI Nutrition International, Brentwood, MO, USA). Animals were fed the assigned diets for 6 weeks. Body weights and food intake were measured weekly. At the end of the intervention a fasting blood sample was collected via tail vein procedure, and animals were euthanized, tissues excised and ovaries collected and fixed in neutral formalin (10%). Animal

care and experimental protocols were conducted in accordance with the Canadian Council of Animal Care and approved by Animal Ethics Committee of the University of Alberta.

3.2.2 Metabolic assessment

3.2.2.1 Fasting plasma biochemical profile

Blood samples were collected after a 16-hour fast, plasma was separated by 10-minute centrifugation (4000 rpm) at 4^0C and the samples were stored at -80^0. Triglyceride (TG) (WAKO, Chemicals USA Inc., Richmond, VA, Catalog No. 461-08992, 461-09092), total cholesterol (WAKO, Catalog No. 439-17501), non-esterified fatty acids (NEFA) (WAKO, Catalog No. 999-34691, 995-34791, 991-34891, 993-35191), low-density lipoprotein (LDL-C) (WAKO, Catalog No. 993-00404, 999-00504) and high-density lipoprotein cholesterol (HDL-C) (WAKO, Catalog No. 997-72591) were measured by a direct colorimetric chemical enzymatic reaction method using commercially available colorimetric assay kits. Plasma glucose concentration was determined using glucose oxidase method (WAKO, Catalog No. 439-90901). Plasma insulin (ALPCO Diagnostics, USA, Catalog No. 80-INSRT-E10) and hormone concentrations; free testosterone (CUSABIO, Wuhan, China, Catalog No. CSB-E05097r), total testosterone (CUSABIO, Catalog No. CSB-E05100r), SHBG (CUSABIO, Catalog No. CSB-E12118r) and estradiol (CUSABIO, Catalog No. CSB-E05110r) were measured using commercially available ELISA kits. All assays were performed according to the manufacturers' instructions. Homeostatic model assessment insulin resistance (HOMA-IR) index was calculated based on fasting insulin and glucose concentrations. Free androgen index (FAI) was calculated based on fasting total testosterone and serum hormone binding globulin (SHBG) concentrations.

3.2.2.2 Plasma apolipoprotein-B48 and B100 quantitation

Plasma concentration of apolipoprotein-B48 (apoB48) and B100 (apoB100) were determined using a Western blotting procedure, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) combined with ECL analysis as described previously (Vine *et al* 2007). A commercially available antibody (Chemicon, Thermo Fisher Sci, Toronto, ON, Canada, Catalog No. AB742)) to apoB was used to identify apo-B48 and apo-B100, visualized using ECL

technique (GE Healthcare, Quebec, QC, Canada, Catalog No. RPN2108) and a charge-coupled device and the Fluor-S Multimanager system. The band thickness and density were determined and quantified using ImageJ software. A known mass of the purified rodent apoB protein was used as a reference standard and was used to quantify plasma apo-B48 and apo-B100 levels as previously established (Vine *et al* 2007).

3.2.2.3 Meal tolerance test

After 5 weeks of intervention, animals were fasted for 16 hours and given a 5.0 g pellet of chow. Blood was taken using an established tail-snip procedure at 0, 30 and 60 minutes after the meal was consumed (Proctor et al 2005) and blood was collected into ETDA-coated microtainer tubes (Vine *et al* 2007). Plasma was separated by 10-minute centrifugation (4000 rpm) at 4^0C and stored at -80^0C for measurement of insulin and glucose concentrations.

3.2.2.4 High-fat meal challenge test

After five weeks of intervention, animals were fasted for 16 hours and given a 5.0 g pellet of meal prepared with 47% milk fat w/w cream and standard rodent chow as previously described (Vine et al 2007). Blood was taken using a tail-snip technique at 0, 2, 4, 6, 8 and 10 hours following the meal. Plasma was separated by 10-minute centrifugation (4000 rpm) at 4^0C and stored at -80^0C for measurement of lipid (TG and cholesterol) and apoB-lipoprotein concentrations.

3.2.2.5 Analysis of postprandial or non-fasting lipid, apoB-lipoproteins, insulin and glucose response

The magnitude of the postprandial response of plasma glucose and insulin following meal tolerance test, and plasma triglyceride, total cholesterol, apo-B48 and apo-B100 following the oral high-fat challenge test was determined by area under the curve (AUC) method using GraphPad Prizm version 5.0a for Mac OC X (GraphPad Software, San Diego, CA, USA) software. AUC represents the total plasma levels of the parameter measured in the postprandial phase, inclusive of fasting and non-fasting response. The incremental AUC (iAUC) was generated by subtracting the fasting plasma concentration of the parameter from the non-fasting plasma concentration to determine the change or response in the parameter following the meal or high-fat meal test.

3.2.2.6 Lymph cannulation procedure

At the end of the intervention (6 wks) half of the animals (n=6 per group) underwent a mesenteric lymph duct cannulation procedure to assess intestinal lipid and chylomicron secretion, as previously described (Lu *et al* 2011). The animals were fasted 16 hours prior to the surgery and anaesthetized with 2-3.5% Isofluorane. Cannulas were inserted into the mesenteric lymph duct and duodenum and the animals allowed to recover postoperatively on duodenal saline (4% w/v glucose) infusion at the 1.5 ml/hr rate overnight using peristaltic pump. The following morning, the saline was infused for a further 5 hours and lymph was collected in EDTA-coated tubes. At 5 hrs animal were duodenally infused with Intralipid (2% v/v in a 4% w/v glucose) for a further 5 hours and lymph collected as previously described (Vine *et al* 2014, Wang *et al* 2012). Lymph TG and total cholesterol were measured using commercially available colorimetric assay kits as described above. Lymph aliquots were stored at -80^{0}C, apo-B48 and apo-B100 were measured using the Western blot procedure coupled with ECL analysis as described above.

3.2.3 Measurement of gene mRNA expression

Quantitative Real-Time PCR (Q-PCR) technique was used to measure relative expression of mRNA for AR, ERα, ERβ, LXRα, PPARα, SREBP1, SREBP2, LDLR, HMGR, apoB, MTP, ACC, FAS, SCAP, DGAT1, DGAT2, IR, MAPK1, AKT2, PTPN1, JNK and GSK3 in liver and enterocytes. Total RNA was extracted form the tissues using TRIzol technique (Invitrogen, Life Technologies, Burlington, ON, Catalog No. 15596-018). Isolated RNA was cleaned using RNeasy MinElute cleanup columns (Qiagen, Catalogue No. 74204). CDNA was synthesized from cleaned RNA using High-capacity cDNA Reverse Transcription kit, MultiScribe Reverse Transcriptase (Invitrogen, Life Technologies, Burlington, ON, Catalogue No. 4311235). Q-PCR was performed using Power SYBR Green mix (Applied Biosystems, Life Technologies, Burlington, ON, Catalogue No. 4367659) and StepOnePlus Real-Time PCR System (Applied Biosystems, Catalogue No. 4376600).

3.2.4 Ovary morphology assessment

Ovaries were longitudinally sectioned into four 4 µm slices and stained with hematoxylin and eosin. All sections were analyzed using inverted birefringence microscope Axio Observer.A1 (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA, Item No. 491911). The quantitative analysis of ovarian follicles at different stages of development and corpora lutea was assessed as described previously (Vine *et al* 2009, Dellmann *et al* 1987, Hirshfield *et al* 1978). In brief, follicles were defined as primary if they had one layer of cuboidal granulosa cells, secondary follicles had multiple layers of granulosa cells, but lacked a fluid-filled antrum, tertiary follicles had a fluid-filled antrum in addition to multiple layers of surrounding granulosa cells, pre-ovulatory follicles had compact multiple-layered granulosa cells and a large fluid filled antrum, atretic follicles exhibited signs of a degenerated oocyte nucleus, folding or discontinuous oocyte membrane, collapse or involution of the antrum, and detachment of the granulosa cells (Vine *et al* 2009, Wright *et al* 1999, Webber *et al* 2003).

3.2.5 Statistical analysis

Statistical analysis was performed using SPSS Statistics software, (IBM Corp., v.20.0.0). All data was tested for normality of distribution using Shapio-Wilk test. In cases where data was not normally distributed, base-10 logarithm of each data value was calculated to normalize the distribution. One-way ANOVA statistical model was used to find difference between the groups with significance set at $p<0.05$. Post-hoc analysis was performed using Tukey's test. The results are expressed as mean±SEM.

3.3 Results

3.3.1 Endocrine profile and reproductive assessment

3.3.1.1 Endocrine hormonal assessment

Endocrine hormone profiles of the PCOS-prone control and treated animals are shown in Table 3-1. There was no significant difference in plasma FT and total T concentrations between lean control and PCOS-prone groups. However, SHBG concentrations were 15% lower in the PCOS-prone groups compared to lean controls. There was no difference in estradiol between PCOS-prone groups and lean controls. However, estradiol concentrations were found to be significantly higher in the metformin treated group compared to the metformin-flutamide combination group. There was no difference in FAI between the groups, however lean control animals had 25% lower FAI compared to PCOS control animals. The group treated with the combination of metformin-flutamide had a "normalized" FAI compared to the lean control group, but this did not reach statistical significance.

Table 3-1. Endocrine hormone profile in PCOS-prone control and treatment groups.

	Lean Control	**PCOS Control**	**PCOS Metformin**	**PCOS Flutamide**	**PCOS Combination**
FT (pg/ml)	0.83±0.16	0.83±0.07	0.93±0.13	1.07±0.20	0.97±0.23
TT (ng/ml)	0.24±0.01	0.24±0.03	0.23±0.02	0.26±0.02	0.20±0.04
SHBG (pg/ml)	480.5±11.8^{a}	411.0±5.3^{b}	414.4±6.6^{b}	407.3±3.5^{b}	407.4±6.3^{b}
Estradiol (ng/ml)	21.6±1.4^{ab}	22.7±1.4^{ab}	28.7±2.5^{a}	23.1±2.6^{ab}	20.7±1.0^{b}
FAI	192±16	254±34	222±22	251±23	186±32

Sample size of n=6 was used for measuring endocrine hormone indices. Values with a common letter are not statistically different ($p<0.05$)

4.3.1.2 Ovarian follicular morphology

Ovarian follicular histological assessment is shown in Table 3-2. PCOS-prone animals had a 3-fold lower frequency of primary follicles compared to the lean control group, whereas the PCOS treated groups tended to have an increase in primary follicle number compared to the PCOS

control group. There was no difference in secondary or tertiary follicle number between groups. Pre-ovulatory follicles were 6-fold lower in number in the PCOS-prone animals compared to lean controls. Interestingly, the number of pre-ovulatory follicles was increased by 30-100% or normalized in PCOS treatment groups compared to PCOS control, although due to high variability in animals this did not reach statistical significance. Interestingly, the flutamide treatment group had a significant increase in pre-ovulatory follicles compared to the PCOS-prone control group. In addition, PCOS-prone animals had five times greater the number of atretic follicles compared to lean controls, whilst all treatment groups showed a significant reduction in the atretic follicle number. No difference in the number corpus luteum and corpus albicans was observed, although the lean control group did appear to have a trend towards an increased number of corpus luteum, indicative of increased ovulation.

Consistent with previous observations in the PCOS-prone group, 60% of animals were observed to have lipid accumulation in the ovary stroma and this was not present in the lean control animals (Shi *et al* 2009). There appeared to be a reduction in lipid deposition in the metformin (20%), flutamide (33%) and combination (33%) treatment groups (see Figure 3-1).

Table 3-2. Ovarian morphology assessment in PCOS-prone control and treatment groups.

	Lean Control	PCOS Control	PCOS Metformin	PCOS Flutamide	PCOS Combination
Primary Follicle	3.4±0.7[a]	1.0±0.3[b]	1.6±0.5[ab]	2.0±0.5[ab]	2.5±0.6[ab]
Secondary Follicle	2.0±0.6	1.6±0.4	1.4±0.5	1.7±0.3	2.0±0.3
Tertiary Follicle	3.2±0.6	3.4±0.5	4.2±0.8	3.8±1.1	5.7±1.6
Pre-ovulatory Follicle	1.2±0.2[ab]	0.2±0.2[a]	1.0±0.3[ab]	0.7±0.3[a]	2.0±0.4[b]
Atretic Follicle	2.6±0.8[a]	12.2±2.5[b]	5.2±1.2[a]	3.2±0.7[a]	5.5±1.0[a]
Corpus Luteum	2.4±0.7	1.6±0.8	1.6±0.4	0.8±0.3	1.0±0.5
Corpus Albicans	3.0±1.1	2.2±0.4	2.4±0.5	1.8±0.5	3.7±0.7

Sample size of n=6 was used for ovarian morphology assessment. Values with a common letter are not statistically different ($p<0.05$)

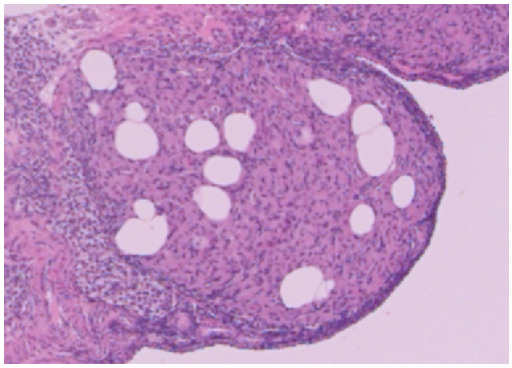
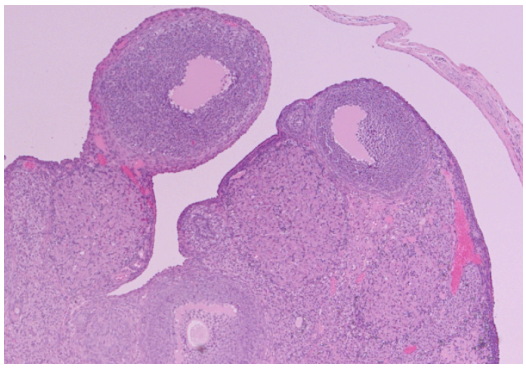
A  B 

Figure 3-1. Ovarian sections from PCOS-prone and lean control rats. Sections are stained with Hematoxylin & Eosin. A: ovary stroma with visible lipid deposits in PCOS-prone groups (magnification 40X); B: lean control with no lipid deposits in ovary stroma (magnification 20X).

3.3.2 Body composition and fasting biochemical parameters

3.3.2.1 Body composition and food intake

Body weight and food intake of the groups are listed in Table 3-3. There was no difference in the final body weight between the PCOS-prone controls and treatment groups. As expected,

lean control animals had significantly lower body weight compared to PCOS-prone animals. Total food intake tended to be lower in the PCOS groups treated with flutamide and combination treatments groups compared with the control and metformin treatment group, although there was no statistical difference in food intake between PCOS-prone groups.

Table 3-3. Body weight and food intake of PCOS control and the treatment groups.

	Lean Control	**PCOS Control**	**PCOS Metformin**	**PCOS Flutamide**	**PCOS Combination**
Final Body Weight (g)	208 ± 4^{a}	376 ± 6^{b}	377 ± 6^{b}	371 ± 6^{b}	378 ± 7^{b}
Food Intake (g/6wks)	585 ± 12^{a}	947 ± 19^{bc}	977 ± 18^{b}	892 ± 19^{cd}	864 ± 19^{d}

Values with a common letter are not statistically different ($p<0.05$)

3.3.3 Fasting plasma biochemical parameters

3.3.3.1 Fasting plasma glucose, insulin and HOMA-IR

Plasma glucose homeostasis profile is shown in Table 3-4. As expected lean control animals had significantly lower fasting plasma glucose and insulin concentrations compared to the PCOS-prone groups. There was no difference in fasting glucose and insulin concentrations between the PCOS-prone groups following treatments. The mean plasma insulin concentration tended to decrease in the metformin, flutamide and the combination treatment groups, however values were not significantly different compared to the PCOS control group. The lean control group had a lower HOMA-IR compared to the PCOS control, as expected ($p<0.001$). The mean HOMA-IR was 50% lower in the PCOS metformin and combination treatment groups compared to the PCOS control, and was 40% lower in the the flutamide treatment group compare to PCOS control, however these differences did not reach statistical significance.

Table 3-4. Fasting plasma glucose homeostasis profile of PCOS control and the treatment groups.

	Lean Control	PCOS Control	PCOS Metformin	PCOS Flutamide	PCOS Combination
Glucose (mg/dl)	96.9±8.1[a]	157.2±10.3[b]	164.1±16.8[b]	154.4±12.9[b]	127.6±7.7[b]
Insulin (ng/ml)	0.32±0.07[a]	3.28±0.73[b]	1.74±0.35[ab]	2.16±0.52[b]	1.89±0.34[b]
HOMA-IR	1.94±0.39[a]	31.56±7.73[b]	15.53±2.91[ab]	25.36±6.18[b]	15.24±2.90[ab]

Values with a common letter are not statistically different ($p<0.05$)

3.3.3.2 Fasting plasma lipids and apoB-lipoproteins

Fasting plasma lipid concentrations are shown in Table 3-5. As expected lean control animals had decreased total plasma TG, TC, FFA, apoB48 and apoB100 levels, compared to the PCOS-prone control group. In addition, HDL-C was higher in the lean control group compared to the PCOS control animals. Total plasma TG concentrations were lowered in the animals treated with flutamide ($p=0.023$) and the flutamide-metformin combination group showed a 30% reduction in plasma TG but this did not reach statistical significance compared to PCOS control ($p=0.053$). Total plasma TG concentration was significantly lower in PCOS flutamide and combination groups compared to the metformin treatment group. There was no significant difference in fasting plasma total cholesterol, non-esterified fatty acid and HDL-C concentrations between the untreated and treated PCOS-prone groups. LDL-C tended to be lower in the PCOS flutamide and combination groups by 30% compared to the lean control, PCOS-prone control and metformin treatment groups, although this did not reach statistical significance. Fasting apoB48 concentrations were reduced by >30% in the metformin-flutamide combination group compared to the metformin treated and the PCOS-prone control groups. Flutamide alone reduced fasting apoB48 concentrations by >25%, but this was not statistically significant. Fasting plasma apoB100 concentrations were not lowered in the metformin or flutamide treatment groups, however the combination treatment significantly lowered apoB100 compared to the PCOS control group.

Table 3-5. Fasting plasma lipid profile of PCOS control and the treatment groups.

	Lean Control	PCOS Control	PCOS Metformin	PCOS Flutamide	PCOS Combination
TG (mg/dl)	19.5±2.8[a]	993.7±129.0[bd]	1031.2±102.8[b]	583.3±47.6[c]	643.1±79.3[cd]
TC (mg/dl)	58.1±6.0[a]	131.0±25.3[b]	138.7±14.4[b]	145.2±19.6[b]	145.1±18.9[b]
NEFA (mmol/l)	0.69±0.08[a]	0.78±0.06[b]	0.88±0.08[b]	1.14±0.19[b]	1.10±0.14[b]
LDL-C (mg/dl)	24.4±3.2	19.2±4.9	24.3±5.6	13.9±1.8	13.4±1.7
HDL-C (mg/dl)	23.4±1.9[a]	15.8±2.9[b]	13.3±3.5[b]	17.9±2.5[b]	17.7±3.5[b]
Apo-B48 (µg/ml)	30±2[a]	620±43[b]	615±49[b]	458±42[bc]	426±44[c]
Apo-B100 (µg/ml)	572±63[a]	2028±316[b]	1657±154[bc]	1388±182[bc]	1185±128[c]

Values with a common letter are not statistically different ($p<0.05$)

3.3.4 Hepatic and intestinal lipid content

The TG and cholesterol content of liver and intestinal epithelial mucosal scrapings are shown in Figure 3-2. Hepatic TG, were 20% lower in the flutamide treatment group compared to the PCOS-prone control and other treatment groups, however this was not statistically significant. Total cholesterol in the liver was not different between groups. There was no significant difference in intestinal TG content between the PCOS-prone groups, although the PCOS-prone control group did tend to have lower TG content. PCOS-prone groups treated with metformin, flutamide and the combination had significantly lower total cholesterol levels compared to lean control animals, and tended to have lower cholesterol compared to the PCOS-control group.

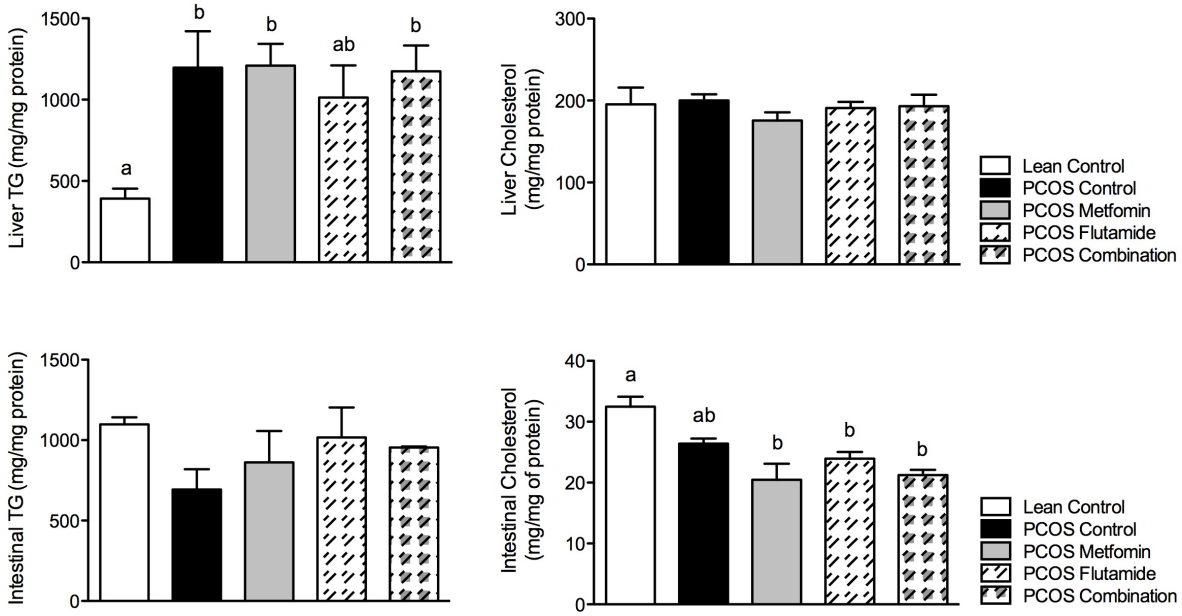


Figure 3-2. Liver and intestinal epithelial mucosa triglycerides and total cholesterol content. Values are expressed relative to total protein content. Sample size of n=6 was used for this measurement. Data is presented as mean±SEM. Bars with a common letter are not statistically different ($p<0.05$).

3.3.5 Postprandial plasma lipids, apoB-lipoproteins, and insulin and glucose response to meal challenge

3.3.5.1 Postprandial plasma insulin and glucose response following a meal challenge

PCOS-prone animals were shown to have an elevated total postprandial (AUC) response in glucose and insulin compared to lean controls, as shown in Figure 3-3 and 3-4. Treatment with metformin and metformin-flutamide combination did tend to lower total postprandial glucose response, however there was no significant difference in the incremental (iAUC) glucose response between all groups. The incremental response in plasma insulin concentration was elevated more than four fold in PCOS-prone animals. Treatment with metformin and flutamide did not modify the postprandial insulin response, however the combination of metformin-flutamide decreased the plasma insulin response towards that observed in lean controls (Figure 3-4).

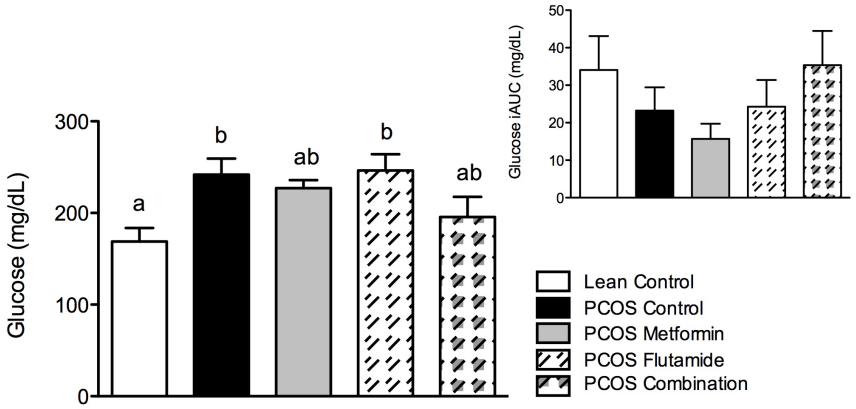


Figure 3-3. The postprandial plasma glucose response (AUC) to an oral meal tolerance challenge in lean and PCOS-prone controls and treatment groups. Sample size of n=6 was used for this measurement. Data is presented as mean±SEM. The total AUC and the change in glucose from fasted concentrations are shown and represented as incremental area under the curve (iAUC). Bars with a common letter are not statistically different ($p<0.05$).

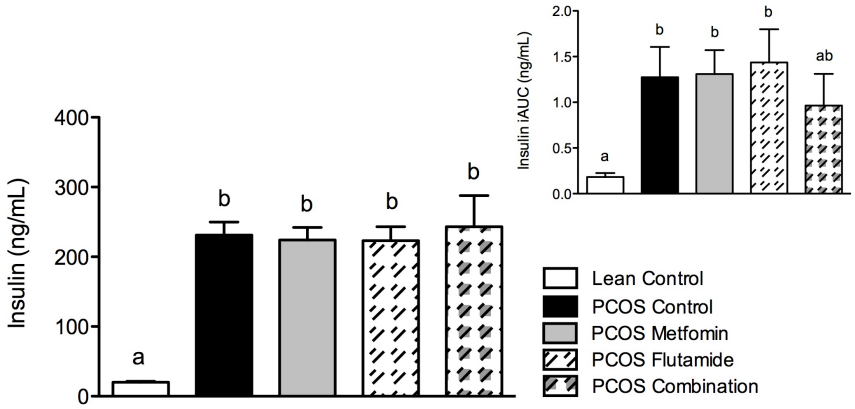


Figure 3-4. The postprandial plasma insulin response (AUC) to an oral meal tolerance challenge in lean and PCOS-prone controls and treatment groups. Sample size of n=6 was used for this measurement. Data presented as mean±SEM. The total AUC and the change in insulin from fasted concentrations are shown and represented as incremental area under the curve (iAUC). Bars with a common letter are not statistically different ($p<0.05$).

3.3.5.2 Postprandial lipid and apoB-lipoprotein response following a high fat meal challenge

Postprandial or non-fasting total postprandial plasma TG, cholesterol, apoB48 concentrations were elevated in PCOS-prone animals compared to lean controls ($p<0.001$), as shown in Figure 3-5 to 3-7. Treatments did not alter total postprandial plasma TG, cholesterol, apoB48 or apoB100 concentrations, as shown in Figure 3-5-3-8. The plasma TG and cholesterol concentration iAUC response tended to be lower by approximately 30% in all treatment groups compared to PCOS control, however this did not reach statistical significance.

The iAUC for ApoB48, a marker of intestinal chylomicron secretion in the postprandial phase, was lowered by approximately 30% in all treatment groups compared to PCOS control but this did not reach statistical significance. There was no difference in total postprandial apo-B100 concentrations between all groups, although apoB100 was elevated by 50% in PCOS-prone groups. The iAUC for plasma apo-B100 concentrations was significantly increased in PCOS-prone animals compared to lean control ($p<0.01$). Treatment with metformin and flutamide did not significantly lower iAUC for apoB100, however the combination treatment reduced postprandial apoB100 by 60% and was similar to lean control concentrations.

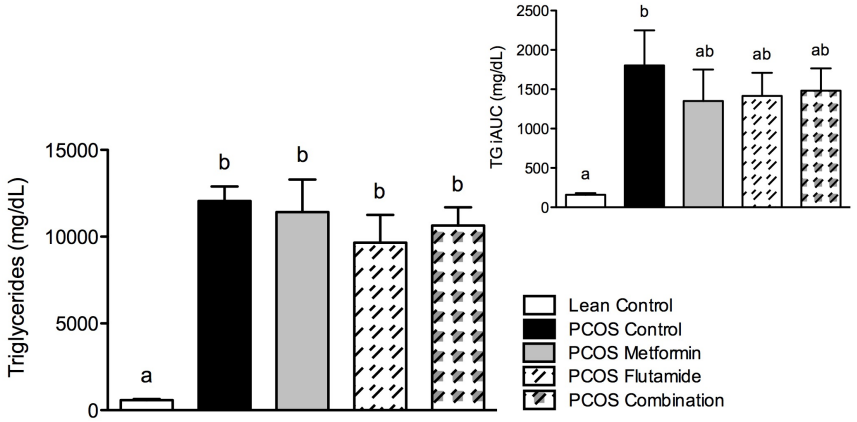


Figure 3-5. The postprandial plasma triglyceride response (AUC) to a high fat meal challenge in lean and PCOS-prone controls and treatment groups. Sample size of n=6 was used for this measurement. Data is presented as mean±SEM. The total AUC and the change in TG from fasted concentrations are shown and represented as incremental area under the curve (iAUC). Bars with a common letter are not statistically different ($p<0.05$).

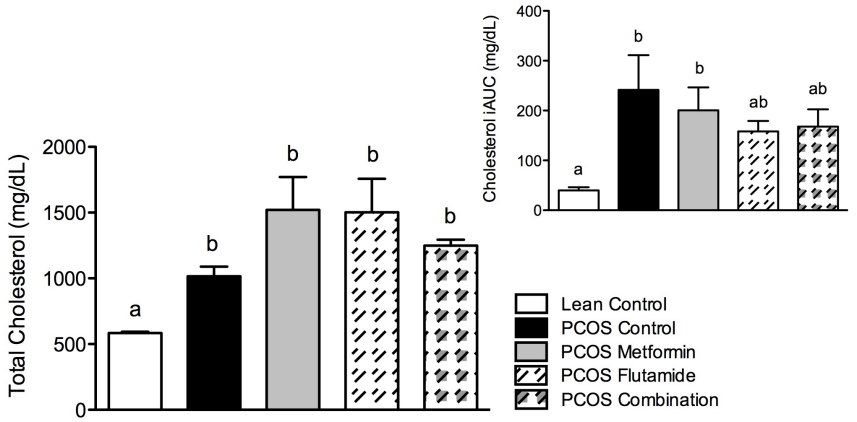


Figure 3-6. The postprandial plasma total cholesterol (AUC) to a high fat meal challenge in lean and PCOS-prone controls and treatment groups. Sample size of n=6 was used for this measurement. Data is presented as mean±SEM. The total AUC and the change in TC from fasted concentrations are shown and represented as incremental area under the curve (iAUC). Bars with a common letter are not statistically different ($p<0.05$).

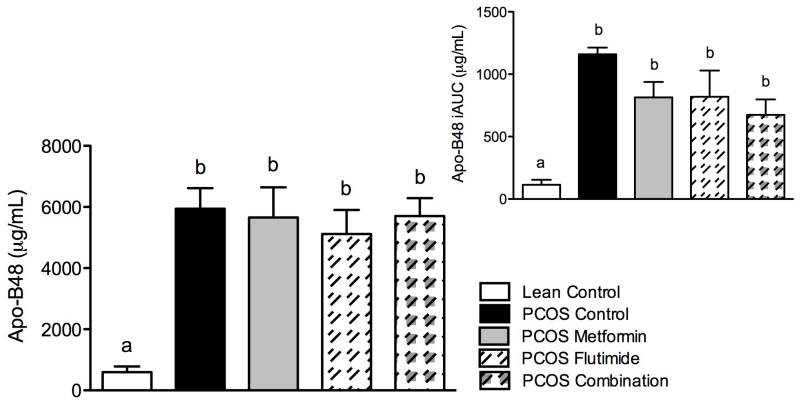


Figure 3-7. The postprandial plasma apoB48 response (AUC) to a high fat meal challenge in lean and PCOS-prone controls and treatment groups. Sample size of n=6 was used for this measurement. Data is presented as mean±SEM. The total AUC and the change in apoB48 from fasted concentrations are shown and represented as incremental area under the curve (iAUC). Bars with a common letter are not statistically different ($p<0.05$).

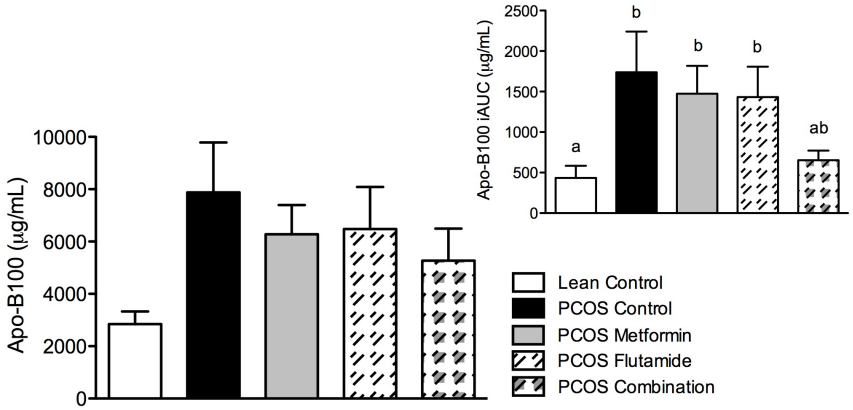


Figure 3-8. The postprandial plasma apoB100 response (AUC) to a high fat meal challenge in lean and PCOS-prone controls and treatment groups. Sample size of n=6 was used for this measurement. Data is presented as mean±SEM. The total AUC and the change in apoB100 from fasted concentrations are shown and represented as incremental area under the curve (iAUC). Bars with a common letter are not statistically different ($p<0.05$).

3.3.6 Intestinal lymph secretion of lipid and apoB-lipoprotein in the fasted and fed state

Intestinal lymph secretion of TG, cholesterol and apo-B48 is shown in Figure 3-9. As expected lean control animals had decreased fasting and non-fasting intestinal lymph secretion of TG, cholesterol and apoB48. Lymph TG in the fasting state were decreased by 75%in the flutamide-treatment group compared to PCOS control, and tended to decrease in PCOS combination group. PCOS animals treated with metformin had similar lymph TG to PCOS control, therefore indicating no effect of metformin on intestinal secretion of TG. As shown on panel B (Figure 3-9), analogous trends were observed for lymph TG secretion in the fed state. There was a 70% and 50% reduction in lymph TG concentration in the fed state with flutamide ($p<0.0001$) and combination ($p=0.01$) treatments, respectively. Cholesterol concentration in the intestinal lymph in the fasted state was 60% lower in the metformin group compared to PCOS control, and this concentration was similar to that observed in the lean control group (panel B, Figure 3-9). This effect was not conserved in the fed state following metformin treatment (panel B, Figure 3-9). Treatment with flutamide and metformin-flutamide combination did also appear to lower cholesterol secretion into the intestinal lymph, however this was not significant and there was no difference in fasting and fed lymph cholesterol concentrations compared to the PCOS-prone group. There was no difference in intestinal lymph secretion of apoB48 in the fasting and fed state in PCOS-prone groups (panel C, Figure 3-9). However, flutamide treatment alone or in combination with metformin reduced the TG/apoB48 ratio, which is representative of the lipid per chylomicron particle, by 75% and 65% (fasted state) and 85% and 70% (non-fasted state) respectively, compared to the PCOS-prone control and metformin alone groups (panel D, Figure 3-9). Furthermore, the cholesterol/apoB48 ratio was reduced by 80%, 70% and 65% in the fasted state with metformin, flutamide and combination treatments, although this did not reach statistical significance (panel E, Figure 3-9). Interestingly, flutamide treatment alone or in combination with metformin decreased cholesterol/apoB48 secretion by 50% and 35% in the fed state in intestinal lymph compared to both the PCOS-prone and lean control groups, however this was significant only in the flutamide treatment group.

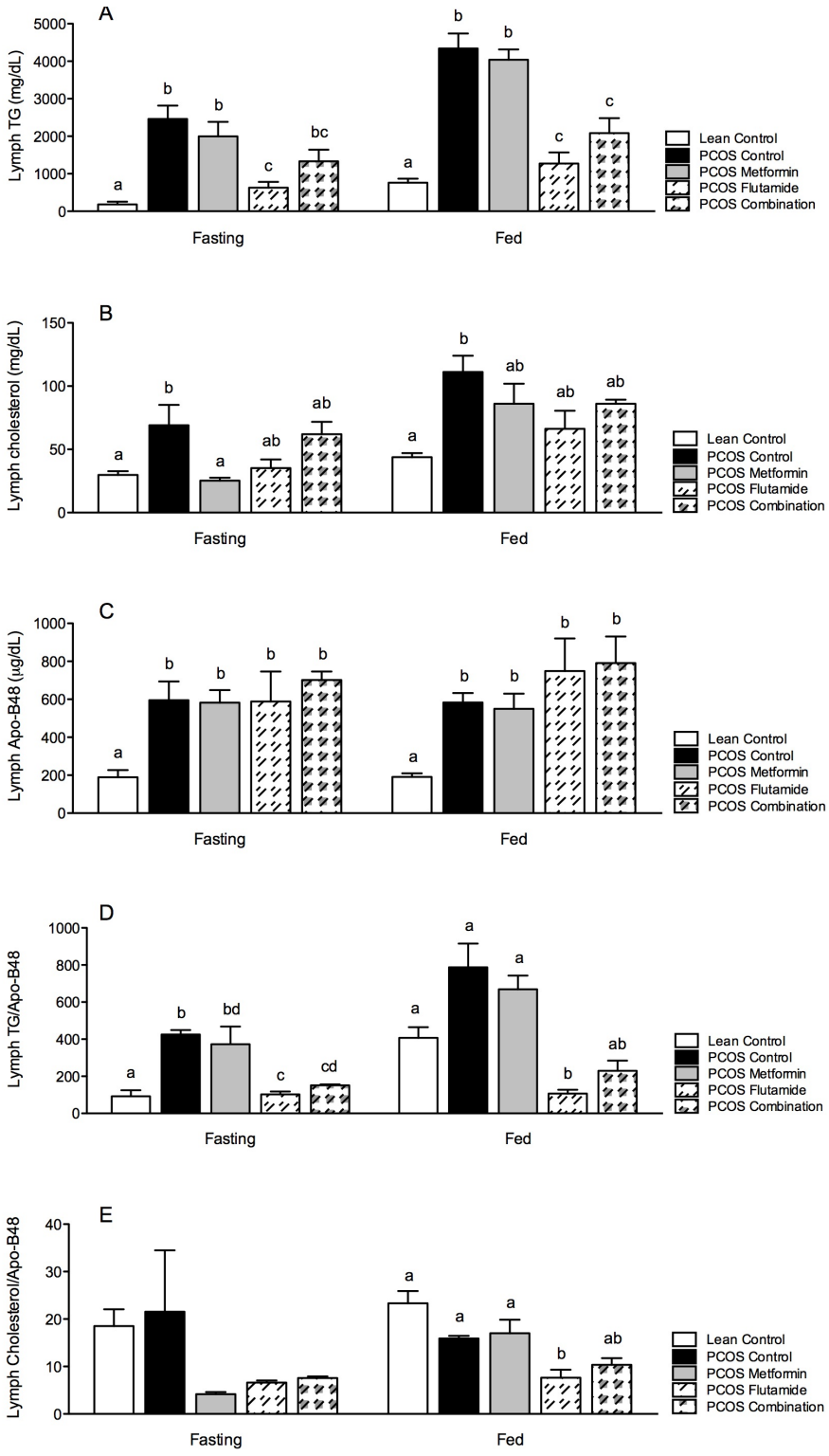


Figure 3-9. The nascent intestinal lymph chylomicron content. Triglyceride (panel A), cholesterol (panel B), apoB48 (panel C), TG/apoB48 (panel D) and TC/apoB48 (panel E) content following saline (fasting state) and Intralipid (fed state) infusion in PCOS-prone control and treatment groups. Sample size of n=6 was used for this measurement. Data is presented as mean±SEM. Bars with a common letter are not statistically different ($p<0.05$).

3.3.7 Hepatic and intestinal gene expression

3.3.7.1 Hepatic and intestinal androgen-estrogen receptor and lipogenic gene mRNA expression

Androgen and estrogen receptor and lipogenic gene mRNA expression were measured in liver and intestine, and the results are shown in Figure 3-10-3-13. Hepatic expression of nuclear receptors AR and ERα are shown in Figure 3-10. AR mRNA expression was not different between groups, however it tended to be approximately 30% higher in PCOS-prone control animals. Treatment with metformin lowered hepatic AR expression by 40% compared to the PCOS-control group, however this was not statistically significant. Treatment with flutamide alone or combination did not alter hepatic AR expression (Figure 3-10). Estrogen receptor alpha (ERα) mRNA was four-fold higher in PCOS-prone animals compared to lean controls. Metformin and metformin-flutamide combination treatments further increased ERα mRNA expression by 35% compared to untreated PCOS-prone group.

PCOS-prone animals exhibited an increase in hepatic mRNA expression of LXRα, SREBP1, low-density lipoprotein receptor (LDLR), HMGR, apoB, ACC, FAS and diacylglycerol acyltransferase 1 (DGAT1) compared to lean controls, as shown in Figure 3-11. Metformin treatment tended to increase LXRα, SREBP1, HMGCR, ACC and FAS compared to PCOS control but only the latter was significantly increased by 35%. Flutamide treatment alone tended to lower SREBP1, LDLR, and HMGCR, and flutamide-metformin combination had no effect on these genes compared to the PCOS control group. The combination of metformin-flutamide significantly increased LXRα and PPARα by 35% and 50% compared to the PCOS control group. Flutamide treatment alone significantly increased DGAT1 mRNA expression by 30%, but not in a combination with metformin or metformin alone (Figure 3-11). Hepatic PPARα mRNA expression tended to be lower in the PCOS-prone group compared to the lean control, and metformin and flutamide treatments lowered PPARα mRNA expression by 40% compared to the lean control group, but not compared to the PCOS-control group. There was no difference in SREBP2 and SCAP mRNA expression observed between groups.

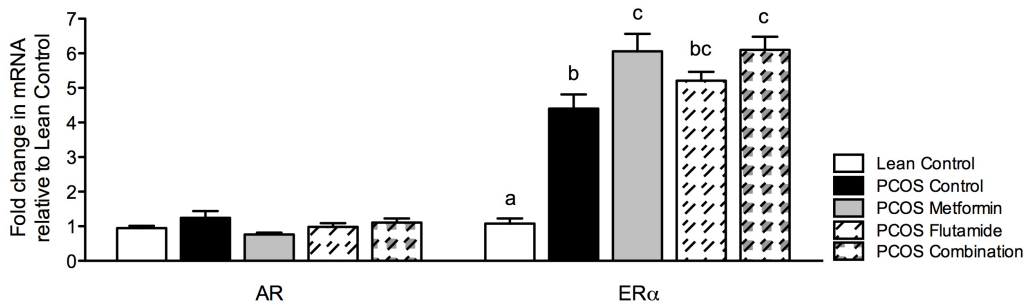


Figure 3-10. Hepatic androgen receptor (AR) and estrogen receptor-α (ERα) mRNA expression in PCOS-prone and treatment groups. Sample size of n=4 was used for this measurement. Data is expressed as a fold change compared to the lean control group and is presented as mean±SEM. Groups with a common letter are not statistically different ($p<0.05$).

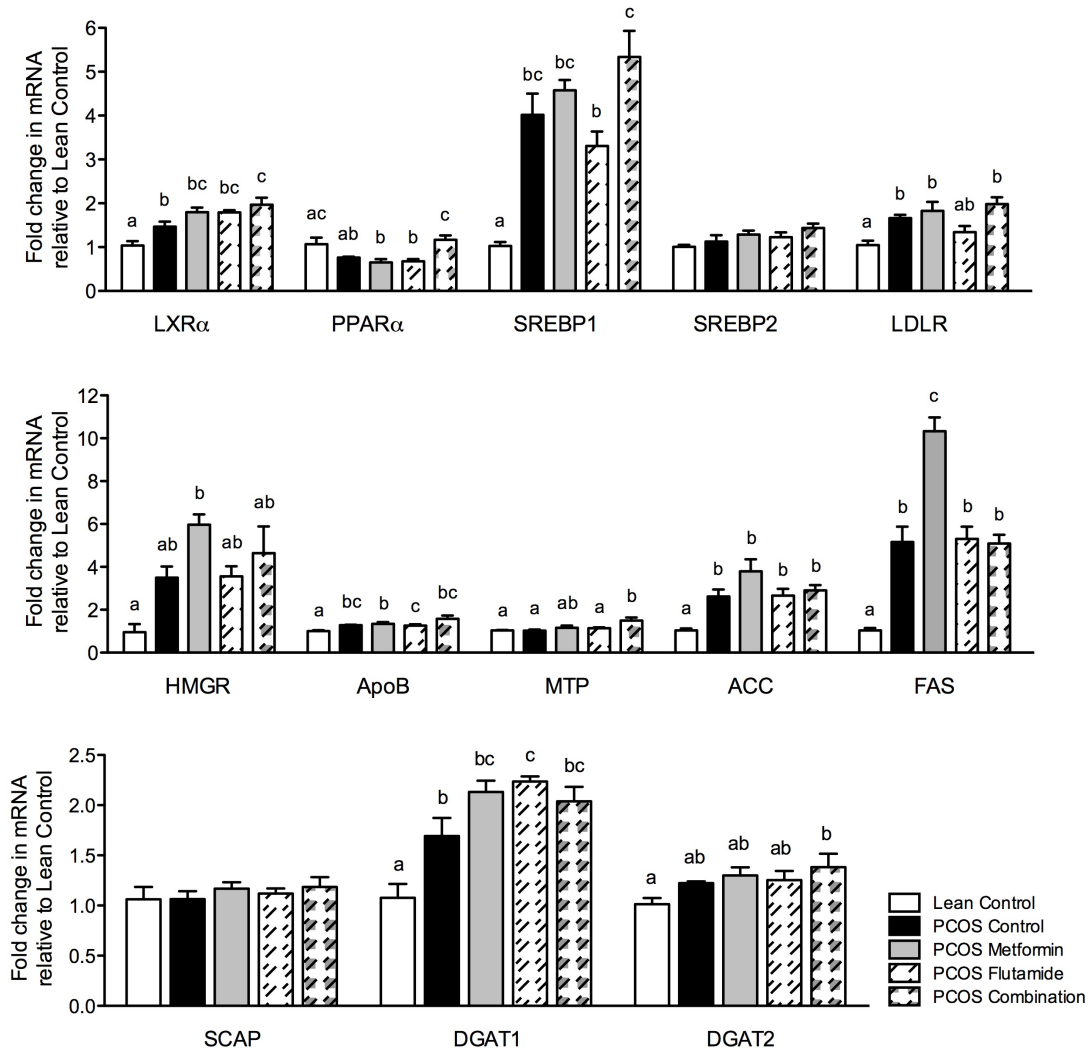


Figure 3-11. Hepatic lipogenic gene mRNA expression in PCOS-prone and treatment groups. Sample size of n=4 was used for this measurement. Data is expressed as a fold change compared to the lean control group and is presented as mean±SEM. Groups with a common letter are not statistically different ($p<0.05$).

Intestinal gene expression is shown in Figure 3-12 and 3-13. AR, ERα and ERβ mRNA expression did not significantly differ between the lean control and PCOS-prone groups, however ERα expression was two-fold higher in the intestine from lean control animals compared to the PCOS-prone group, and this result is in contrast to that observed in the liver. Metformin and flutamide treatments reduced AR mRNA expression, however this was only reached significance in the metformin-flutamide treatment group. ERβ mRNA expression was unchanged by metformin and flutamide treatments, whilst metformin-flutamide combination significantly decreased ERβ mRNA expression by more than 60% compared to the PCOS-prone control and other treatment groups.

The PCOS-prone control group showed increased mRNA expression of SREBP2, LDLR and apoB compared to lean controls, consistent with higher plasma and intestinal lipid and apoB-lipoprotein secretion in the lymph. Intestinal mRNA expression of LXRα was significantly reduced in PCOS-prone animals compared to lean controls by 25%, and was restored to lean control expression with metformin treatment alone. Flutamide treatment alone tended to lower SREBP2 mRNA expression compared to the PCOS control group. PPARα, SREBP1, HMGR, FAS, SCAP and DGAT1 mRNA levels did not differ significantly between groups. Microsomal triglyceride transfer protein (MTP) mRNA expression was increased by 20% and 15% in animals treated with metformin and flutamide alone, but not in the animals treated with combination. ACC mRNA expression was significantly different between metformin and flutamide alone groups, with elevated expression in the metformin and lowered expression in the flutamide treatment groups.

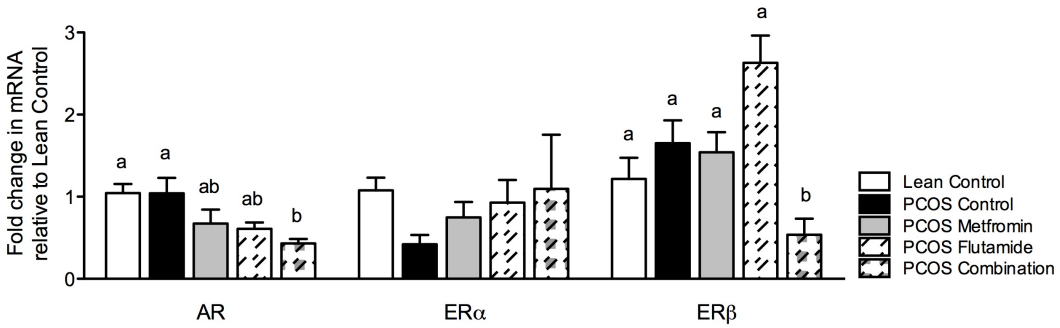


Figure 3-12. Intestinal gene expression of androgen receptor (AR), estrogen receptor α (ERα) and β (ERβ) in PCOS-prone and treatment groups. Sample size of n=4 was used for this measurement. Data is expressed as a fold change compared to the lean control group and is presented as mean±SEM. Groups with a common letter are not statistically different ($p<0.05$).

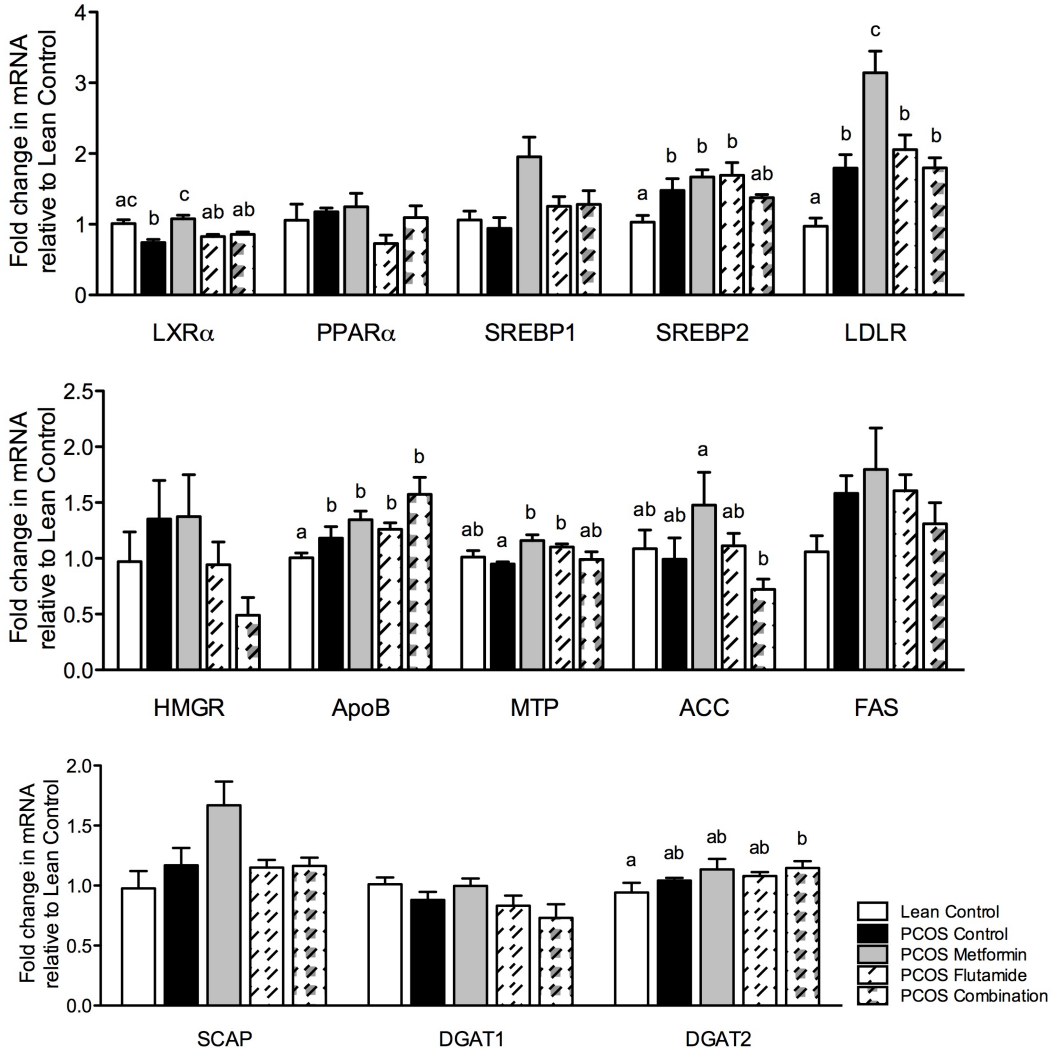


Figure 3-13. Intestinal lipogenic gene mRNA expression in PCOS-prone and treatment groups. Sample size of n=4 was used for this measurement. Data is expressed as a fold change compared to the lean control group and is presented as mean±SEM. Groups with a common letter are not statistically different ($p<0.05$).

3.3.7.2 Hepatic and intestinal insulin signaling gene mRNA expression

Hepatic and intestinal insulin signaling gene mRNA expression is shown in Figure 3-14 and 3-15. The expression of insulin receptor (IR) in liver was upregulated by 30% with the metformin treatment and significantly greater by 2-fold in the flutamide and the combination treatments. Mitogen activator protein kinase 1 (MAPK1) mRNA was 30%, 40% and 50% higher in the metformin only, flutamide only and the metformin-flutamide combination treated groups, respectively. Protein kinase B (a.k.a. AKT2) expression was also increased by all treatments by 40% (metformin), 90% (flutamide) and 80% (metformin and flutamide combination) compared to the PCOS-prone control group. No change in the expression of tyrosine-protein phosphatase non-receptor type 1 (PTPN1), c-Jun N-terminal kinase (JNK) and glycogen synthase kinase 3 (GSK3) were observed following treatments (Figure 3-14). There was a trend, however, of JNK to be higher by 45% in PCOS-prone control animals compared to lean controls, and all treatments were lowered to the level of the lean control group. GSK3 was similar between lean control, PCOS-prone control and metformin, and in flutamide and combination groups GSK3 was reduced by 30% and 50%, respectively compared to untreated PCOS-prone animals, however this did not reach statistical significance.

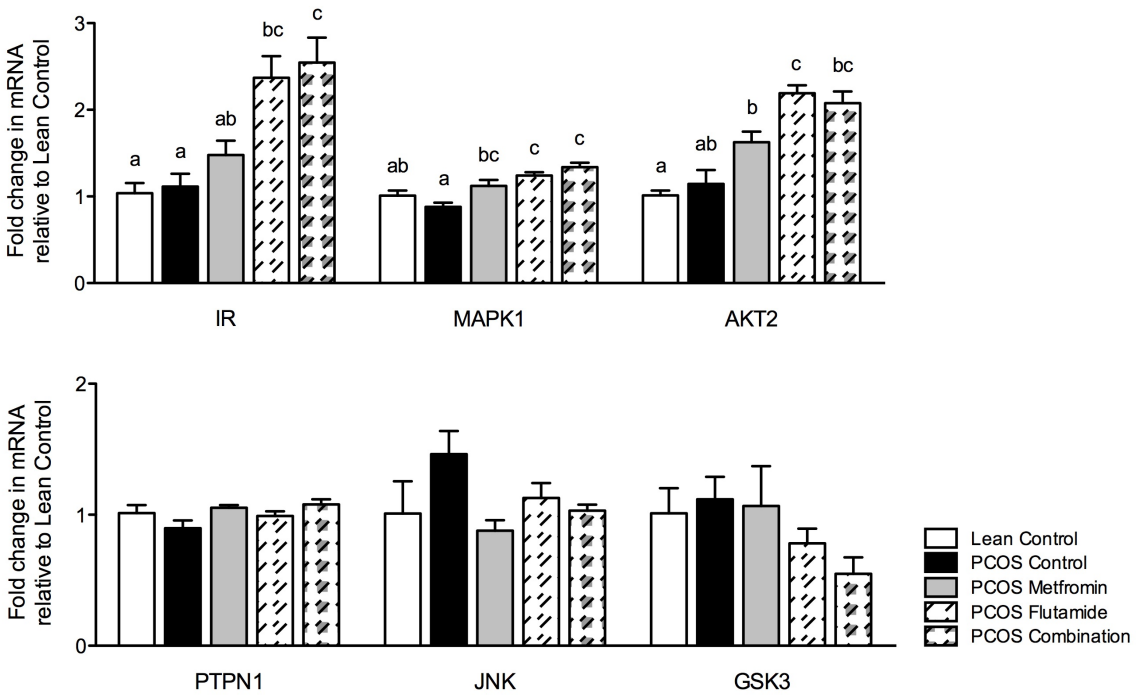


Figure 3-14. Hepatic insulin signaling gene mRNA expression in PCOS-prone and treatment groups. Sample size of n=4 was used for this measurement. Data is expressed as a fold change compared to the lean control group and is presented as mean±SEM. Groups with a common letter are not statistically different ($p<0.05$).

Insulin receptor expression was significantly lower in the intestine of PCOS-prone groups by 50% compared to the lean control group and the treatments were observed to have no effect on the mRNA expression of this protein (Figure 3-15). Similarly, MAPK1, AKT2 and PTPN1 mRNA levels were found to be lower in PCOS-prone animals. Flutamide treatment significantly lowered MAPK1 expression by 30% and flutamide-metformin reduced MAPK1 expression further by 30%. Flutamide and metformin treatments did not have an effect on AKT2 expression independently, but in combination reduced AKT2 by 50% (Figure 3-15). PTPN1 and JNK mRNA expression was also significantly reduced with flutamide-metformin combination treatment by 30% and 40%, respectively, compared to PCOS-prone control group. However, there was no statistical difference between lean control and PCOS-prone groups in JNK and GSK3 mRNA expression. GSK3 expression tended to be lowered with flutamide and combination treatments by 30% and 50%, respectively, compared to the PCOS-prone control groups, but this was not statistically significant.

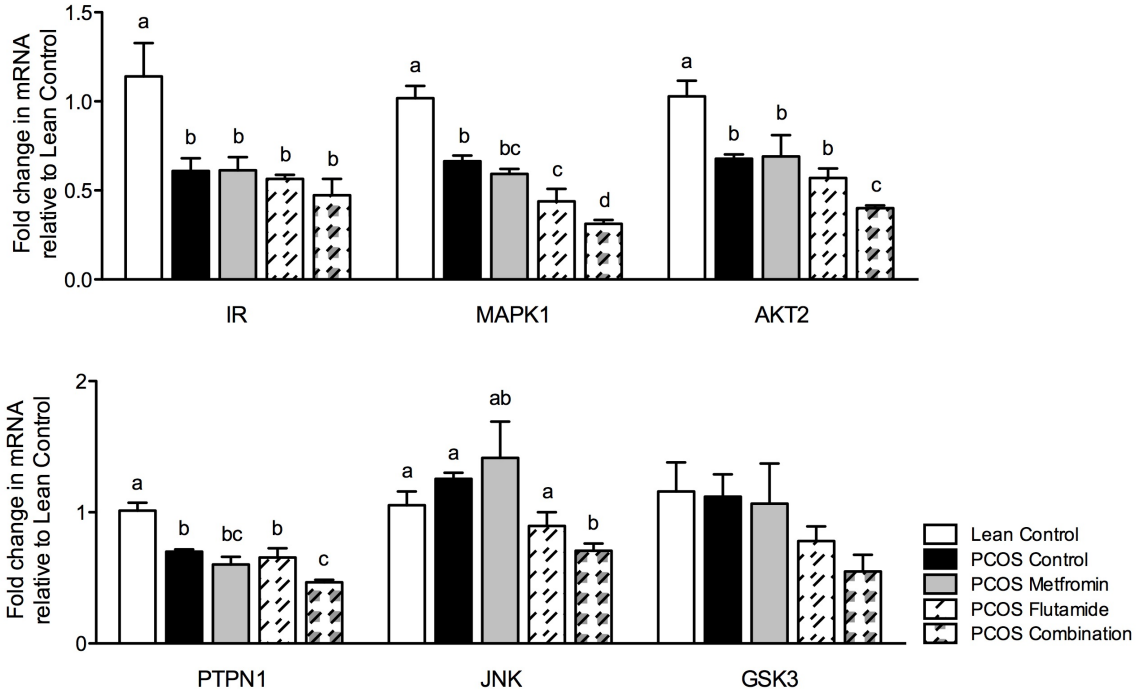


Figure 3-15. Intestinal insulin signaling gene mRNA expression in PCOS-prone and treatment groups. Sample size of n=4 was used for this measurement. Data is expressed as a fold change compared to the lean control group and is presented as mean±SEM. Groups with a common letter are not statistically different ($p<0.05$).

3.4 Discussion

Hyperinsulinemia and hyperandrogenemia have been proposed to be involved in the pathophysiology of cardiometabolic risk, in particular atherogenic dyslipidemia, which affects approximately 70% of adolescents and adults with PCOS (Coviello *et al* 2006, Legro *et al* 2001, Wild *et al* 2012). The aim of this study was to investigate the effects and mechanisms of action of the insulin sensitizer metformin, the androgen receptor antagonist flutamide and combination metformin-flutamide therapy, on fasting and non-fasting plasma lipid, apoB-lipoprotien and insulin-glucose metabolism, and endocrine-reproductive indices in the PCOS-prone JCR:LA-*cp* rodent model. The major findings of this study appear to be consistent with clinical findings for metformin and flutamide treatments (Bargiota *et al* 2012, Diamanti-Kandarakis *et al* 1998, Gambineri *et al* 2004, 2009, Ibanez *et al* 2000). Metformin improved fasting insulin and HOMA-IR, and FAI and ovarian follicular development in PCOS-prone animals. On the other hand, flutamide treatment improved fasting plasma TG, apoB48 and apoB100-lipoproteins, and this effect was most pronounced in the PCOS-prone animals treated with a combination of flutamide-metformin. In addition, effects of flutamide, alone or in combination were associated with intestinal secretion of chylomicrons with reduced TG and TC per apoB48 particle. Interestingly, these findings did not appear to be associated with significant modifications in plasma testosterone, FAI or mRNA expression of lipogenic, AR or ER gene expression. However, metformin-flutamide appeared to target select genes, such as insulin receptor, MAPK1 and AKT2 associated with hepatic and intestinal insulin-glucose signaling.

3.4.1 The effect of metformin, flutamide and metformin-flutamide combination on endocrine-reproductive indices in PCOS-prone JCR:LA-cp rats

Endocrine hormone profiles were similar in lean and PCOS-prone animals with no significant difference observed in serum TT and FT or estradiol concentrations. The animals at the end of the intervention were 16 weeks old, and previously our laboratory has reported that lean and PCOS-prone JCR:LA-*cp* rodents have similar levels of TT at 6 weeks and similar E_2 levels at 12 weeks of age (Shi *et al* 2009). Androgen concentrations may not significantly elevate until

animals are >12 weeks and/or into late adulthood, although ovarian morphology and acyclicity are present (Shi *et al* 2009). Plasma SHBG concentrations were significantly lower in PCOS-prone groups and did not change following treatment, however the FAI tended to be lower in the metformin and metformin-flutamide combination treatment group compared to the PCOS-prone control group. A recent meta-analysis by Tang and colleagues reported an attenuating effect of metformin on serum FT (WMD=-0.60; 95%CI; P<0.001), but no effect on serum SHBG concentration in women with PCOS (Tang *et al* 2012). Although studies to date report inconsistent findings, some studies have demonstrated that metformin may reduce steroidogenesis in the ovary and adrenal gland via inhibition of key steroidogenic enzymes, therefore reducing levels of FT, E_2, DHEAS and FAI in plasma (Nestler *et al* 1997, Palomba *et al* 2009, Kolodziejejczyk *et al* 2000, Nestler *et al* 1996, 1997, Velazquez *et al* 1994, Ehrmann *et al* 1997a, Marca *et al* 1999, Vrbikova *et al* 2001). In this study metformin alone did not lower serum testosterone but did tend to lower FAI, and this trend was also seen in the combination treatment. In PCOS, 17,20-lyase and 17α-hydroxylase, enzymes essential for testosterone synthesis, have been reported to be dysregulated in PCOS ovaries (Rosenfield *et al* 1990, Nestler *et al* 1997), and flutamide has been shown to inhibit 17α-hydroxylase and 17,20-lyase activity in rat testis *in vitro* (Ayub *et al* 1987). Clinically, flutamide treatment has been demonstrated to lower plasma TT, FT, DHEA, androstenedione and SHBG concentrations in PCOS after short (8 weeks) and long term (18 months) treatment, and to increase the number of ovulatory cycles (De Leo *et al* 1998, Eagleson *et al* 2000, Ibanez *et al* 2000, Paradisi *et al* 2013). However, the results of the present study do not support flutamide inhibition of T synthesis, at least as measured by plasma TT and free T. On the other hand, flutamide inhibition of AR may mediate other effects on steroidogenic pathways such as decreased ACTH-stimulated adrenal androgen production (DHEAS, FT) and 17β-hydroxysteroid dehydrogenase enzyme activity, but these were not measured in this study (Vrbikova *et al* 2004). Metformin has been proposed to improve insulin sensitivity, which mediates a downregulation of 17,20-lyase and 17α-hydroxylase enzymes in ovarian androgen synthesis (Ehrmann *et al* 1997b, Biallargeon *et al* 2003). Therefore, metformin and metformin-flutamide combination may mediate effects through insulin regulation of ovarian androgen synthesis but this was not

measured directly in this study. It has been shown that atretic follicles have higher testosterone/E_2 ratio, and it has been proposed that androgen-induced follicular atresia opposes estradiol-driven granulosa cell proliferation and follicular development (Uilenbroek *et al* 1980. This is may occur through AR binding leading to reduced ovulation rate and impaired cycle profile in PCOS (De Leo *et al* 1998). Although the cyclicity profile was not measured in this study, the number of atretic follicles was significantly reduced following flutamide treatment.

Hyperinsulinemia is a common metabolic feature of PCOS and may act to upregulate ovarian testosterone synthesis, impair follicular growth and ovulation rate (Pirwany *et al* 1999). In the ovary insulin enhances LH-induced androgen synthesis and secretion from theca cells, and increases granulosa cell sensitivity to FSH which enhances atretic and cystic follicle formation, and increases ovarian volume (Giallauria *et al* 2009). The ovaries from PCOS-prone animals were observed to have five times more atretic follicles compared to lean control rats. Clinically, an increased number of atretic follicles and small cysts is a common feature in PCOS (Takahashi *et al* 1994). Both insulin and androgens upregulate IGF1 receptor expression, which stimulates primordial follicle recruitment into the growing follicle pool and IGF1 prevents follicle apoptosis in the follicular phase which leads to follicular atresia (Giallauria *et al* 2009, Cheng *et al* 2002). Metformin and flutamide have been shown to restore cyclicity and the number of ovulatory cycles in PCOS patients (De Leo *et al* 1998, Gambineri *et al* 2004, Paradisi *et al* 2013). In fact, the AR is abundantly expressed in primary and secondary, but not in atrial containing follicles, such as tertiary and pre-ovulatory follicles (Cheng *et al* 2002). It has been shown that androgen action in granulosa cells switches from stimulatory to inhibitory as follicles mature, and a later reduction in AR number in granulosa cells prevents this inhibitory action of androgens in primate ovaries (Hillier *et al* 1997). During these latter cycle stages, androgens promote follicle atresia and AR has been shown to be highly expressed in atretic follicles in mouse ovaries (Cheng *et al* 2002), suggesting that this failure to reduce AR number in maturing follicles may contribute to follicular atresia in PCOS. Although we did not measure mRNA AR or IGF1 expression in the ovary, increased AR expression and possibly IGF1 may help to explain the higher number of atretic follicles observed in PCOS-prone animals, and a normalization of

atretic follicle number compared to that of lean controls following metformin and flutamide treatment.

3.4.2 Beneficial effect of metformin and metformin-flutamide combination on insulin-glucose metabolism in PCOS-prone JCR:LA-cp rats

Metformin treatment alone reduced fasting plasma insulin and HOMA-IR by approximately 50% in PCOS-prone animals. However, metformin did not reduce insulin secretion following a meal, although glucose response tended to decrease. Clinically, metformin is prescribed routinely in PCOS individuals with impaired insulin-glucose metabolism (Al-Nozha *et al* 2013, Bargiota *et al* 2012, Harborne *et al* 2005, Tang *et al* 2012). Metformin has been shown to inhibit mitochondrial respiratory-chain complex-1 resulting in increased ADP-to-ATP ratio, which leads to activation of AMPK (Viollet *et al* 2012). AMPK phosphorylates insulin receptor and tyrosine kinase as well as downstream targets, insulin receptor substrate (IRS) 1 and 2, therefore restoring insulin signaling in muscles and the liver (Chopra *et al* 2012, Jakobsen *et al* 2001, Cheng *et al* 2006, Yuan *et al* 2003). In addition, AMPK activation has been shown to increase GLUT1 membrane translocation in human hepatocytes (Gunton *et al* 2003). Metformin also is proposed to inhibit hepatic gluconeogenesis due to of AMPK activation, which acts to decrease activity of catabolic pathways utilizing ATP such as gluconeogenesis and lipidogenesis (Viollet *et al* 2012). It has been shown that MAPK1 activation is downregulated in insulin resistance and activation of this pathway regulates insulin receptor expression (Zhang *et al* 2011). In this study metformin treatment increased hepatic MAPK1 mRNA expression by 20%, AKT2 and JNK by 40% and IR levels by 30%, compared to the PCOS-prone control group. AKT2 is an important regulator of insulin signaling and AKT2 activation promotes translocation of glucose transporters to the plasma membrane (Gonzalez *et al* 2006, Gezginci-Oktayoglu *et al* 2013). Increase in JNK expression and activation of the associated kinase pathway has been shown to increase serine phosphorylation of IRS-1 leading to impaired insulin signaling (Yang *et al* 2008). In turn, AMPK was shown to activate JNK and suppress phosphatase and tension homologue (PTEN) phosphorylation, which is a negative regulator of insulin signaling. Hence, inhibition of PTEN restores AKT2 activation and is linked to improved insulin signaling in cells and

responsiveness to insulin (Lee *et al* 2011). It is unknown whether metformin has a direct effect on the expression and/or activation on these insulin-glucose signaling proteins. However, it is known that metformin activates AMPK and its pathway signaling is independent on the insulin receptor/AKT2 and MAPK1 cascade; however, there are some cross-interactions. Therefore, metformin effect on AKT2, JNK and MAPK1 protein level and activity could be investigated in future studies.

Interestingly, PCOS prone animals exhibited an increase in hepatic ERα expression. ERα has been proposed to regulate insulin-glucose homeostasis and ERα knock-out (αERKO) mice develop insulin resistance (Bryzgalova *et al* 2006). Metformin, flutamide and combination treatments were shown to increase ERα mRNA expression in PCOS-prone animals. A study by Barros and colleagues reported that ERα activates genes associated with insulin-glucose metabolism such as PPARγ and GLUT4 in skeletal muscle and adipose tissues, resulting in an increase in tissue glucose uptake (Barros *et al* 2009). However, liver-specific αERKO were shown to be metabolically normal suggesting that hepatic ERα may not influence whole-body insulin-glucose metabolism (Matic *et al* 2013). Flutamide treatment alone lowered fasting plasma insulin by 30%, but this was not statistically significant. Flutamide had a more pronounced effect on insulin-glucose metabolism in combination with metformin in PCOS-prone rats. These results are consistent with clinical studies, in which flutamide alone does not attenuate insulin-glucose response and provides no additional benefit to lower fasting and non-fasting insulin and glucose concentrations (Gambineri *et al* 2004, Sahin *et al* 2004). It has been shown that plasma insulin concentrations positively correlate with decreased SHBG and increased testosterone concentrations in PCOS women (Burghen *et al* 1980, Dunaif 1997). Testosterone acts via the AR to elicit metabolic effects and AR polymorphisms, such as shorter $(CAG)_n$ repeat length, and these are associated with increased transcriptional activity of androgen receptor (Beilin *et al* 2000). Shorter CAG length of AR in addition to increased circulating plasma testosterone has been positively correlated with insulin concentrations in women with PCOS (Mohlig *et al* 2006). At this point, the mechanism linking AR activation to insulin resistance is poorly understood. In the metformin-flutamide treated group AKT2 and MAPK1 expression was

increased and AR mRNA expression was decreased. Furthermore, AKT2 has been shown to phosphorylate AR at Ser^{213} and Ser^{791} residues. This phosphorylation allows AR to be recognized by Mdm2 E3 ligase and undergo further ubiquitination and proteosomal degradation (Lee *et al* 2003). This may lead to a decrease in cytoplasmic AR levels and decreased androgen binding to AR. Therefore, improved insulin sensitivity in JCR:LA-*cp* PCOS-prone animals treated with metformin and flutamide combination could be due to synergistic effects on insulin signaling pathways.

3.4.3 Beneficial effect of flutamide and metformin-flutamide combination on fasting and non-fasting plasma lipids and apoB-lipoproteins in PCOS-prone JCR:LA-cp rats

The JCR:LA-cp model of PCOS and cardiometabolic risk has previously been established, and demonstrates similar fasting and postprandial plasma lipid profiles to that observed in women with PCOS (Shi *et al* 2009, Bahceci *et al* 2007, Jovanovic *et al* 2010, Sidhwani *et al* 2011, Silva Dantas *et al* 2013, Velazquez *et al* 2000). As expected, PCOS-prone rats had increased plasma concentrations of apoB-lipoproteins, TG and total cholesterol in the fasting and non-fasting states. Flutamide treatment independently reduced fasting plasma triglycerides by 40%, and although not significantly, tended to decrease apoB-lipoproteins and LDL-C by approximately 25% and 30%, respectively. These findings are consistent with clinical studies, which have shown flutamide reduces fasting plasma lipids, including TG, total cholesterol and LDL-C (Bahceci *et al* 1999, Gambineri *et al* 2009, Diamanti-Kandarakis *et al* 1998, Ibanez *et al* 2000). This is the first study to show that flutamide treatment may have the potential to attenuate the non-fasting lipid response in the PCOS condition, as flutamide attenuated iAUC for plasma TG, total cholesterol or apoB48-lipoprotein by 30-40%. Although these findings were not significant due to the variability observed in animal responses, collectively this data supports a role of the AR in lipogenic pathways.

Findings from clinical studies using metformin in PCOS have shown no effect to beneficial effects on plasma lipids, including TG, total cholesterol and LDL-C (Aghahosseini *et al* 2010, Moghetti *et al* 2000, Sahin *et al* 2007, Velazquez *et al* 1994). Different doses, length of

treatment and heterogeneity of patient population could result in variable results among the studies However, metformin has been shown to improve postprandial TG, VLDL, LDL and chylomicron apoB-lipoprotein concentrations in T2D patients challenged with a high fat meal (Eleftheriadou *et al* 2008). Metformin-flutamide treatment significantly reduces fasting plasma TG after 12 mths treatment, albeit under conditions of dietary caloric restriction (Gambineri *et al* 2009), and metformin-flutamide combined with an oral contraceptive reduces LDL-C and increases HDL-C, but does not reverse OC-induced hypertriglyceridemia (Ibanez *et al* 2004). In this study metformin treatment did not impact postprandial plasma lipids and apoB-lipoproteins in the PCOS-prone animals in this study. However, the metformin-flutamide combination reduced fasting plasma TG, apoB48 and apoB100, and tended to decrease LDL-C concentrations. Combination treatment appeared to further reduce fasting plasma apoB48 and apoB100 by 10-30% compared to metformin or flutamide alone. This attenuating effect is mainly due to flutamide action since metformin alone did not improve plasma lipids significantly.

3.4.4 Attenuating effect of metformin, flutamide and metformin-flutamide combination on intestinal lipid and apoB48 secretion in fasting and non-fasting states in PCOS-prone JCR:LA-cp rats

Intestinal lymph secretion of TG and cholesterol were reduced in the fasting and non-fasting state with flutamide treatment, but this did not reach statistical significance. However, the TG/apoB48 and TC/apoB48 ratios, which represent the lipid associated with each chylomicron particle, were significantly reduced in animals treated with flutamide. This may represent a reduction in atherosclerotic risk as it has been shown previously that less lipidated chylomicron particles are lipolyzed and cleared at a higher rate from the circulation (Martins *et al* 1996). It has been demonstrated that cholesterol dense CM remnants readily permeate the arterial wall and are atherogenic, therefore particles with reduced cholesterol content and increased rate of remnant clearance may contribute to a reduction in atherogenic risk (Proctor *et al* 2002, 2004). Interestingly, metformin treatment decreased cholesterol, rather than TG, secretion into lymph in the fasted state, and reduced TC/apoB48 ratio to similar levels of the lean control animals.

However, metformin-flutamide treatment did not appear to have any additional benefit on intestinal secretion of lipids and chylomicron-apoB48, suggesting that the AR does play an independent regulatory role in intestinal lipid secretion. It has been proposed IR results in intestinal over-secretion of chylomicrons (Federico *et al* 2006, Martins *et al* 1994, Vine *et al* 2007) and enterocytes from IR hamsters have decreased protein expression and activity of of IRS-1, AKT and increased protein expression and activity of protein tyrosine phosphatase non-receptor type 1 (PTPN1) (Federico *et al* 2006). Metformin treatment did not attenuate enterocyte mRNA expression of these genes and there was no reduction in intestinal lipid or chylomicron-apoB48 secretion in PCOS-prone. However, intestinal MAPK1, AKT2, PTPN1 and JNK expression were reduced with metformin-flutamide combination, suggesting a possible role of AR in the regulation of these genes that could mediate reductions in intestinal lipid secretion associated with insulin signaling in PCOS-prone animals. Further studies could examine protein and or phosphorylated protein modulation to determine the role of AR in regulating insulin signalling pathways.

3.4.5 The intestinal and hepatic AR, ER and lipogenic gene expression is altered with metformin, flutamide and metformin-flutamide combination treatment in PCOS-prone JCR:LA-cp rats

Flutamide, metformin and combination treatments lowered intestinal and tended to lower hepatic mRNA AR expression. This is in contrast to other cell culture studies that have shown flutamide upregulates AR mRNA expression (Hackenberg *et al* 1992, Lee *et al* 2003). Furthermore, our study showed flutamide treatment had no significant effect on FT, TT or FAI. To date no other studies have reported changes in AR expression in-vivo in response to flutamide. Further investigations are required to clarify the dose and action of flutamide, and changes in androgens associated with modulation of the AR, and downstream interaction with lipogenic genes in this rodent model. Despite AR inhibition normalizing the aberrant lipid profile in clinical PCOS conditions, whole-body AR knock-out female mice have increased fasting plasma TG, FFA, insulin and glucose, and increased hepatic TG accumulation (Lin *et al* 2005). Hepatic PPARα expression was reduced in AR-knock out mice suggesting a role of AR in

regulation fatty acid oxidation (Lin *et al* 2005). Collectively, data to date suggests that presence of functional AR is required for normal energy, insulin-glucose and lipid metabolism in females, however in the present study hepatic TG and lipogenic gene expression were not significantly altered by flutamide treatment.

Metformin decreased intestinal secretion of cholesterol in PCOS-prone animals to the level of lean animals in the fasted state. This may be related to LXRα regulation of cholesterol homeostasis, as LXRα mRNA expression was significantly increased in the enterocytes of metformin-treated animals. Intestine-specific LXRα activation has been shown to lower the intracellular cholesterol pool in the enterocyte, which reduces cholesterol incorporation into chylomicrons and secretion of cholesterol into lymph (Kruit *et al* 2006). LXR activation has been shown to increase LDLR degradation in hepatocytes and enterocytes, thus reducing LDL-C uptake into cells (Engelking *et al* 2012, Zhang *et al* 2012). In enterocytes, SREBP is responsible for LDLR expression and opposes LXR-mediated LDLR degradation (Engelking *et al* 2012). However metformin treatment did not alter SREBP2 mRNA expression in enterocytes suggesting the LDLR mRNA increase was a result of a compensatory mechanism opposing LXR induction of LDLR degradation or LDLR expression may have been upregulated by SREBP2. Therefore, further investigation of this mechanism is required to investigate protein expression of these enzymes in enterocytes to further elucidate metformin's action on the lipogenic pathways.

Enterocyte ERβ expression was significantly reduced by the combination treatment, but no effect was observed with metformin or flutamide treatment alone. Little is known regarding ERβ, lipid and insulin-glucose metabolism, particularly in conditions of PCOS, in which there is hyperandrogenemia and insulin resistance. Ovariectomized ERβ knock-out mice have been reported to have restored insulin-glucose metabolism, and normalized TG and cholesterol accumulation in adipocytes. These results suggested that ERβ deficiency plays a cardiometabolic protective role (Foryst-Ludwig *et al* 2008).

Hepatic AR expression tended to be increased in PCOS-prone animals, and AR expression in all treatment groups was lowered by 20-30% compared to the PCOS-prone control group but these findings were not significant. However, this data does support the potential involvement of the AR in regulation of lipogenic pathways, as flutamide inhibits activation of the AR preventing binding to androgen response elements on target genes. Indeed, activation of the AR at least in cancer cell lines has been shown to activate SREBP1, and cholesterol and TG synthesis (Heemers *et al* 2006). Our results showed flutamide reduced the expression of hepatic SREBP-1c and ApoB mRNA which may reflect the decrease in fasting total plasma TG and apoB-lipoproteins, however no change in hepatic mRNA of FAS or ACC genes involved in TG synthesis was observed. Total cholesterol was not reduced with flutamide treatment, and LDL-C was reduced by 30% but this did not reach significance and HMG-CoA expression was not altered. This effect could be due to LXR-mediated degradation of LDLR as it has been shown to increase inducible degrader of the low-density lipoprotein receptor (IDOL) (Zhang *et al* 2012). LXRα mRNA expression was increased with flutamide and metformin-flutamide combination, though protein levels were not measured in this study. Metformin has been proposed to activate AMPK, resulting in downregulation of lipogenic genes, such as SREBP1, HMGR and ACC, to reduce hepatic TG and cholesterol synthesis (Mihaylova *et al* 2011). However, no effect of metformin administration alone on hepatic lipogenic gene expression was observed in this study, but only in combination with flutamide. Metformin seemed to have an additive benefit with flutamide and further decreased fasting plasma apoB48 and apoB100, further supporting a possible interaction between insulin-signaling and AR. Hepatic PPARα expression was significantly increased in flutamide-metformin combination compared to control, suggestive of an increase in fatty acid oxidation, which may limit fatty acid availability for TG synthesis and incorporation into apoB-lipoproteins. In contrast expression of SREBP1 mRNA tended to be elevated and MTP mRNA expression was significantly upregulated with the combination treatment, which requires further investigation. An alternative possibility, although not measured in this study, is that the AR may interact with other nuclear transcription factors such as the glucocorticoid receptor (GR). The AR has shown to form a heterodimer with GR in a CV-1b monkey kidney cell line, which resulted in mutual inhibition of transcription activation of

both receptors and this effect may expand to the entire superfamily of these nuclear receptors such as ER, thyroid hormone (TR) and vitamin D receptors (Chen *et al* 1997). Genetic studies on GR receptor polymorphism in PCOS have reported GR playing an important role in insulin sensitivity and lipid metabolism (Maciel *et al* 2014), suggesting that impaired glucocorticoid signaling may contribute to metabolic aberrations in PCOS, but this requires further investigation.

3.5 Conclusion

The Insulin-sensitizing agent metformin, the AR antagonist flutamide and the combination treatment were shown to selectively improve metabolic and endocrine aberrations associated in JCR:LA-*cp* rodent model of PCOS. Metformin improved insulin sensitivity and decreased intestinal-chylomicron cholesterol secretion, which may in part be explained by upregulation in insulin sensing genes such as MAPK. Flutamide was shown to improve fasting plasma TG and apoB-lipoprotein concentrations, and intestinal-chylomicron TG and TC secretion, and these results are consistent with the hypolipidemic benefits of flutamide observed in clinical studies (Diamanti-Kandarakis *et al* 1998, Gambineri *et al* 2004, Ibanez *et al*, 2000). Furthermore, these findings highlight the effect of inhibition of the AR on reducing intestinal lipid secretion associated with apoB-48-chylomicrons and atherogenicity of the particles secreted. Indeed, this effect may be explained by flutamide targeting hepatic SREBP1 and apoB expression, however further studies are required to explore the effect of treatments on lipogenic gene protein expression and activation of the key regulatory enzymes such as AKT2, SREBP1, LXRα and MTP. The effects of flutamide appeared to be associated with potential downregulation of the AR, independent of changes in androgen metabolism, such as plasma free testosterone, and SHBG. Further investigations of protein and enzyme expression in androgen metabolism are required to elucidate the AR regulation of these pathways in the JCR:LA-cp rodent model of PCOS.

4. Investigation of the effects of dietary nicotinic acid to improve atherogenic blood lipid profile, insulin-glucose metabolism and reproductive indices in the JCR:LA-*cp* rodent model of polycystic ovarian syndrome

4.1 Introduction

Dyslipidemia is recognized as a major cardiometabolic risk factor in Polycystic Ovary Syndrome (PCOS), as it affects up to 70% of adolescent and adult women and predisposes them to the development of cardiovascular disease (Legro *et al* 2001, Coviello *et al* 2006, Wild 2012). A cluster of factors contributes to the dyslipidemic profile in PCOS. It includes elevated plasma triglycerides (TG) and apoB-lipoprotein concentrations in the fasting and non-fasting state, as well as increased fasting total cholesterol and low-density lipoprotein cholesterol (LDL-C) (Jovanovic *et al* 2010, Talbott *et al* 1995, Wild *et al* 2011, 2012). Women with PCOS also tend to have lowered high-density lipoprotein cholesterol (HDL-C) and reduced HDL-C particle size (Jovanovic *et al* 2010, Meirow *et al* 1996, Sighwani *et al* 2011). Postprandial lipemia caused by an increase in triglyceride-rich lipoprotein secretion and reduced clearance of these lipoproteins is a major factor exacerbating CVD risk (Hyson *et al* 2003, Roche *et al* 2000). Interestingly, both obese and normal weight PCOS subjects are prone to developing an atherogenic lipid profile, which has been associated with subclinical cardiovascular dysfunction including left ventricular and endothelial function and increased carotid intimal media thickness (Alipour *et al* 2008, Guerchi *et al* 2000, Jackson *et al* 2012, Orio *et al* 2004, Talbott *et al* 2000). Indeed, plasma TG are elevated in PCOS independent of body weight (Wiltgen *et al* 2010). The metabolic syndrome, insulin resistance and obesity, are co-associated with PCOS diagnosis and are proposed to contribute and exacerbate the dysregulation of lipid metabolism (Bahceci *et al* 2007, El-Mazny *et al* 2010, Glueck *et al* 2005, Kalra *et al* 2006, Rocha *et al* 2011, Talbott *et al* 2001, Xiang *et al* 2012). In addition, hyperandrogenemia has been proposed to facilitate an increase in lipidogenesis and dyslipidemia in PCOS, and free T or FAI at >50th percentile is associated with an exacerbated lipid profile (Carmina *et al* 2005, Dejager *et al* 2002, Lambrinoudaki *et al* 2006).

Diet and lifestyle modification strategies are considered the primary interventions to target improvements in the endocrinological and metabolic milieu associated with PCOS, including

lowering androgens to restore cyclicity and anovulation, insulin sensitivity and lower plasma lipids (Moran *et al* 2011, 2013). Hyperinsulinemia and insulin resistance is likely to contribute to atherogenic dyslipidemia in PCOS (Diamanti-Kandarakis *et al* 2007, Wild *et al* 2011). Insulin resistance is associated with increased lipogenesis and overproduction of hepatic (apoB100-containing VLDL) and intestinal (apoB48-containing chylomicron) triglyceride rich lipoproteins (TRL's) and decreased clearance of the remnants of these apoB-TRL's from the circulation (Eckel *et al* 1984, Garg 1996, Yki-Jarvinen *et al* 1984, Phillips *et al* 2002). Insulin sensitizers are commonly used to treat insulin resistance in PCOS, however they may or may not improve dyslipidemia in PCOS (Bargiota *et al* 2012, Diamanti-Kandarakis *et al* 2010). Thiazolinediones (glitazones) have been shown to improve insulin sensitivity, but have neutral or attenuating effect on plasma non-esterified fatty acids (NEFA) and plasma TG concentrations (Bargiota *et al* 2012, Pasquali *et al* 2006, Bargiota *et al* 2012, Koo *et al* 2007). However, these drugs are contraindicated in women with PCOS due to potential unwanted side-effects such as weight gain, adverse effect on fetal development, increased incidence of cardiac insufficiency and decreased bone density (Du *et al* 2012). Similarly, metformin is prescribed most commonly in PCOS to target improvements in insulin sensitivity, though metformin use does not concomitantly mediate improvements in blood lipids (Bargiota *et al* 2012). Evidence supports the use of metformin to restore insulin sensitivity, and restore cyclicity and ovulation, possibly via reductions in ovarian steroidogenesis (Baptiste *et al* 2010, Baillargeon *et al* 2005, 2006, Diamanti-Kandarakis *et al* 1998, Velazquez *et al* 1997). Lowering plasma insulin and/or androgen concentrations may mediate decreases in plasma lipids, but this is not observed in all cases, and 5-8% of individuals experience metformin intolerance (Lord *et al* 2003).

Lipid-lowering medications such as statins (HMG-CoA reductase inhibitors) and anti-androgens have been used to target PCOS-associated dyslipidemia. Statins have been shown to decrease plasma LDL-C, total cholesterol and TG concentration (Duleba *et al* 2012, Raja-Khan *et al* 2011, Sathyapalan *et al* 2009). However, intervention studies with statins have reported adverse effects on insulin sensitivity, which may offset potential improvements in lipid metabolism (Banaszewska *et al* 2007, Raja-Khan *et al* 2011). Furthermore, the use of statins in young women is contraindicated as statins may induce fetal malformations, congenital anomalies and

other teratogenic effects (Godfrey *et al* 2012, Kazmin *et al* 2007). In addition, statins have been proposed to have diabetogenic effects and may induce glycemia (Goldstein *et al* 2013). Flutamide, the androgen receptor antagonist, has also been used in PCOS to reduce hyperandrogenemia, improve reproductive indices and plasma lipids (Bahceci *et al* 1999, Gambineri *et al* 2009, Diamanti-Kandarakis *et al* 1998, Paradisi *et al* 2013). Similarly, flutamide is potentially teratogenic and hepatotoxic, and is also not recommended as a lipid lowering treatment in young women with PCOS (Kassim *et al* 1997, Ohbuchi *et al* 2009, Wysowski *et al* 1996). Steroidal anti-androgens such as cyproterone acetate, which suppress LH secretion and binds androgen receptor, and spironolactone, competitive inhibitor of aldosterone, have been used to improve androgens, hirsutism and ovulation rate (Azziz *et al* 2000, Lumachi *et al* 2003). However these interventions do not necessarily target atherogenic dyslipidemia in PCOS and cyproterone acetate may induce body weight gain (Bargiota *et al* 2012). Collectively, treatments to target dyslipidemia in PCOS are currently not considered safe or necessarily efficacious in all adolescents and young women with PCOS. Therefore dietary approaches using lipid-lowering nutrients have been proposed as a safe alternative to target atherogenic dyslipidemia in PCOS.

Niacin or nicotinic acid (NA) is a well-established lipid-lowering vitamin, which has been used clinically for over 50 years to treat dyslipidemia and reduce the risk of cardiovascular disease (Altschul *et al* 1955). Nicotinic acid therapy has been shown to reduce incidence of myocardial infarction, stroke and coronary artery revascularization procedures for worsening ischemic symptoms (Duggal *et al* 2010, Lavigne *et al* 2013). Nicotinamide is another form of niacin (or vitamin B3), though it does not exhibit lipid-lowering or cardioprotective effects compared to nicotinic acid (Bogan *et al* 2008, Carlson *et al* 1963). Nicotinic acid has been shown to lower fasting plasma TG and LDL-C, and to elevate HDL-C in dyslipidemic individuals (Canner *et al* 2006, Grundy *et al* 2002). Nicotinic acid also has anti-inflammatory properties as it reduces nitric oxide synthase activity and C-reactive protein concentration in plasma (Yu *et al* 2007). Nicotinic acid therapy has been shown to reduce carotid-media thickness and restore endothelial function in patients with metabolic syndrome, coronary heart disease and type 2 diabetes (Holzhauser *et al* 2011, Meyers *et al* 2004, Thoenes *et al* 2007, Wu *et al* 2010). The

mechanisms of niacin's hypolipidemic effects remain unclear, although inhibition of hepatic DGAT2 to reduce TG synthesis and increased post-translational apoB degradation resulting in lower VLDL production have been proposed (Ganji *et al* 2004, Jin *et al* 1999). Some clinical studies show that nicotinic acid may adversely impact insulin-glucose metabolism by increasing fasting glucose concentrations. Although, this glycemic effect may be outweighed by the positive lipid lowering effects to reduce long term CVD risk (Grundy *et al* 2002, Goldberg *et al* 2008). In PCOS, niacin treatment may be a potential dietary-nutraceutical approach to lower blood lipids. A very recent study by Aye and colleagues was the first to explore the effects of extended release niacin with laropiprant (a prostaglandin D_2 receptor antagonist which reduces niacin-induced flushing symptoms) treatment for 12 weeks on insulin-glucose and lipid metabolism in non-insulin resistant PCOS patients (Aye *et al* 2014). As expected, niacin improved fasting plasma TG, total cholesterol, LDL-C and HDL-C concentration, and reduced postprandial TG response following a mixed meal. In this study niacin treatment elevated fasting plasma insulin and HOMA-IR, but did not adversely affect postprandial insulin response following a meal. Fasting and postprandial glucose concentration remained unchanged with NA treatment. No effect of niacin on FAI and androgen levels was observed in this study (Aye *et al* 2014). To date, no other studies have reported the effects of nicotinic acid on plasma lipids, insulin-glucose metabolism or reproductive-endocrine parameters in PCOS. The discovery of the niacin receptor, PUMA-G in the rodent and GPR109A in humans (Tunaru *et al* 2003, Wise *et al* 2003), and the development of slow release formulas has maintained the viability of nicotinic acid as a potential lipid lowering therapy in PCOS. However, the mechanisms and physiological effects on hepatic and intestinal lipid metabolism remain unclear. Our laboratory has recently shown that niacin is an effective lipid-lowering agent in the male JCR:LA-*cp* rodent model of the metabolic syndrome in which nicotinic acid supplementation has been shown to lower fasting plasma TG, total cholesterol and apoB48 (Mangat *et al* 2013).

The aim of this study was to investigate the effects of nicotinic acid, and a combination of niacin and metformin on fasting and non-fasting lipid, insulin-glucose metabolism and reproductive indices in an established rodent model of PCOS, the JCR:LA-cp rodent. It was hypothesized that niacin independently and in combination with metformin can improve atherogenic lipid profile

and work synergistically with metformin to improve both insulin resistance and dyslipidemia in the JCR:LA-*cp* PCOS-prone rodent model.

4.1 Methods

4.2.1 Animal model and study design

Female JCR:LA-*cp* rats, n=48, were raised in the established breeding colony at the University of Alberta. PCOS-prone rats, n=36, were homozygous for the recessive *cp* gene (*cp/cp*) and lean control animals, n=12, were either homozygous normal (+/+) or heterozygous (+/?). Rats were weaned at 21 days of age and housed in the 12/12-hour reversed light cycle to allow establishing a normal diurnal cycle. At 10 weeks of age, rats were transferred to individual ventilated cages from the breeding colony housing. Lean control and PCOS-prone rats had ad libitum access to the randomly assigned diets. The chow for all animal groups was prepared using standard dry chow powder (5001, PMI Nutrition International, Brentwood, MO, USA). At 10 weeks of age, PCOS-prone rats were randomly assigned to one of the three treatment groups or a control group. PCOS-prone control animals (n=12) were fed a standard chow diet. The treatment groups of PCOS-prone rats were fed a diet supplemented with nicotinic acid, 10 mg/kg of chow (1% of food intake comprised nicotinic acid w/w or equal to 800 mg/kg of body weight), (n=12) or with niacin plus metformin, 10 mg/kg of chow (nicotinic acid) and 300 mg/kg of body weight (metformin), (n=12). The nicotinic acid dose used was based on previous studies in the male JCR:LA-cp genonotype in which a reduction in plasma and intestinal lipids (Mangat *et al* 2013). Metformin dose selection was described in Chapter 3, Section 3.2.1. Animals were fed the assigned diets for 6 weeks. Body weights and food intake were measured weekly.

At 5 weeks following the dietary intervention, a meal tolerance test or an oral fat challenge test was administered to assess plasma insulin-glucose metabolism and lipid and apoB-lipoprotein metabolism, respectively. All blood samples were taken using a tail snip procedure and collected into ETDA-coated Microtainer tubes as previously described (Vine *et al* 2007). At the end of the dietary intervention, half of the animals (n=6 per group) underwent a mesenteric lymph duct cannulation surgery to assess intestinal lipid metabolism. Fasting and non-fasting states were mimicked by saline and intralipid infusions as described in Chapter 3 (Vine *et al* 2014, Wang *et al* 2012). Fasting blood sample were collected via tail vein procedure or a cardiac puncture upon completion of the study. Animals were euthanized and ovaries were collected

and fixed in neutral formalin (10%). Animal care and experimental protocols were conducted in accordance with the Canadian Council of Animal Care and approved by Animal Ethics Committee of the University of Alberta.

4.2.2. Metabolic assessment

See Methods in Chapter 3 (Section 3.2.2). However, in this study animals were fasted for 24 hours prior to the oral high-fat meal challenge, meal tolerance tests and before euthanasia at the end of the dietary intervention.

4.2.3. Reproductive assessment

4.2.3.1 Estrus cyclicity assessment

Collection of vaginal smears was performed daily for 10 days prior to the end of the intervention to determine the estrous cycle phase of each animal. The four stages of reproductive cycle, proestrus (P), estrus (E), metestrus (M), and diestrus (D) were determined by Evans blue staining of vaginal smears and the microscopic assessment of the predominant cell type, as shown in Figure 4-1. Animals were considered to have normal cycle if at least three out of four cycle stages (P, E, M, D) were present within four to five consecutive days.

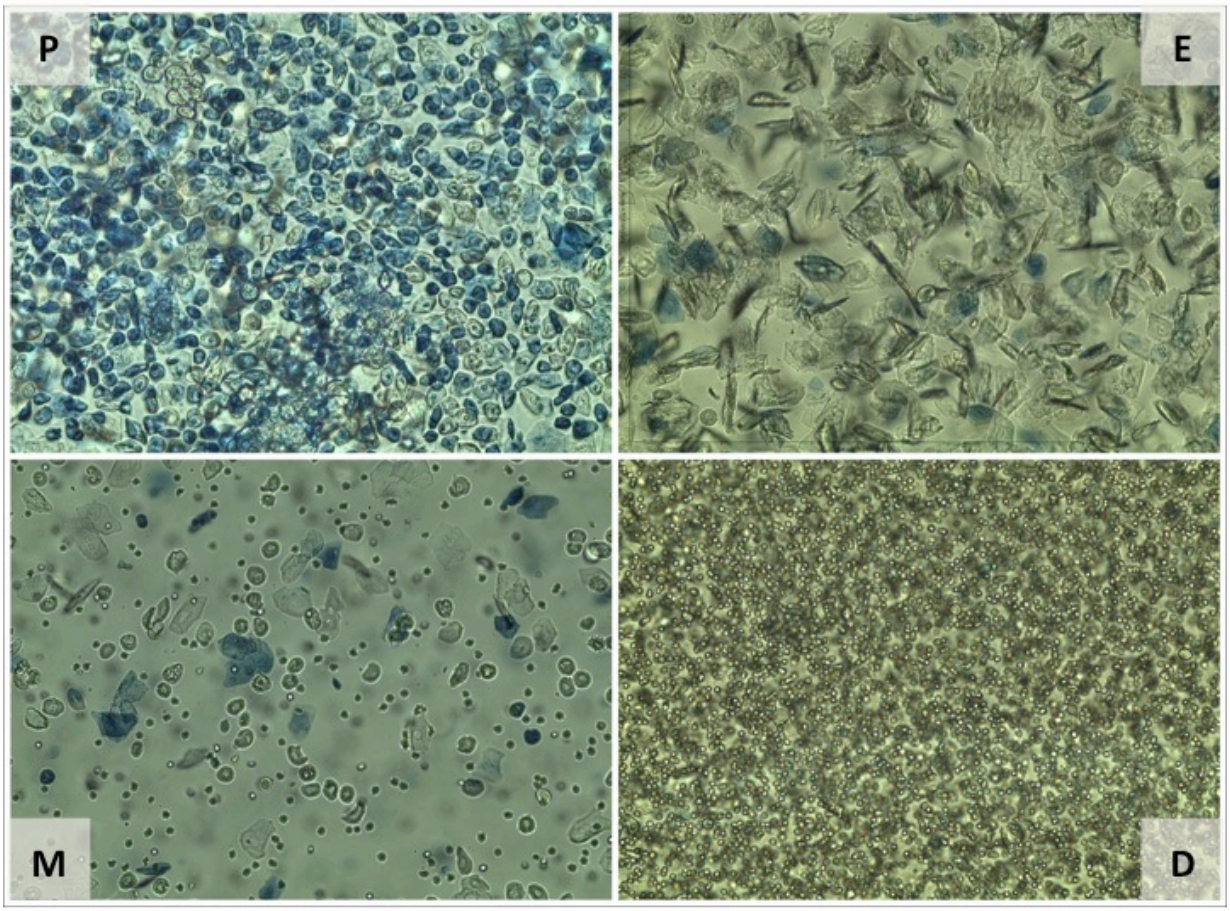


Figure 4-1. Photomicrographs of stained vaginal smears from JCR:LA-cp rats at four estrus cycle stages (magnification 20X). P, proestrus; E, estrus; M, metestrus; D, diestrus.

4.2.3.2 Ovary morphology assessment

See Methods in Chapter 3 (Section 3.2.4)

4.2.4 Statistical analysis

See Methods in chapter 3 (Section 3.2.5)

4.3 Results

4.3.1 Endocrine profile and reproductive assessment

4.3.1.1 Endocrine hormonal assessment

Endocrine hormone profile is shown in Table 4-1. There was no significant difference in free testosterone (FT) and total testosterone (T), SHBG or estradiol concentrations between groups. However, FT was 25% higher in PCOS-prone control animals, and niacin and niacin-metformin combination groups tended to have lower and similar FT concentrations to the lean control group. Free androgen index was not significantly different between lean and PCOS-prone control groups and intervention groups, although the intervention groups did tend to have a lower FAI by approximately 5-20%.

Table 4-1. Endocrine hormone profile of lean and PCOS controls, and niacin-metformin treatment groups.

	Lean Control	**PCOS Control**	**PCOS Niacin**	**PCOS Niacin+Metformin**
Free Testosterone (pg/ml)	0.44±0.04	0.55±0.07	0.43±0.05	0.44±0.03
Total Testosterone (ng/ml)	0.66±0.07	0.68±0.07	0.57±0.07	0.69±0.06
SHBG (pg/ml)	460.7±1.7	456.6± 11.6	469.6±13.5	482.4±19.4
Estradiol (ng/ml)	9.9±1.7	10.3±1.6	10.7±0.9	11.2±1.1
FAI	613±53	582±59	476±60	555±56

Sample size of n=6 was used for measuring endocrine hormone indices. Values with a common letter are not statistically different ($p<0.05$)

4.3.1.2 Ovarian follicular morphology

There was no difference in the frequency of each type of follicle identified between control and treatment groups (Table 4-2). Treatment with niacin-metformin combination tended to increase the number of corpus luteum and reduce corpus albicans in the ovary stroma by 80% compared to PCOS-prone control animals. Rodents normally have an estrous cycle every 4-5

days as shown in the lean control group. Estrous cycle frequency was significantly increased by 50% in niacin and niacin-metformin combination treatment groups.

Table 4-2. Ovarian morphology assessment in lean and PCOS controls, and the treatment groups.

	Lean Control	**PCOS Control**	**PCOS Niacin**	**PCOS Niacin+Metformin**
Primary Follicle	2.5±0.6	1.7±0.6	1.7±0.3	2.8±0.8
Secondary Follicle	2.3±0.4	2.8±0.6	2.8±0.5	2.3±0.7
Tertiary Follicle	2.5±0.7	4.2±0.8	4.7±0.6	4.0±0.9
Pre-ovulatory Follicle	1.2±0.4	1.2±0.4	1.2±0.5	1.2±0.3
Atretic Follicle	2.3±0.3	3.0±0.6	3.8±0.5	3.0±0.9
Corpus Luteum	1.3±0.6	2.5±0.5	2.3±0.7	4.5±1.2
Corpus Albicans	3.7±0.6	4.0±0.5	2.8±0.5	2.5±0.4
Estrus Cycles (per 10 days)	1.9 ± 0.10^{a}	1.0 ± 0.1^{b}	1.5 ± 0.2^{c}	1.5 ± 0.2^{c}

Sample size of n=6 was used for measuring ovarian morphology assessment. Values with a common letter are not statistically different ($p<0.05$)

4.3.1.3 Estrus cycle assessment

Estrous cycle patterns of lean and PCOS-prone controls and treatment groups are depicted in Figure 4-2. Two animals representative of each group were selected and the cycle patterns were plotted. Eleven out of twelve lean control animals had two cycles per ten-day period, and PCOS-prone control animals showed irregular or arrested estrous cycle patterns.

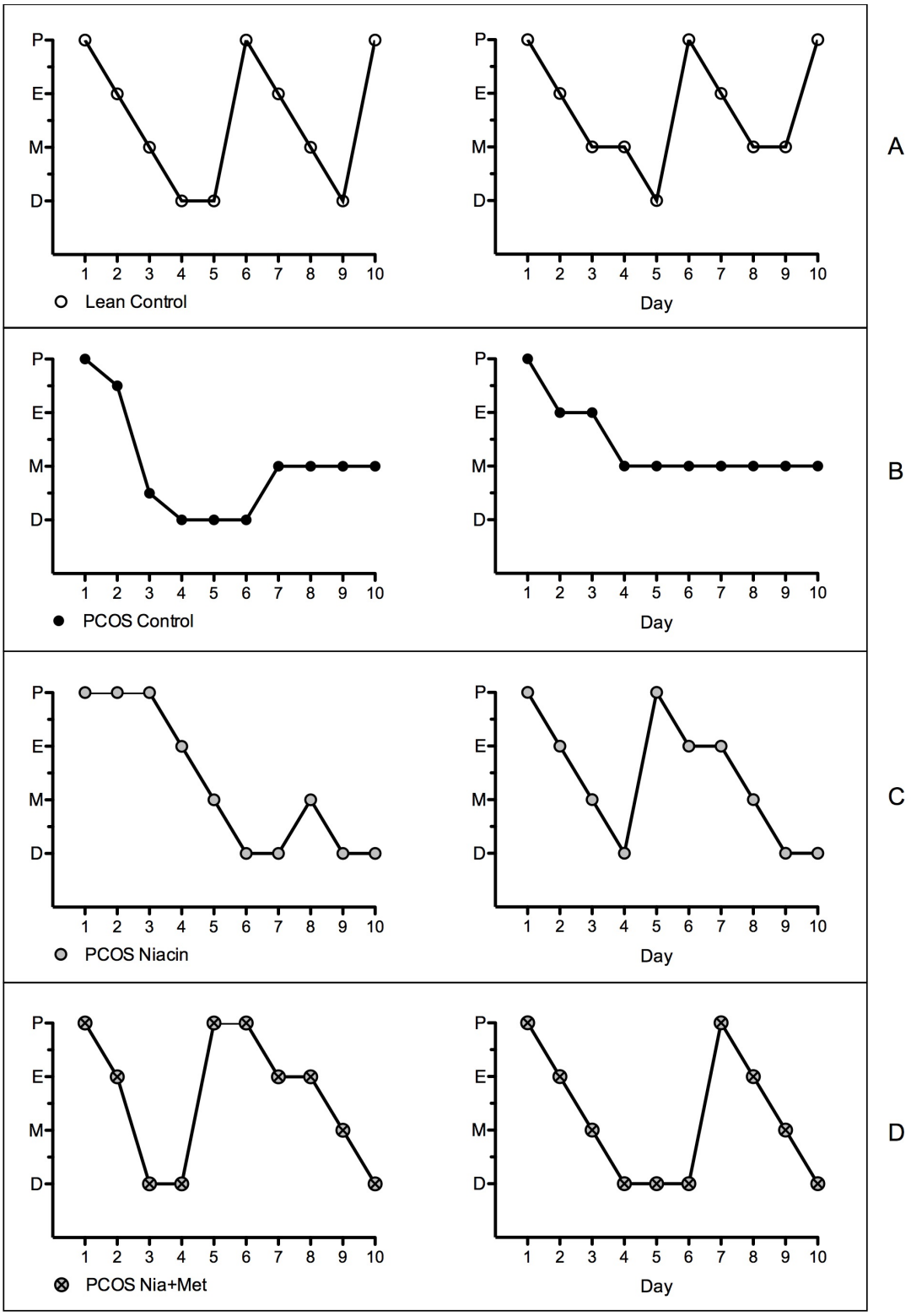


Figure 4-2. Representative estrous cycle patterns of lean control, PCOS-prone and treatment groups. Estrous cycles of two animals per group are presented on each panel A-D. Lean Control (panel A), PCOS-prone Control (panel B), PCOS Niacin (panel C), PCOS Niacin + Metformin (panel D). Cycle stages labeled as P (Proestrous), E (Estrous), M (Metaestrous), D (Diestrous).

4.3.2 Body composition and fasting biochemical parameters

4.3.2.1 Body weight and food intake

Body weight and food intake of control and treatment groups are listed in the Table 4-3. There was no significant difference in the final body weight and food intake in the PCOS-prone groups. However, niacin treatment alone and niacin-metformin was shown to reduce body weight gain compared to the PCOS-prone control group. However, the food intake was lower in both niacin and combination treatment groups, which may explain the lower body weight gain (p=0.011 and 0.04 respectively).

Table 4-3. Body weight and food intake of lean and PCOS controls and the treatment groups.

	Lean Control	**PCOS Control**	**PCOS Niacin**	**PCOS Niacin+Metformin**
Final Body Weight (g)	223 ± 4^{a}	406 ± 8^{b}	389 ± 9^{b}	388 ± 9^{b}
Body Weight Gain (g)	32 ± 3^{a}	120 ± 3^{b}	91 ± 3^{c}	100 ± 7^{c}
Food Intake (g/wk)	114 ± 1^{a}	177 ± 4^{b}	161 ± 5^{c}	158 ± 5^{c}

Values with a common letter are not statistically different ($p<0.05$)

4.3.2.2 Fasting plasma glucose, insulin and HOMA-IR

Fasting plasma glucose and insulin concentrations are shown in Table 4-4. As expected, lean control animals had significantly lower fasting plasma glucose and insulin concentrations compared to PCOS-prone groups ($p<0.001$). The mean fasting plasma glucose and insulin concentrations were lowered 15 and 60%, respectively, in the niacin-metformin combination group compared to the PCOS-prone group. Niacin alone did not seem to have a significant effect on insulin and glucose metabolism in PCOS-prone animals. HOMA-IR was 60% lower in the combination treatment group compared to the PCOS-prone group (p=0.04). Niacin treatment alone tended to decrease HOMA-IR by 20%, however this did not reach statistical significance.

Table 4-4. Fasting plasma glucose, insulin and HOMA-IR in lean and PCOS controls, and the niacin and metformin treatment groups.

	Lean Control	PCOS Control	PCOS Niacin	PCOS Niacin+Metformin
Glucose (mg/dl)	146.8±10.1[a]	191.1±7.3[b]	178.2±4.9[bc]	164.3±4. 6[ac]
Insulin (ng/ml)	0.21±0.04[a]	2.19±0.51[b]	1.96±0.33[bc]	0.89±0.18[ac]
HOMA-IR	1.85±0.49[a]	26.91±6.82[b]	21.58±3.79[ab]	8.96±1.79[a]

Values with a common letter are not statistically different ($p<0.05$)

4.3.2.3 Fasting plasma lipids and apoB-lipoproteins

Fasting plasma lipid profile is shown in Table 4-5. As expected PCOS-prone animals had increased fasting plasma TG, TC, apoB48 and apoB100 concentrations compared to lean control animals. Fasting plasma TG concentrations were not significantly different between PCOS-prone groups. Niacin treatment alone and niacin-metformin combination did not significantly improve fasting plasma TG, TC, apoB48 or apoB100 concentrations. However fasting plasma TG tended to decrease by 30% and apoB48 by 20%, following niacin and niacin-metformin combination treatments in PCOS-prone animals compared to the PCOS-prone control group. All groups had similar concentrations of LDL-C, HDL-C and non-esterified fatty acids, however, PCOS-prone animals tended to have higher NEFA (approximately 45%) and lower HDL-C levels (25%) compared to lean control animals, and treatments had no effect on these fasting plasma lipids.

Table 4-5. Fasting plasma lipid profile of lean and PCOS controls, and treatment groups.

	Lean Control	PCOS Control	PCOS Niacin	PCOS Niacin+Metformin
TG (mg/dl)	84.1±9.4[a]	1080.0±94.5[b]	960.9±151.2[b]	711.0±61.9[b]
NEFA (mmol/l)	0.61±0.12	0.90±0.11	0.96±0.12	0.94±0.14
TC (mg/dl)	74.0±2.7[a]	103.3±8.5[b]	120.9±9.0[b]	125.5±8.9[b]
LDL-C (mg/dl)	17.1±1.0	15.2±1.1	16.2±1.7	13.9±1.2
HDL-C (mg/dl)	41.6±1.38[a]	30.0±3.6[ab]	32.1±3.7[b]	24.5±4.8[ab]
Apo-B48 (µg/ml)	25.7±1.7[a]	520.7±44.1[b]	579.5±55.3[b]	425.5±51.5[b]
Apo-B100 (µg/ml)	793±50[a]	1966±137[b]	2052±135[b]	1909±184[b]

Values with a common letter are not statistically different ($p<0.05$)

4.3.3 Postprandial plasma lipids, apoB-lipoproteins, and insulin and glucose response to meal challenge

4.3.3.1 Postprandial plasma insulin and glucose response to an oral meal challenge

PCOS-prone animals were shown to have elevated total postprandial response in plasma glucose and insulin (total area under the curve, AUC) compared to lean control animals, as shown in Figures 4-3 and 4-4. Treatment with niacin-metformin combination improved the postprandial plasma glucose response (AUC, $p=0.019$) and was comparable to the response observed in lean control animals. Niacin treatment alone did not change or adversely alter the postprandial plasma glucose response to an oral meal challenge. The incremental plasma glucose response (iAUC) was not different between lean and PCOS-prone groups, although animals treated with niacin and niacin-metformin combination treatment did show a 40% and 30%, respectively, lower plasma glucose response compared to the PCOS-prone control group. The postprandial plasma insulin response (AUC) was significantly lower in both niacin (45%) and combination (55%) treatment groups compared to the PCOS-prone group. The iAUC insulin response was significantly lowered by 30% in the niacin and 60% in the niacin-metformin combination group, compared to the PCOS-prone control group. Niacin treatment alone

reduced the iAUC insulin response by 40% towards that observed in lean controls, however this did not reach statistical significance (Figure 4-4).

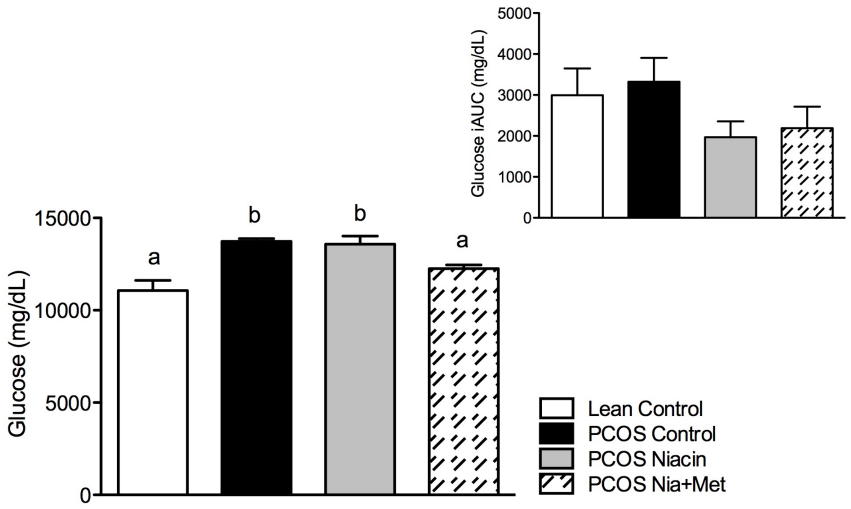


Figure 4-3. The postprandial plasma glucose response (AUC) to an oral meal tolerance challenge in lean and PCOS-prone controls and treatment groups. Sample size of n=6 was used for this measurement. Data is presented as mean±SEM. The total AUC and the change in glucose from fasted concentrations are shown and represented as incremental area under the curve (iAUC). Bars with a common letter are not statistically different ($p<0.05$).

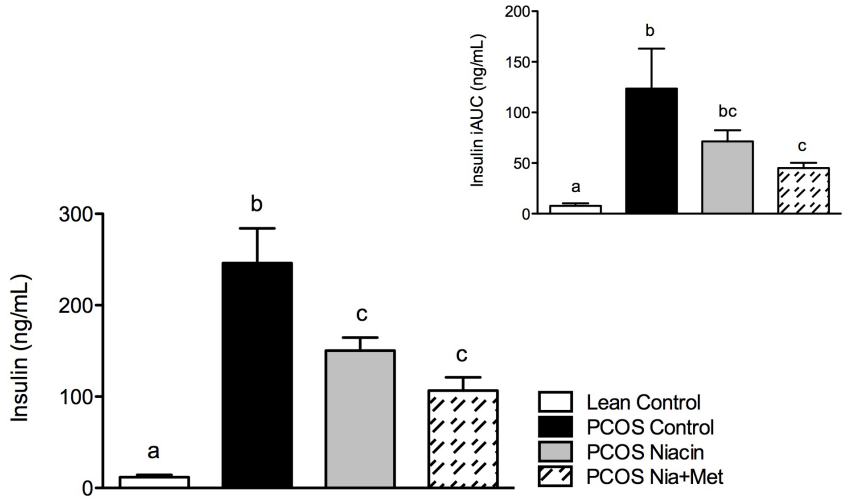


Figure 4-4. The postprandial plasma insulin response (AUC) to an oral meal tolerance challenge in lean and PCOS-prone controls and treatment groups. Sample size of n=6 was used for this measurement. Data presented as mean±SEM. The total AUC and the change in insulin from fasted concentrations are shown and represented as incremental area under the curve (iAUC). Bars with a common letter are not statistically different ($p<0.05$).

4.3.3.2 Postprandial lipid response following a high fat meal challenge

Total postprandial (AUC) plasma TG and cholesterol concentrations following a high fat meal challenge were significantly elevated in PCOS-prone animals compared to lean controls, consistent with previous results in this animal model as shown in Figure 4-4 and 4-5 (Chapter 3, Vine et al 2014). Treatments did not significantly improve postprandial plasma TG and cholesterol AUC response; however the plasma TG response tended to be 30% lower in the combination niacin-metformin treated group compared to PCOS-prone controls. Similarly, the plasma TG incremental response (iAUC) tended to be lower in the combination treatment group as shown in Figure 4-5. Incremental response in plasma cholesterol concentration (iAUC) was more than five fold higher in PCOS-prone animals, however treatments with niacin and niacin-metformin combination did not significantly improve the total AUC or cholesterol iAUC response in PCOS-prone animals.

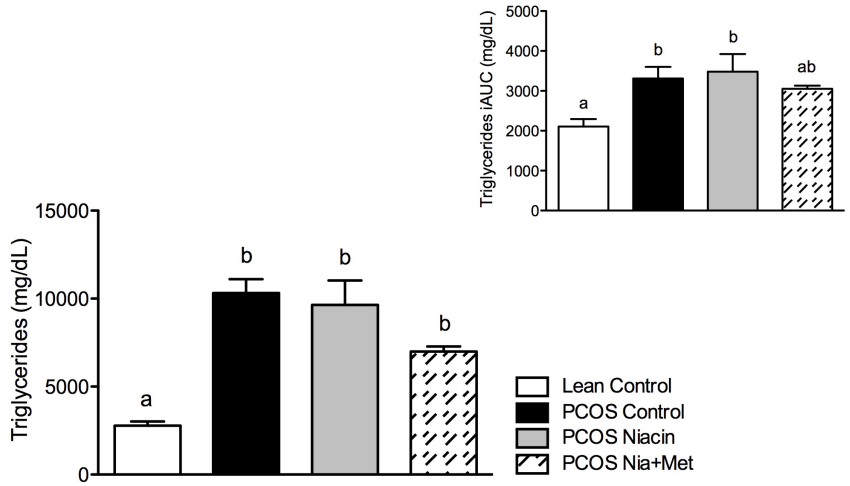


Figure 4-5. The postprandial plasma triglyceride response (AUC) to a high fat meal challenge in lean and PCOS-prone controls and treatment groups. Sample size of n=6 was used for the assessment. Data is presented as mean±SEM. The total AUC and the change in TG from fasted concentrations are shown and represented as incremental area under the curve (iAUC). Bars with a common letter are not statistically different ($p<0.05$).

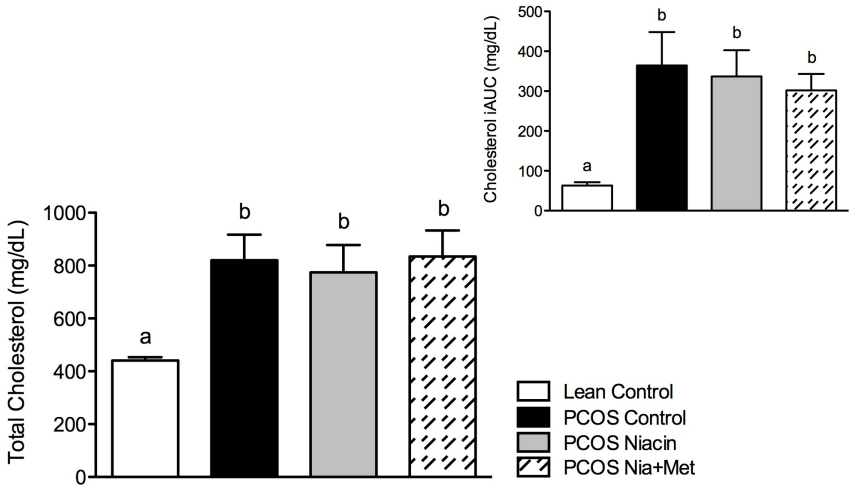


Figure 4-6. The postprandial plasma total cholesterol response (AUC) to high fat meal challenge in lean and PCOS-prone controls and treatment groups. Sample size of n=6 was used for the assessment. Data is presented as mean±SEM. The total AUC and the change in TC from fasted concentrations are shown and represented as incremental area under the curve (iAUC). Bars with a common letter are not statistically different ($p<0.05$).

4.3.4 Intestinal lymph secretion of lipid and apoB lipoproteins in the fasted and fed state

Intestinal lymph secretion of TG, cholesterol, apoB48 are shown in Figure 4-6. Lean control animals had decreased intestinal lymph concentration of TG (approximately 40-80%) and cholesterol (approximately 15-40%) in the fasting and non-fasting states compared to PCOS-prone groups, however this difference was not statistically significant. Niacin treatment alone tended to lower intestinal TG secretion approximately 35% in the fasting and 30% in the non-fasting state. The niacin-metformin combination treatment reduced intestinal TG secretion 50% in the fasting state and 30% in the non-fasting state. Intestinal chylomicron-apoB48 secretion was improved in both fasting (30%) and non-fasting (25%) conditions following niacin treatment, and in the niacin-metformin group apo-48 there was a trend towards decreased chylomicron-apoB48 secretion. Despite net lipid intestinal secretion, cholesterol and TG, being lower in lean control animals, the TG/apoB48 was 3-fold greater in lean controls in the non-fasting state compared to PCOS-prone animals ($p<0.001$), but there was no difference under fasting conditions. Similarly, lean control animals had an increased ratio of cholesterol/apo-B48 ratio in both the fasted and non-fasted states compared to PCOS-prone animals ($p<0.001$). The TG and cholesterol secreted per chylomicron-apoB48 particle was not different between treatment groups in the fasted state or non-fasted state.

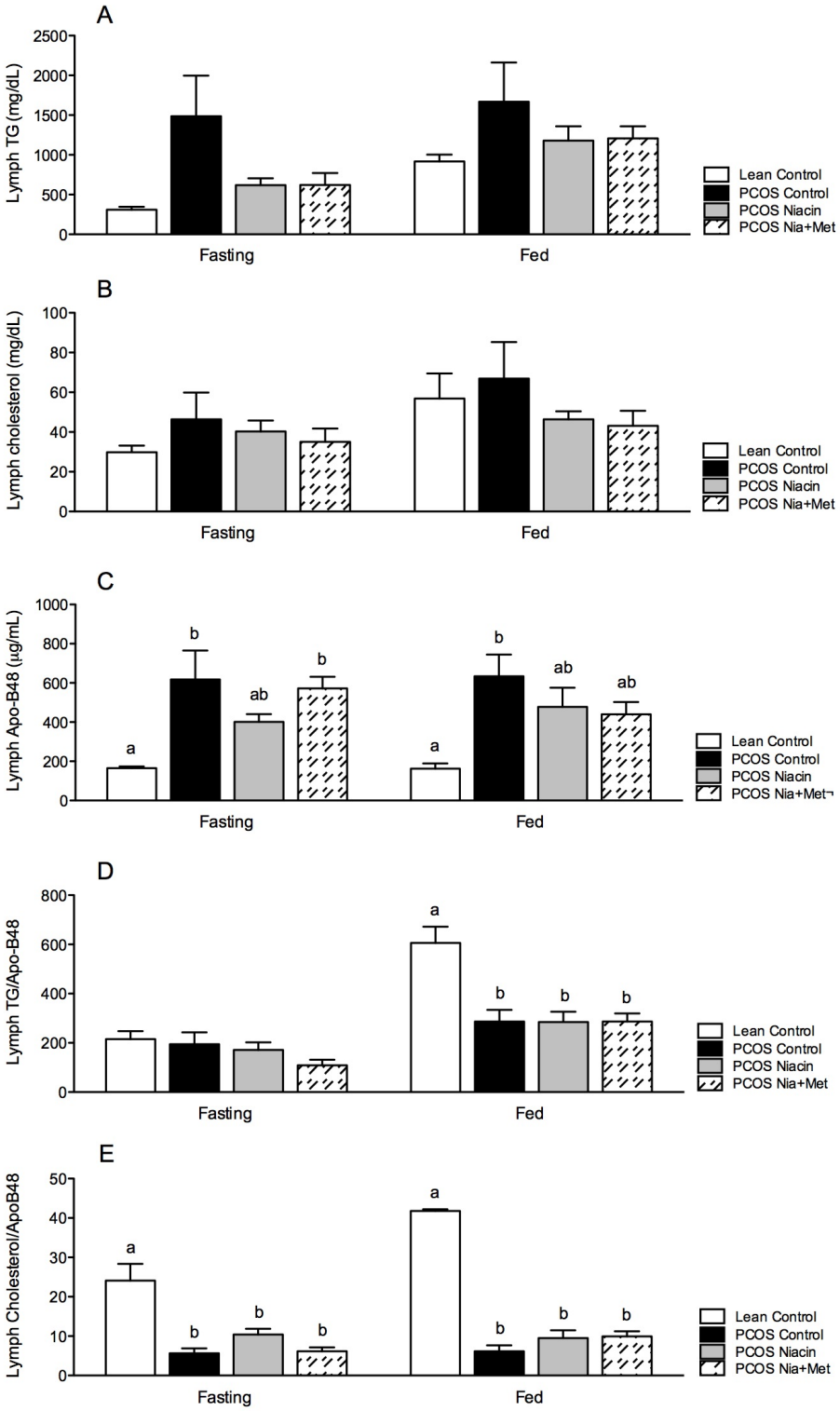


Figure 4-7. The nascent Intestinal lymph chylomicron content. Total triglyceride (panel A), cholesterol (panel B), apo-B48 (panel C), TG per Apo-B48 (panel D) and cholesterol per Apo-B100 (panel E) content following saline (fasting state) and Intralipid (fed state) infusions. Sample size of n=6 was used for the assessment. Data is represented as mean±SEM. Bars with a common letter are not statistically different ($p<0.05$).

4.4 Discussion

Atherogenic dyslipidemia and insulin resistance are major cardiovascular disease risk factors affecting more than 70% of adolescents and adults with PCOS (Legro *et al* 2001). The aim of this study was to explore the effect of dietary nicotinic acid, a potent lipid-lowering vitamin, and a combination of niacin with the insulin-sensitizer metformin on plasma lipids, insulin-glucose metabolism and endocrine-reproductive indices in the JCR:LA-*cp* PCOS-prone animal model. Clinically, niacin has been prescribed to dyslipidemic individuals to reduce fasting plasma TG, TC and LDL-C, and to increase HDL-C (Linke *et al* 2009, King *et al* 1994). Metformin is prescribed to women with PCOS to improve insulin resistance and to lower fasting plasma insulin concentrations (Bargiota *et at* 2012). This is the first study to examine the effects of niacin on cardiometabolic risk factors, including lipid and apoB-lipoprotein metabolism, in a rodent model of PCOS. In this study, niacin alone and in combination with metformin improved estrus cyclicity without significantly altering plasma testosterone, FAI and ovarian morphology. Niacin alone attenuated postprandial insulin response, whereas metformin-niacin combination improved both, fasting and postprandial plasma insulin and glucose concentrations, and HOMA-IR. Both treatments did not significantly alter fasting and postprandial lipids and apoB-lipoprotein concentrations, however there was a trend towards reducing intestinal secretion of TG and apoB48 with both niacin and niacin-metformin combination.

4.4.1 The effect of niacin and niacin-metformin combination on endocrine-reproductive indices in PCOS-prone JCR:LA-cp rats

The JCR:LA-cp rodent model presents with acyclicity, anovulation and metabolic aberrations at 6 weeks of age, although the full PCOS phenotype including hyperandrogenism may not present until well into adulthood. Therefore, the heterogeneity observed in the plasma androgen profile is expected in the JCR:LA-cp model in this young adult stage. Plasma free testosterone concentration did tend to be higher in the PCOS-prone animals by 20%, compared to lean controls, and both niacin and niacin-metformin combination treatments lowered FT, but these findings were not statistically significant due to variability. SHBG tended to increase and the FAI decrease in both the niacin and niacin-metformin treatment groups compared to the PCOS-

prone group, although these findings were also not significant. Aye et al recently reported that dietary nicotinic acid had no significant effect on serum androgens in PCOS patients. However, FAI decreased by 15% from baseline, which is consistent with the findings in this study (Aye *et al* 2014). Tang and colleagues reported in a recent meta-analysis that metformin has an attenuating effect on serum FT, but no effect on serum SHGB levels in PCOS patients (Tang *et al* 2012). It has been demonstrated that metformin reduces ovarian P450c17α enzyme activity and therefore may reduce free testosterone synthesis (Palomba *et al* 2009). Ovarian P450c17α activity was not measured in this study but may be useful to measure in future studies. In addition, no significant difference in ovary follicular morphology was observed between the groups, although the number of corpus luteum was increased in the niacin-metformin group, and corpus albicans were reduced in both the niacin and niacin-metformin treatment groups, suggesting trends for improvement in cyclicity but the mechanisms remain unknown. Importantly, the number of estrus cycles was restored by 50% in the animals treated with niacin and combination treatments. These results are consistent with clinical findings in which metformin therapy improves menstrual cyclicity in PCOS (Essah *et al* 2006). Metformin treatment for 12 wks has also been shown to improve ovulation rate in normoandrogenic females (Carmina *et al* 2004) supporting the notion that metformin directly regulates ovary androgen production in human theca cells (Attia *et al* 2001, Palomba *et al* 2009). The mechanisms of niacin and metformin's action on endocrine-androgen metabolism and ovarian physiology remain unclear and further investigations are needed to understand these pathways in the JCR:LA-cp model.

4.4.2 Beneficial effect of metformin and metformin-flutamide combination on insulin-glucose metabolism in PCOS-prone JCR:LA-cp rats

Niacin alone did not have a significant effect on fasting insulin-glucose parameters in the JCR:LA-*cp* rodent model of PCOS. Niacin has been associated with negative to neutral effects on insulin-glucose metabolism (Aye *et al* 2014, Goldberg *et al* 2000, Grundy *et al* 2002). Niacin alone decreased postprandial insulin response by 50% compared to PCOS-prone controls. It has been shown in females and males with the metabolic syndrome that extended-release niacin (1000 mg/day) for 6 months improved insulin sensitivity in isolated adipocytes and there was a

15% reduction in postprandial glucose response and HOMA-IR, athough these results were not statistically significant (Linke *et al* 2009). Westphal and colleagues have shown that niacin has the potential to raise adiponectin levels through niacin receptor (GPR109A) expression in isolated adipocytes in men with the metabolic syndrome. Higher adiponectin secretion could be beneficial to insulin sensitivity (Plaisance *et al* 2009, Westphal *et al* 2008), however there was no favorable effect on insulin-glucose metabolism upon 6 weeks of niacin treatment in these dyslipidemic males. Another study by Pan and colleagues noted a significant reduction in hemoglobin A_{1c} in patients with type 2 diabetes treated with 1000-4000 mg/d of extended-release niacin (Pan *et al* 2002). However, extended-release niacin-laropiprant combination exacerbated HOMA-IR in women with PCOS and had a neutral effect on postprandial glucose response. In this study in the JCR:LA-cp rodent model of PCOS, the niacin-metformin combination did show beneficial effects on insulin-glucose metabolism. In the fasted state, combination treatment significantly lowered plasma glucose and insulin by 15% and 60%, respectively. The combination improved the fasting plasma HOMA-IR by nearly 70%. Clinically, metformin is prescribed routinely to individuals with impaired insulin-glucose metabolism and has been shown to lower both, fasting and postprandial insulin-glucose concentrations (Aghahosseini *et al* 2010, Al-Nozha *et al* 2013, Bargiota *et al* 2012, Harborne *et al* 2005). This clinical data is consistent with findings in Chapter 3, where it has been shown that metformin treatment alone reduces fasting plasma insulin and HOMA-IR. Postprandially, nicotinic acid-metformin combination treatment, had a greater effect to reduce insulin response compared to metformin alone (Chapter 3), suggesting that niacin may have a additive role to improve insulin-glucose in female PCOS-prone rats. Metformin has been proposed to increase insulin receptor activation, leading to upregulated GLUT-mediated uptake of glucose into cells, as demonstrated in human hepatocytes (Gunton *et al* 2003). In addition, metformin has been shown to lower the rate of hepatic gluconeogenesis via inhibition of mitochondrial respiratory-chain complex 1, thus increasing the ADP/ATP ratio and activating AMPK, therefore inhibiting catabolic pathways that utilize ATP such as gluconeogenesis (Viollet *et al* 2012). Metformin may improve insulin sensitivity in muscle tissue and decrease intestinal glucose absorption (Bargiota *et al* 2012). Our results for niacin-metformin treatment in the JCR:LA-*cp* PCOS-prone model are

consistent with clinical improvements in fasting insulin-glucose and with findings in the JCR:LA-cp model in Chapter 3. Niacin may have additive effects with metformin or neutral effects on insulin-glucose metabolism, and this could be confirmed by exploring protein or mRNA expression of genes involved in insulin-glucose metabolism in future studies.

4.4.3 The effect of niacin and niacin-metformin combination on fasting and non-fasting plasma lipids and apoB-lipoproteins in PCOS-prone JCR:LA-cp rats

Nicotinic acid supplementation alone did not significantly attenuate fasting and postprandial plasma TG, total cholesterol and apoB-lipoproteins in the JCR:LA-*cp* rats. These PCOS-prone animals were treated with niacin from 10 wks of age, which is clinically equivalent to adolescence or pre-adulthood. At 10 wks of age these animals already have established dyslipidemia and metabolic dysregulation (Shi *et al* 2009). Commencing dietary treatment in younger animals, post weaning at 4 wks (childhood) or at 6-8 wks (young adolescence) may have been more effective in attenuating or preventing the development of dyslipidemia in this animal model. Furthermore, a longer intervention period and/or increased animal numbers to improve statistical power of the study is likely to be required in order to observe significant improvements in plasma lipids in this animal model. Indeed clinical studies in PCOS, diabetes and metabolic syndrome show improvements in plasma lipids following 12-week interventions with niacin (Aye *et al* 2014, King *et al* 1994, Lamon-Fava *et al* 2008, Scoffone *et al* 2013, Pang *et al* 2014). In addition, it has been shown that higher doses of niacin enhance the effect of niacin on fasting plasma TG, cholesterol, LDL-C and HDL-C in dyslipidemic males (Chapman *et al* 2010). A minimum dose of 1000 mg/day appears essential to reduce plasma TG, whereas a 500 mg dose reduces plasma TG by 5% and is not statistically significant (Chapman *et al* 2010). A 3000 mg/day dose has been shown to result in >40% reduction in plasma TG concentrations. In some studies the niacin dose was titrated and increased until a desired improvement in atherogenic dyslipidemia was achieved (Linke *et al* 2009, Pan *et al* 2002). In this study PCOS-prone animals received 800 mg of nicotinic acid per kg of body weight, a dose that is approximately 10 times higher than prescribed clinically. However, JCR:LA-*cp* PCOS-prone animals are highly dyslipidemic and are likely to require aggressive treatment to reduce plasma lipids. In addition, a dose of 400 mg/kg body weight was not effective to treat dyslipidemia in the male JCR:LA-*cp*

phenotype with the metabolic syndrome, whereas a dose of 800 mg/kg reduced fasting plasma TG, cholesterol and apoB48 concentrations. Niacin or nicotinic acid treatment (fast-acting) for 12 wks has been shown to improve postprandial TG response in hyperlipidemic men following a high-fat meal (King *et al* 1994). In a recent study by Aye and colleagues nicotinic acid was shown to improve both fasting and non-fasting TG concentration, and to reduce fasting total cholesterol, LDL-C and HDL-C in PCOS women without hyperinsulinemia (Aye *et al* 2014). The results of the present study in the JCR:LA-cp PCOS-prone rodent model are consistent with niacin improving fasting plasma TG (approximately 10%), but our results did not reach statistical significance, and there was no change in other lipid parameters. The beneficial effect of niacin on plasma lipid levels may be explained by multiple mechanisms. Nicotinic acid acts on adipocytes through GPR109A receptor activation and subsequent decrease in lipolysis and FFA mobilization in adipose tissue leading to a decrease substrate supply for hepatic VLDL synthesis, thus reducing plasma LDL and TG levels (Kei *et al* 2012). In addition, niacin was shown to directly inhibit hepatic DGAT2 thus potentially reducing TG synthesis (Ganji *et al* 2004). With a decrease in substrate TG supply for apoB-lipoprotein assembly, post-translational apoB rate of degradation is thought to be increased, and this may occur without changes in apoB and MTP mRNA expression, as shown in HepG2 cell line (Jin *et al* 1999).

The results of this study showed that the niacin-metformin combination treatment reduced fasting plasma TG concentrations by 35%, although this did also not reach statistical significance. There is some evidence that metformin may reduce plasma TG levels by suppressing FFA synthesis in liver and increasing fatty acid oxidation in the insulin resistant state (Cleasby *et al* 2004), but this mechanism has not been investigated in PCOS. Clinically, metformin treatment did not show consistent results in improving dyslipidemia, such as TG, total cholesterol and LDL-cholesterol in patients with PCOS (Al-Nozha *et al* 2013, Bargiota *et al* 2012, Harborne *et al* 2005). The findings of this study show metformin in conjunction with niacin has a more pronounced effect on fasting plasma TG concentrations compared to niacin alone and this appears to be associated with a reduction in intestinal TG secretion and improvements in insulin-glucose metabolism, as fasting insulin and HOMA-IR were significantly improved in this treatment group. Non-fasting or the postprandial TG and cholesterol response

following a high fat meal challenge were not different between niacin treated and PCOS-prone control animals. The niacin-metformin combination reduced the TG response > 30% in PCOS-prone animals compared to controls, although this was not significant.

Postprandial apoB48 and apoB100 concentrations were not measured in the study. It is well established that PCOS-prone JCR:LA-*cp* animals have increased apoB-lipoprotein response after an oral fat meal challenge (Chapter 3, Shi *et al* 2009). In this study, niacin and niacin-metformin treatments did not attenuate postprandial TG and cholesterol responses. These results were consistent with the results from male JCR:LA-*cp* rats treated with niacin (unpublished data), in which no reduction in postprandial TG, total cholesterol and apoB-lipoproteins was observed. Therefore, apoB-lipoprotein concentrations in response to the high fat meal were not measured in this study.

4.4.4 The effect of niacin and niacin-metformin combination on intestinal lipid and apoB48 secretion in fasting and non-fasting states in PCOS-prone JCR:LA-cp rats

These preliminary findings using niacin also suggest niacin and niacin-metformin combination treatment reduces intestinal secretion of TG, which may contribute to the lower plasma TG concentrations observed in this animal model of intestinal lipemia (Vine *et al* 2014). Furthermore, treatment with niacin did not adversely affect insulin-glucose metabolism in this model, but rather tended to improve these indices, and this improvement was enhanced in the niacin-metformin intervention. Niacin treatment tended to lower intestinal secretion of TG and apoB48 by approximately 30% in both the fasting and non-fasting states, but this also did not reach statistical significance. Although intestinal lipogenic gene expression was not measured in this study, these observations could be a consequence of direct inhibition of DGAT2 and increased apoB degradation, as mentioned earlier. Our laboratory has recently shown that niacin increases PPARα and ABCA1 mRNA expression in cultured primary enterocytes (unpublished data).

Interestingly, the amount of TG and cholesterol per apoB48 particle was lower in PCOS-prone animals compared to lean controls in the fed state, suggesting PCOS-prone animals secreted

less lipidated chylomicron particles. TG per apoB48 did not vary between the groups in the fasting state. This data is not consistent with previous lymph secretion experiments (Chapter 3, Shi *et al* 2009), where PCOS-prone animals have increased TG/apoB48 and TC/apoB48 ratios compared with lean controls in the fasted and non-fasted state. Despite inconsistency in the ratio aspect, intestinal secretion of TG and apoB48 was higher in PCOS-prone animals compared to lean controls in fasting and non-fasting state and this trend is similar to what was observed before (Chapter 3).

Niacin has been found to decrease expression of genes involved in lipolysis such as hormone sensitive lipase (Karpe *et al* 2004), carnitine palmitoyl transferase 2 and fatty acid synthase (FAS) (Linke *et al* 2009), which are regulated through the niacin receptor, GPR109A. Interestingly, FAS inhibition in rodents has been shown to decrease food intake leading to weight loss due to decreased neuropeptide Y (NPY) signaling in the hypothalamus. Hence, lower food intake in niacin and niacin-metformin combination treatment groups (25% and 17%, respectively), in addition to a 5% weight reduction in these groups, may be related to nicotinic acid inhibiting FAS expression and NPY signaling via leptin-independent pathways (Loftus *et al* 2000). In future studies these genes involved in lipolysis, fatty acid metabolism, food intake and the niacin receptor expression could be examined to elucidate potential mechanisms of niacin reducing food intake and body weight. Furthermore, reduced expression of FAS and the body weight loss could contribute to reduced postprandial hyperinsulinemia in niacin-treated animals. Weight loss may decrease abdominal fat mass, insulin resistance and hyperandrogenemia and improve atherogenic blood lipid profile (Moran *et al* 2006). In addition, short-term interventions have shown a positive effect on weight, insulin-glucose metabolism and plasma lipids with moderate to severe caloric restriction (Moran *et al* 2006, Norman *et al* 2002). In the PCOS-prone animals niacin was shown to modestly reduce free testosterone, and this reduction could play a role in attenuating plasma TG concentration due to decreased androgen receptor activation as discussed in Chapter 3.

4.5 Conclusion

The present study has shown that nicotinic acid alone was not effective at reducing fasting and postprandial plasma lipids and lipoproteins in the JCR:LA-*cp* rodent model of PCOS. Niacin treatment tended to lower intestinal TG and aboB48 secretion in fasting and non-fasting states. Despite nicotinic acid clinical effectiveness to treat dyslipidemia, the female JCR:LA-*cp* rodent model may require higher treatment dose initiated at earlier age and may require longer intervention length. Nicotinic acid, however, improved plasma insulin response to a meal tolerance test, and with metformin addition, there was also a significant improvement in fasting plasma insulin, glucose and HOMA-IR. Therefore, the results have suggested that niacin may have an additive role to improve insulin-glucose metabolism. Niacin-metformin combination treatment tended to lower fasting and postprandial plasma TG, and fasting apoB48 concentrations. Therefore, niacin-metformin combination could be a potential, safe treatment for dyslipidemia in PCOS.

5. Summary and Final Discussion

5.1 Summary of Primary Aims, Hypotheses and Results

The primary aim of this thesis was to understand the physiological and mechanistic contribution of hyperandrogenemia and hyperinsulinemia to cardiometabolic risk, in particular atherogenic dyslipidemia, and to investigate the effect of dietary niacin to improve atherogenic dyslipidemia in the JCR:LA-*cp* rodent model of PCOS. The primary hypothesis was that treatment with the insulin sensitizer metformin, the androgen receptor antagonist flutamide, and nicotinic acid would independently improve atherogenic dyslipidemia, and these compounds in combination with metformin would improve insulin resistance to modulate cardiometabolic risk in the JCR:LA-*cp* rodent model of PCOS. In Chapter 3, flutamide treatment alone and in combination with metformin was shown to improve fasting plasma TG, apoB48- and apoB100-lipoproteins but had no significant impact on postprandial or non-fasting response in lipid parameters following a high-fat meal challenge. Metformin treatment alone tended to decrease fasting plasma apoB100, but did not have an attenuating effect on other plasma lipid parameters. However, metformin treatment alone and in combination with flutamide improved fasting insulin and HOMA-IR, and this was associated with increased mRNA expression of hepatic insulin receptor and AKT2, and decreased mRNA expression of JNK. Flutamide had an attenuating effect on insulin and HOMA-IR only in combination with metformin. The results from the lymph cannulation studies demonstrated that the JCR:LA-*cp* rats treated with metformin had decreased intestinal secretion of TC, whereas flutamide treatment decreased intestinal TG secretion in the fasting and fed states. Flutamide-metformin combination treatment did not provide additional improvement in atherogenic dyslipidemia, suggesting the androgen receptor and testosterone may modulate lipid metabolism to a greater extent than hyperinsulinemia in the JCR:LA-cp model of PCOS. In Chapter 4, the results showed that nicotinic acid treatment alone improved the postprandial plasma insulin response to a meal tolerance test (MTT), but did not alter plasma insulin concentrations in the fasting state. The combination of nicotinic acid and metformin improved fasting plasma insulin, glucose, HOMA-IR, and postprandial insulin and glucose response to a MTT. Fasting and non-fasting plasma

lipids were not modulated by nicotinic acid or nicotinic acid-metformin treatment, however there was a trend for these treatments to reduce intestinal TG and apoB48 secretion. Therefore, the results from this study did not support the hypothesis that nicotinic acid would improve on plasma lipid and apoB-lipoprotein metabolism in the JCR:LA-cp model of PCOS.

The secondary aim of this study was to explore the effects of modulating androgens and insulin (Chapter 3), and the effects of dietary niacin (Chapter 4) on reproductive-endocrine effects in the JCR:LA-*cp* rodent model of PCOS. It was hypothesized that metformin, flutamide and nicotinic acid treatments and combination treatments of flutamide or nicotinic acid with metformin would improve reproductive-endocrine indices in the JCR:LA-*cp* rodent model of PCOS. Metformin, flutamide and nicotinic acid treatments alone, and metformin-flutamide and niacin-metformin combination treatments were observed to have no significant effect on the endocrine-hormonal profile in the JCR:LA-cp rodent model of PCOS. However, all treatments tended to lower free androgen index (FAI), and nicotinic acid and niacin-metformin combination decreased FT by 20%. Ovarian follicular morphology improved with metformin and flutamide treatments, characterized by a decreased number of atretic follicles compared to untreated PCOS-*prone* animals. The regularity of the estrus cycle was assessed in niacin and niacin-metformin treated animals, and was found to be significantly improved in both treatment groups. Collectively, these results were associated with improvements in both hyperandrogenemia indices and insulin resistance, suggesting both these pathways play a role in modulating ovarian follicular morphology and estrus cyclicity in the JCR:LA-cp model of PCOS.

5.2 General Discussion

One of the major aims of this study was to explore the effects of modulating androgens and insulin, and dietary niacin on reproductive-endocrine indices in the JCR:LA-*cp* animal model of PCOS. Metformin, flutamide and nicotinic acid treatment had a minimal effect on endocrine hormone profile as shown in Chapters 3 and 4, respectively.

5.2.1 Effects of metformin, flutamide and nicotinic acid on reproductive-endocrine indices

Although not significant, metformin treatment and metformin-flutamide combination tended to lower FAI by 10-25%. Interestingly, metformin treatment alone increased serum estradiol (E2) concentration by 20%, suggesting that metformin improved P450 aromatase enzyme activity, which converts androstenedione (A) to estrone and testosterone (T) to E2. It has been shown that P450 aromatase enzyme activity is reduced in PCOS ovaries leading to an increase in A and T (Baptiste *et al* 2010, Norman *et al* 2007, Wood *et al* 2003). Therefore, improvement in insulin resistance and/or direct metformin action on the P450 aromatase enzymatic activity in the ovary may be responsible for this effect. Ovarian follicular morphology was improved with both metformin and flutamide treatments as indicated by a reduction in the number of atretic follicles, and these results suggest the role of the AR and improved insulin signaling in reducing follicular atresia.

Niacin and niacin-metformin treatments improved plasma FT concentration (by 20%) and estrus cyclicity in the PCOS-prone JCR:LA-*cp* animals. Both treatments also had an attenuating effect on insulin-glucose metabolism, suggesting niacin independently and in combination with metformin elicit effects on ovarian function and endocrine hormonal profile via insulin. The direct role of insulin in endocrine and ovary function was not the focus of this thesis. Protein expression and activity of key enzymes in ovarian androgen synthesis pathways could be measured in future studies to examine the role of insulin further in follicular and cystic development in the ovary.

5.2.2 Effects of metformin and flutamide on insulin and glucose metabolism and the associated mechanisms

Although PCOS is the most common endocrine disorder in females (March *et al* 2010, Moran *et al* 2013), the etiology of PCOS has been poorly studied and the exact mechanisms of how hyperandrogenemia and insulin resistance contribute to dyslipidemia in PCOS are yet to be elucidated (Alexander *et al* 2009, Cussons *et al* 2007, Polotsky *et al* 2012). To date, several

studies in cancer cell models have addressed the role of the androgen receptor in lipidogenesis, but no studies have examined the role of the AR in PCOS (Heemers *et al* 2006).

In Chapter 3, metformin and flutamide treatments were used to target hyperinsulinemia and hyperandrogenemia, respectively, to assess the contribution of these two pathways in modulating atherogenic dyslipidemia in PCOS (as proposed in Figure 1-8). The results showed the effect of metformin was consistent with clinical studies to improve fasting plasma insulin and HOMA-IR, however metformin had no effect on plasma lipids and apoB-lipoprotein metabolism. In this study, metformin appeared to improve insulin signaling cascades, as hepatic mRNA expression of insulin receptor and AKT2 were increased and JNK decreased, which collectively may have improved glucose transport into the cell and suppressed hepatic glucose production (Gonzalez *et al* 2006, Gezginci-Oktayoglu *et al* 2013, Lee *et al* 2011). These findings most likely are associated with the improved fasting plasma insulin and HOMA-IR, however phosphorylation and activation experiments of insulin-signaling proteins are needed to explore these mechanisms further. The main mechanism of metformin action has been proposed to be through activation of AMPK, which reduces hepatic lipid synthesis and gluconeogenesis (Viollet *et al* 2012), however AMPK phosphorylation and activation were not measured in this study.

Flutamide alone did not significantly improve insulin-glucose concentrations and HOMA-IR, but there was a significant increase in hepatic MAPK1 and AKT2 mRNA expression. In prostate cancer cell culture studies, increased PI3K/Akt signaling induces AR expression and activation by posttranslational modification and reduced co-repressor activity (Ghosh *et al* 2003, Lonergan *et al* 2011). In our study, despite an increase in AKT2 expression, AR mRNA tended to decrease and the antagonistic action of flutamide was effective in reducing plasma TG and lipogenic gene expression (as discussed below) (Figure 5-1). This suggests that AKT2 mRNA expression may not have an effect on hepatic AR expression and activity in JCR:LA-cp PCOS model. Interestingly, metformin and flutamide treatments had contrasting effects on intestinal insulin signaling gene expression compared to the liver. Intestinal expression of MAPK1, AKT2, PTPN1 and JNK was reduced with metformin-flutamide combination, but not with metformin and flutamide independently, suggesting a possible synergistic role of AR and IR in the regulation of these

genes in the intestine. The association between AR activation and IR has been explored in androgen treated female rats, and human adipocytes and muscle tissue. Testosterone and DHT-treated female rats exhibited reduced whole-body insulin sensitivity and a 50% reduction in insulin-mediated glucose uptake in muscle tissue, and this was proposed to be associated with reduced glycogen synthase activity (Holmang *et al* 1990). Adipocytes isolated from premenopausal women were treated with testosterone and showed a marked reduction in glucose uptake and this was associated with impaired insulin-stimulated protein kinase C (PKC) phosphorylation, but not IRS-1, PI3K or Akt activation (Corbould 2007). Female AR knockout (ARKO) mice studies have shown that these animals have impaired hepatic insulin signaling, which was proposed to be linked to a decreased PI3K activity and increased PTPN1 activity, a negative regulator of IRS-1 downstream signaling (Lin *et al* 2008). However, the exact mechanism of how AR regulated the activity of these enzymes was not fully investigated. In the present study in the JCR:LA-*cp* model of PCOS, AR antagonism did not impair insulin signaling, suggesting AR inhibition has different effects and mechanisms compared to complete AR deletion. PTPN1 mRNA expression was not significantly changed with the treatments, and IRS-1 and PI3K expression and activation were not measured, and these could be measured in future studies. Testosterone has been shown to have a bidirectional dose-dependent effect in preadipocyte and myoblast cell lines, with lower T concentrations being stimulatory to IRS-1 and GLUT4 protein expression (Chen *et al* 2006). Therefore, future studies are essential to elucidate the role of different doses of T and mechanisms of action further. These could include examining protein abundance and phosphorylated protein modulation to determine the role of the AR in regulating insulin-signaling mechanisms in the liver and intestine.

Metformin-flutamide combination treatment significantly reduced intestinal AR mRNA by 50% as well as MAPK1, AKT2, PTPN1 and JNK mRNA by 30-50% compared with PCOS-prone animals. These results suggest that there is a possible interaction between AR and intestinal insulin signaling pathways, however flutamide did not have any additional benefit to metformin's attenuating effect in insulin sensitivity. In addition, gene expression experiments in adipose and muscle tissues would be beneficial to further understand the role of insulin resistance and the

androgen receptor in these tissues and how these tissues may be associated with whole body insulin-glucose and lipid metabolism, including glucose-fatty acid utilization and fatty acid release from these tissues. in the JCR:LA-cp rodent model of PCOS

5.2.3 Effects of metformin and flutamide on lipid and lipoprotein metabolism and the associated mechanisms

Flutamide improved fasting plasma TG, apoB48 and apoB100, but had no significant effect on plasma lipids postprandially following a high-fat meal. Hepatic lipogenic gene expression was not significantly altered, suggesting that AR inhibition may not directly regulate mRNA expression of these genes. Activation of AR by T and DHT has been shown to upregulate SREBP1 and 2 mRNA and protein expression, and maturation, SCAP and nSREBP fragments in prostate cancer cell cultures. Other lipogenic gene mRNA expression such as ACC, FAS and HMGR were found to be upregulated in these cancer cell models (Heemers *et al* 2006). It has been proposed that AR transcriptionally regulates SCAP, which mediates SREBP precursor translocation to the Golgi for activation. An androgen response element sequence has been identified on the SCAP gene (Heemers *et al* 2001, 2004). However, in the present study no changes in SREBP1, SREBP2, ACC, FAS or SCAP were observed following inhibition of the AR. Future studies could measure protein levels of these genes to determine the direct effects of AR inhibition by flutamide. Plasma apoB100 concentrations were reduced in the fasting state. SREBP1 mRNA expression was lowered, but not significantly, and apoB mRNA expression in the liver was not altered following flutamide treatment. These results suggest inhibition of the AR and lowering of plasma apoB100 concentrations may be through other mechanisms, such as apoB degradation or clearance mechanisms associated with apoE/C expression on these lipoproteins, but these mechanisms were not measured in this study. LDLR mRNA and protein expression and activation in HepG2 cell line were shown to be increased by 17β-estradiol-driven ER activation, but this effect is antagonized by activated AR (Croston *et al* 1997). In the present study, flutamide did not have a significant effect on LDLR mRNA expression.

Despite lower plasma apoB48 concentrations, apoB48 secretion from the intestine and apoB mRNA expression in enterocytes did not appear to be altered with flutamide treatment.

Flutamide treatment alone and metformin-flutamide combination did reduce lymphatic TG secretion in fasting and fed states, but intestinal lipogenic gene mRNA expression was not alterated. A potential role of ERα and ERβ in lipid metabolism could also explain a reduction in intestinal TG and TG/apoB48 secretion. ERα mRNA was increased 2-fold in flutamide-treated animals, although this was not significant. ERα activation by phytoestrogens has been shown to lower fasting plasma TC, and TG and TC content in liver, WAT and muscle tissues (Cederroth *et al* 2008). This lipid-lowering effect of ERα was proposed to be due to higher energy expenditure and increased lipid oxidation in these tissues. It has been found that PPARα mRNA expression and AMPK and ACC phosphorylation were increased in estrogen-treated female mice. In addition, genes involved in peroxisomal fatty acid oxidation and mitochondrial biogenesis were increased in liver and adipose tissue (Cederroth *et al* 2008, Foryst-Ludwig *et al* 2010). Therefore, measuring oxidative metabolism markers such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) may be useful to determine if fatty acid oxidation plays an attenuating role in modulating hepatic and intestinal lipid synthesis. In addition, AR has been shown to decrease ER activity, at least in HepG2 cell line, either by direct contact with ER or competing for common cofactors (Croston *et al* 1997), however the exact mechanism of AR/ER interaction is yet to be elucidated. Therefore, future studies could examine protein expression with acute flutamide treatment, possibly in cell culture models or in lymph cannulation studies, to determine the direct role of AR in lipid metabolism in the intestine. For example, the role of AR in intestinal lipid secretion has not been previously studied, therefore primary enterocyte and hepatocyte cell culture studies treated with metformin, flutamide and their combination could help to elucidate the mechanisms further. Measuring protein expression of key regulatory proteins such as SREBP, PPAR, PGC-1α, LDLR and receptors AR and ER in these studies could be done to elucidate if mRNA changes are predictive of protein expression and changes in lipid synthesis and lipoprotein secretion.

The results from present study have shown that metformin alone had no effect on lipogenic gene expression in the liver and intestine. Interestingly, metformin treatment alone improved intestinal cholesterol secretion and this may warrant further investigation. Nuclear receptor

LXRα expression was significantly increased in metformin-treated animals. LXRα regulates cholesterol homeostasis in enterocytes, and LXRα activation has been shown to reduce cholesterol incorporation into chylomicrons (Kruit *et al* 2006). Therefore, future studies could explore the effect of metformin on intestinal LXRα protein abundance and activation, and cholesterol metabolism. Overall, metformin improved insulin sensitivity in the JCR:LA-*cp* model of PCOS but did not significantly improve dyslipidemia, which is consistent with clinical findings in PCOS (Tang *et al* 2012).

In present study, JCR:LA-*cp* animals were treated with metformin doses higher than used clinically in women with PCOS. The animals were given 300 mg/kg/d of metformin, which is 20-30 times higher than doses used in the clinical setting. The JCR:LA-*cp* model used in this thesis is highly insulin resistant has pre-established dyslipidemia at 10 weeks of age, therefore a high dose was chosen to elicit improvements in cardiometabolic risk. Previous experiments in these PCOS-prone rats have shown that metformin (300 mg/kg/d) improved fasting plasma insulin concentrations and reduced fasting plasma TG and TC (Wang *et al* 2012). These animals were younger (8 wks of age) and the treatment was for 4 wks. In male streptozotocin-induced diabetic rats, 500 mg/kg/d metformin treatment for two weeks improved fasting and postprandial plasma glucose, and fasting plasma TG concentration (Zhang and Tan 2000). Therefore, in future experiments to increase metformin dose could be an option as the JCR:LA-*cp* female rats have severe dyslipidemia and insulin resistance.

Flutamide was given at 10 mg/kg/d, which is comparable to doses prescribed clinically. Daily doses of 250-750 mg/d have been shown to reduce fasting plasma TG, TC and LDL-C in women with PCOS (as shown in Chapter 1, Table 1-7). Previously, 10 mg/kg/d of flutamide administered in JCR:LA-*cp* PCOS-prone rats for 4 weeks reduced fasting plasma apoB48 and apoB100, but did not reduce fasting plasma TG, TC, LDL-C (Wang *et al* 2012). In the current study flutamide treatment was commenced at 10 wks of age for 6 weeks, therefore animals were 2 wks older and although treated for 2 more wks than the previous experiments, this did not impact plasma lipids (Wang *et al* 2012). A flutamide dose of 10 mg/kg/d for 12 months was effective at

improving leptin signaling in the hypothalamus in female rats, however no changes were observed in insulin sensitivity, and plasma lipids were not measured (Feng *et al* 2011). Doses of 50 and 100 mg/kg/d increased plasma E2 and adrenal weight, and decreased ovary weight in these rats (Feng *et al* 2011). Therefore in the future studies the treatment regimen could be initiated at 8 wks of age or earlier and for a prolonged time period because clinical studies and rodent studies suggest that a reduction in plasma lipids in conditions of PCOS may require 12 months treatment with flutamide (as shown in Chapter 1, Table 1-7).

The JCR:LA-*cp* rats have a defect in the *ob*R gene, which leads to a malfunction of the leptin receptor and diminished leptin signaling (Shi *et al* 2009). Diminished leptin signaling in the hypothalamus leads to hyperphagia and excess weight gain. Interestingly, women with PCOS have been shown to have increased circulating leptin concentrations and impaired leptin signaling (Jacobs and Conway 1999, Brzechffa *et al* 1996, Mantzoros *et al* 1997). In insulin-sensitive tissues such as liver, muscle and WAT, leptin has been shown to activate MAPK, PI3K/Akt, AMPK and decrease activity of ACC and increase fatty acid oxidation (Bjorbaek and Kanh 2004). Therefore, impaired leptin signaling may result in decreased insulin signaling, fatty acid oxidation, and increased lipogenesis. In the JCR:LA-*cp* model of PCOS there may be an interaction between the AR, insulin and leptin signaling pathways, but the latter were not explored in this study.

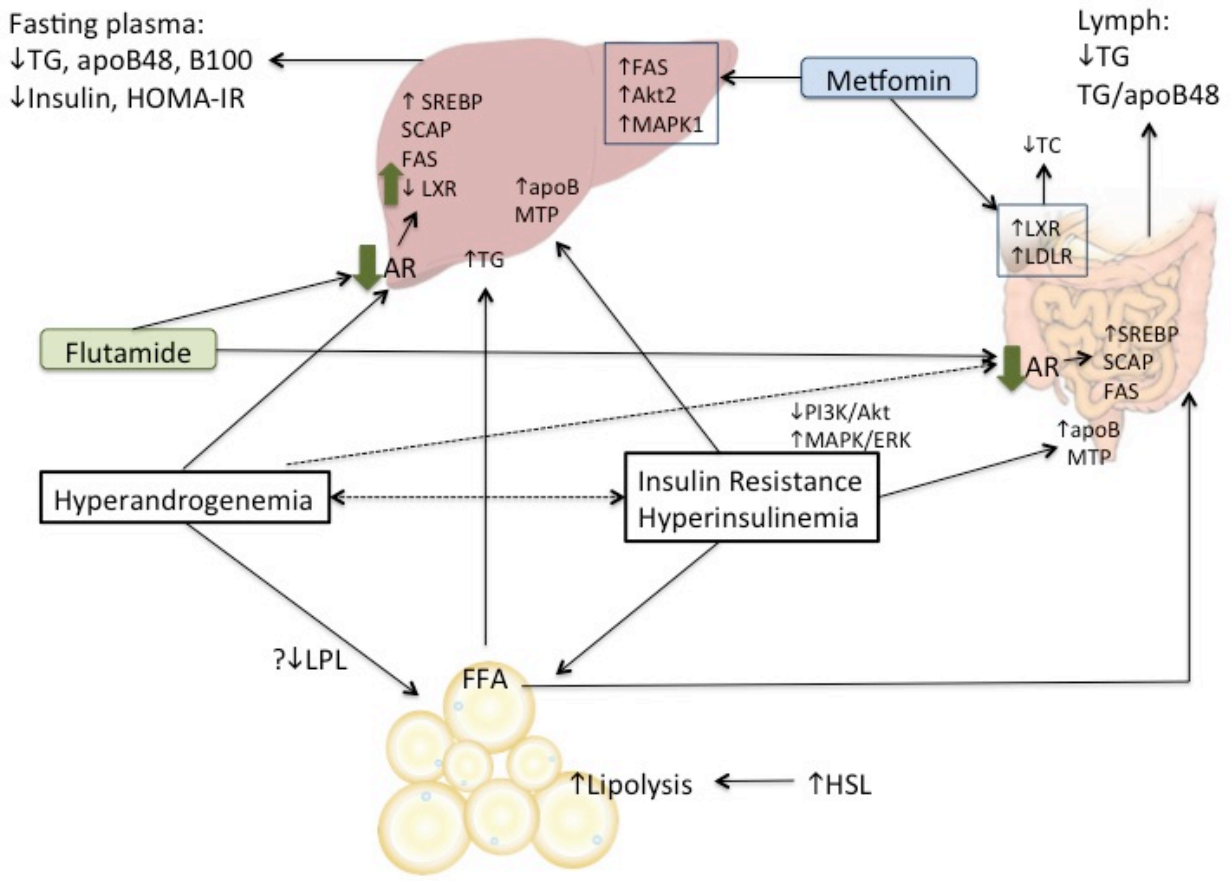


Figure 5-1. Proposed mechanisms of dyslipidemia and the effect of flutamide and metformin on pathways of insulin and lipid metabolism in the JCR:LA-cp rodent model of PCOS. Insulin resistance and hyperandrogenemia induce lipolysis in adipose tissue leading to increased free fatty acid (FFA) release into plasma, increasing substrate availability for triglyceride (TG) synthesis in the liver and intestine for triglyceride-rich apoB-lipoprotein assembly. Flutamide treatment reduced androgen receptor (AR) expression in the liver and intestine and decreased fasting plasma TG, apoB48 and apoB100 and reduced intestinal TG secretion. Metformin increased expression of fatty acid synthase (FAS), protein kinase B (Akt2) and mitogen activated protein kinase (MAPK) 1 expression in liver, and liver X receptor and low density lipoprotein receptor (LDLR) mRNA expression. Metformin treatment improved fasting plasma insulin and HOMA-IR, and reduced total cholesterol (TC) secretion from the intestine. Abbreviations: ERK, extracellular signal-regulated kinase; FAS, fatty acid synthase; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; MTP, microsomal triglyceride transfer protein; PI3K, phosphoinositide 3-kinase; SCAP, SREBP cleavage activating protein; SREBP, sterol regulatory element-binding protein. (Adapted from Diamanti-Kandarakis *et al* 2007).

5.2.4 Niacin and niacin-metformin combination effects on insulin and glucose, and lipid and lipoprotein metabolism

There has been no therapy developed that is safe and effective to target atherogenic dyslipidemia and cardiovascular disease risk in PCOS, thus nicotinic acid has been proposed as a promising dietary alternative to modulate dyslipidemia in young reproductive women with PCOS. Metformin has been used in this study in conjunction with nicotinic acid to target both dyslipidemia and insulin resistance, as nicotinic acid is known to have neutral to adverse effects on insulin-glucose metabolism (Aye *et al* 2014, Goldberg *et al* 2008, Grundy *et al* 2002).

Nicotinic acid alone improved postprandial insulin response to a meal challenge, but had no significant effect on fasting and postprandial plasma lipids and apoB-lipoprotein metabolism (Figure 5-2). Although not significant, nicotinic acid tended to decrease intestinal TG and apoB48 secretion in both the fasting and non-fasting states. This trend is consistent with findings in the male JCR:LA-*cp* phenotype in which the same dose of niacin and length of feeding intervention was implemented (Mangat *et al* 2013). It was hypothesized that niacin would improve atherogenic dyslipidemia in the JCR:LA-cp model of PCOS. The non-significant results observed in this study are in contrast to the established beneficial effects of nicotinic acid on fasting and non-fasting plasma TG, and total cholesterol in dyslipidemic patients (Aye *et al* 2014, King *et al* 1994, Plaisance *et al* 2008, Scoffone *et al* 2013, Pang *et al* 2014). The JCR:LA-*cp* animals in this study had established dyslipidemia at the start of the dietary intervention at 10 wks, and fasting plasma TG, apoB48 and apoB100 are 13-, 21- and 2.5-fold higher compared to lean controls. Therefore, commencing treatment in younger animals may have been more effective at targeting dyslipidemia in this rodent model. Although the nicotinic acid dose used in this study was 10 times higher (mg/kg body weight) than used clinically, it may not have been a substantial pharmacological dose to attenuate dyslipidemia in this severe hyperlipidemic animal model. Some studies in diabetic patients with MetS have used a titration approach in which the dose of niacin is increased gradually until the desired lowering in plasma lipids is achieved (Linke *et al* 2009, Pan *et al* 2002). A nicotinic acid treatment dose of 1000 mg/d in women with PCOS was shown to decrease fasting plasma TG, TC and LDL-C, however these

patients were either normolipidiemic or did not have severe dyslipidemia. Studies in our laboratory in male JCR:LA-*cp* animals using the same nicotinic acid dose show significant decreases in fasting plasma TG, TC and apoB48, however these animals had less severe dyslipidemia than the female genotype. Specifically, the female PCOS-prone JCR:LA-*cp* animals had more than two- and five-fold higher plasma TG and apoB48 concentrations compared to male rats with MetS. Therefore PCOS-prone females may require a higher nicotinic acid dose and/or a longer treatment intervention period to achieve reductions in plasma lipids and apoB-lipoprotein concentrations.

Interestingly, metformin-niacin combination reduced fasting plasma insulin and HOMA-IR 40-50% more, compared to metformin alone or metformin-flutamide, suggesting role of niacin in attenuating insulin resistance in this animal model of PCOS. To explore the mechanism of nicotinic acid in insulin-glucose metabolism in PCOS-prone JCR:LA-*cp* animals, mRNA and protein expression of key insulin-signaling genes in the liver, muscle and adipose is required, but these were not measured in this study. Nicotinic acid treatments have been shown to increase adipocyte insulin sensitivity and increase adiponectin secretion in men with MetS (Plaisance *et al* 2009, Westphal *et al* 2008). Adipocyte cell culture studies have shown that through GPR109A receptor, nicotinic acid inhibits adenylyl cyclase and decreases TG lipolysis and FFA release from adipose tissue (Ahmed *et al* 2009) (Figure 5-2). Chronic elevated FFA from adipose tissue is linked to insulin resistance in peripheral tissues and the liver, and is associated with decreased GLUT4 translocation and glycolysis in the muscle and increased hepatic glucose production (Boden and Shulman 2002). Therefore, nicotinic acid may lower adipose FFA release and alter insulin signaling pathways to improve IR in the JCR:LA-cp model of PCOS (Figure 5-2), but this requires further investigation.

The nicotinic acid administered in this study was similar to the commercially available clinical extended-release nicotinic acid formulas. The chow pellets were enriched with nicotinic acid and the animals consumed a constant amount of nicotinic acid, approximately 300mg/d for >12 hrs in the dark cycle. In liver and plasma, the half-life of nicotinic acid is approximately one hour, and it is metabolized and excreted in approximately 2-4 hours following an injection of

500 mg/kg nicotinic acid, as show in rodents (Petrack *et al* 1966). Therefore, the results from this study reflect a chronic dietary effect of nicotinic acid. However animals were fasted 24 hours prior to sacrifice, blood and tissue collection and whether this fasted period affects the parameters measured is unknown. In future experiments, nicotinic acid could be administered together with the MTT, oral fat meal challenge tests, and saline and intralipid solutions in lymph cannulation experiments, in order to assess if acute nicotinic acid administration has an attenuating effect on insulin, glucose, lipid and apoB-lipoprotein secretion in PCOS-prone JCR:LA-*cp* rats.

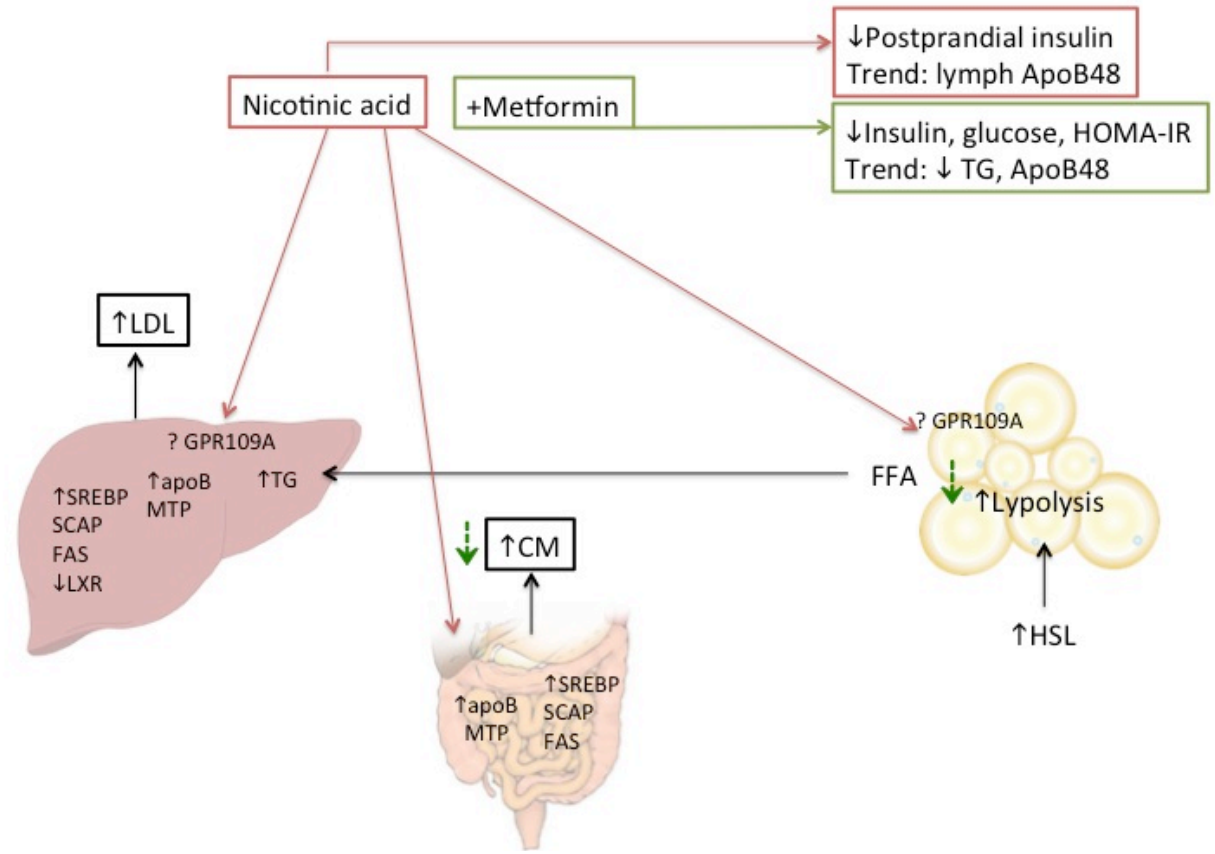


Figure 5-2. The effect of nicotinic acid and nicotinic acid-metformin combination on insulin-glucose and lipid metabolism in the JCR:LA-cp rodent model of PCOS. Nicotinic acid improved postprandial insulin concentration, whereas addition of metformin improved fasting insulin and glucose and tended to lower fasting and postprandial plasma triglycerides (TG) and lymphatic apoB48 secretion. FAS, fatty acid synthase; FFA, free fatty acids; HSL, hormone-sensitive lipase; LXR, liver X receptor; MTP, microsomal triglyceride transfer protein; SCAP, SREBP cleavage activating protein; SREBP, sterol regulatory element-binding protein.

5.3 Conclusion

The JCR:LA-*cp* model of PCOS was used to explore the role of hyperinsulinemia and hyperandrogenemia in the development of atherogenic dyslipidemia, and to assess the effectiveness of nicotinic acid to target cardiometabolic risk, particularly dyslipidemia in PCOS. The PCOS-prone JCR:LA-*cp* rodent is a model of severe atherogenic dyslipidemia and insulin resistance, and more aggressive treatment regimes may be required in future studies to improve these indices. Alternatively, this model offers similarities to clinical conditions of PCOS in which patients have severe atherogenic dyslipidemia and IR, and are resistant to dietary and lifestyle approaches, and pharmaceutical interventions are required. Nicotinic acid lowered postprandial insulin response and attenuated fasting plasma insulin, glucose and HOMA-IR in combination with metformin treatment. Nicotinic acid did not improve fasting or postprandial lipid concentrations, although niacin-metformin combination tended to lower fasting plasma TG and apoB48 concentrations, and intestinal TG and apoB48 secretion. This data suggests niacin-metformin combination treatment may be a potential treatment for dyslipidemia but requires further investigation. Metformin treatment attenuated insulin resistance and this was associated with improvements in the mRNA expression of insulin signaling genes, however metformin had no effect on lipid and apoB-lipoprotein metabolism. Flutamide treatment improved fasting plasma TG and apoB-lipoproteins and lymphatic TG secretion, however there was no change observed in hepatic or intestinal lipogenic gene expression. These results suggest other mechanisms associated with AR signaling may be involved, such as pathways involved in lipid oxidation, apoB-lipoprotein clearance and degradation. Flutamide-metformin combination treatment did not provide additional improvement in atherogenic dyslipidemia, suggesting the androgen receptor and testosterone may modulate lipid metabolism to a greater extent than hyperinsulinemia in the JCR:LA-cp model of PCOS.

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