MOLECULAR INTERACTIONS OF POLYCYSTIC OVARIAN SYNDROME (PCOS)
WITH METABOLIC SYNDROME AND NON-ALCOHOLIC FATTY LIVER
DISEASE (NAFLD)

by

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A Thesis
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Science
Biology

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Date: March 4th
Spring Semester 2013
George Mason University
Fairfax, VA
Molecular Interactions of Polycystic Ovarian Syndrome (PCOS) with Metabolic Syndrome and Non-alcoholic Fatty Liver Disease

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

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Spring Semester 2013
George Mason University
Fairfax, VA
DEDICATION

This is dedicated to my loving husband and my two wonderful children, Daniel and Andre.
ACKNOWLEDGEMENTS

I would like to thank my family, my advisor, Dr. Baranova, my lab members at GMU-TRI-Inova Fairfax Hospital, especially Dr. Birerdinc, who have made this happen. Dr. Cox, Dr. Luchini, and the other members of my committee were of invaluable help. Special thanks to the Center for Liver Disease of Inova Fairfax Hospital for providing samples and well-equipped laboratory in which to work. Finally, thanks go out to the University Dissertation & Thesis Services, especially Ms. Sally R. Evans, in assisting the thesis format review.
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<thead>
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<th>Acetyl-CoA carboxylase alpha</th>
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Very-low-density lipoprotein .......................................................... VLDL
Variable number of tandem repeats ........................................ VNTR
Zucker diabetic fatty .................................................................... ZDF
ABSTRACT

MOLECULAR INTERACTIONS OF POLYCYSTIC OVARIAN SYNDROME (PCOS) WITH METABOLIC SYNDROME AND NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD)

Thuy Phuong T. Tran, MS

George Mason University, 2013

Thesis Director: Dr. Ancha Baranova

Obesity is a common factor involved in both polycystic ovarian syndrome (PCOS) and non-alcoholic fatty liver disease (NAFLD). Obesity causes NAFLD and aggravates hirsutism and menstrual disorder in polycystic ovary syndrome (PCOS). Recent findings suggest that women with PCOS may be at risk for developing NAFLD and conversely, NAFLD patients may be a risk for PCOS. To assess the literature for associations between PCOS and NAFLD at molecular level, we performed a systematic review of peer-reviewed articles related to PCOS and NAFLD. Articles were summarized and grouped according to different sections defining interactions of PCOS with metabolic syndrome and NAFLD as well as common risk factors, pathogenic pathways and treatment options. Based on the association of PCOS and other metabolic abnormalities, such as insulin resistance, hyperandrogenism, obesity and NAFLD, the PCOS candidate genes have been proposed. Closer scrutiny of these genes placed most of their proteins at the crossroads of three highly inter-related conditions: metabolic syndrome, obesity and
NAFLD. This made us to postulate that PCOS is, in fact, the ovarian manifestation of metabolic syndrome, similarly to NAFLD that is currently recognized as the hepatic manifestation of metabolic syndrome. PCOS and NAFLD conditions may co-exist and may respond to similar therapeutic strategies.

In order to untangle the complex relationship between PCOS and NAFLD experimentally, we analyzed serum biomarkers of apoptosis, select adipokines and mRNA profile in the visceral adipose tissue of obese patients. Two clinical cohorts were compared: one with both PCOS and NAFLD that have not yet progressed to NASH according to their liver biopsies (N=12) and another, a BMI-matched non-PCOS non-NASH NAFLD control cohort (N=12). The total serum levels of apoptotic biomarker M30 were significantly elevated in PCOS patients with liver steatosis as compared to non-PCOS NAFLD controls (P < 0.02), pointing that androgen-dependent proapoptotic PCOS environment may directly contribute to NAFLD progression in these patients. Similarly, hyperandrogenism may explain an observed PCOS-specific decrease (P < 0.04) in adipose LDLR mRNA expression that may be connected to the proneness of PCOS patients with concomitant liver disease to progress to NASH. The levels of mRNA encoding angiogenesis-associated GSK-3B interacting protein Ninein were significantly increased in PCOS adipose (P < 0.007). In entire NAFLD cohort, the levels of the Resistin were positively correlated with expression levels of LDLR and prothrombin time. In regards to all these parameters, the studies of larger cohorts of PCOS patients are needed, including subgroups without any histological sign of liver disease and those without an excess of androgens.
CHAPTER 1

Systematic Review the Association of Polycystic Ovarian Syndrome with Metabolic Syndrome and Non-Alcoholic Fatty Liver Disease

One of the most common disorders for women at child-bearing age is polycystic ovarian syndrome (PCOS), which affects not only hormones that regulate the normal development of eggs in the ovaries but also other metabolic pathways.\(^1\) The prevalence of this disorder is estimated at 5\% to 10\% of women at reproductive age. However, the true prevalence rates for PCOS could be even higher with the use of the recently adopted Rotterdam criteria, increasing this rate, in community settings, to around 18\%.\(^2\) Importantly, PCOS has been noted to affect 28\% of obese women, but only 5\% of lean women.\(^2,3\)

According to the Rotterdam criteria, a positive diagnosis of PCOS can be made if at least two of the following criteria are met: chronic oligo-ovulation, clinical or biochemical hyperandrogenism and ultrasonic evidence of polycystic ovaries.\(^1\) The cause of PCOS is currently unknown; therefore no preventive measures can be implemented. PCOS is thought to be initiated by low levels of follicle stimulating hormone (FSH) and high levels of androgen, which are detected in the majority of cases. Hyperandrogenism causes the follicles in the ovaries to develop poorly. Consequently, the eggs in these follicles cannot mature and give rise to the cysts seen in patients with PCOS.\(^1\)
We performed a systematic review using Medline search (1985–2010) for peer-reviewed articles related to PCOS and NAFLD. The articles were summarized and grouped according to different sections defining interactions of PCOS with metabolic syndrome and NAFLD. In addition, articles dealing with common risk factors for both PCOS and NAFLD were summarized. Finally, articles related to pathogenic pathways and treatment options are reviewed.

Information retrieved from reviewed articles is summarized in the following sections.

**Section One**

**PCOS and metabolic syndrome**

Various epidemiological studies have established a close association between body fat mass and an increased risk of developing a variety of chronic disorders including cardiovascular diseases and non-alcoholic fatty liver disease (NAFLD). The excessive body fat, especially visceral fat, contributes to the development of a complex network of potentially serious clinical conditions such as insulin resistance, glucose intolerance, dyslipidaemia, elevated blood pressure, impaired fibrinolysis and endothelial dysfunction. This constellation of risk factors are recognized as components of what is now called Metabolic Syndrome (MS). The most common description of MS includes visceral obesity, insulin resistance, dyslipidaemia, and hypertension. The pathogenetic mechanism of MS resides within a triangle of the following factors: (i) insulin resistance, (ii) hyperinsulinemia, and (iii) glucose intolerance, which are mainly due to defect(s) in the insulin signal transduction pathways.
Along with the epidemic of obesity, the prevalence of MS is increasing worldwide, both in the developing and developed countries. As noted previously, MS is associated with a risk of cardiovascular disease and is a common early abnormality in the development of type 2 diabetes. In addition, MS plays a well-recognized role in the development of obstructive sleep apnea, erectile dysfunction, polycystic ovary syndrome and malignant tumors.

Insulin resistance (IR), a hallmark of metabolic syndrome, is observed in about 50% to 80% of women with PCOS. In PCOS, IR is more than a biomarker of the disease, but is rather an active contributor to its pathogenesis. Insulin receptors are abundant in ovaries; dysregulation of insulin signaling in theca cells augments the production of androgens. Obesity aggravates the clinical presentation of PCOS. In fact, the prevalence of hirsutism and menstrual disorders is greater in the obese as compared to non-obese PCOS subjects. However, even in lean women, PCOS is often accompanied by abnormalities of insulin secretion and higher basal blood glucose than weight-matched controls. In cases when lean women with PCOS maintain normal sensitivity to insulin, adiponectin biosynthesis deficiency or chronic low level inflammation is often present.

The molecular underpinnings of insulin resistance (IR) in PCOS are related to a variety of defects, including post-binding receptor failure and insulin signaling defect at the level of glucose transport in skeletal muscle. Specifically, the cause of IR in skeletal muscle might be due to low levels of Insulin receptor substrate 1 (IRS-1) expression,
impaired IRS-1 phosphorylation, reduced activity of the serine/threonine kinase AKT2 and altered glucose transporter GLUT4 translocation to the plasma membrane.\textsuperscript{15} However, some studies have shown that muscular IR in PCOS results from both intrinsic factors (genetically determined defects in insulin signalling) and extrinsic conditions pertinent to environmental exposures (obesity, ovarian dysfunction).\textsuperscript{15}

**Section Two**

**PCOS and non-alcoholic fatty liver disease**

Non-alcoholic fatty liver disease (NAFLD) is now considered to be one of the most common forms of chronic liver disease in the Western world. It occurs in an estimated 25\% to 30\% of the US general population, whereas its potentially progressive form, non-alcoholic Steatohepatitis (NASH) is reported in 2–3\% of the population. Although the most common explanation for the increased prevalence of NAFLD is the increased prevalence of obesity, the risk of developing NAFLD and NASH is not limited to the overweight and obese individuals.

Non-alcoholic fatty liver disease refers to a clinico-pathologic spectrum of conditions ranging from simple steatosis (simple fatty liver) to NASH, involving inflammation and some evidence of liver cell damage, and in some cases, cirrhosis, which is advanced scarring of the liver.\textsuperscript{16} From the liver disease standpoint, simple, or bland, steatosis is a relatively benign condition. Its clinical manifestations are usually absent or subtle and it usually comes to medical attention incidentally when aminotransferase levels are found to be elevated or a radiographic study reveals that the liver is fatty.\textsuperscript{17} It is often noted that simple steatosis rarely progresses to NASH and may
even be reversible. A few recent longitudinal studies with paired liver biopsies showed that the progression to NASH may occur in a proportion of patients with bland steatosis.\textsuperscript{18,19} NASH, in turn, can potentially progress to cirrhosis, decompensated liver disease and hepatocellular carcinoma. The long-term follow-up evaluation of NAFLD patients revealed that NASH patients have increased liver-related mortality compared with non-NASH patients. In addition, patients with both NAFLD and type II diabetes are especially at risk for liver-related mortality.\textsuperscript{20,21}

Insulin resistance (IR) is the key event linking NAFLD to MS.\textsuperscript{22} The epidemiology, pathogenesis and approach to treatment of NAFLD follow the same trends as all other metabolic disorders.\textsuperscript{23,24} Clinical features of the metabolic syndrome (obesity, diabetes mellitus, or hyper-triglyceridemia) are commonly observed in NAFLD. Moreover, primary NAFLD is now considered the hepatic manifestation of metabolic syndrome.\textsuperscript{24} NAFLD is an early predictor of metabolic disorders in general, particularly in the normal-weight population.\textsuperscript{25}

Although PCOS and NAFLD do share a common attribute in regards to their pathogenesis, insulin resistance, the link between the two diseases has not been obvious until the first case was described in 2005. A young female patient with PCOS, at an outpatient Gastroenterology Clinic, was found to have both insulin resistance and severe NASH in her liver biopsy.\textsuperscript{26} This interesting case opened a new path into the investigation of the association of PCOS and NAFLD through their link to increased risk of metabolic disorders, such as insulin resistance and abdominal adiposity.\textsuperscript{27} Elevated alanine
aminotransferase (ALT) serum levels are a common finding in PCOS.\textsuperscript{27} Moreover, in PCOS women with abnormal ALT, insulin sensitivity is markedly decreased (P < 0.001). One study found that 55\% (48/88) of PCOS women had both hepatic steatosis and high HOMA-IR scores (P = 0.033).\textsuperscript{28}

Another study found that 41\% (17/41) of women with PCOS had concomitant NAFLD as diagnosed by hepatic steatosis and abnormal ALT levels, whereas the incidence of NAFLD in the weight and age matched non-PCOS control group was only 19\% (6/31, P < 0.05).\textsuperscript{29} Some studies have also shown that for obese PCOS patients, the rates of co-diagnoses with NAFLD or NASH are even higher.\textsuperscript{30} Even stronger associations between NAFLD and PCOS has been described when screening for PCOS was performed in the NAFLD cohort. A recently published study of 14 NAFLD female patients of reproductive age (22–45) revealed that 71\% (10/14) of these patients matched the 2003 Rotterdam diagnosis criteria for PCOS.\textsuperscript{31} Nevertheless, it should be noted that in the cohort of young and lean PCOS patients, no evidence of NAFLD were found.\textsuperscript{32} Despite its important contribution to the literature, this study lacked follow-up and therefore, the question of whether PCOS patients are predisposed to NAFLD at older age remains open.

Although insulin resistance is primarily associated with obesity, clinical evidence has revealed that insulin resistance exists in both obese and lean PCOS patients.\textsuperscript{27} Importantly, when insulin resistance was studied in young, lean individuals, net muscle glycogen synthesis in the group with IR was found to be lower by 61\% as compared with
the age-BMI matched, insulin sensitive control group. On the other hand, net hepatic triglyceride synthesis was about 2.5-fold greater in the IR group as compared to the insulin sensitive group. Moreover, hepatic de novo triglycerides lipogenesis was increased by 2.2-fold in the IR group (15.7 +/- 1.5%) compared with the insulin sensitive group (7.2 +/- 0.7%, P = 0.00005). These data suggested that in young, lean insulin sensitive individuals, energy is stored mostly in the muscle and liver glycogen, whereas in young lean insulin-resistant individuals, the energy is mainly diverted from muscle glycogen synthesis into liver triglyceride synthesis, resulting in increased fat production in the liver and leading to hepatic steatosis. This finding also suggests that skeletal muscle’s insulin resistance, which is common in PCOS patients, predisposes them to having hepatic IR, which is strongly linked to non-alcoholic fatty liver disease (NAFLD).

The above findings suggest that women with PCOS probably are at an increased risk for developing NAFLD and conversely, women with NAFLD may be at risk for having PCOS. In addition, it is reasonable to propose that women with central obesity are at a higher risk for both NAFLD and PCOS. However, for reasons yet unknown, some women develop obesity and never develop PCOS, whereas others develop obesity and then develop PCOS and/or NAFLD. Below, we will review the molecular genetics and environmental interactions in PCOS and try to dissect these facets for possible interplay between PCOS, NAFLD and metabolic syndrome (Figure 1).
Figure 1. An interplay between various components of the PCOS phenotype

Section Three
The role for environment factors in PCOS and NAFLD

The prevalence of PCOS is increased in obese as compared to lean women (30% vs. 10%). The strongest contributing factors to the phenotypes of obesity and insulin resistance are poorly balanced diet and physical inactivity. Although an increasing number of studies stress the very strong association between PCOS, insulin resistance and obesity as well as co-association of insulin resistance and obesity, these conditions, to some degree, remain independent from each other. Some obese patients are insulin sensitive and there are lean patients who have substantial resistance to insulin. However, being overweight with a body mass index (BMI) greater than 25 kg/m², especially with
visceral fat deposits, substantially increases the risk of insulin resistance, metabolic disorders and reproductive abnormalities consistent with a PCOS phenotype. Similar to having PCOS, being overweight or obese can predispose one to NAFLD. Depending on the particular diagnostic criteria, the prevalence of NAFLD in obese cohorts is estimated to be between 60% and 90%. Once excessive adiposity is established, the more profound the associated metabolic abnormalities the more likelihood of patients having the progressive form of NAFLD or non-alcoholic steatohepatitis (NASH).

Section Four
Molecular genetics of PCOS

Until recently, the molecular genetics of PCOS have been poorly understood. Pedigree studies indicate that the inheritance mode of PCOS is autosomal dominant: the disorder is transmitted to both sons and daughters, but the clinical phenotype only occurs in women. Incidentally, women from PCOS families have been shown to have both insulin resistance and high levels of androstenedione in their blood. Other factors such as diet, lifestyle and medications can contribute to the progression of the disorder as well. By using several molecular methods, such as cDNA microarray analysis, linkage studies and mutation analysis as well as case–control association studies, some genes associated with PCOS have been identified. However, dysregulation of one of these candidate genes alone is not sufficient to cause PCOS. As the pathogenesis of PCOS usually involves multiple pathways, ranging from insulin synthesis, androgen hormone production, follicle development and obesity; so far, studies of PCOS have focused on candidate genes from these pathways. Among the genes, most often
investigated in the study of PCOS are the steroid biosynthesis-related cytochrome genes
*CYP11A* and *CYP17*, *FEM1A* gene encoding for ischaemia-related mitochondrial protein, the obesity associated gene (*FTO*); several genes involved in the insulin pathway (*AKT2* and *INSR*), in leukotriene metabolism (*ALOX15*), and the androgen signalling pathway (*SGTA*). In addition, some other genes were found to be differentially expressed in relevant tissues of the PCOS patients, including ones encoding for insulin signalling components (*IRS-1, IRS-2, GLUT4*), sex hormone-binding globulin (*SHBG*), Adiponectin and inflammatory cytokines (*IL-6, IL-18, TNFα*). Functions of PCOS related genes are summarized in Table 1.

<table>
<thead>
<tr>
<th>PATHWAYS INVOLVED</th>
<th>GENE NAMES</th>
<th>PCOS</th>
<th>NAFLD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steroid biosynthesis pathway</td>
<td><em>CYP11A</em></td>
<td>Elevates testosterone levels</td>
<td>Suppressed in response to fenofibrate treatment in parallel with alleviation of steatosis</td>
</tr>
<tr>
<td></td>
<td>Both <em>CYP11a</em> transcription and the development of the endocrine pancreas is regulated by GATA-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>CYP17</em></td>
<td>Is elevated in ovaries and associated with IR</td>
<td>Is attenuated by adiponectin, known anti-NAFLD/NASH factor</td>
</tr>
<tr>
<td></td>
<td><em>SRD5A</em></td>
<td>Produces hyperandrogenism, lowers cortisol/cortisone ratio</td>
<td>Is sensitive to pioglitazone, liver fat accumulation is associated with lower cortisol/cortisone ratio</td>
</tr>
<tr>
<td>Androgen signaling pathway</td>
<td><em>SHBG</em></td>
<td>Levels are decreased in PCOS, thus, increasing bioavailability of androgens</td>
<td>Levels are decreased in steatosis</td>
</tr>
<tr>
<td>Obesity associated gene</td>
<td><em>SGTA</em></td>
<td>Promotes apoptosis</td>
<td>Promotes apoptosis, a hallmark of NASH</td>
</tr>
<tr>
<td></td>
<td><em>FTO</em></td>
<td>influences both hyperandrogenemia and impairment of glucose tolerance</td>
<td>Predisposes to NAFLD (in PCOS population)</td>
</tr>
</tbody>
</table>
particularly, in muscle IR

| Leukotriene metabolism related genes | ALOX15 | Contributes to inflammation seen in PCOS; augments the IR | When disrupted, prevents NAFLD in model animals |
| FEM1A | Mutated in some PCOS patients | Participates in anti-inflammatory signaling of prostaglandin E2 that alleviates liver damage by ROS. |
| FEM1B | Variants are associated with both reduced likelihood of PCOS and lower IR | Binds SREBPs that are central effectors in NAFLD and NASH inflammatory mediators. Mediates apoptosis. |
| Adipokines and cytokines | IL-6, IL-18, hs-CRP, TNFα, TNFR2 | Elevated (especially in obese PCOS) | Known to increased the risk of NAFLD |
| Adiponectin | Both total and HMW adiponectin levels are lowered in PCOS | Both total and HMW Adiponectin levels are lowered in NAFLD/NASH |
| Insulin signaling pathway | Insulin (INS), INSR, IRS-1, IRS-2, AKT2, GLUT4 | An involvement of insulin signaling is well known pathogenetic cornerstone for both PCOS and NAFLD |

**Subsection One**

*Enzymes participating in the steroid biosynthesis pathway*

In PCOS, an increased androgen production is dependent on the increased activity of three enzymes, two cytochromes – *CYP11A* and *CYP17* – and Steroid 5-α Reductase (*SRD5A*). As can be seen from observations outlines below, all three of these enzymes possess steatogenic or diabetogenic properties.

*CYP11A* encodes a cholesterol side chain cleavage enzyme participating in the androgen biosynthesis by converting cholesterol to pregnenolone, the common steroid precursor. *CYP11A* contains a common polymorphism, specifically, a variable number of
tandem repeats (VNTR) site that is associated with elevated serum testosterone levels in PCOS.\textsuperscript{47} In addition, the upregulation of GATA-6, which is involved in the \textit{CYP11A} transcription, is also associated with PCOS.\textsuperscript{48} GATA-6 also regulates the development of the endocrine pancreas and interacts with Nkx2.2, a critical islet transcription factor.\textsuperscript{49} Interestingly, \textit{CYP11A} is suppressed in response to fenofibrate, a hypolipidemic ligand for PPAR\(\alpha\) used in the treatment of hypertriglyceridemia and combined hyperlipidemia, being particularly effective in lowering the plasma triglyceride and cholesterol levels.\textsuperscript{50} Incidentally, fenofibrate also alleviates steatosis in Zucker diabetic fatty (ZDF) rats, while not changing either energy intake or expenditure or the progression of diabetes.\textsuperscript{51}

The levels of \textit{CYP17} mRNA encoding for 17-\(\alpha\) hydroxylase, yet another steroidogenic enzyme, are increased in ovaries of PCOS women.\textsuperscript{52} This increase in expression may be caused by the activity of many factors that affect the transcriptional and post-translational regulation of \textit{CYP17} in the thecal cells;\textsuperscript{53} one of these factors is insulin. Interestingly, obese PCOS carriers of hyperactive A2 allele of \textit{CYP17} show an odds ratio of 9.1 (confidence interval, 3.0–27.4; \(P < 0.0001\)) for developing insulin resistance.\textsuperscript{54} Adiponectin, generally a beneficial Adipokine, typically deficient in obese and insulin-resistant individuals, decreases insulin-induced androstenedione production by attenuation of IGF-I-induced LHR, \textit{CYP11A1} and \textit{CYP17A1} gene expression in theca cells.\textsuperscript{55}

Steroid 5-\(\alpha\) reductase (\textit{SRD5A}) encodes an enzyme that converts testosterone into the more potent androgen, dihydrotestosterone and, as well, reduces cortisol. The
decrease of cortisol levels in the blood stimulates ACTH-dependent steroidogenesis and produces hyperandrogenism.\textsuperscript{56} In PCOS, an increase in activity of 5-alpha reductase in the liver, skin and follicles was observed.\textsuperscript{57} The levels of \textit{SRD5A mRNA} are also elevated in patients with PCOS.\textsuperscript{58} The metabolic abnormalities frequently seen in PCOS patients are tightly linked to increased cortisol elimination. In particular, \textit{SRD5A} activity correlated with BMI, insulin levels and HOMA scores.\textsuperscript{59} The activity of 5-alpha reductase is sensitive to the antidiabetic drug pioglitazone\textsuperscript{60} and to weight loss.\textsuperscript{61} Even more interesting is that higher urinary excretion of 5-\textit{α} reduced cortisol metabolites is associated with indices of obesity, and liver fat accumulation, a hallmark of NAFLD with a lowered ratio of cortisol/cortisone metabolites.\textsuperscript{62} 

\textbf{Subsection Two}

\textit{Androgen signaling pathway}

Sex hormone-binding globulin (SHBG) is a glycoprotein that binds to both testosterone and estradiol and influences the function of these hormones. The levels of SHBG have been shown to negatively correlate with insulin levels\textsuperscript{46} and even were suggested as a surrogate marker of insulin resistance. In PCOS patients, due to their increased insulin levels, the SHBG levels are reduced significantly, leading to the release of more androgens, which causes the characteristic hyperandrogenemia of the disorder.\textsuperscript{41,63} Importantly, SHGBs levels are lower in IR PCOS vs. non-IR PCOS patients.\textsuperscript{63} Moreover, in patients with steatosis, SHBG levels are also decreased,\textsuperscript{27,64} possibly reflecting known abnormalities associated with NAFLD that influence SHBG secretion by the liver, such as obesity, central adiposity and insulin resistance.
Small glutamine-rich tetracopeptide repeat containing protein α gene (SGTA) is a member of the androgen receptor chaperone-co-chaperone complex in the androgen signaling pathway, which binds to the androgen receptor (AR) and restrains its activity by holding it in cytoplasm. Over expression of SGTA results in the inhibition of AR bioactivity whereas reduced SGTA expression increases AR activity and promiscuous activation of the AR by non-classical ligands (e.g. progesterone). Goodarzi and colleagues investigated variants of the SGTA gene within the PCOS patient population and found that haplotype-1 was associated with PCOS risk and haplotype-2 was associated with increased insulin resistance, a feature of PCOS. Experimental evidence suggests that the SGTA gene may increase PCOS risk by promoting apoptosis through enhanced DNA fragmentation, chromosome misalignment and mitotic arrest. Importantly, enhanced apoptosis is a characteristic of both PCOS and NAFLD, in particular, of NASH variety.

Subsection Three
Fat mass and obesity associated gene

The fat mass and obesity associated gene (FTO) has been shown to be associated with obesity in humans in several genome-wide association scans. The relevance of FTO to PCOS is suggested by the high prevalence of obesity in PCOS, about 60% to 70%. The function of FTO was not known until recently, when a transcriptional coactivator activity of FTO was demonstrated for both unmethylated and methylation-inhibited CCAAT/enhancer binding proteins (C/EBPs)-dependent gene promoters. Consistent with its C/EBP enhancer function, mutation of FTO leads to temporal progressive loss of
adipose tissue in experimental animals.\textsuperscript{72}

Variants within the \textit{FTO} gene influence both hyperandrogenemia and impairment of the glucose tolerance parameters in women with PCOS.\textsuperscript{73} Tan \textit{et al.} determined that the \textit{FTO} genetic variants appear to have a greater impact on obesity and related traits in PCOS than in other phenotypes.\textsuperscript{43} These and other studies \textsuperscript{74} demonstrated that the predisposition to common obesity also result in altered susceptibility to PCOS, confirming the mechanistic link between these conditions.

Importantly, \textit{FTO} is equally involved in muscle IR. The expression of \textit{FTO} in skeletal muscle from type 2 diabetic patients is elevated, but could be normalized by thiazolidinedione treatment.\textsuperscript{75} Given the link between muscle IR and liver IR\slash steatosis discussed above, \textit{FTO} overexpression common in PCOS may contribute to the predisposition to NAFLD in this population.

\textbf{Subsection Four}
\textit{Leukotriene metabolism related genes}

One of the studies of the skeletal muscles from PCOS women employed gene set enrichment analysis (GSEA) to get an insight into the early stages of IR and found the systemic IR-associated changes in the expression of genes involved in mitochondrial oxidative metabolism and evidence of abnormal lipid metabolism.\textsuperscript{76} 12\slash 15-Lipoxygenase (\textit{ALOX15}) is one of the lipid metabolism genes up-regulated in the omental fat of PCOS patients.\textsuperscript{44} As a large number of lypoxygenase-oxidised fatty acids become leukotrienes, which are natural chemical substances in the body that promote inflammatory response to
damage, an impaired lipoxygenase function can theoretically contribute to the inflammatory condition seen in PCOS.  

Incidentally, it was shown that the lipoxygenase encoded by ALOX15 augments the resistance to insulin, whereas its inhibition enhances the action of insulin in rat models of insulin resistance and type 2 diabetes.  

12/15-lipoxgenase deficient mice are resistant to streptozotocin-induced diabetes. Targeted deletion of ALOX15 also protects non-obese diabetic (NOD) mice from autoimmune diabetes. The antidiabetic action of ALOX15 deletion is explained by the failure to augment the production of IL-12 in macrophages and induce apoptosis in β-cells of the pancreas.

Importantly, disruption of ALOX15 protects apolipoprotein E-deficient (ApoE-/-) mice against the development of NAFLD. These mice show reduced serum alanine aminotransferase levels; decreased hepatic steatosis, inflammation and macrophage infiltration; and decreased fatty acid synthase, TNF-α, monocyte chemo-attractant protein-1 (MCP-1), interleukin (IL)-18 and IL-6 expression.

The FEM1A gene is a homologue of fem-1 sex determination gene of C. elegans; it is highly expressed in human skeletal and cardiac muscle, brain, liver and in the ovaries; it localizes within mitochondria. Mouse homologue of FEM1A is expressed in androgen-producing secondary interstitial cells, with a marked increase in expression after puberty, consistent with a key feature of PCOS – ovarian hyperandrogenism. In C. elegans, fem-1 determines the development of the male phenotype. Therefore, it is likely that human homologue of fem-1 contributes to the hyperandrogenic features of PCOS.
Moreover, the *FEM1A* gene locates to chromosome 19p13.3 and it has been confirmed by several studies that the microsatellite D19S884 on chromosome 19p13.2 is a potential PCOS susceptibility locus. In a study performed on five Caucasian PCOS patients from Mississippi, a heterozygous germline missense mutation in a conserved amino acid within *FEM1A*, *H500Y* (substitutes a tyrosine (Y) for a histidine (H) at codon 500), was found.

Interestingly, *FEM1A* was independently discovered in a yeast two-hybrid based screening for proteins that directly interact with the cytoplasmic tail of the EP4 receptor for prostaglandin E2 (PGE2) in human macrophages, where it participates in anti-inflammatory signaling of prostaglandin E2. PGE2 has a prominent antioxidant effects in the liver where it decreases the production of hydroxyl radicals, lipoperoxides, conjugated dienes, malonic dialdehyde and carbonyl-containing products of lipid peroxidation. In addition, PGE2 normalizes activity of the liver microsomal monooxygenase system components responsible for the free oxygen radical production and alleviates liver damage in cirrhotic rats. In addition to its PGE2 augmenting action, *FEM1A* directly interacts with NF-kappaB1 p105/p50 in macrophages, and, in a concentration-dependent manner, inhibits NF-kappaB1 activation induced by various pro-inflammatory stimuli.

*FEM1B* gene, an orthologue of the *C. elegans* feminization factor 1 (*Fem-1b*) is a binding partner for PHTF1, a transcription factor encoding a gene identified as susceptibility factors for type 1 diabetes in humans. *FEM1B* is a proapoptotic protein
that interacts with apoptosis-inducing proteins Fas, tumour necrosis factor receptor-1 (TNFRI) and apoptotic protease activating factor-1 (Apaf-1) and mediates proteasome inhibitor-induced apoptosis.\textsuperscript{88} It also can serve as an adaptor protein that links CHK1 and Rad9 thus facilitating check-point signaling induced by replication stress.\textsuperscript{89} FEM1B polymorphisms are associated with both a reduced likelihood of PCOS and lower insulin resistance.\textsuperscript{82} This fact is not surprising as Fem1b-knockout mice display abnormal glucose tolerance that is due predominantly to defective glucose-stimulated insulin secretion.\textsuperscript{90}

**Subsection Five**

*Inflammatory mediators*

As mentioned above, obese PCOS patients have high levels of pro-inflammatory mediators, such as high sensitive C-reactive protein (hs-CRP), interleukin 6 (IL-6) and IL-18, which make them vulnerable to an increased risk of non-alcoholic fatty liver disease (NAFLD).\textsuperscript{91} C-reactive protein (hs-CRP), one of the acute phase proteins known as a vascular inflammatory marker, rises dramatically in response to inflammation due to an increase in the circulating level of interleukin 6 (IL-6), which is produced mainly by macrophages and adipocytes. The level of hs-CRP is significantly elevated in PCOS patients.\textsuperscript{92} However, some other studies report that serum hs-CRP levels increase with obesity rather than with the presence of PCOS itself. Therefore, an elevation of hs-CRP in PCOS women is mostly related to their increased BMI.\textsuperscript{93}

Other inflammatory mediators, such as interleukin 18 (IL-18), interleukin 6 (IL-6), tumour necrosis factor α (TNFα) and type 2 TNF receptor (TNFR2) are also
reportedly elevated in PCOS,\textsuperscript{41, 94} and are strongly associated with central fat excess in PCOS patients.\textsuperscript{95} As both \textit{hs-CRP} and \textit{IL-6} are considered strong risk markers for NASH and severity of liver fibrosis, these findings may point towards a mechanism for increased risk of NAFLD and NASH in PCOS patients.\textsuperscript{96, 97} Furthermore, the increased levels of \textit{TNFα} induced insulin resistance is a common feature in NASH and PCOS.\textsuperscript{97, 98} All together, these observations indicate that multifaceted inflammatory processes may be the underlying causes of the high prevalence of type 2 diabetes and steatohepatitis seen in PCOS patients.

\textbf{Subsection Six}

\textit{Adiponectin}

Adiponectin, a peptide hormone, is secreted by adipocytes and is the only adipokine down-regulated in obesity.\textsuperscript{45} Clinical studies have shown that PCOS patients, especially those with obesity, type 2 diabetes and insulin resistance have significantly decreased levels of adiponectin as compared to weight-matched controls.\textsuperscript{45} Importantly, the high-molecular weight (HMW) form of adiponectin that most closely correlates with insulin sensitivity was even more substantially down-regulated in PCOS women than total adiponectin.\textsuperscript{98} The lower levels of total and, specifically, HMW adiponectin in PCOS patients may explain the very high risk for PCOS patients of being prone to metabolic syndrome and diseases of the NAFLD spectrum and point at a possibility of adipocyte dysfunction underlying the pathology of PCOS.
Subsection Seven
INS, INRS, AKT2, GLUT4 and IRS genes

It has been known that hyperinsulinemia and insulin resistance are common features of PCOS patients with or without obesity. Hence, several genes involved in insulin secretion and action have been proposed as candidate genes in PCOS pathogenesis.\(^\text{31}\)

Insulin, encoded by gene \textit{INS}, is a hormone secreted by the β cells of the pancreas to maintain normal blood glucose levels by regulating cellular glucose uptake, carbohydrate, lipid and protein metabolism.\(^\text{99}\) In PCOS, these functions of insulin may be disturbed by defects in insulin signaling, resulting in insulin resistance and hyperinsulinemia. Through a family-based association study using DNA obtained from 1723 individuals in 412 families with 412 index cases and 43 PCOS sisters, Urbanek \textit{et al.} found that there was an association between D19S884 allele 8 with higher fasting insulin levels and homoeostasis model assessment for IR (HOMA-IR)) in lean PCOS women BMI <25 kg/m\(^2\).\(^\text{100}\) In addition to regulating blood glucose levels, insulin also stimulates the growth and replication of cells in the ovaries. With excess of insulin in the bloodstream, the cells of the ovaries are stimulated to overproduce androgens, which lead to the hyperandrogenism seen in PCOS.

The defects of \textit{INSR} gene that encodes for the insulin receptor are an uncommon cause of diabetes; however, conditional knockouts of this gene in various murine tissues served as an indispensable tool in understanding insulin resistance, often producing surprising effects. For example, knockout of \textit{INSR} in white adipose tissue protects mice
against obesity, whereas its elimination in brown adipose lead to the development of β-cell failure (see for detailed analysis). By using microsatellite markers, analysis of linkage and family-based association, it was concluded that the INSR gene marker D19S884, which is located 1 cM telometric to the INS gene, is significantly associated with PCOS ($\chi^2 = 11.85; P < 0.0006$). Another study carried out by Siegel et al. found an association between a C/T single nucleotide polymorphism (SNP) at the tyrosine kinase domain of INSR with PCOS in lean patients.

As compared to the insulin receptor itself, two of its substrate proteins, IRS-1 and IRS-2, critical to signal transduction in insulin target tissues, garnered larger attention as both PCOS and NAFLD candidates. For example, IRS-1 polymorphisms were associated with increased susceptibility to PCOS in many independent studies. One suggested mechanism linking IRS-1 and PCOS involves an co-stimulatory interaction between testosterone and insulin that promotes the serine phosphorylation of IRS-1 Ser(636/639), that, in turn, influence the phosphorylation of its downstream targets, particularly, AKT, mTOR and S6K.

Both IRS-1 and IRS-2 also play important roles in the control of hepatic metabolism, with IRS-1 more closely linked to glucose homoeostasis and IRS-2 more closely linked to lipid metabolism. Importantly, decreased IRS-1 was also associated with a trend towards increased blood glucose, whereas knockdown of IRS-2 resulted in the upregulation of lipogenic enzymes and increased hepatic lipid accumulation. In our own study of phosphoproteomic endpoints in morbidly obese patients who underwent
liver biopsy, two components of the insulin signaling pathway, AKT kinase and IRS1 were identified as independent predictors of NASH.69

*AKT2* is one of the three conserved genes that encode isoforms of the protein kinase B, which participates in the insulin pathway, mitogenic signaling and apoptosis. Out of three isoforms, *AKT2* is the one most relevant to metabolic syndrome and the related chronic diseases. Experimental evidence shows that *AKT2* is involved in insulin signaling in adipose tissue and is required for the relocation of *GLUT4* to the cell membrane in response to insulin.107 In diabetes type 2, activation of *AKT* by insulin is impaired. Overexpression of activation-impaired *AKT2* blocks insulin signaling and reduces glucose disposal,108 both of which are features of PCOS.

Upon analysis of the variants of the *AKT2* gene in PCOS and control populations, it was revealed that the minor alleles of rs3730051 and rs8100018 as well as their haplotypes are significantly associated with PCOS.109 The leading hypothesis that connects *AKT2* defects with PCOS, although, is not insulin related, but rather focused on its apoptosis suppressing qualities. Overexpression of *AKT2* reduces the rate of apoptosis in many tissues, including ovaries. This feature may contribute to the abnormal growth of follicle cells, eventually, leading to the forming of polycystic ovaries. The effect of *AKT2* on the rate of apoptosis may also increase the number of steroidogenic cells,110 thus, increasing total androgen production by the ovary and resulting in hyperandrogenism, a predominant characteristic of PCOS.

Importantly, *AKT2* is the predominant isoform of *AKT* in the liver. *AKT2* was
recently shown to be the key mediator for PIP3-induced accumulation of lipids in the liver, manifesting as NAFLD in model animals.111 Humans with post insulin receptor defects in AKT2 signaling also manifest increased lipogenesis and elevated liver fat content accompanied by an increase in triglyceride-enriched VLDLs, hypertriglyceridemia and low HDL cholesterol levels.112 Interestingly, other studies showed that AKT2 is required for hepatic lipid accumulation in obese, insulin-resistant states induced by high-fat diet feeding; thus indicating that AKT2 is a requisite component of the insulin-dependent regulation of lipid metabolism.113 In either instance, deregulation of AKT2 seems detrimental in both NAFLD and PCOS.

In addition to the components of the insulin signaling cascade described above, PCOS patients demonstrate changes in the levels or the functionality of the down-stream components of the insulin signaling cascade, including the glucose transporter 4 (GLUT4), which is necessary for glucose uptake into the cell. In particular, GLUT4 levels are reduced both in endometrial tissue13 and in adipose.114 It is well known that insulin resistance in adipocytes leads to down-regulation of GLUT4; the SLC2A4 gene that encodes GLUT4 is a part of the intra-hepatic gene network associated with NAFLD in obese subjects.115

Section Five
Bariatric surgery in PCOS and NAFLD

Both PCOS and NAFLD/NASH conditions are confounded by insulin resistance and by obesity. The impact of the removal of one or another of these factors on each of these two disorders is worthwhile to note. In PCOS patients, use of insulin sensitizers,
primarily Metformin, results in the reduction of glucose levels and the attenuation of insulin resistance that are often paralleled by improvement of the distinctive features of PCOS – hyperandrogenism, irregularity of menses and ovulatory dysfunctions.\textsuperscript{116} Likewise, weight loss-centred interventions often results in similar improvements; however, responsiveness to weight loss in overweight/obese PCOS women varies considerably.\textsuperscript{117} In a number of studies, it has been reported that obese women with PCOS, on average, display more severe phenotype and experience more profound impairment of fertility as compared to the normal-weight PCOS patients, even if the latter group has the same degree of insulin resistance. It seems that the primary underlying cause of low menstrual frequency observed in a subset of PCOS patients is obesity and not insulin resistance. In line with this hypothesis, a randomized, placebo-controlled, double-blind study unequivocally showed that Metformin did not improve the degree of weight loss or menstrual frequency in obese patients with PCOS, while weight loss alone through lifestyle changes substantially improves menstrual frequency.\textsuperscript{118}

In obese patients, weight loss can be achieved by lifestyle modification / behavioral therapy or by bariatric surgery. Out of these two options, bariatric surgery is the most informative one as it produces relatively fast resolution of insulin resistance that precedes actual loss of adiposity.

Increasingly, bariatric surgery is being applied to women of reproductive age who fail to achieve a loss of weight through dietary modification and exercise. The majority of the weight loss occurs in the first year, when pregnancy avoidance is recommended.\textsuperscript{119}
Therefore, in the PCOS population, the effects of insulin resistance abatement and loss of adiposity are difficult to discern. Nevertheless, bariatric surgery was shown to improve many aspects of reproduction, including menstrual regularity,\textsuperscript{120} clinical and biochemical hyperandrogenism\textsuperscript{121} and successful conception.\textsuperscript{122}

Similar to PCOS, both liver steatosis and NASH improve after successful weight loss. This improvement is usually accompanied by alleviation of both metabolic parameters and levels of inflammatory mediators. Weight loss improves histological disease activity in NASH in a dose dependent manner; however, more than 50% of patients fail to achieve their BMI targets and more than 50% of patients fail to achieve target weight loss (reviewed in\textsuperscript{123,124}). Although lack of randomized controlled trials evaluating, any bariatric procedure may have led to some bias,\textsuperscript{125} there are increasing evidence that weight loss induced by bariatric procedures could be beneficial for patients with NASH.\textsuperscript{20,126} As NAFLD was recently proposed as the hepatic manifestation of metabolic syndrome, it seems appropriate to establish PCOS as the ovarian manifestation of the same spectrum of metabolic diseases and expect the improvement of its manifestations after bariatric surgery.

Section Six
Conclusions

Polycystic ovary syndrome is a complex disorder, in which multiple genetic, metabolic and hormonal controls fail to interact properly and produce the hallmark symptoms of the disease. Based on the high association of PCOS and other metabolic abnormalities, such as insulin resistance, hyperandrogenism, obesity and non-alcoholic fatty liver disease, the
candidate genes, which are related to those metabolic pathways, have been proposed as PCOS causative genes. Close scrutiny of these PCOS candidates identified that most of their protein products are located at the crossroad of three highly relevant conditions, metabolic syndrome, obesity and NAFLD. In all studied populations, the prevalence of both PCOS and NAFLD rises proportionally to the degree of insulin resistance and gain of adiposity. It seems that both NAFLD and PCOS are often accompanied or augmented by two interrelated physiological states i.e. metabolic syndrome and obesity. As NAFLD was recently proposed as the hepatic manifestation of metabolic syndrome, it seems highly plausible that PCOS is the ovarian manifestation of the same spectrum of metabolic diseases (Figure 2) and consequently, expect improvement of this condition following bariatric surgery.

Figure 2. Both NAFLD and PCOS are at the crossroad of two highly relevant conditions, metabolic syndrome and obesity.
CHAPTER 2

Molecular Signature of Adipose in Patients with both Non-Alcoholic Fatty Liver Disease (NAFLD) and Polycystic Ovarian Syndrome (PCOS)

Although both PCOS and NAFLD progress through the requisite steps of central obesity and insulin resistance, the two disorders are not always present in tandem. Research on the commonalities of these two disorders has yielded conflicting conclusions, often confounded by the additional complications posed by the concurrent presence of metabolic syndrome. Despite these unresolved issues, it is evident that PCOS and NAFLD share some of the metabolic pathways and are influenced by both obesity and insulin resistance. Untangling this complex relationship may provide better options for the treatment of these diseases, as well as the prevention of associated risk factors.

An aberrant adipokine production in visceral adipose tissue has been implicated in both metabolic syndrome and the pathogenesis of NAFLD/NASH.\textsuperscript{127,128} So far, however, there is a scarcity of information on PCOS related gene expression patterns in adipose. In this experimental study, we analyze mRNA profile in the visceral adipose tissue of obese patients with NAFLD and concomitant PCOS as compared to these with NAFLD only. To further support our findings, we augmented our data by the protein expression studies using serum samples collected from same patients.
Section One
Materials and Methods

Subsection One
Sample Collection and Storage
This study used visceral adipose tissue and serum samples previously collected from obese patients at the time of their bariatric surgeries after informed consent and immediately frozen with liquid nitrogen before storage at -80°C. Clinical data and routine laboratory data was obtained at the time of surgery. Each patient also underwent a liver biopsy, which was read by the study pathologist. The study was approved by the Institutional Review Board of Inova Fairfax Hospital.

Subsection Two
mRNA Extraction
Aurum™ Total RNA Fatty Acid and Fibrous Tissue Kit (Bio-Rad, Hercules, California) was used to efficiently isolate excellent quality mRNA from the selected human adipose tissue samples. Per extraction, ~100mg of human adipose tissue was removed from the -80°C freezer and placed in 1mL of PureZOL™ RNA isolation reagent into a 2.0 ml microcentrifuge tube and subsequently disrupted and homogenized by using rotor-stator homogenizer. Samples were then, incubated with the lysate (PureZOL™ RNA isolation reagent) at room temperature for 5 minutes. 200μL of chloroform was added; samples were covered and shaken vigorously for 15 seconds. The mixture was incubated at room temperature for 5 minutes while periodically mixing the sample. Samples were then centrifuged at 12,000 x g for 15 min at 4°C. Without
disturbing the interphase, the aqueous phase was transferred immediately into fresh 2.0 ml tubes. Equal volumes of 70% ethanol were added to the tube and mixed thoroughly. The resulting lysate/ethanol mixture was passed through a filter cartridge into a collection tube via centrifugation. The flow-through was discarded and the filter was washed with 700μL low stringency wash solution followed by high stringency wash solution. Lastly, mRNA was eluted from the filter into a clean capped tube with 30μL of preheated 70°C Elution Solution and was immediately placed on ice for mRNA quality confirmation.

**Subsection Three**

*mRNA Quality Confirmation*

The quality of the extracted adipose tissue mRNA was confirmed prior to storage by spectrophotometer by insuring that the A260/A280 ratio was between 1.8 and 2.0. In addition, the presence of intact RNA was also confirmed via 1% agarose gel electrophoresis. If the RNA sample was intact, a sharp 28S and 18S rRNA bands were clearly visible on the gel and the 28S rRNA band was about 2 times more intense than the 18S rRNA band.

**Subsection Four**

*First strand cDNA synthesis*

Freshly extracted RNA samples of good quality and large concentrations were processed to synthesize cDNA using RT² First Strand Kit (Qiagen, Valencia, CA). Reverse transcription reactions were performed using approximately 1.5 μg of total RNA. Reactions were heated at 42° C for 5 min in a total volume of 10.0 μl in the presence of 2.0 μl of 5X gDNA Elimination Buffer and chilled on ice immediately for at least 1 min. Then, a RT cocktail consisting of 4 μl of BC3 (5X Reverse Transcription Buffer 3), 2 μl
of RE3 (RT enzyme mix 3), 1 µl P2 (Primer and External Control Mix) and 3 µl nuclease-free H₂O was added. The mixture was then incubated at 42°C for exactly 15 min and then immediately stopped by heating to 95°C for 5 minutes. 91 µl of H₂O was added to each 20-µl of cDNA synthesis reaction. All extracted RNA and subsequent cDNA were stored in -20°C for short-term storage and -80°C for long-term storage.

**Subsection Five**

*Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)*

Target genes for this study were selected among regulatory genes directly or indirectly involved in the insulin signaling. Hence, the candidate genes were picked from the carbohydrate metabolism pathway including: fructose-1,6-bisphosphatase 1 (*FBP1*), glycogen synthase kinase-3β (*NIN* or *GSK3B*) and protein phosphatase 1 (*PPP1CA*); from the lipid metabolism pathway: low density lipoprotein receptor (*LDLR*), sterol regulatory element binding transcription factor 1 (*SREBF1*), acetyl-CoA carboxylase alpha (*ACACA*), sorbin and SH3 domain containing 1 (*SORBS1*), RAF proto-oncogene serine/threonine-protein kinase (*RAF1*); and target genes for peroxisome proliferator-activated receptor subfamily of nuclear receptors, such as acyl-CoA oxidase 1 (*ACOX1*) and peroxisome proliferator-activated receptor gamma (*PPARG*).

Endogenous reference genes, RNA polymerase II (*RPRII*), Beta-2-microglobulin (*B2M*), and β–Actin (*ACTB*) were selected based on previously published studies and profiled simultaneously.

PCR primers for *ACTB*, *B2M*, *FBP1*, *NIN*, *PPP1CA*, *LDLR*, *SREBF1*, *ACACA*, *RAF1*, *ACOX1*, *SORBS1* and *PPARG* were purchased at Real Time Primers, USA.
Primers for the *RPII* and *SORBS1* were custom designed using Oligo Perfect Designer software (Invitrogen, USA) and synthesized at Invitrogen, USA. Gene functions and primer sequences are summarized in Table 2.

**Table 2.** Sequences of primers used in qRT-PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward (F)</th>
<th>Reverse (R)</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-CoA Carboxylase Alpha (ACACA)</td>
<td>5’ CCCAGATTCTGCAGTTAAGA -3’</td>
<td>5’ - CAT CCACATGT AAG CAC CAA -3’</td>
<td>Long-chain fatty acid synthesis</td>
</tr>
<tr>
<td>Sterol Regulatory Element Binding Transcription Factor 1 (SREBF1)</td>
<td>5’ TACATTGGCTTTGCTAAGA -3’</td>
<td>5’ - GTCAGGGTCC TCCACCTC -3’</td>
<td>Transcriptional activator for lipid homeostasis</td>
</tr>
<tr>
<td>Peroxisome Proliferator-Activated Receptor Gamma (PPARG)</td>
<td>5’ - CCC AAG TTT GAG TTT GCT GT -3’</td>
<td>5’ - AAC AGC TGT GAG GAC TCA GG3’</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>Glycogen Synthase Kinase 3 Beta interaction protein ninein (NIN)</td>
<td>5’ - GAA TGT GGA TGG AGA GAT GC</td>
<td>5’ - ATT GCT GAT GAG GTT GTG GT 3’</td>
<td>Glycogen metabolism</td>
</tr>
<tr>
<td>RAF proto-oncogene serine/threonine-protein kinase (RAF1)</td>
<td>5’ - ATC CGA ATG CAT AAC AA 3’</td>
<td>5’ - AAG ATC TGG GGA GGA ATA TC3’</td>
<td>Regulator in cell fate decisions</td>
</tr>
<tr>
<td>Fructose-1, 6-Bisphosphatase 1 (FBP1)</td>
<td>5’ - TCA ACT GCT TCA TGG TGG AC -3’</td>
<td>5’ - CGT AGA CCA GAG TGC GAT GA3’</td>
<td>Gluconeogenesis regulator</td>
</tr>
<tr>
<td>Acyl-CoA Oxidase 1 (ACOX1)</td>
<td>5’ - CTG AAG GCT TTC ACC CCA CTG CTG -3’</td>
<td>5’ - CAT GCC ACA CAC CAA CTT CT -3’</td>
<td>Fatty acid Beta-Oxidation pathway</td>
</tr>
<tr>
<td>Low Density Lipoprotein Receptor (LDLR)</td>
<td>5’ - CCA AAC CCC TAA ACT CAG GA 3’</td>
<td>5’ - AAG TGG CAT CAT TGG GTG AA 3’</td>
<td>Receptor-mediated endocytosis of LDL</td>
</tr>
<tr>
<td>Sorbin and SH3 Domain containing 1 (SORBS1)</td>
<td>5’ - CTGCAAGCCCCACAGTTTCCAGT -3’</td>
<td>5’ - CGAGCAAGCTTTCCCTCCCCGC -3’</td>
<td>Insulin-stimulated glucose transport</td>
</tr>
<tr>
<td>Protein phosphatase 1 (PPP1CA)</td>
<td>5’ - ACC TGC AGT CTA TGG AGC AG 3’</td>
<td>5’ - TAG CCG TCT TCT ACC ACC TG -3’</td>
<td>Glycogen metabolism</td>
</tr>
</tbody>
</table>
Gene expression levels were quantified by qRT-PCR using the gene-specific primers with individual Tm’s between $58^\circ$ C and $60^\circ$ C. Primer specificity was validated by both melting curve analysis and gel electrophoresis. The real-time PCR mixtures containing 1 μl of the RT sample, 400 nM each of forward and reverse primers (Qiagen, Valencia, CA) and 2X SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Hercules, CA) were carried out in a total volume of 10 μl. Reactions were performed in a 96-well format in the BioRad CFX96 Real Time System (BioRad Laboratories, Hercules, CA). After melting curve based quality control, the RT-PCR data were transformed using the $\Delta$Ct method.

Subsection Six
Enzyme-linked immunosorbent assay (ELISA)

Previously collected and frozen serum samples were used to determine levels of the cytokines of interest by sandwich ELISA (Total CK18 M65 and Caspase Cleaved CK 18 Prototype M30 - Peviva, Bromma, Sweden; Human Adiponectin and Human Resistin – R&D Systems, Minneapolis, MN; Insulin EIA – Alpco Diagnosis, Salem, NH). The standards, antigen control and serum samples were added simultaneously with a horseradish peroxidase enzyme labeled monoclonal antibody (MAb) to the 96-well microplate coated with a MAb specific for the relevant antigen. The microplate was then incubated on an orbital microplate shaker (700-900 rpm) for 2 hours at room temperature. After the first incubation was complete, the wells were washed with Wash Buffer and blotted dry. The substrate was added and the microplate was again incubated on an orbital microplate shaker for an addition of 30 minutes while the plate was protected from light. Once the second incubation period was complete, the Stop Solution was added and
the optical density (OD) was measured using the ELx800 spectrophotometer at 450nm with a reference wavelength of 620nm. The intensity of the color generated was directly proportional to the amount of relevant cytokines, or IgG in the sample.

Section Two
Data analysis

To compare the expression levels of individual mRNAs, the statistical analysis by non-parametric Mann-Whitney test was performed. The analysis also included Spearman rank correlation tests and multiple regression analysis (MATLAB®, The MathWorks, Inc. Natick, MA). P values < 0.05 were considered significant.

For co-correlated genes (ACACA, SREBF1, FBPI, PPPICA, ACOX1 and PPARG), the factor analysis was performed with default rotation parameter and specified single factor output. The estimates of factor loadings were based on data from all subjects. The number of factors was estimated using the maximum likelihood method. The matrices of factor loadings were rotated by orthogonal transformation to simplify the resulting structure and ensure the independency of the factors. The common factor obtained through this approach was subject to a univariate correlation analyses with Glucose level, Insulin level, individual Adipokine levels and HOMA scores.

Section Three
Results

Subsection One
Clinical and biochemical characteristics of PCOS-NAFLD patients and controls

For this study we selected a cohort of biopsy-proven NAFLD patients (N=24) with (N=12) or without (N=12) PCOS. In all cases, histological assessment of liver biopsies had excluded NASH, thus an extent of NAFLD in all cases was limited to the
steatosis of the liver. Patients with PCOS and without PCOS were matched for age, BMI, AST and ALT levels as well a number of other parameters (Table 3). Diagnosis of PCOS was established according to Rotterdam diagnosis criteria and a combination of laboratory tests. Non-PCOS causes of anovulation and infertility were ruled out. The clinical and biochemical descriptions are provided in Table 3.

Table 3. Clinical and biochemical characteristics of PCOS-NAFLD patients and controls.

<table>
<thead>
<tr>
<th></th>
<th>non-PCOS (n=12)</th>
<th>PCOS (n = 12)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>37.6 +/- 10.0</td>
<td>35.2 +/- 9.60</td>
<td>NS</td>
</tr>
<tr>
<td>Heights, cm</td>
<td>165.0 +/- 11.0</td>
<td>168.1 +/- 3.9</td>
<td>NS</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>127.4 +/- 11.6</td>
<td>138.7 +/- 39.4</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/cm²)</td>
<td>44.1 +/- 3.9</td>
<td>45.0 +/- 3.6</td>
<td>NS</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>23.5 +/- 15.00</td>
<td>23.3 +/- 10.4</td>
<td>NS</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>28.7 +/- 21.9</td>
<td>29.9 +/- 15.3</td>
<td>NS</td>
</tr>
<tr>
<td>AST/ALT</td>
<td>0.96 +/- 0.35</td>
<td>0.85 +/- 0.24</td>
<td>NS</td>
</tr>
<tr>
<td>TCHOL (mg/dL)</td>
<td>187.3 +/- 31.2</td>
<td>178.5 +/- 34.9</td>
<td>NS</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>101.4 +/- 27.5</td>
<td>108.1 +/- 37.3</td>
<td>NS</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>43.6 +/- 11.1</td>
<td>54.2 +/- 14.6</td>
<td>NS</td>
</tr>
<tr>
<td>TRIG (mg/dL)</td>
<td>177.1 +/- 110.52</td>
<td>133.7 +/- 34.8</td>
<td>NS</td>
</tr>
</tbody>
</table>

Subsection Two

*Gene expression levels of Glycogen Synthase Kinase 3β interacting protein ninein (NIN) and Low-Density Lipoprotein Receptor (LDLR) are altered in PCOS NAFLD group*

To identify specific components of the insulin signaling pathway that could potentially be involved in the pathogenesis of PCOS in NAFLD patients, the expression levels of ten target genes related to insulin regulation were investigated: *FBPI, NIN, PPP1CA, LDLR, SREBF1, ACACA, RAF1, ACOX1, SORBS1* and *PPARG*.
Among these genes, expression levels of *NIN* and *LDLR* mRNA were significantly altered in the PCOS NAFLD group as compared to the non-PCOS NAFLD group (upregulated 1.65 folds with $p < 0.007$ and downregulated 0.51 folds with $p < 0.04$, respectively) (Figure 3).

**Figure 3.** Differential gene expression of *NIN* and *LDLR* genes in PCOS and non-PCOS (Fold change: 1.65, $p = 0.007$ and 0.51, $p = 0.04$, respectively)
The gene expression levels for the rest of the target genes were not significantly different between cohorts (Table 3).

Table 4. Spearman correlation coefficient (r) with p < 0.05

<table>
<thead>
<tr>
<th>Correlation coefficient (r)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistin - HOMA</td>
<td>0.66</td>
</tr>
<tr>
<td>Resistin - Insulin</td>
<td>0.67</td>
</tr>
<tr>
<td>Resistin - PT</td>
<td>0.42</td>
</tr>
<tr>
<td>LDLR - Resistin</td>
<td>0.721</td>
</tr>
<tr>
<td>LDLR – PT</td>
<td>0.89</td>
</tr>
<tr>
<td>PPARG - ACACA</td>
<td>0.74</td>
</tr>
<tr>
<td>PPARG – SREBF1</td>
<td>0.83</td>
</tr>
<tr>
<td>PPARG – FBPI</td>
<td>0.88</td>
</tr>
<tr>
<td>PPARG - PPP1CA</td>
<td>0.70</td>
</tr>
<tr>
<td>PPARG – ACOXI</td>
<td>0.90</td>
</tr>
<tr>
<td>ACOXI - ACACA</td>
<td>0.66</td>
</tr>
<tr>
<td>ACOXI - SREBF1</td>
<td>0.87</td>
</tr>
<tr>
<td>ACOXI - FBPI</td>
<td>0.87</td>
</tr>
<tr>
<td>PPP1CA - ACACA</td>
<td>0.90</td>
</tr>
<tr>
<td>PPP1CA - SREBF1</td>
<td>0.78</td>
</tr>
<tr>
<td>PPP1CA - FBPI</td>
<td>0.70</td>
</tr>
<tr>
<td>ACACA - FBPI</td>
<td>0.75</td>
</tr>
<tr>
<td>ACACA - SREBF1</td>
<td>0.71</td>
</tr>
</tbody>
</table>

Subsection Three

Caspase cleaved CK18 levels are elevated significantly in PCOS group

Since cell death is implicated in both PCOS and NAFLD, we also compared the serum biomarkers of cell death in both PCOS and non-PCOS NAFLD patients. The
serum total cytokeratin 18 (M65) levels reflect the amount of total epithelial cell death, regardless of the cause of death; the caspase cleaved cytokeratin 18 (M30) is a biomarker of apoptosis. The serum M65 levels in the PCOS NAFLD and the non-PCOS-NAFLD groups were similar (Table 4), while the levels of M30 were higher in PCOS–NAFLD group as compared to that in the nonPCOS-NAFLD group (224.93 U/L +/- 111.87 U/L vs. 119.07 U/L +/- 7.94 U/L, P < 0.002) (Table 5 and Figure 4).

**Figure 4.** Caspase-cleaved CK18 (M30) levels in PCOS and nonPCOS groups
Table 5. Adipokine and Gene Expression levels in PCOS-NAFLD group and non-PCOS NAFLD

<table>
<thead>
<tr>
<th></th>
<th>Non-PCOS (n=12)</th>
<th>PCOS (n=12)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>M65, U/L</td>
<td>284.18 +/- 73.81</td>
<td>299.92 +/- 98.87</td>
<td>NS</td>
</tr>
<tr>
<td>M30, U/L</td>
<td>119.07 +/- 7.94</td>
<td>224.93 +/- 111.87</td>
<td>0.02</td>
</tr>
<tr>
<td>Adiponectin, µg/ml</td>
<td>6.88 +/- 4.50</td>
<td>6.98 +/- 2.34</td>
<td>NS</td>
</tr>
<tr>
<td>Resistin, ng/ml</td>
<td>14.25 +/- 5.50</td>
<td>11.17 +/- 4.17</td>
<td>NS</td>
</tr>
<tr>
<td>Insulin (MicroIU/ml)</td>
<td>16.9 +/- 11.9</td>
<td>21.7 +/- 23.7</td>
<td>NS</td>
</tr>
<tr>
<td>ACACA, Art. units</td>
<td>0.32 +/- 0.22</td>
<td>0.30 +/- 0.17</td>
<td>0.77</td>
</tr>
<tr>
<td>SREBF1, Art. units</td>
<td>0.56 +/- 0.43</td>
<td>0.67 +/- 0.35</td>
<td>0.39</td>
</tr>
<tr>
<td>NIN, Art. units</td>
<td>0.74 +/- 0.37</td>
<td>1.25 +/- 0.64</td>
<td>0.009</td>
</tr>
<tr>
<td>SORBS1, Art. units</td>
<td>0.00173 +/- 0.00101</td>
<td>0.00216 +/- 0.00124</td>
<td>0.44</td>
</tr>
<tr>
<td>FBP1, Art. units</td>
<td>0.77 +/- 0.46</td>
<td>0.55 +/- 0.35</td>
<td>0.21</td>
</tr>
<tr>
<td>PPP1CA, Art. units</td>
<td>5.61 +/- 3.05</td>
<td>5.37 +/- 1.88</td>
<td>0.71</td>
</tr>
<tr>
<td>RAF1, Art. units</td>
<td>1.58 +/- 0.84</td>
<td>1.55 +/- 0.73</td>
<td>0.94</td>
</tr>
<tr>
<td>ACOXI, Art. units</td>
<td>3.82 +/- 3.91</td>
<td>3.66 +/- 3.27</td>
<td>0.86</td>
</tr>
<tr>
<td>PPARG, Art. units</td>
<td>8.96 +/- 8.29</td>
<td>10.56 +/- 10.49</td>
<td>0.80</td>
</tr>
<tr>
<td>LDLR, Art. units</td>
<td>0.45 +/- 0.38</td>
<td>0.21 +/- 0.16</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Subsection Four

Resistin levels correlate with expression of LDLR, prothrombin time and measures of insulin resistance

The serum levels of the Resistin, the Adiponectin, and the Insulin were not significantly different between PCOS and non-PCOS cohorts (Table 4). The levels of the Resistin were positively correlated with expression levels of LDLR, prothrombin time, serum levels of insulin and HOMA scores. Additionally, levels of LDLR mRNA were also correlated with prothrombin time (Table 3). Prothrombin time (PT) is the blood test that
measures how long it takes blood to clot. An abnormal PT can be caused by liver disease or injury or by treatment with blood thinners.

**Subsection Five**

*Levels of mRNAs encoding proteins involved in glucose metabolism and lipid metabolism co-correlate with each other*

*PPARG* and *ACOX1* are regulators of adipocyte differentiation that have been implicated in both obesity and type 2 diabetes. In our study, expression levels of *PPARG* showed significant positive correlation with levels of mRNAs encoded by genes involved in fatty acid metabolism (*ACACA, r = 0.742, p < 0.014*), cholesterol metabolism (*SREBF1, r = 0.830, p < 0.006*), glucose metabolism (*FBP1, r = 0.883, p < 0.003*) and glycogen metabolism (*PPP1CA, r = 0.700, p < 0.043* and *ACOX1, r < 0.903, p = 0.001*).

Expression levels for mRNAs of *ACOX1, SREBF1, FBP1, ACACA* and *PPP1CA* genes were co-correlated (Table 3). This co-correlation was observed in both PCOS and non-PCOS groups and was confirmed by factor analysis, where p-values for co-correlation to common factor varied from < 0.3 to < 1.56e⁻⁵. None of the other measured parameters, including Glucose, Insulin, Adipokine levels and HOMA scores, were co-correlated to the expression levels for this group of genes.
Section Three
Discussion

Recent studies have revealed that both PCOS and NAFLD are highly associated with insulin resistance, metabolic syndrome and obesity.\textsuperscript{131} This is especially true for women with visceral obesity.\textsuperscript{43,44} As women with PCOS may be at increased risk for developing NAFLD and conversely, women with NAFLD may be at risk for PCOS, the screenings for co-morbidities were proposed in respective cohorts.\textsuperscript{131}

In our previous study we showed that despite similar clinical and laboratory profiles to the obese controls enrolled at a time of bariatric surgery, PCOS patients seem to be more prone to have histologic NASH.\textsuperscript{132} In this study we specifically selected the population of patients with both PCOS and NAFLD that have not yet progressed to NASH according to their liver biopsies (N=12) and BMI-matched this population with non-PCOS non-NASH NAFLD controls (N=12). The levels of Adiponectin, Resistin, Insulin and HOMA scores as well as number of other clinical and biochemical parameters were not significantly different between these two groups of patients.

Surprisingly, the total serum levels of Caspase-cleaved CK18 (M30) were significantly elevated in PCOS patients without NASH, pointing that apoptosis is more prominent in steatotic livers of patients with PCOS as compared to the livers of patients with NAFLD but no PCOS. This observation confirms findings of Tan et al who compared serum apoptosis biomarkers in 186 PCOS patients and 73 age-matched controls, and found that M30 levels are higher in PCOS patients than in normal controls even after correction for BMI and suggested that NASH should be prevalent in PCOS cohorts.\textsuperscript{43} However, our study takes this investigation one step further, as its design
specifically excluded patients with histologically confirmed NASH, thus indicating that an increase in apoptosis is early feature of NAFLD observed in PCOS subjects, or that an overall increase in apoptosis is a general feature of PCOS. One of the most prominent features of PCOS is a hyperproduction of Androgens, like Testosterone, dihydrotestosterone (DHT) and Dehydroepiandrosterone (DHEA), known proapoptotic agents that were shown to act upon many types of peripheral cells, including hepatocytes.\textsuperscript{133} For example, DHT administration results in apoptosis of androgen-sensitive liver cells, that is, at least in part, realized through PKR/eIF2\(\alpha\)/GADD153 cascades.\textsuperscript{135} It is possible that the patients with concomitant NASH and liver steatosis are more prone to the development of NASH as androgen instigated apoptotic processes in their livers actively contribute to the progression of NAFLD. To further investigate this hypothesis, the study of larger cohorts of PCOS patients is needed, including subgroups without any histological sign of the liver disease and these without an excess of androgens.

Our present study also provides interesting insights into the PCOS-associated changes in the expression levels of genes involved in specific aspects of the lipid metabolism and the carbohydrate metabolism in adipose. Importantly, in adipose tissue specimen collected from PCOS patients, the decreased mRNA levels for LDL receptor (\textit{LDLR}) were observed. An expression of \textit{LDLR} gene is a subject of estrogen control that is maintained through the estrogen-responsive region adjacent to the sterol response element within \textit{LDLR} promoter. In addition to that, in hepatocytes, the androgen receptor agonists were shown to attenuate the estrogen-induced up-regulation of \textit{LDLR}.\textsuperscript{134} It seem
that hyperandrogenic phenotype of PCOS may have direct suppressive influence on 
LDLR levels both in adipocytes and in the liver. As the LDL receptor plays a major role 
in the clearance of apoB and apoE-containing lipoproteins, its downregulation should 
prolong the plasma half-life of VLDL and LDL and, therefore, have steatogenic effects.

In accordance with this logic, Ldlr−/− mice have increased sensitivity for oxLDL-
induced inflammation, apoptosis and fibrosis. On diabetogenic, cholesterol-augmented 
diet, Ldlr−/− mice develop a distinct hepatic phenotype characterized by increased 
inflammation and oxidative stress. Thus, an observed decrease in LDLR mRNA in 
PCOS might be connected to the proneness of PCOS patients with concomitant liver 
disease to the progression to NASH.

The only other mRNA that was differentially expressed in PCOS adipose was one 
encoding for centrosomal protein Ninein (NIN). NIN was selected for this study as it 
interacts with kinase GSK3beta that is hyperactivated and resistant to down-regulation by 
insulin in adipocytes of women with PCOS. As of now, the connection of the Ninein 
to PCOS phenotype is unclear. One possible clue that can make this connection is that the 
Ninein is critical for formation of the vascular tubes. An observed increase in 
expression of Ninein aligns well with hypervascularity of the ovarian theca interna and 
stroma commonly observed in patients with PCOS.

When PCOS and non-PCOS cohorts were compared, no difference in the levels of 
Resistin, Adiponectin and Insulin were observed, thus, confirming the findings of 
others, and pointing that the levels of these hormones are defined by underlying 
metabolic state and patient’s BMI, rather than by PCOS. On the other hand, when entire
cohort of NAFLD patients was analyzed together, the levels of *LDLR* expression in adipose were positively correlated with serum levels of the Resistin. This is an important observation as Resistin was recently shown to downregulate an *LDLR* at the protein level *in vitro*, both in HepG2 and in primary hepatocytes, through an increase in cellular expression of the recently identified protease, PCSK9, which enhances intracellular LDLR lysosomal degradation.\(^{141}\) Thus, our study indicate that the same phenomenon may take place *in vivo*, and highlights Resistin as possible therapeutic target to be manipulated in patients with elevated serum LDL levels.

Additionally, both the *LDLR* mRNA expression and the Resistin levels were correlated with the measure of the extrinsic pathway of coagulation, a prothrombin time (PT). An increase in PT is common in insulin resistance and in PCOS that are both regarded as low-grade systemic coagulation conditions.\(^{142,143}\) Recently, Resistin was shown to induce a procoagulant state in HUVECs by inducing both an expression of tissue factor (TF) and an activity of factor Xa.\(^{144}\) Correlative observations made in metabolic syndrome cohort showed that Resistin is strongly associated with hypercoagulative and hypofibrinolitic activities.\(^{145}\) It seems that inflammation-associated increase in the release of the Resistin into circulation might contribute to the prothrombotic state observed under diabetic conditions.

The limitation of our study, as with most molecular studies, is small sample size, which may contribute to the lack of statistically significant differences of group comparison analysis of changes in gene expressions as well as limit our analysis of
correlations. However, in our design we attempted the mitigation of these limitations by matching the PCOS and non-PCOS cohorts by the histology of their liver.

**Section Four**

Conclusions

In conclusion, here we report that the total serum levels of apoptotic biomarker M30 are significantly elevated in PCOS patients with liver steatosis as compared to non-PCOS NAFLD controls, pointing that androgen-dependent proapoptotic PCOS environment may directly contribute to NAFLD progression in these patients. Similarly, hyperandrogenism may explain an observed decrease in adipose LDLR mRNA expression that may be connected to the proneness of PCOS patients with concomitant liver disease to the progression to NASH. Additionally, our study points that inflammation-associated increase in the release of the Resistin into circulation might contribute to the prothrombotic state observed under conditions associated with insulin resistance, including PCOS.
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CURRICULUM VITAE

Thuy Phuong T. Tran received her Bachelor of Science from George Mason University in 2009. She has started graduate study at GMU since 2011 and has been working on her thesis at the Center for the Study of Chronic Metabolic Diseases at GMU-Inova Translational Research Facility at Inova Fairfax Hospital. She has just received the Outstanding Graduate Student Scholar Award for the 2012-2013 academic year. She also was a summer intern at United State Patent and Trade Mark Office (PTO) in 2011.