

**THE GENETICS OF INSULIN RESISTANCE: ANALYSIS OF THE PEROXISOME  
PROLIFERATOR-ACTIVATED RECEPTOR PATHWAY**

by

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Polycystic ovary syndrome (PCOS) is a heterogeneous familial disorder characterized by insulin resistance and an increased risk of type 2 diabetes mellitus (T2DM), a disorder with epidemic public health significance. The aim of this dissertation was to determine the risk of T2DM among Caucasian and African American women with PCOS compared to controls and to assess potential genetic variants that may affect development of T2DM. T2DM was defined as a fasting plasma glucose level  $\geq 126$  mg/dL or self-report of physician diagnosis. Genetic variants analyzed for association with PCOS and subclinical coronary heart disease (CHD) risk measures were the peroxisome proliferator-activated receptor-gamma (PPAR- $\gamma$ ) single nucleotide polymorphism (SNP) P12A, insulin receptor substrate-1 (IRS-1) SNP G972R, one novel SNP of lipoprotein lipase (LPL), and three novel SNPs from acetyl-CoA carboxylase-beta (ACC- $\beta$ ). Significant association of genotype frequency with PCOS was determined by Pearson's  $\chi^2$  tests. Generalized linear modeling was utilized to test for association of genotype with subclinical measures of CHD, including insulin resistance (HOMA-IR) and C-reactive protein (CRP). The 8-year prevalence of T2DM was 13.4% in PCOS cases and 5.8% in controls. After adjusting for age and BMI, women with PCOS had an estimated 2-fold risk of developing T2DM compared to normal control women. When stratified by body mass index (BMI) and controlling for age, PCOS cases with BMI  $\geq 35$  kg/m<sup>2</sup> were estimated to have 5x higher risk of developing T2DM. There were no significant associations between genotype frequencies and PCOS for Caucasian or African American subjects. However, the G972R variant of IRS-1 and PCOS significantly interacted to affect CRP concentrations indicating that cases with the R allele had significantly elevated CRP compared to all other permutations of G972R and PCOS status interaction. The

final CRP model explained 22% of variability in CRP concentrations. In conclusion, the significant risk of T2DM attributed to women by PCOS was not explained by genetic SNPs analyzed here, however, a significant association of G972R and G972RxPCOS interaction with CRP concentrations was found, further supporting the growing body of evidence of associations between insulin resistance and systemic inflammation.

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## PREFACE

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## **1.0 INTRODUCTION**

Polycystic ovary syndrome (PCOS) is a heterogeneous disorder characterized by chronic anovulation, hyperandrogenism (HA), and insulin resistance (IR). The estimated prevalence is 5-10% among women of reproductive age (1). Given this high prevalence and the association of PCOS with an increased risk to develop coronary heart disease (CHD) and type 2 diabetes mellitus (T2DM), women with PCOS may represent a large, unique group of women at high-risk for the development of CHD. Thus, understanding the etiology of PCOS may have a large public health impact for women.

### **1.1 PHENOTYPIC ASPECTS OF PCOS**

Risk factors associated with PCOS (i.e., elevated cholesterol, elevated low density lipoprotein (LDL) decreased high density lipoprotein (HDL), and decreased insulin sensitivity) are associated with increased risk of developing T2DM (2, 3) and an adverse cardiovascular risk profile (4-6). Talbott and colleagues (7) evaluated the age-specific CHD risk profiles in women with PCOS and age- and neighborhood-matched controls. A total of 244 cases and 244 controls (mean age = 36 years) were compared across four specific age groups (19-24 years, 25-34 years, 35-44 years, and 45+ years). Compared to controls, PCOS women had substantially higher LDL and total cholesterol levels at each age group under 45+ years after adjustment for body mass index (BMI), hormone use, and insulin levels. After age 45, little difference was noted between cases and controls. Furthermore, a recent study examining carotid intima-media thickness (IMT) in PCOS demonstrated increased IMT in PCOS women compared to their age-matched controls (0.75 vs. 0.70, n = 105) (8). These studies suggest that PCOS women exhibit significantly

adverse lipid and CHD risk factor profiles even at the younger ages, implying that there may be an underlying genetic disorder.

In determining CHD risk factors that may be under, at least, partial genetic control, hyperinsulinemic IR (HI/IR) associated with PCOS may defer increased risk of CHD to women with PCOS (4). (See [Appendix C](#) for an in-depth discussion of insulin action in PCOS.) Within the cluster of defining characteristics of PCOS (e.g., elevated androgen levels and chronic anovulation), HI/IR appears to be a central mediating factor (9). One major hypothesis of how HI/IR may be related to elevated rates of CHD is based on the insulin-glucose-androgen pathway. Elevated insulin levels promote increased ovarian theca cell androgen secretion. The resulting HA may then directly or indirectly suppress ovulation at the level of the ovary (10). Higher levels of insulin may also promote obesity by interfering with signaling pathways in fatty acid (FA) metabolism with the end result being an overweight phenotype.

Research during the past several years has led to an explosion in the understanding of adipose tissue and the active role it plays in insulin sensitivity (See [Appendix B](#) for a discussion of Cellular Aspects of Insulin Resistance). Thiazolidinedione compounds (glitazones) have insulin sensitizing effects among individuals with T2DM. Troglitazone has been shown to ameliorate IR in skeletal muscle cells when present in co-culture with adipocytes (11) and to improve ovulation, hirsutism, HA, and IR in women with PCOS (12). Studies have identified that one target molecule for the glitazones is the nuclear hormone receptor peroxisome proliferator-activated receptor gamma (PPAR- $\gamma$ ); a transcription factor activated by various FA and FA metabolites (PPAR- $\gamma$  agonists). The role of PPAR- $\gamma$  as a critical modulator of fat cell differentiation and function provides a direct link between FA concentrations and the regulation of gene transcription in adipocytes (13). Glitazones have two main effects on IR: (1) decreasing serum free fatty acid (FFA) and triglyceride levels and (2) increasing adipogenesis. Studies have shown that treatment of IR rodents with potent PPAR- $\gamma$  agonists increase the number of small (insulin sensitive) adipocytes while decreasing the number of large (insulin insensitive) adipocytes in white adipose tissue depots (13), thereby increasing insulin sensitivity. Also, smaller white adipocytes utilize more glucose and secrete fewer FAs and less transcription nuclear factor alpha (TNF $\alpha$ ), a proinflammatory cytokine, than large white adipocytes.

## 1.2 GENOTYPIC ASPECTS OF PCOS

Clinical observations and family studies have indicated a genetic predisposition toward the development of PCOS and several candidate PCOS susceptibility genes have been explored with limited success (14-16). Efforts to establish a definitive mode of inheritance have been challenging for several reasons as recently noted by Legro (17). Firstly, PCOS is associated with infertility, which makes it difficult to find a large family in which PCOS is highly prevalent. Secondly, assigning phenotypes to certain family members (e.g., premenarchal girls, postmenopausal women, and men) is not straightforward and reliance of self-reported information may be inaccurate. Thirdly, varying conclusions reached by different studies may merely reflect differences in ascertainment and disease heterogeneity. Recent evidence suggests that PCOS may be autosomal dominant and studies using both genetic association and linkage mapping techniques have continued to examine the genetic background of potential phenotypic pathways often displayed in PCOS (i.e., the metabolic/gonadotropic/reproductive axis dysfunction). A review of pertinent genetic studies of PCOS using both linkage and association techniques are reviewed in [Appendix A](#).

## 1.3 SELECTED CANDIDATE GENES

With the exception of the IRS-1 gene, this study will focus on genes in the PPAR- $\gamma$  pathway because this pathway has biological relevance to FA metabolism and HI/IR (18). Research on the PCOS phenotype has indicated that IR may be related to abnormal signal transduction downstream of PPAR- $\gamma$  (13).

Four candidate genes have been selected to be examined for association with PCOS and PCOS-related phenotypes. These candidate genes are: PPAR- $\gamma$ , lipoprotein lipase (LPL), acetyl-CoA carboxylase beta (ACC- $\beta$ ), and IRS-1.

### 1.3.1 PPAR-Gamma

The PPAR- $\gamma$  is a receptor that binds peroxisome proliferators, such as the glitazones and FAs. The P12A (or Pro12Ala) polymorphism of this gene is associated with insulin sensitivity in various populations. The Ala-allele of the PPAR- $\gamma$  polymorphism is associated with improved whole body insulin sensitivity among both Swedish Caucasians (19) and middle-aged and elderly Finns (20). Witchel et al. (21) found that in children and adolescent girls, the P12A polymorphism might be a genetic marker indicating increased risk for obesity persisting into adolescence.

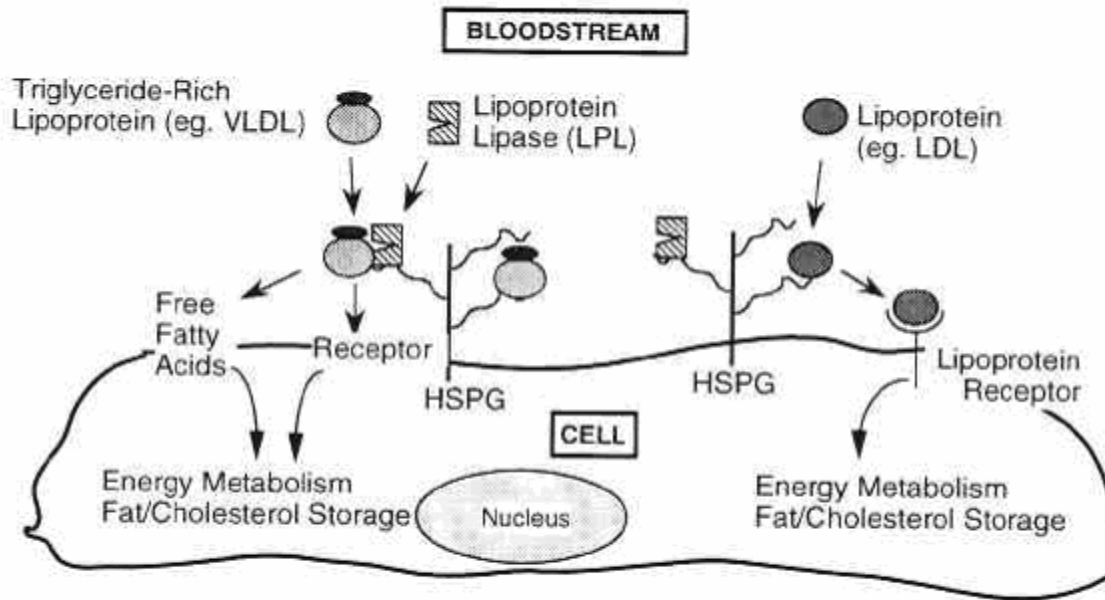
In contrast, Beamer et al. (22) showed that subjects with at least one Ala allele had a significantly higher mean BMI than subjects homozygous for the Pro allele. Several studies have been published on the P12A polymorphism with conflicting results, some finding significant results (23, 24) and some finding no significant differences (40) between carriers of the P12A polymorphism and non-carriers, warranting its inclusion in this study.

Recently, two cross-sectional studies attempted to elucidate the relationship between the P12A polymorphism and insulin sensitivity in women with PCOS. Korhonen et al. (23) genotyped 135 PCOS-affected women and 115 healthy control subjects for the P12A polymorphism and found a significantly different allele distribution between cases and controls. PCOS cases had a significantly lower frequency of the Ala isoform. They concluded PPAR- $\gamma$  may play a role in the pathogenesis of PCOS and the Ala isoform is most likely protective against the development of PCOS. This study supports the inclusion of PPAR- $\gamma$  in the current research, but focused its population on Finnish women and results may have been due to geographic isolation. The current study will include, not only women of other ethnicities, but their family members so linkage can be studied for determining mode of inheritance. Another study of P12A in women with PCOS recruited 218 PCOS-affected women of varying races (Caucasian, African American, Hispanic, South Asian, and Middle Eastern) to examine how the Ala isoform influenced insulin resistance in women compared to women with the Pro allele (24). Twenty-eight (12.8%) of these women had the Ala allele, all in the heterozygous state. Nondiabetic Caucasians with an Ala allele (Pro/Ala) were more insulin sensitive than those in the Pro/Pro group. The authors concluded the Ala isoform in P12A modified insulin resistance in Caucasian women. One limitation of this study was the inability of the authors to use data

from all ethnicities originally included. Due to the Ala allele being present in only African Americans, Caucasians, and Hispanics, the other ethnicities were excluded from Ala frequency analysis. Due to too few subjects with the Ala allele in the African American and Hispanic groups, only Caucasian women were included in the Ala comparison analysis.

### **1.3.2 Lipoprotein lipase**

LPL is a serine esterase expressed in adipocytes and striated muscle. PPAR- $\gamma$  selectively induces the expression of LPL in adipose tissue without changing its expression in muscle tissue. LPL is located on the luminal surface of capillary endothelial cells and is involved in lipid transport (Figure 1-1). Many cell types synthesize LPL, including macrophages, skeletal muscle cells and cardiac muscle cells with, its highest expression level found in adipose tissue. All these tissues have a high demand for fatty acids (25). LPL's main function is the hydrolysis of triglycerides in triglycerides-rich lipoproteins, such as chylomicrons and very low density lipoproteins (26). The released free fatty acids (FFAs) are oxidized to generate ATP in muscle, re-esterified and stored in adipose tissue or are secreted in milk by the mammary gland. Hence, LPL is pivotal in lipoprotein and energy metabolism



**Figure 1-1. Lipoprotein lipase in the blood**

(Reprinted from the internet. No copyright.)

Glucose and long chain fatty acids (LCFAs) are competitive substrates in insulin-dependent tissues (27) and FFAs greatly interfere with glucose utilization. Boden et al. (28) demonstrated a negative dose-dependent relationship between plasma FFA concentrations and glucose uptake. The reciprocal relationship between plasma FFA and insulin stimulated glucose uptake may be particularly important in obese patients, with therapy directed toward lowering high plasma FFA concentration having a beneficial effect on glucose tolerance. Roden et al. (29) suggested that an increased FFA concentration causes insulin resistance by both inhibition of glucose transport or phosphorylation and through subsequent reduction in rates of glucose oxidation and muscle glycogen synthesis.

LPL activity has been shown to increase as women enter menopause, predisposing postmenopausal women to gain body fat (30). Kim et al. (31) found in their study with transgenic mice that induced tissue-specific overexpression of LPL caused tissue-specific IR. In skeletal muscle, IR was associated with decreases in insulin-stimulated glucose uptake, while, in liver, IR was associated with the impaired ability of insulin to suppress endogenous glucose

production associated with defects in insulin activation of insulin receptor substrate-2-associated phosphatidylinositol 3-kinase activity. The role of LPL has not been examined in women with PCOS even though it has a pivotal role in fat/energy metabolism like that similar to the PCOS phenotype.

### **1.3.3 Acetyl-CoA carboxylase-Beta**

Acetyl-Coenzyme A carboxylase (ACC) is a complex multifunctional enzyme system that has not yet been studied in women with PCOS. ACC is a biotin-containing enzyme whose activation increases malonyl-CoA activity, the rate-limiting step in fatty acid synthesis, and increases circulating FA levels. The beta form (ACC- $\beta$ ) may be involved in the provision of malonyl-CoA or in the regulation of FA uptake and oxidation by mitochondria. ACC- $\beta$  is relevant to this study because it has been identified as perhaps being critical for its role in FA oxidation (30). Since insulin sensitivity depends upon skeletal muscle reactivity in women with PCOS, ACC- $\beta$  may be dysfunctional in women with PCOS and their family members.

When FFAs are released by increased LPL activity, FA accumulates intracellularly and promotes beta-oxidation. Enhanced beta-oxidation leads to an accumulation of acetyl-CoA and, thus, acetyl-CoA carboxylase, which then inhibits pyruvate dehydrogenase activity resulting in decreased glucose oxidation via the Krebs cycle (32). This “lipid signaling model of insulin secretion” (33) is proposed to end in decreased insulin sensitivity, like that seen in women with PCOS.

### **1.3.4 Insulin Receptor Substrate-1**

The IRS-1 protein functions immediately downstream of the insulin receptor. A common polymorphism, G972R (or Gly972Arg), is a mild loss-of-function mutation that has been associated with decreased insulin sensitivity (34) and T2DM (35). Recently, this polymorphism has been associated with phenotypic features of PCOS as well (34), (36). Ibanez et al. (37) found that among girls with premature pubarche due to premature adrenarche, the frequency of this variant was increased. Since premature pubarche precedes the development of PCOS in

some girls, the increased frequency of the IRS-1 variant suggests that it may play a role in PCOS. Conversely, a study by Ehrmann et al. (38) of the IRS-1 polymorphism G972R found no association of this polymorphism with any clinical or hormonal measure in 227 nondiabetic Caucasian and African American PCOS cases. However, since the G972R allelic frequencies in this population were 0.95(Gly) and 0.05(Arg), the ability to analyze differences in clinical or hormonal parameters between groups might have been severely impaired by low allele number in the Arg group.

The IRS-1 gene has also been selected due to its potential interaction with PPAR- $\gamma$ . Stumvoll et al. (39) studied the gene-gene interaction between the P12A variant of PPAR- $\gamma$  and the G972R variant of IRS-1. Significant increases in insulin sensitivity were found between the X/Ala and Pro/Pro carriers within the Arg972 background that was not present in the whole population or within carriers of the Gly972 background. They concluded that both genotypes were modifiers of insulin sensitivity and suggested that the Ala allele of PPAR- $\gamma$  becomes particularly advantageous within the background of the possibly disadvantageous G972R polymorphism and the P12A effect becomes more detectable. Since we postulate a polygenic mode of inheritance, gene-gene interaction of IRS-1 and PPAR- $\gamma$  is relevant to the study of insulin resistance in PCOS-affected women and their families.

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## **2.0 RISK OF TYPE 2 DIABETES MELLITUS AND IMPAIRED GLUCOSE FUNCTIONING AMONG PCOS CASE AND CONTROLS SUBJECTS: RESULTS OF AN EIGHT-YEAR FOLLOW-UP**

### **2.1 ABSTRACT**

Background: Polycystic ovary syndrome (PCOS) is a heterogeneous familial disorder characterized by chronic anovulation, hyperandrogenism/hyperandrogenemia, and insulin resistance. PCOS is associated with an increased risk of developing type 2 diabetes mellitus (T2DM) and may be associated with an increased risk of coronary heart disease.

Study Design: Longitudinal cohort analysis

Specific Aims: The aim of this analysis was to determine the risk of T2DM among women with PCOS compared to controls over an 8-year period.

Methods: Ninety-seven women with PCOS and 95 controls were followed prospectively and assessed for risk of T2DM using Kaplan-Meier survival analysis and Cox proportional hazards regression modeling. Baseline measures of insulin sensitivity, blood lipids, and obesity were assessed as covariates of T2DM development.

Results: At baseline, PCOS cases were significantly heavier than controls (body mass index,  $p < 0.0001$ ) and had a higher cardiovascular disease (CVD) risk profile with significantly higher triglycerides ( $p = 0.0002$ ), lower HDL levels ( $p = 0.003$ ), and higher LDL levels ( $p = 0.07$ ). Insulin sensitivity, measured by glucose:insulin ratio ( $p = 0.003$ ) and the homeostasis model of assessment (HOMA) ( $p = 0.002$ ), was significantly lower in PCOS cases than controls. In survival analysis, the 8-year event-free rate of T2DM was 86.6% in PCOS cases compared to 94.2% in controls ( $p = 0.05$ ). After adjusting for age and BMI, women with PCOS had an estimated 2-fold risk of developing T2DM compared to normal control women (adj. HR=2.00,

p=0.22). When stratified by BMI, PCOS cases with BMI  $\geq 35$  kg/m<sup>2</sup> were estimated to be at 5.1 times higher risk of developing T2DM (95% CI: 1.67-15.78, P=0.004), whereas PCOS women with BMI < 35 were at similar risk (adj. HR=1.45, P=0.56) compared to control subjects.

Conclusions: Women with PCOS have significantly greater risk of developing T2DM compared to age-adjusted control women. The risk of T2DM is 5 times greater in obese women with PCOS. Thus, our results emphasize the importance of lifestyle interventions with weight loss to lower the risk of T2DM among women with PCOS.

## 2.2 INTRODUCTION

Polycystic ovary syndrome (PCOS) is a heterogeneous familial disorder characterized by chronic anovulation, hyperandrogenism (HA), and insulin resistance (IR). Numerous clinical studies have demonstrated that hyperinsulinemia and insulin resistance are central to the pathophysiology of PCOS. With weight loss or use of medications that decrease insulin resistance, insulin and androgens concentrations decrease (1). The estimated prevalence of PCOS is approximately 5-10% among women of reproductive age in the United States (2). Women with PCOS have an increased risk to develop T2DM (3, 4). In four studies which utilized oral glucose tolerance tests (OGTT) to assess for impaired glucose tolerance, the prevalence or incidence of T2DM was increased in women with PCOS (5-8) (See Table 2-1).

To date, there have been only four studies assessing incidence or prevalence of T2DM among PCOS cases. Legro et al (6) cross-sectionally studied 254 women with PCOS from two populations (mean ages 27 and 28 years) and 80 control women (mean age 30 years). All subjects underwent a standard oral glucose tolerance test (OGTT). Overall, the authors found that 38.6% (n = 98) of pcos women had either impaired glucose tolerance (IGT) (31.1%) or T2DM (7.5%) compared to 14.0% of controls with IGT. No controls presented with T2DM. In a second cross-sectional study, Weerakiet et al. (8), the prevalence of IGT and T2DM was investigated among 79 Asian women with PCOS (mean age: 28 years) who were administered an OGTT. Overall prevalence of IGT was 20.3% and of T2DM was 17.7%.

The remaining two investigations were longitudinal studies of prevalence and incidence of T2DM among PCOS-affected women. Ehrmann et al. (5) investigated IR at baseline among 122 women with clinical and hormonal evidence of PCOS by standard OGTT. At that time, 12 presented with non-insulin dependent diabetes mellitus (NIDDM) (9.8%). Follow-up recruitment of this original cohort was conducted among women without NIDDM at baseline (n = 110) and 25 (23%) women from the original cohort were reassessed (mean follow-up: 2.4 years). Of the 25 women seen at follow-up, those who presented with normal glucose concentrations at baseline were distributed as follows at their second visit: 5 remained (45%) normoglycemic, 5 (45%) developed IGT, and 1 (9%) developed NIDDM. Of the remaining 14 with IGT at baseline, 3 reverted to a normoglycemic state (21%), 7 developed IGT (50%), and 4

developed NIDDM (29%). Incidence rates among baseline normoglycemic subjects was 6 cases in 17.75 person years and among baseline IGT subjects was 4 in 10.75 person-years.

Norman et al (7) prospectively followed 67 PCOS cases (mean baseline age: 32.5 years) with normal glucose concentrations (n = 54) or IGT (n = 13) as determined by a 75-g glucose tolerance test (mean follow-up: 6.2 years). Among women normoglycemic at baseline, 5 (9%) developed IGT and 4 (8%) progressed to NIDDM. Among women with IGT at baseline, 7 (54%) developed NIDDM. Of the two incidence studies, this analysis was most similar to that conducted by Norman et al. in its longitudinal aspect.

Risk factors associated with an adverse cardiovascular risk profile, i.e., elevated cholesterol, elevated LDL, decreased HDL, and decreased insulin sensitivity, are often observed in women with PCOS (9-11). Specifically, Talbott and colleagues (12) compared the age-specific coronary heart disease risk profiles in women with PCOS to those in age- and neighborhood-matched controls. After adjustment for BMI, hormone use, and insulin levels, women with PCOS had substantially higher LDL and total cholesterol levels at each age group less than 45+ years compared to controls. After age 45, significant differences disappeared for LDL and total cholesterol levels, but remained significant for other measures assessed including BMI, triglycerides, and blood pressure. Furthermore, a recent study examining carotid intima-media thickness (IMT), a phenotypic marker for atherosclerosis, in women over age 45 with PCOS demonstrated increased IMT in women with PCOS compared to similarly-aged controls (0.78 vs. 0.70;  $p=0.005$ ) (13). These studies suggest a latency effect of PCOS on adverse lipid and coronary heart disease risk factor profiles at relatively young ages.

The previous two studies of incidence of T2DM have some limitations. Neither study had controls by which to compare increased rates of development of T2DM among PCOS-affected women. Furthermore, the women in the previous studies are relatively young and may not yet have been exhibiting the full impact of adverse metabolic functioning of the PCOS/BMI interaction. There was also a limitation of small proportions of subjects with follow-up assessment in both studies.

To address these limitations, the present analysis of 97 PCOS cases and 95 age- and neighborhood-matched controls followed prospectively examined the time to development of T2DM over an 8 year time span. The natural history of PCOS is, therefore, important in determining the true risk of developing T2DM among PCOS-affected women. The aim of this

analysis was to confirm that the risk to develop T2DM was increased in women with PCOS and to identify additional predictive factors.

## 2.3 METHODS

### 2.3.1 Subjects

The present analysis was conducted using women recruited for the Cardiovascular Health and Risk Measurement Study (CHARM) (NIH NHLBI 446640-10). The CHARM study was established in 1992 to investigate cardiovascular risk in women. Due to previous doctor diagnosis of PCOS, the women recruited for CHARM were considered at high-risk for developing CVD. Women diagnosed with PCOS between 1970 and 1993 (median age: 35.5 at the time of recruitment) were identified from the records of an academic reproductive endocrine practice located at Magee-Womens Hospital, Pittsburgh, PA. The presumptive clinical diagnosis of PCOS was made if there was a history of chronic anovulation in association with either (A) clinical evidence of androgen excess (hirsutism) or biochemical evidence of an elevated total testosterone concentration ( $>57.64$  ng/dl (2nmol/l)). Eligible women (N = 496) were contacted by phone between 1992 and 1994 for a telephone interview (n = 184) and for further recruitment for a clinical visit (n = 312). During that time, age ( $\pm 5$  years)- and race-matched neighborhood control subjects were selected and recruited using a combination of voter's registration tapes for 1992 from the Greater Pittsburgh area and Cole's Cross Reference Directory of Households and were similarly recruited. After initial phone contact, 244 PCOS-affected women and 244 controls completed a clinic visit where they consented to a fasting blood draw, waist and hip measurements, standard blood pressure assessment and a questionnaire-based interview.

In 2001-02, 104 cases and 96 controls were re-evaluated and baseline measures were repeated. Due to differential follow-up and eligibility glucose requirements for this analysis, matching was broken between cases and controls. The follow-up visit included medical history and diagnosis of T2DM made by a physician and year of diagnosis (fasting glucose  $\geq 7.0$  mmol/l

or  $\geq 126$  mg/dL). Women who failed to report a year of diagnosis (N=5) were assigned one occurring at midpoint between last year seen and year of follow-up through linear interpolation. Of the 200 women seen at follow-up, 8 (7 cases, 1 control) were excluded prior to analysis due to baseline-assessed physician diagnosed IDDM (n=1) or T2DM (n=3) or on the basis of baseline glucose  $\geq 126$  mg/dl (n=2),  $<30$  mg/dl (n=1) or missing glucose value at baseline (n=1). Patients presenting with IFG (fasting glucose between 90 and  $<110$  mg/dL) at baseline were included in follow-up. The present analysis is comprised of the remaining 192 women with prospective follow-up data (97 cases and 95 controls). Included in this analysis are 174 Caucasian (81 cases, 93 controls) and 18 African American (16 cases, 2 controls) women. Written, informed consent, as approved by the University of Pittsburgh Institutional Review Board, was obtained from all participants in this analysis.

### **2.3.2 Laboratory Analysis**

All blood lipid assessments and fasting glucose were measured at the Heinz Nutrition Laboratory under the direction of Dr. Rhobert Evans. The laboratory is carefully monitored and participates in the Centers for Disease Control standardization programs.

#### **2.3.2.1 Blood lipids**

High density (HDL) and low density (LDL) lipoproteins were determined after selective precipitation by heparin/manganese chloride and removal by centrifugation of very low density (VLDL) (14). Duplicate samples, standards and control sera were included in each run. The coefficient of variation between runs was 2.1%. Triglycerides were determined enzymatically using the procedure of Bucolo et al. (15). Duplicate samples, standards and control sera were included in each run. The coefficient of variation between runs was 1.7%.

#### **2.3.2.2 Insulin and glucose measurement**

Baseline serum insulin levels were measured using competitive RIA (Diagnostic Products Corp, Malvern, PA) (16)). There was no cross-reactivity with C-peptide or glucagon; however, there

was 40% cross-reactivity with proinsulin. The interassay coefficient of variation range was 4.9 - 10.0%. Glucose was quantitatively determined by an enzymatic determination read at 340/380 nm with a procedure utilizing the coupled enzyme reactions catalyzed by hexokinase and glucose-6-phosphate dehydrogenase (17). The coefficient of variation between runs was 1.8%.

Fasting glucose and insulin were used to assess the glucose:insulin ratio (GIR) and homeostasis assessment model (HOMA), measures of insulin resistance. Insulin resistance was defined as GIR < 4.5 mg/dl over  $\mu\text{U}/\text{mL}$  or an elevated HOMA score. In HOMA, values are calculated from the fasting concentrations of insulin and glucose using the following formula: (fasting serum insulin ( $\mu\text{U}/\text{mL}$ ) x fasting plasma glucose (mmol/L))/22.5 (18). HOMA ( $\mu\text{U}/\text{mL}$  x mmol/L) has been shown to be significantly correlated with clamp IR in a large number of subjects with both normal and impaired glucose tolerance (19, 20) and with the index of sensitivity obtained from the fasting intravenous glucose tolerance testing among normal and insulin resistant volunteers, as well as diabetics (21).

Normal glucose sensitivity was defined as a fasting glucose concentration < 110 mg/dl. Impaired fasting glucose (IFG) was defined as a fasting glucose concentration  $\geq 110$  and < 126. Development of T2DM from baseline (year 0) to follow-up (year 8) was defined as either physician diagnosis of T2DM between the initial and follow-up visits or a follow-up fasting glucose level  $\geq 126$  mg/dl.

### **2.3.3 Data analyses**

Baseline characteristics were compared between cases and controls by use of  $\chi^2$  tests for categorical variables and Wilcoxon one-way analysis of variance for continuous variables (due to skewed distributions). The cumulative incidence of T2DM was estimated by the Kaplan-Meier methods and compared between cases and controls by the log-rank statistic. Cox proportional hazards regression was used to estimate the adjusted hazard ratio of developing T2DM in relation to PCOS. Ties were handled using the Breslow statistic. Covariates of insulin sensitivity in women with PCOS (i.e., age and BMI) and variables that differed in prevalence between cases and controls upon univariate analyses (i.e., race, systolic blood pressure) were evaluated for confounding, with age and BMI ultimately included in adjusted models. Race was not significantly associated with insulin sensitivity and was excluded from further analysis.

Systolic blood pressure was not included as a covariate due to its strong correlation with BMI ( $r = 0.54$ ;  $p < 0.001$ ), and the limited number of variables efficiently controlled for given the sample size of 192 women. All analyses were performed using SAS, version 8 (SAS Institute, Inc., Cary, NC).

## 2.4 RESULTS

### 2.4.1 Baseline characteristics in Women with PCOS cases and Controls

At baseline, PCOS cases were significantly younger than controls ( $38.0 \pm 5.9$  vs.  $40.0 \pm 5.2$  years;  $p = 0.017$ ), as well as heavier (BMI  $31.6 \pm 9.6$  vs.  $26.2 \pm 6.0$  kg/m<sup>2</sup>,  $p < 0.0001$ ; WHR  $0.82$  vs.  $0.75$ ;  $p < 0.0001$ ) (Table 2-2). Race was distributed differently among cases and controls with 83.5% of cases and 97.9% of controls being Caucasian ( $p = 0.0006$ ). Mean systolic blood pressure was significantly greater among cases than in controls ( $115 \pm 16$  vs.  $110 \pm 13$  mmHg;  $p = 0.009$ ), while diastolic blood pressure was not significantly different between women with PCOS and control subjects.

Women with PCOS had significantly higher triglyceride concentrations ( $117 \pm 83$  vs.  $77 \pm 34$  mg/dl;  $p = 0.0002$ ), lower HDL concentrations ( $52 \pm 14$  vs.  $58 \pm 14$  mg/dl;  $p = 0.003$ ), and higher LDL concentrations ( $122 \pm 30$  vs.  $113 \pm 28$  mg/dl;  $p = 0.07$ ) suggestive of a higher CVD risk profile. The age at which menses ceased was significantly lower among cases than controls ( $38.7$  vs.  $44.9$  years;  $p = 0.02$ ); the reasons for which were similar between cases (8 by surgery, 6 natural menopause, 1 from drug therapy, and 2 via accidents) and controls (10 by surgery, 12 natural menopause, 1 due to drug therapy, and 2 missing) ( $p = 0.30$ ) (Data not shown). Baseline hormone use, defined as either oral contraceptive or hormone replacement therapy, (16.5% vs. 18.9%;  $p = 0.65$ ) and rates of smoking (17.5% vs. 16.8%;  $p = 0.97$ ) were similar between cases and controls.

Insulin sensitivity, as measured by fasting insulin (17.1 vs. 11.6  $\mu$ U/ml;  $p = 0.0003$ ), GIR (6.3 vs. 7.8 mg/dl over  $\mu$ U/ml;  $p = 0.003$ ), and HOMA-IR (3.6 vs. 2.4 mmol/l x  $\mu$ U/ml;  $p =$

0.002), was significantly lower in PCOS cases than controls. In contrast, fasting glucose levels were similar between PCOS women and control women ( $p = 0.69$ ), a result due, at least in part, to the requirement that all subjects in this analysis at year 0 (baseline measurement) had a glucose level below 126 mg/dl.

African American cases and controls were assessed separately for baseline characteristics and there were no statistically significant differences (Data not shown). However, it should be noted that larger mean values were found in African American subjects compared to the entire population with regard to BMI (cases: 38.9, controls: 41.5 kg/m<sup>2</sup>), SBP (cases: 126.6, controls: 111.0 mmHg), fasting insulin (cases: 20.0, controls: 16.5  $\mu$ U/ml), and HOMA-IR (cases: 4.2, controls: 3.5).

#### **2.4.2 HOMA-IR Levels Between PCOS Cases and Controls by BMI**

To investigate the relationship between HOMA-IR and PCOS status by body weight (Figure 2-1), subjects were categorized into three groups according to baseline BMI as follows: normal [BMI < 25 kg/m<sup>2</sup>], overweight and obese [ $25 \leq \text{BMI} < 35 \text{ kg/m}^2$ ], and morbidly obese [BMI  $\geq 35 \text{ kg/m}^2$ ]. Whereas there was essentially no difference in HOMA scores between PCOS cases and controls in normal and overweight/obese women, HOMA scores were markedly, albeit non-significantly, higher in morbidly obese PCOS women compared to morbidly obese controls.

#### **2.4.3 Incidence of T2DM Among Control and PCOS Case Subjects Over Time**

Among the 189 subjects with normal glucose levels (<110 mg/dl) at baseline (94 cases and 95 controls), 10 PCOS (10/94; 10.6%; 9 Caucasian, 1 AA) and 5 control women (5/95; 5.3%; 5 Caucasian) developed T2DM over an 8-year time span. Additionally, 6 cases (6/94; 6.2%; 6 Caucasian) and 2 controls (2/95; 2.1%; 2 Caucasian) developed IFG in that same time period (Data not shown). All participants with IFG at baseline (N=3 cases) subsequently developed T2DM (100%). In survival analysis, the overall 8-year event-free rate of T2DM was 86.6% in PCOS cases compared to 94.2% in controls ( $p = 0.05$ ) (Figure 2-2).

Using baseline glucose levels to stratify PCOS case and control subjects, those subjects with a baseline glucose level < 85 (N= 68 and 67, respectively) had similar rates of freedom from development of T2DM over 8 years of follow-up (92.6% and 95.2%, respectively;  $p = 0.48$ ) (Figure 2-3, top panel). Conversely, cases and controls with baseline glucose measurements  $\geq 85$  mg/dl (N=29 and 28, respectively) had diverging rates of freedom from T2DM over the same follow-up. Only 72.4% of women with PCOS were free from T2DM at follow-up compared to 91.6% of controls ( $p = 0.04$ ) (Figure 2-3, bottom panel) indicating not only increased risk of developing T2DM, but the risk of developing T2DM faster than their unaffected counterparts.

#### **2.4.4 Hazard ratios of Incident T2DM by PCOS status**

Adjusting for age and BMI, women with PCOS had an estimated 2-fold risk of developing T2DM compared to control women (adjusted HR=2.00, 95% confidence interval (95% CI): 0.67-5.99,  $P=0.22$ ) (Table 2-3). When stratified by baseline glucose (< 85 mg/dl;  $\geq 85$  mg/dl), the unadjusted HR was elevated in cases (HR = 4.29). After adjustment for age and BMI, the HR was attenuated (HR = 2.38;  $p = 0.31$ ) indicating similar risk. Furthermore, considerable overlap in the relatively wide confidence intervals was consistent with the interpretation of similar risk when comparing PCOS case and control subjects. In contrast, there was a strong indication that the effect of PCOS on developing T2DM was modified by BMI. Compared to control subjects, PCOS cases with BMI  $\geq 35$  were estimated to be at 5.1 times higher risk of developing T2DM (95% confidence interval: 1.67-15.78,  $P=0.004$ ), whereas PCOS women with BMI < 35 were at similar risk (adj. HR=1.45, 95% confidence interval: 0.41-5.08,  $P=0.56$ ). These data indicate that, in women with PCOS, the risk of developing T2DM is increased in the presence of morbid obesity.

## 2.5 DISCUSSION

It has been recognized that women with PCOS have an increased risk to develop diabetes. Incidence rates of T2DM in two prior studies were 9% (5) and 16% (7) among women with PCOS at baseline, regardless of basal glucose tolerance. Even though these studies had small cohort sizes, the risk of developing T2DM starting from either IGT or normal glucose tolerance were similar to the 13.4% rate of progression found in our population.

These analyses offer insight into the natural development of T2DM in women with PCOS due to both their older age at first visit (38.0 years for cases and 40.0 years for controls) and their length of follow-up time (8 years) (i.e., age at follow-up: cases =  $46.6 \pm 5.98$  years, controls =  $48.1 \pm 5.36$  years;  $p = 0.08$ ). BMI as a contributing factor to development of T2DM in PCOS-affected women, as found in this analysis, was supported in studies of both incidence and prevalence of T2DM (5, 7). This study demonstrated that BMI appears to significantly interact with PCOS to affect risk of T2DM. The fact that women with PCOS had substantially higher BMI is both a strength and limitation in these analyses as it is both a confounder and an effect modifier of PCOS on development of T2DM. The inclusion of controls in this analysis allows interpretation of the effect of increased BMI and PCOS separately and through interaction. Specifically, BMI is not the only contributing factor to the development of T2DM. Compared to controls (HR=1.0), while PCOS alone does confer a 50% higher risk of developing T2DM in this analysis, a much higher ~5.1-fold risk is observed in morbidly obese women.

One possible explanation for the increased incidence of T2DM found in PCOS cases is the association of insulin resistance with polycystic ovary syndrome. Approximately 50% to 70% of affected women have IR (22). Compared to the prevalence of IR found in the US general population that amount of insulin resistance within this subgroup results in a 2- to 4-fold higher risk among PCOS cases for development of IR (23), which itself is a risk factor for the development of T2DM.

Another factor which may contribute to increased risk of T2DM is the hyperinsulinemia that co-exists with insulin resistance in PCOS-affected women. One major hypothesis of how HI/IR is related to the PCOS phenotype is based on the insulin-glucose-androgen pathway. Elevated glucose levels may produce secondary HI in an attempt to decrease circulating glucose

levels. HI may then create a state of IR by over-stimulating insulin-sensitive tissues (i.e., the androgen-secreting ovarian theca cell) in an attempt to produce enough insulin to subdue rising glucose levels at the periphery causing increased androgen production. The resulting HA may then directly or indirectly suppress ovulation at the level of the ovary (24). Androgen levels in women with PCOS have been positively correlated with measures of hyperinsulinemia in several studies (25-29) and, thus, may be associated with development of T2DM.

The main limitation of these analyses is the small cohort size so our results from these analyses must be tempered. A second limitation is the reliance of development of T2DM on self-reported diagnosis. To verify accuracy of self-report, all subjects were asked to bring current medications with them to each clinic visit as well as being asked date of diagnosis and length of medication use. Another limitation of this analysis is the inclusion of women taking hormones (OC/HRT).

In summary, women with PCOS had significantly greater risk of developing T2DM compared to age-adjusted control women. Risk of future development of T2DM in PCOS-affected women seems to be greatly modified by obesity. Future studies of incidence of T2DM related to polycystic ovary syndrome should focus on larger groups of older women followed through premenopausal, perimenopausal, and menopausal stages of development. In addition, our results suggest that extensive weight control efforts be made among women with PCOS to minimize the propensity to develop insulin resistance and T2DM.

**Table 2-1. Studies of Incidence and Prevalence of T2DM Among PCOS-Affected Women**

<p><b>Ehrmann (1999) (5)</b></p> <p><u>Objective:</u> To characterize the prevalence and incidence of glucose intolerance in a cohort of women with PCOS.</p>	<p><u>Population:</u> 122 women with clinical and hormonal evidence of PCOS</p> <p><u>Methods:</u> All women had a standard oral glucose tolerance test (OGTT) with measurement of glucose and insulin levels. At follow-up, 25 of the original cohort of women were subsequently re-evaluated to characterize the natural history of glucose tolerance in PCOS.</p>	<p><u>Results:</u> Glucose tolerance was abnormal in 55 (45%) of the 122 women with 43 (35%) having impaired glucose tolerance (IGT) and 12 (10%) having NIDDM at the time of initial study. After a mean follow-up of <math>2.4 \pm 0.3</math> years (range 0.5–6.3), 25 women had a second OGTT. Of the 11 normoglycemic (NG) at baseline, 5 remained NG (45%), 5 had IGT (45%) and 1 had NIDDM (9%). Of the 14 women with IGT at baseline, 3 (20%) became NG, 7 (50%) had IGT, and 4 (30%) had NIDDM.</p>
<p><b>Legro (1999) (6)</b></p> <p><u>Objective:</u> To determine the prevalence of glucose intolerance and parameters associated with risk for glucose tolerance among PCOS-affected women.</p>	<p><u>Population:</u> 254 PCOS women, aged 14-44 yr</p> <p><u>Methods:</u> All women were prospectively evaluated at 2 centers (n = 110) for regional diversity. A subset of PCOS women were compared to 80 control women of similar weight, race, and age. Participants were administered a standard OGTT.</p>	<p><u>Results:</u> The prevalence of glucose intolerance was 31.1% impaired glucose intolerance and 7.5% T2DM. In non-obese PCOS women (body mass index, <math>&lt;27 \text{ kg/m}^2</math>) had 10.3% IGT and 1.5% T2DM. The prevalence of glucose intolerance was significantly higher in PCOS vs. control women (<math>\chi^2 = 7.0</math>; <math>P = 0.01</math>; odds ratio = 2.76; 95% confidence interval = 1.23-6.57).</p>
<p><b>Norman (2001) (7)</b></p> <p><u>Objective:</u> To determine the prevalence of glucose intolerance among PCOS-affected women</p>	<p><u>Population:</u> 67 PCOS cases</p> <p><u>Methods:</u> All participants received a standard OGTT and lipids assessment at baseline and at follow-up after an average time of 6.2 years. All women followed prospectively had normal glucose tolerance (n = 54) or IGT (n = 13) at the start of the study.</p>	<p><u>Results:</u> Change in glycemic control from baseline was frequent, with 5/54 (9%) of normoglycemic women at baseline developing IGT and a further 4/54 (8%) developing T2DM. For women with IGT at baseline, 7/13 (54%) had NIDDM at follow-up. Body mass index (BMI) at baseline was an independent significant predictor of adverse change in glycemic control.</p>
<p><b>Weerakiet (2001) (8)</b></p> <p><u>Objective:</u> To determine prevalence of glucose metabolism abnormalities in Asian women with PCOS and to assess the different impact of using 1985 and 1999 WHO and ADA criteria for the diagnosis of T2DM.</p>	<p><u>Population:</u> 79 PCOS cases</p> <p><u>Methods:</u> All women underwent a standard OGTT. Fasting insulin and testosterone levels were also measured.</p>	<p><u>Results:</u> Prevalence of IGT and T2DM was 22.8 and 15.2% with the 1985 WHO criteria, and 20.3 and 17.7% according to the 1999 WHO consultation criteria, respectively. Using ADA criteria, fasting glucose levels determined a prevalence of 6.3% for T2DM. PCOS cases with glucose metabolism abnormalities had higher BMI and elevated fasting glucose and 2-h post-load glucose levels than those with NGT. The prevalence of glucose intolerance was significantly positively associated with BMI.</p>

**Table 2-2. Prevalence of Baseline Characteristics Among Caucasian Subjects**

<b>Baseline Characteristic</b>	<b>Cases (N = 97)</b>	<b>Controls (N = 95)</b>	<b>P- Value</b>
Age (mean years $\pm$ SD)	38.02 (5.89)	39.97 (5.21)	<b>.017</b>
Body mass index (mean $\pm$ SD)	31.56 (9.55)	26.22 (6.00)	<b>&lt;.0001</b>
Waist:Hip ratio	.82 (.10)	.75 (.06)	<b>&lt;.0001</b>
Race (n)	-----	-----	<b>.0006</b>
African-American	16	2	-----
Caucasian	81	93	-----
SBP (mean mmHg $\pm$ SD)	115.40 (15.75)	109.74 (12.57)	<b>.009</b>
DBP (mean mmHg $\pm$ SD)	73.48 (11.23)	70.23 (7.86)	.096
Triglycerides (mean mg/dl $\pm$ SD)	116.61 (82.52)	76.51 (34.03)	<b>.0002</b>
HDL (mean mg/dl $\pm$ SD)	51.57 (13.93)	57.53 (14.10)	<b>.003</b>
LDL (mean mg/dl $\pm$ SD)	121.79 (29.61)	113.26 (27.99)	.07
Smoking (%)	17.53	16.84	.97
Taking hormones (OC/HRT; %)	16.49	18.95	.65
Fasting glucose (mean mg/dl $\pm$ SD)	82.29 (13.54)	81.54 (7.95)	.691
Fasting insulin (mean $\mu$ U/ml $\pm$ SD)	17.09 (11.73)	11.61 (4.08)	<b>.0003</b>
Glucose:Insulin ratio (mean $\pm$ SD)	6.26 (2.66)	7.75 (2.20)	<b>.0003</b>
HOMA-IR (mean $\pm$ SD)	3.62 (3.14)	2.37 (.92)	<b>.002</b>

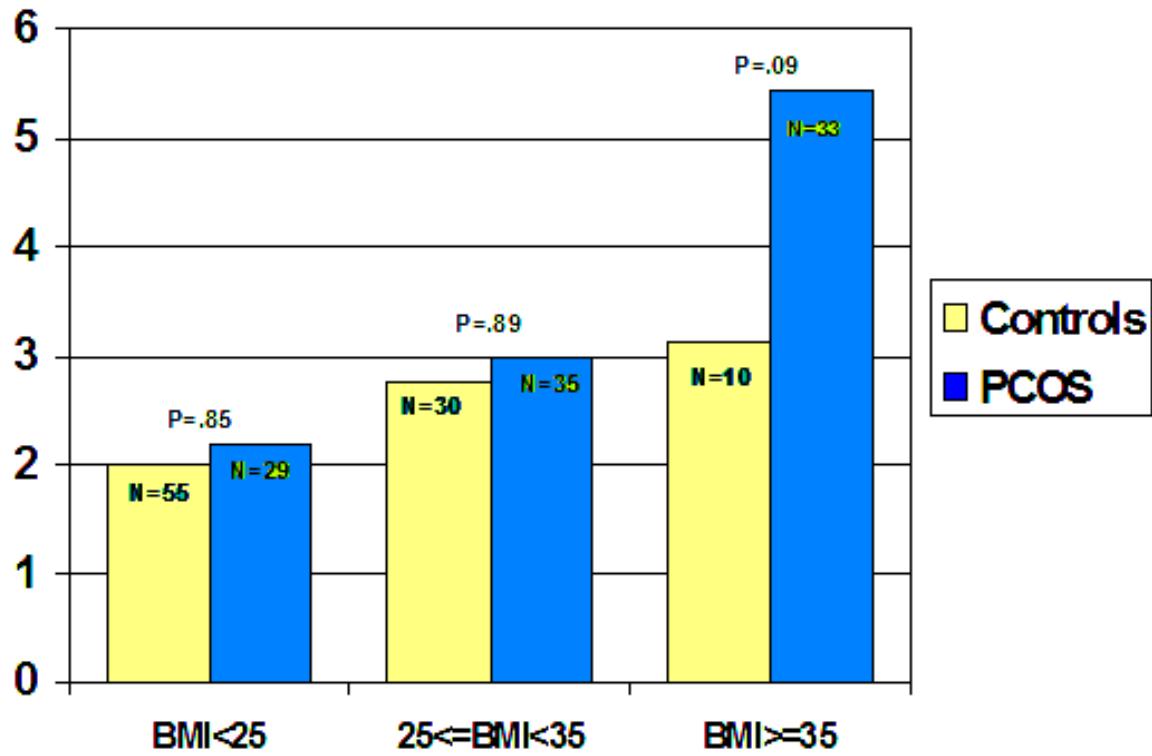
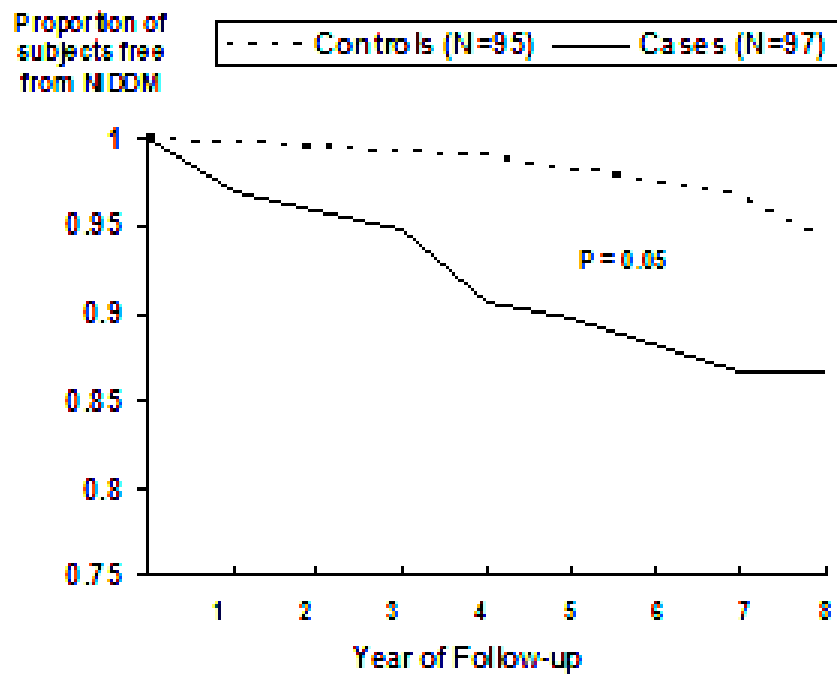
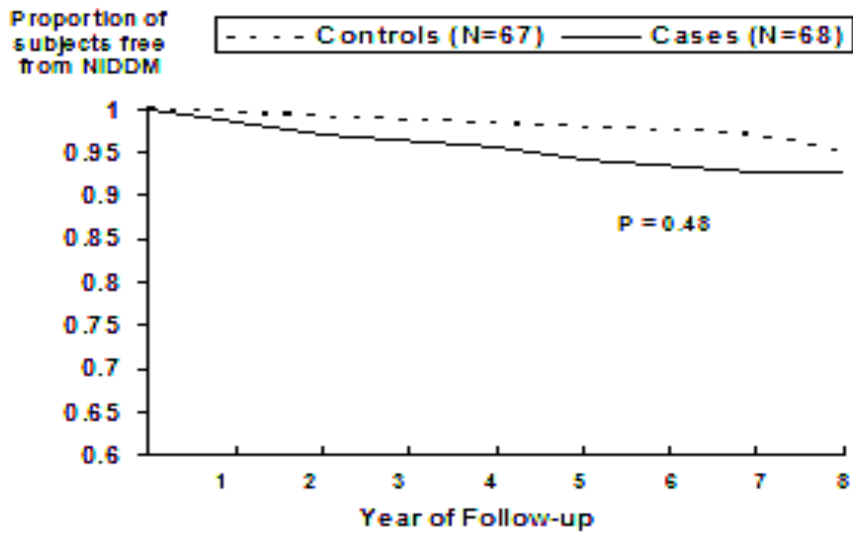


Figure 2-1. Average HOMA Scores Among PCOS Cases and Controls By BMI Strata



**Figure 2-2. Proportion of PCOS Cases and Controls Free of T2DM By Year of Follow-up**

### BASELINE GLUCOSE < 85



### BASELINE GLUCOSE > 85

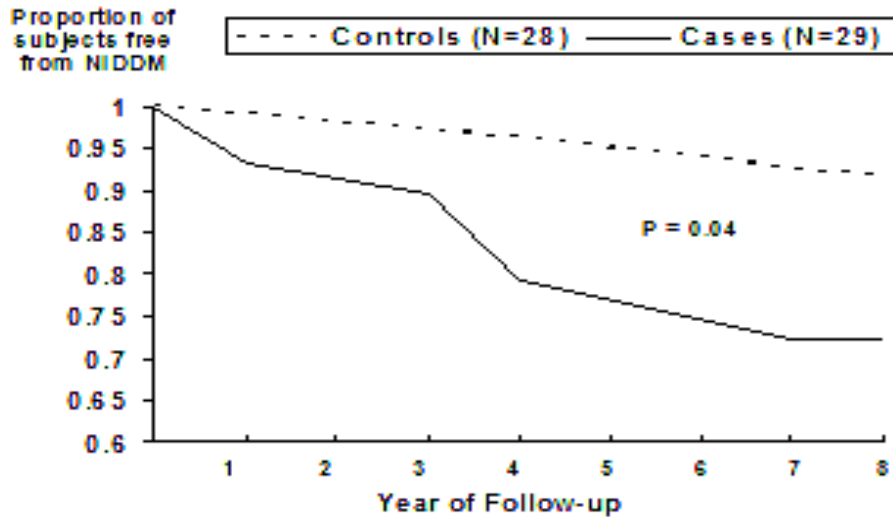


Figure 2-3. Proportion of PCOS Cases and Controls Free of T2DM By Year of Follow-up Stratified by Baseline Glucose

**Table 2-3. Hazard Ratios of Incident T2DM by PCOS Status**

Subject Group	N	Incidence Rate	Unadj. HR	Adj. HR	95% C.I.	P – value
All Subjects						
Controls	95	5.8%	1.0	-----	-----	-----
Cases	97	13.4%	2.66	2.00 <sup>a</sup>	0.67 – 5.99	0.22
Baseline glucose < 85						
Controls	67	4.8%	1.0	-----	-----	-----
Cases	68	7.3%	1.67	1.35 <sup>a</sup>	0.30 – 6.15	0.70
Baseline glucose ≥ 85						
Controls	28	8.4%	1.0	-----	-----	-----
Cases	29	27.6%	4.29	2.38 <sup>a</sup>	0.44 – 12.91	0.31
<b>PCOS/BMI Interaction</b>						
Controls, BMI<35	85	6.4%	1.0	-----	-----	-----
Controls, BMI ≥ 35	10	0%	0.0	-----	-----	-----
Cases, BMI<35	64	7.8%	1.34	1.30 <sup>b</sup>	0.37 - 4.56	0.68
Cases, BMI ≥ 35	33	24.2%	4.67	4.61 <sup>b</sup>	1.50 – 14.15	<b>0.008</b>
All Controls	95	5.8%	1.0	-----	-----	-----
Cases, BMI < 35	64	6.0%	1.49	1.45 <sup>b</sup>	0.41 – 5.08	0.56
Cases, BMI ≥ 35	33	21.3%	5.20	5.14 <sup>b</sup>	1.67 – 15.78	<b>0.004</b>

<sup>a</sup>Adjusted for age, body mass index

<sup>b</sup>Adjusted for age

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### **3.0 GENE EFFECTS OF ACETYL-COA CARBOXYLASE BETA AND LIPOPROTEIN LIPASE AMONG WOMEN WITH POLYCYSTIC OVARY SYNDROME: NO EVIDENCE OF AN ASSOCIATION**

#### **3.1 ABSTRACT**

Introduction: One potential mechanism leading to insulin resistance is ectopic fat storage of fat in muscle and liver. Since one characteristic of women affected by polycystic ovary syndrome (PCOS) is insulin resistance, we speculated that genetic variants in genes encoding proteins involved in fat metabolism could be considered as candidate genes for PCOS. Genes included in these analyses are the P12A variant of PPAR- $\gamma$ , the G972R variant of insulin receptor substrate-1, three single nucleotide polymorphisms (SNPs) of acetyl-coA carboxylase beta (ACC- $\beta$ ), and one SNP of lipoprotein lipase (LPL).

Methods: DNA was assessed for 305 Caucasian and African American (AA) PCOS cases and controls (148 PCOS, 157 controls). Case and control frequencies for each allele, P12A/G972R combinations and the ACC- $\beta$  gene were computed and compared by use of  $\chi^2$  tests. Linkage disequilibrium within the ACC- $\beta$  locus was calculated for all pairs of SNPs. A non-parametric T5 statistic was used to test for significant ACC- $\beta$  haplotype frequency differences between cases and controls.

Results: There were no significant differences in allele frequency for any genotypes between Caucasian cases and controls. However, the G194216A variant of ACC- $\beta$  allele frequency was significantly different among AA cases and controls. Linkage disequilibrium was significant between two ACC- $\beta$  SNPs, T204540C and G194216A in both Caucasian and AA subjects. When comparing PCOS cases to same race controls, ACC- $\beta$  haplotype frequencies were similarly distributed. However, not surprisingly, the distribution of ACC- $\beta$  haplotype

frequencies were significantly different between AA and Caucasian subjects ( $p < 0.001$ ). When comparing the P12A/G972R combinations, only the Ala/Ala genotype/Gly/Gly genotype combination presented with a potential association (OR=4.37; 95% CI: 0.42 – 216.99) in Caucasian subjects, but was too rare (4 cases and 1 control) to truly assess its impact.

Discussion: Allele frequencies for P12A, G972R, ACC- $\beta$  SNPs (T204540C, G194216A, and G263491A), and LPL SNP A7634966C were not significantly different between controls and PCOS-affected women. There were also no significant associations of the ACC- $\beta$  haplotype or P12A/G972R combined genotypes with PCOS. Future studies may be necessary to validate the results of this study, especially regarding ACC- $\beta$  whose effects on PCOS merit further study.

## 3.2 INTRODUCTION

Polycystic ovary syndrome (PCOS) is characterized by chronic anovulation, hyperandrogenism (HA), and insulin resistance (IR). Affected women may be obese and manifest dyslipidemia. One hypothesis for causal mechanisms leading to decreased insulin sensitivity in humans is ectopic fat storage of lipids in skeletal muscle or liver rather than just adipose tissue. This theory is based on findings of increased triglyceride content in the skeletal muscle of subjects with obesity or type 2 diabetes (T2DM) (1) as well as increased intramuscular triglyceride levels found in non-obese, insulin resistant, first-degree relatives of type 2 diabetics (2). These results suggest that fat deposition within skeletal muscle may be an early change in body composition associated with insulin resistance, obesity and type 2 diabetes rather than a later development resulting from excess adiposity. The similar phenotype (i.e., abdominal obesity, insulin resistance, and elevated lipid levels) shared by polycystic ovary syndrome and T2DM raises the possibility that ectopic fat storage occurs in women with PCOS as well as type 2 diabetics.

Since PCOS is a familial disorder, genes associated with lipogenesis are of interest due to their potential connection with both fat deposition and insulin sensitivity. The peroxisome proliferator-activated receptor gamma (PPAR- $\gamma$ ) is a nuclear transcription factor activated by thiazolidinediones (TZDs) and specific fatty acids (3). This factor plays a major role in adipogenesis and influences fatty acid metabolism and insulin resistance/hyperinsulinemia (4). Once activated, it heterodimerizes with the retinoid X receptor and binds to the peroxisome proliferator receptor elements of DNA to promote transcription of numerous target genes (5). PPAR- $\gamma$ 2 is expressed in adipose tissue where it plays a key role in regulation of adipogenic differentiation (6) and energy storage (5). The loss-of-function P12A polymorphism of PPAR- $\gamma$ 2 has been studied in association with T2DM in several populations with inconsistent findings. Reports suggest an association of decreased risk of T2DM in carriers of the P12A variant (7), an association of increased risk of development of T2DM among P12A carriers (8, 9) or a lack of association of the P12A variant with T2DM (10-14).

Studies published on the association of the P12A polymorphism with PCOS (15, 16) have also been inconclusive. Korhonen et al. (16) genotyped 135 PCOS-affected women and 115 healthy controls from Finland for the P12A polymorphism and found a significantly different allele distribution between cases and controls. PCOS cases had a significantly lower frequency of the Ala isoform. They concluded PPAR- $\gamma$ 2 may play a role in the pathogenesis of PCOS and the Ala isoform is most likely protective against the development of PCOS. Conversely, Orio et al. (15) investigated the P12A polymorphism in 100 PCOS-affected women and 100 controls from Italy matched for age and body mass index and found no association with PCOS. The inconsistency of these studies supports further investigation of the potential role of the P12A variant of PPAR- $\gamma$ 2 among women with PCOS.

A common polymorphism of the insulin receptor substrate-1 (IRS-1), G972R, is a mild loss of function mutation that has been associated with decreased insulin sensitivity (17), T2DM (18), and PCOS (17, 19). Furthermore, the P12A and G972R genotypes may interact to effect insulin sensitivity. Stumvoll et al. (20) studied the gene-gene interaction between the P12A variant of PPAR- $\gamma$  and the G972R variant of IRS-1 among 318 normoglycemic, unrelated volunteers. Insulin sensitivity was significantly greater in individuals carrying the heterozygous or homozygous Ala allele versus Pro/Pro homozygotes ( $p=0.01$ ) when compared within the X/Arg background. This association was not observed in the whole population or within the Gly/Gly background. The authors concluded that the Ala12 allele of PPAR- $\gamma$  may become particularly advantageous in individuals with decreased insulin sensitivity, i.e., heterozygous or homozygous carriers of the Arg allele. Unlike previous investigations of P12A and G972R genotype frequencies among women with polycystic ovary syndrome, this study includes separate analyses of Caucasian and African American women with PCOS and control subjects.

Lipoprotein lipase (LPL), a serine esterase expressed in adipocytes and striated muscle, plays a pivotal role in fat and energy metabolism. LPL's main function is the hydrolysis of triglycerides in triglyceride-rich lipoproteins, such as chylomicrons and very low density lipoproteins (21). Genetic variants of the LPL gene have been associated with risks for components of the metabolic syndrome (22). The potential role of the LPL gene has not been examined in women with PCOS.

Thiazoladinediones produce their insulin sensitizing effects partly by inducing mitochondrial carnitine palmitoyl transferase 1 (CPT1) activity (23). CPT1 activity is increased through the inhibition of malonyl-CoA, which itself can play a pivotal role in glucose-sensitive insulin secretion. Acetyl-Coenzyme A carboxylase (ACC) is a complex multifunctional enzyme system resulting in increased activity of malonyl-CoA, the rate-limiting step in fatty acid synthesis. The beta form (ACC- $\beta$ ) may be involved in the provision of malonyl-CoA or in the regulation of fatty acid uptake and oxidation by mitochondria and is, thus, critical for its role in fatty acid oxidation (24). A loss-of-function mutation in the ACC- $\beta$  gene could potentially increase overall insulin sensitivity through decreased malonyl-CoA production, as is seen in TZD treatment. Since insulin resistance is prevalent among PCOS-affected individuals, genetic variants of the ACC- $\beta$  gene may be associated with PCOS. Furthermore, it has been postulated that insulin resistance may be related to gene transcription downstream of PPAR- $\gamma$  (25), such as the LPL and ACC- $\beta$  genotypes selected for this analysis.

The specific aims of this analysis are 1) To test the association of P12A of PPAR- $\gamma$ , G972R of IRS-1, ACC- $\beta$  SNPs (G263491A, T204540C, and G194216A) and LPL SNP A7634966C with polycystic ovary syndrome among Caucasian and African American case and control subjects; 2) to evaluate the association of the ACC- $\beta$  haplotype with polycystic ovary syndrome among Caucasian and African American case and control subjects; and 3) to examine the association of P12A/G972R interaction among Caucasian and African American polycystic ovary syndrome cases and controls.

### **3.3 METHODS**

#### **3.3.1 Subjects**

The present analysis was conducted using women recruited for the Cardiovascular Health and Risk Measurement Study (CHARM). The CHARM study was established in 1992 to

investigate the effect of polycystic ovary syndrome on cardiovascular risk factors and associated disease (CVD) in women. Due to previous medical diagnosis of PCOS, the population of women recruited for CHARM were considered at high-risk for developing CVD. Women diagnosed with PCOS between 1970 and 1993 who were at least 30 years of age at the time of recruitment were identified from the records of an academic reproductive endocrine practice located at Magee-Womens Hospital, Pittsburgh, PA. The clinical diagnosis of PCOS was made if there was (1) a history of chronic anovulation in association with either (A) clinical evidence of androgen excess (hirsutism) or biochemical evidence of an elevated total testosterone concentration ( $>57.64$  ng/dl (2nmol/l)) or (B) a ratio of luteinizing to follicle stimulating hormone  $> 2.0$ . Eligible women were contacted by phone between 1992 and 1994 for a telephone interview and for further recruitment for a clinical visit. During that time, age ( $\pm 5$  years)- and race-matched neighborhood control subjects were selected using a combination of voter's registration tapes for 1992 from the Greater Pittsburgh area and Cole's Cross Reference Directory of Households and were similarly recruited. After initial phone contact, 244 PCOS-affected women and 244 controls completed a clinical visit where they received a fasting blood draw, waist and hip measurements, standard blood pressure assessment and a questionnaire-based interview.

In 1996-1999, the same population of women was re-contacted for a second clinical visit also including a fasting blood draw, waist and hip measurements, standard blood pressure assessment and a questionnaire-based interview. Of the original 488 women seen between 1992 and 1994, 335 were enrolled for a second clinical visit. At this second visit, 329 women consented to a blood draw for DNA analysis. After genomic DNA extraction, 24 samples were devoid of leukocytes and were unusable for further analyses. The present analysis is comprised of the remaining 305 follow-up visit women (148 cases and 157 controls), of which 252 were Caucasian and 53 were African American. All participants gave written, informed consent as approved by the Institutional Review Board of the University of Pittsburgh.

### 3.3.2 Genotype Analyses

Genomic DNA was assessed for blood samples drawn from 305 CHARM study case and control subjects seen at the second visit between 1996 and 1999. Buffy coats were collected from 20 cc whole blood from each CHARM individual seen at the second visit and immediately frozen at  $-80^{\circ}\text{C}$  at the University of Pittsburgh, Graduate School of Public Health, Heinz Nutrition Laboratory. Genomic DNA was subsequently extracted in 2004 using established methods (26), and was available on 305 individuals. Ambiguous samples were analyzed a second time. **PPAR- $\gamma$  variant P12A**: Molecular genetic analysis of PPAR- $\gamma$  variant P12A was performed using the polymerase chain reaction (PCR) primers, sense (5'-GGCCAATTCAAGCCCAGTC-3') and anti-sense (5'-GATATGTTTGCAGACAGTGTATCAGTGAAGGAATCGCTTCCG-3'), producing a 270-bp PCR product. Carrier status of the P12A variant of the PPAR-gamma gene was determined by restriction fragment length polymorphism (RFLP) analysis (27). **IRS-1 variant G972R**: Genetic analysis of the IRS-1 variant G972R was performed using PCR primers sense (5'-CTTCTGTCAGGTGTCCATCC-3') and anti-sense (5'-TGGCGAGGTGTCCACGTAGC-3'). Identification of the IRS-1 variant G972R involved *Bst*NI restriction enzyme digestion of a 262-bp PCR product. Carrier status of the G972R variant of the IRS-1 gene was determined by restriction fragment length polymorphism (RFLP) analysis (28). **ACC- $\beta$** : Three SNPs were identified from the ACC- $\beta$  gene located on chromosome 12. PCR primers for these novel SNPs were as follows: *SNP rs2268403* (A/G) (sense – AGGGAAGAGGCCATTTTCGTTGGTA-3' and anti-sense – 5'-GGGTTCTTGGCTGT-GAACCAAACA-3'), *SNP rs2268393* (C/T) (sense – 5'-TGCCA-GTTGCACAGAATTCCAA-CC-3' and anti-sense 5'-ACAATGGGAACAGCT-ACACCACCT-3'), and *SNP rs3742023* (A/G) (sense – 5'-ATTACCTTGCTCGTCC-TGTCACCA-3' and anti-sense – 5'-TATGAGGTAAAGCCAGGCTGTCC) were identified and created using the Primer Quest primer creation program on Integrated DNA Technologies website ([www.idtdna.com](http://www.idtdna.com)). Thermocycling conditions for all ACC- $\beta$  SNPs were  $94^{\circ}\text{C}$  for 3 minutes, followed by 30 cycles of  $94^{\circ}\text{C}$  for 30 seconds,  $60^{\circ}\text{C}$  for 30 seconds,  $72^{\circ}\text{C}$  for 1 minute, finalized by a 7 minute soak at  $72^{\circ}\text{C}$ . Restriction enzymes used for each SNP were *EarI*, *AfeI*, and *NcoI*, respectively (New England Biolabs, Inc., Beverly, MA). PCR thermocycling of ACC- $\beta$  variant rs2268403 created a 474-bp product in which an *EarI*

restriction site presented concurrently with the G → A change at nucleotide 194216 to generate the G194216A mutation (rs2268403). After *EcoRI* digestion at 37°C for 2 hours and 3% agarose gel electrophoresis, the expected product sizes were 368 and 106 bp for the G194216 variant; 368, 199, 169, and 106 bp for the heterozygote; and 199, 169, and 106 for G194216A (Figure 3-1). After thermocycling of ACC-β SNP rs2268393, the PCR product was 225-bp and the sequence contained an *AfeI* restriction site introduced by the T → C variant at nucleotide 204540 to generate the T204540C mutation. Digestion by *AfeI* (37°C for 2 hours) produced the expected lengths of 225 bp for the T204540; 225, 118, and 107 bp for heterozygotes; and 118 and 107 bp for the T204540C mutation (Figure 3-2). PCR thermocycling of ACC-β SNP rs3742023 created a 213-bp fragment containing an *NcoI* restriction site introduced by the G → A change at nucleotide 263491. Expected product sizes were 213 for the G263491 homozygous; 213, 150, and 63 for the heterozygote; and 150 and 63 for the G263491A mutation (Figure 3-3). **LPL**: One SNP on the lipoprotein lipase gene on chromosome 8 was also analyzed. LPL SNP rs3735964 was assessed by PCR thermocycling with Primer Quest primers identified and created by Integrated DNA Technologies sense (5'-TGCAATGAGCCAGATGGAGTACCA-3') and anti-sense (5'-TGCTGAAGGACA-ACACACATGCAG-3'). PCR thermocycling of rs3735964 created a 237-bp product in which an *EcoRI* restriction site presented concurrently with the A → C change at nucleotide 7634966 to generate the A7634966C mutation. After *EcoRI* digestion at 37°C for 2 hours and 3% agarose gel electrophoresis, the expected product sizes were 237 bp for the A7634966 variant; 237, 167, and 70 bp for the heterozygote; and 167 and 70 bp for A7634966C (Figure 3-4).

### 3.3.3 Data analyses

Allele frequencies: Allele frequencies for each SNP were computed by gene counting and compared between cases and controls by use of  $\chi^2$  tests. Genotype conformation to Hardy-Weinberg equilibrium proportions were tested using Fisher's exact test. All single nucleotide polymorphisms in this study were in Hardy-Weinberg equilibrium (<http://ihg.gsf.de/cgi-bin/hw/hwa1.pl>). Haplotype estimation: Linkage disequilibrium, or  $D'$ , was calculated using the R/Genetic Analysis Package for all pairs of SNPs within the ACC-β locus. Subsequently,

haplotype frequencies for ACC- $\beta$  were estimated using the PHASE software program (29, 30) and tested for significant difference between same-race cases and controls using  $\chi^2$  tests. PHASE uses the expectation-maximization algorithm to obtain maximum-likelihood estimates of haplotype frequencies. The association of pairwise comparisons of ACC- $\beta$  SNPs in case and control subjects was tested using Fisher's exact test (31, 32). Haplotypes are described here by a three-digit code, where the first digit indicated the allele present in T204540C, the second indicated G194216A, and the last referred to G263491A. A "0" in T204540C meant the estimated allele present was a "T" and a "1" represented a "C". In G194216A and G263491A, a "0" represented a "G", and a "1" indicated an "A" allele. For example, an ACC- $\beta$  haplotype of "100" meant that the subject has the "C" allele for T204540C, a "G" allele for G194216A, and a "G" allele for G263491A. Association analyses: A non-parametric T5 statistic, which is implemented in the EH program (33, 34), was used to test for significant differences in haplotype frequencies between cases and controls (35). To compute T5, EH was run separately for cases, for controls, and for cases and controls combined. Each run produced a log-likelihood, the combination of which is used to compute the T5 statistic. Under the hypothesis of allowable allelic association, T5 is defined as  $2[\ln(L_{\text{case}}) + \ln(L_{\text{control}}) - \ln(L_{\text{combined}})]$ , and has an approximate  $\chi^2$  distribution with df equal to number of haplotypes tested. StatXact was used to test for significance between ACC- $\beta$  haplotype combinations among PCOS cases and controls and to calculate odds ratios with 95% confidence intervals.

## 3.4 RESULTS

### 3.4.1 Estimated Allele Frequencies Among Caucasian and AA Subjects

As can be seen from Table 3-1, there were no significant differences in allele frequency between cases and controls among Caucasian subjects. Among African American subjects, the G194216A SNP of ACC- $\beta$  was borderline statistically significant ( $p=0.05$ ) with 100% of

cases compared to 91% of controls having the more common G allele. There were no other significantly different allele frequencies between African American cases and control subjects.

### **3.4.2 Linkage Disequilibrium between ACC-Beta SNPs**

When assessing LD between the three ACC- $\beta$  SNPs, T204540C and G194216A seemed to have significant linkage in both Caucasian and African American subjects (Table 3-2).  $D'$ , the standardized measure of linkage disequilibrium, for T204540C and G194216A was 0.969 among Caucasians and 0.998 among African Americans.

### **3.4.3 Estimated haplotype frequencies for ACC-Beta**

When comparing women with PCOS to control women of the same race, ACC- $\beta$  haplotype frequencies showed similar distribution (Table 3-3). The most common haplotype among Caucasian cases and controls and African American cases was 010 for G194216A, T204540C, and G263491A, respectively, with 36.1% of Caucasian cases, 37.2% of Caucasian controls, and 50.3% of African American cases with the observed haplotype. Among African American controls, the 000 haplotype was most common and was observed in 35.4% of this subgroup, while the 010 haplotype was found in 34.8%. The 111 haplotype, though represented in this table was not found in any individuals.

Even though haplotype distributions were not significantly different between same-race cases and controls, the distribution of haplotype frequencies were significantly different between African American and Caucasian subjects ( $p < 0.001$ ), which was not unexpected given the common finding that genotype frequencies often vary between individuals of different ethnic backgrounds.

#### **3.4.4 The Association of ACC-Beta Haplotypes with PCOS**

Among Caucasian and African American subjects, there were no significant associations of any ACC- $\beta$  haplotype with polycystic ovary syndrome (Table 3-4). A potential association of the 010/010 haplotype combination was present among African American women only (OR=5.56; 95% CI: 0.92 – 57.95), but failed to reach significance due to the small sample of African American women in this analysis and the rarity of the haplotype (8 cases and 2 controls). When testing for an overall association of the ACC- $\beta$  haplotype with polycystic ovary syndrome using results from EH program analysis (Table 3-5), there was no evidence of an association among Caucasian ( $p = 0.50$ ) or African American women ( $p = 0.25$ ).

#### **3.4.5 The Interaction Between P12A and G972R genotypes with PCOS**

The P12A variant of the PPAR-gamma gene and the G972R variant of the IRS-1 gene combinations were analyzed to determine if an interactive effect of their combined genotypes was present when comparing PCOS-affected women and control subjects (Table 3-6). The Pro/Pro and Pro/Ala genotypes did not seem to have any interactive effect with the IRS-1 gene in association with PCOS within Caucasian or African American subjects. The Ala/Ala genotype/ Gly/Gly genotype combination may have a potential association (4.37 (0.42 – 216.99)) with PCOS within Caucasian subjects, but was too rare (4 cases and 1 control) for this analysis to truly assess its impact. Within African American participants, there were no significant differences between the combination genotype frequencies in comparing PCOS cases and controls, but it is noteworthy that the combined P12A/G972R genotype frequencies seemed to be distributed differently between Caucasian and African American subjects.

### 3.5 DISCUSSION

In summary, allele frequencies for P12A, G972R, the three ACC- $\beta$  SNPs (T204540C, G194216A, and G263491A), and LPL SNP A7634966C were not significantly different between controls and PCOS-affected women. There were also no significant associations of the ACC- $\beta$  haplotype or combinations of ACC- $\beta$  haplotypes with PCOS. Furthermore, the P12A and G972R combined genotypes frequencies did not seem to interact with case/control status. Significant results in previous studies could have been due to differing PCOS diagnostic criteria. due to differing PCOS diagnostic criteria.

The potential for association of PCOS with PPAR- $\gamma$  and associated genes was grounded not only in the significant findings of similar studies among diabetic populations (7, 36), but also upon knowledge of cellular mechanisms by which insulin resistance may occur; insulin resistance greater than anticipated for BMI is typical for women with PCOS. Although the precise molecular mechanism responsible for insulin resistance in obesity remains to be elucidated, current evidence suggests that elevated free fatty acids are major players in this association. Evidence for this association is comprised from various sources, as follows: 1) most obese people have elevated FFA plasma levels (37, 38) and 2) both chronic and acute (39-42) plasma FFA elevations produce acute insulin resistance. One hypothesis to explain the relationship between FFAs and insulin resistance is that ectopic fat storage of fat impairs insulin signaling (43-46). Results of studies have suggested that FFA may produce insulin resistance by protein kinase C activation and this may occur via serine/threonine phosphorylation of the insulin receptor and/or IRS-1, which has been shown to inhibit insulin signaling. Using the hypothesis of intramuscular triglyceride stores as a marker of insulin resistance, an association between the IRS-1 loss-of-function mutation is not unexpected among women with PCOS, especially given the strong genetic basis for its development as demonstrated by family studies (47-52) and presence of insulin resistance among lean women with PCOS (53-56).

PPAR- $\gamma$  has a central role in adipogenesis (57-59) based on two main processes. It functions as a transcription factor that alters expression of genes involved in adipogenesis and energy metabolism. As such, it promotes increased expression of target genes that promote fatty acid trapping and storage in adipocytes, such as fatty acid binding protein (59), LPL

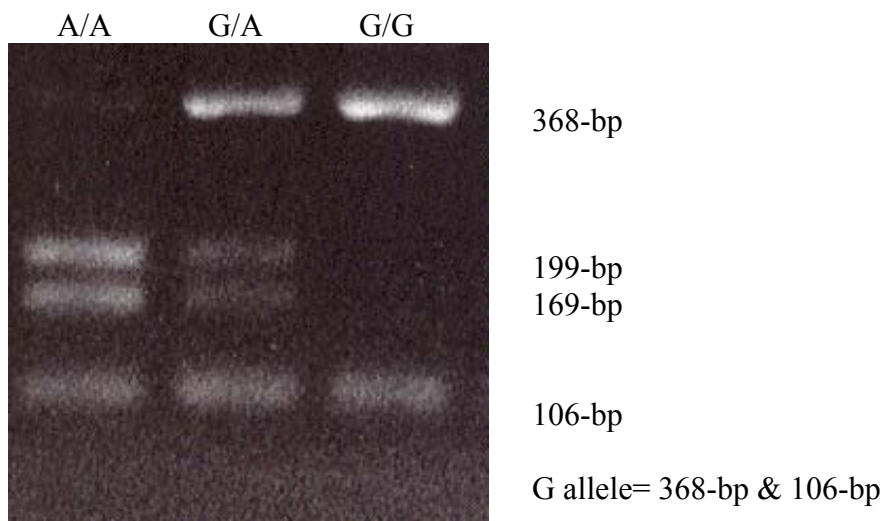
(60), and acyl-CoA synthase (61). Among other actions, it also represses genes that induce lipolysis and the release of fatty acids, such as the beta3-adrenergic receptor (62) and cytokines leptin (63, 64) and TNF- $\alpha$  (65, 66). These results can be supported by the demonstrated effects of TZDs on PPAR- $\gamma$  activation (67, 68). Treatment with TZDs seems to favor redistribution of white adipose tissue, with decreased visceral depots relative to subcutaneous fatty regions (67-70). This fat cell redistribution includes a shift in the cell type population resulting in more small adipocytes and fewer large, insulin insensitive adipocytes (71-73). By decreasing insulin resistance through use of TZDs, androgen concentrations decrease leading to ovulation and fertility in women with PCOS. The PCOS phenotype seems to be intricately bound to fatty acid metabolism through the PPAR-gamma pathway. The several unsuccessful attempts to identify a “PCOS gene” has led to reconsideration of PCOS as a polygenic multifactorial disorder with phenotypic and genotypic heterogeneity.

As one gene whose transcriptional activity is regulated by PPAR-gamma, LPL expression is attenuated through hormones, notably insulin, and this directly impacts fatty acid utilization (74, 75). Specifically, fasting promotes decreased LPL activity in adipose tissue and increased activity in cardiac tissue, while feeding causes increased adipose enzyme and decreased muscle LPL (75-77). LPL expression and variants affecting its expression are further regulated by disease states, notably atherosclerosis and diabetes (78-80, 83). Transcriptional control of LPL also impacts fatty acid usage. Metabolites that induce LPL gene transcription include the peroxisome proliferator’s response element in liver and adipose tissues and in macrophages in response to fibrates, some fatty acids, glucose, and TZDs (60, 79, 81). Decreased LPL activity has been seen in individuals with type 2 diabetes and insulin resistance (82-85). Furthermore, the resultant decrease in LPL activity contributes to hypertriglyceridemia, decreased HDL levels, and increased risk of coronary heart disease (86). Since LPL is regulated by diabetes as well as other diseases, a mutation affecting the activity of the LPL gene would not be surprising among PCOS-affected women. This analysis did not find such an effect, but it cannot be ruled out as only one SNP was used to test our hypothesis.

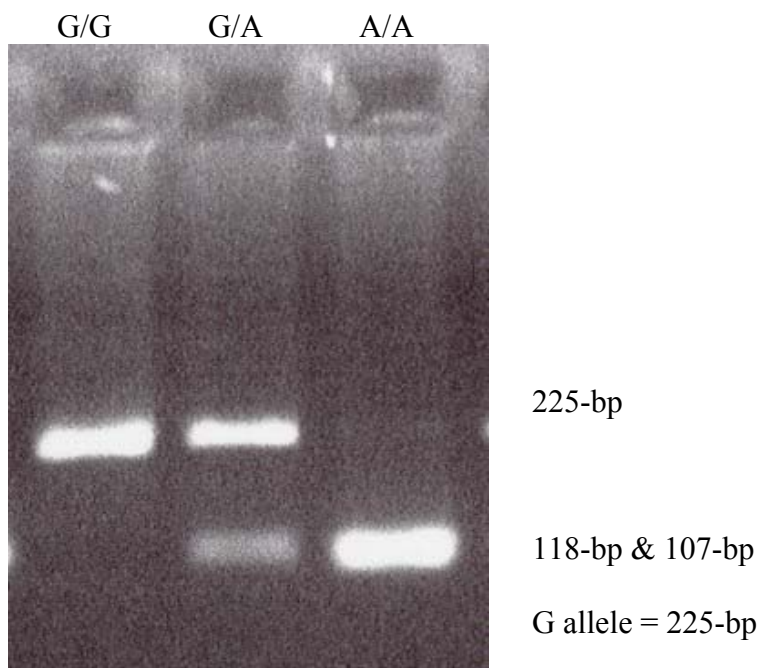
A second, complementary theory of how obesity impacts insulin sensitivity is as a fuel partitioning disorder. According to Neel’s hypothesis of the thrifty genotype (87), the ability to store excess energy was advantageous in ancestral societies subjected to periods of

starvation. This hypothesis purports that multiple cellular mechanisms are present to sense increased availability of food and to trigger biological responses designed to most efficiently store energy. Malonyl-CoA has been identified as a biochemical sensor (88) believed to switch from fatty acid to glucose oxidation. During states of high concentrations of glucose and insulin, malonyl-CoA accumulation inhibits CPT1 and reduces lipid oxidation, preferring lipid storage into triglycerides. By virtue of the effect malonyl-CoA on LCFA transport into mitochondria, it has been shown to regulate intracellular FA oxidation in several tissues, including the liver (89), muscle (90), the pancreatic beta-cell (91), and endothelium (92) and probably works similarly in the adipocyte (93) and the central nervous system. ACC- $\beta$  has a direct link to fatty acid utilization through its control over malonyl-CoA production. Its indirect relationship with PPAR-gamma through CPT1 makes it highly feasible as a candidate gene affecting expression of the PCOS phenotype, yet an effect of ACC- $\beta$  was not seen in this population. This was the first study to associate ACC- $\beta$  with PCOS and we found that it seemed not to be associated with case status among Caucasians. There was a borderline significantly higher allele frequency for the common G allele of the G194216A SNP among African American cases compared to controls (1.00 vs. 0.91), a result due to the absence of the less common allele among cases. Furthermore, the ACC- $\beta$  haplotype had no association with PCOS demonstrating an overall lack of association. Results from this population regarding the ACC- $\beta$  gene will need to be validated before conclusions can be reached, especially for the G194216A polymorphism.

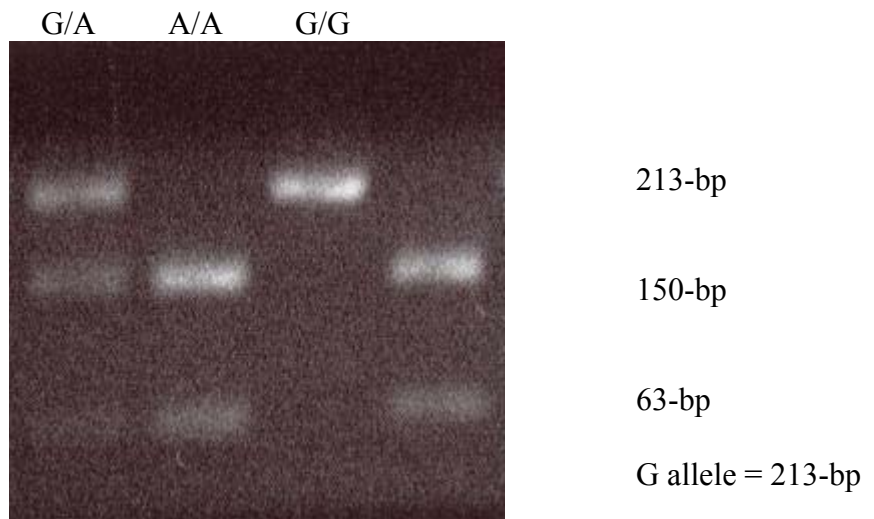
The main limitation of this analysis was the lack of power needed to detect a difference in allele frequencies among PCOS cases and controls. The lack of significance found between cases and controls for all genotypes could be more due to small sample size than an actual lack of significance. Furthermore, the sample size could prevent detection of a minor weak effect. Using these analyses most common allele frequencies, the current sample size of Caucasian subjects had 67% power to detect a 15% allele frequency difference between cases and controls and 37% power to detect a 10% difference in allele frequencies. The borderline significant 9% difference in allele frequency between African American cases and controls had a 10% power to detect, suggesting this result may need to be repeated in a larger population for validity. Future studies may be necessary to validate the results of this study, especially regarding ACC- $\beta$  whose effects on PCOS merit further study.



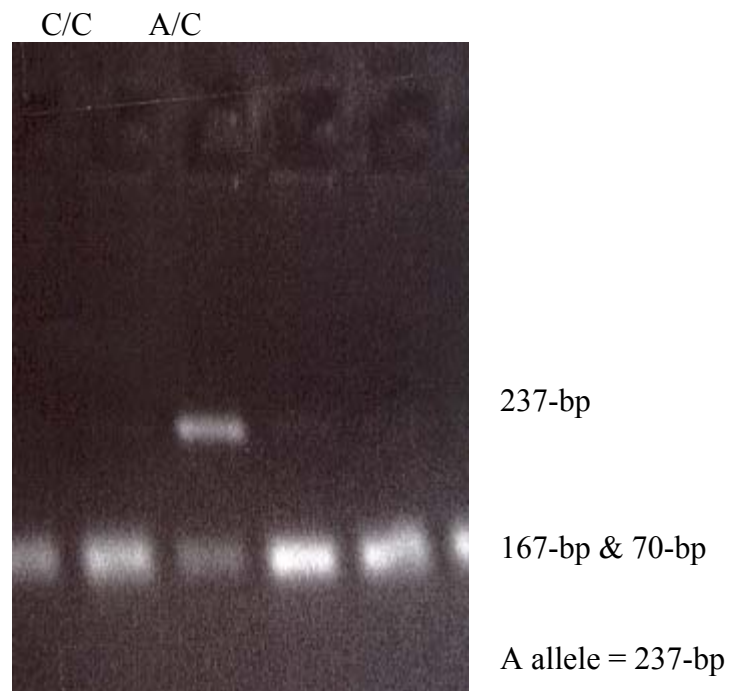
**Figure 3-1. Gel electrophoresis of ACC-Beta SNP G194216A**



**Figure 3-2. Gel electrophoresis ACC-Beta SNP T204540C**



**Figure 3-3. Gel electrophoresis of ACC-Beta SNP G263491A**



**Figure 3-4. Gel electrophoresis of LPL SNP A7634966C**

**Table 3-1. Estimated Allele 1 Frequencies in Caucasian and AA PCOS Cases and Controls**

<b>Allele 1 Frequency (N) In Caucasian subjects<sup>b</sup></b>				
<b>SNP</b>	<b>Cases (n = 244)</b>	<b>Controls (n = 260)</b>	$\chi^{2(a)}$	<b>P-value</b>
PPAR- $\gamma$ (P12A)	0.80 (196)	0.81 (210)	0.93	0.62
IRS-1 (G972R)	0.92 (225)	0.93 (242)	1.88	0.45
ACC- $\beta$ (G263491A)	0.68 (166)	0.67 (173)	0.64	0.73
ACC- $\beta$ (G194216A)	0.83 (202)	0.82 (213)	0.77	0.69
ACC- $\beta$ (T204540C)	0.55 (134)	0.54 (141)	0.07	0.98
LPL (A7634966C)	0.91 (221)	0.90 (234)	1.65	0.56
<b>Allele 1 Frequency (No./Total) In African American subjects<sup>b</sup></b>				
<b>SNP</b>	<b>Cases (n = 52)</b>	<b>Controls (n = 54)</b>	$\chi^{2(a)}$	<b>P-value</b>
PPAR- $\gamma$ (P12A)	0.96 (50)	0.98 (53)	0.39 <sup>a</sup>	0.61
IRS-1 (G972R)	0.94 (49)	0.87 (47)	1.52	0.58
ACC- $\beta$ (G263491A)	0.81 (42)	0.78 (42)	0.5	0.88
ACC- $\beta$ (G194216A)	1.00 (52)	0.91 (49)	5.32 <sup>a</sup>	<b>0.05</b>
ACC- $\beta$ (T204540C)	0.60 (31)	0.50 (27)	1.14	0.58
LPL (A7634966C)	0.90 (47)	0.91 (49)	0.004	1.00

- a. Pearson  $\chi^2$  used instead of Fisher's exact for  $\chi^2$  value only
- b. Allele 1 defined as most common allele - The ACC- $\beta$  single nucleotide polymorphisms (G263491A, G194216A, and T204540C) were also assessed for their most common allelic frequencies (G, G, and C, respectively) and were not found to be significantly different between case and control subjects ( $p = 0.73$ ,  $p = 0.69$ , and  $p = 0.98$ , respectively). The LPL SNP, A7634966C, was similarly distributed between PCOS cases and controls with 91% of cases and 90% of controls having the more common C variant ( $\chi^2 = 1.65$ ;  $p = 0.56$ ).

**Table 3-2. Pairwise Linkage Disequilibrium between ACC-Beta SNPs in Caucasian Subjects**

Pairwise Comparison	Caucasian				African American		
	PCOS	Controls			PCOS	Controls	
	P-value	P-value	D'		P-value	P-value	D'
G263491A vs. G194216A	0.18	0.83	0.329		-----	1.00	0.027
G263491A vs. T204540C	0.99	0.62	0.055		0.3	0.25	0.011
G194216A vs. T204540C	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>0.969</b>		-----	0.36	<b>0.998</b>

P-values determined using Two-sided Fisher's Exact test

D' calculated using R/gap

**Table 3-3. Estimated ACC-Beta Haplotype Frequencies in Caucasian and AA Subjects**

ACC- $\beta$ Haplotypes			Caucasian Case Frequencies (n=122)	Caucasian Control Frequencies (n=130)
G194216A	T204540C	G263491A		
0	0	0	0.182 (22)	0.170 (22)
0	0	1	0.107 (13)	0.108 (14)
0	1	0	0.361 (44)	0.372 (48)
0	1	1	0.177 (22)	0.169 (22)
1	0	0	0.128 (16)	0.118 (16)
1	0	1	0.033 (4)	0.061 (8)
1	1	0	0.009 (1)	0.001 (0)
1	1	1	0.001 (0)	0.001 (0)
			AA Case Frequencies (n=26)	AA Control Frequencies (n=27)
0	0	0	0.304 (8)	0.354 (10)
0	0	1	0.099 (3)	0.078 (2)
0	1	0	0.503 (13)	0.348 (9)
0	1	1	0.093 (2)	0.127 (3)
1	0	0	-----	0.053 (2)
1	0	1	-----	0.014 (0)
1	1	0	-----	0.021 (1)
1	1	1	-----	0.003 (0)

**Table 3-4. Association of ACC-Beta Estimated Haplotype Combinations with PCOS**

<b>Haplotypes (G194216A, T204540C, G263491A)</b>		<b>Cases (n=122)</b>	<b>Controls (n=130)</b>	<b>OR<sup>a</sup></b>	<b>Exact 95% C.I.</b>
11x	xxx	34 / 88	38 / 92	0.94	0.52 – 1.68
10x	xxx	81 / 41	82 / 48	1.16	0.67 – 2.01
1xx	xxx	39 / 83	38 / 92	1.14	0.64 – 2.02
0xx	xxx	25 / 97	31 / 99	0.82	0.43 – 1.56
100	xxx	39 / 83	41 / 89	1.02	0.58 – 1.80
110	xxx	1 / 121	0 / 130	-----	-----
101	xxx	1 / 121	6 / 124	0.17	0.01 – 1.45
<b>African American subjects</b>					
<b>Haplotypes (G194216A, T204540C, G263491A)</b>		<b>Cases (n=26)</b>	<b>Controls (n=27)</b>	<b>OR<sup>a</sup></b>	<b>Exact 95% C.I.</b>
11x	xxx	6 / 20	8 / 19	0.71	0.17 – 2.88
10x	xxx	17 / 9	16 / 11	1.3	0.37 – 4.60
1xx	xxx	3 / 23	2 / 25	1.63	0.17 – 20.97
0xx	xxx	15 / 11	18 / 9	0.68	0.19 – 2.39
100	xxx	0 / 26	4 / 23	0	0.00 – 1.50
110	xxx	0 / 26	0 / 27	-----	-----
101	xxx	0 / 26	1 / 26	-----	-----

<sup>a</sup>The reference group for each odds ratio estimate is the remaining number of subjects without the haplotype combination being evaluated. Run using StatXact.

**Table 3-5. Association of ACC-Beta Haplotype Frequencies among Caucasian and AA Subjects**

<b>Association in Caucasians</b>				
<b>Status</b>	<b>N</b>	<b>Ln(L)</b>	<b><math>\chi^2</math></b>	<b>P-value</b>
PCOS cases	122	-299.57	39.3	0.5
Controls	130	-322.83	47.49	
PCOS + controls	252	-625.4	80.94	
<b>Association in African Americans</b>				
PCOS cases	26	-45.19	1.56	0.25
Controls	27	-59.88	4.04	
PCOS + controls	53	-109.9	4.07	

Calculated using t5 statistic provided by EH software package

**Table 3-6. Association of P12A/G972R genotype combinations with PCOS**

<b>Genotype Combination</b>	<b>Caucasian Cases (n=122)</b>	<b>Caucasian Controls (n=130)</b>	<b>OR<sup>a</sup> (95% CI)</b>
11/11	64/58	70/60	0.94 (0.56 – 1.60)
11/12	12/110	12/218	1.07 (0.42 – 2.73)
11/22	2/120	0/130	-----
12/11	37/85	41/89	0.94 (0.53 – 1.67)
12/12	3/119	5/125	0.63 (0.10 – 3.33)
22/11	4/118	1/129	4.37 (0.42 – 216.99)
22/12	0/122	1/129	-----
<b>Genotype Combination</b>	<b>AA Cases (n = 26)</b>	<b>AA Controls (n=27)</b>	<b>OR<sup>a</sup> (95% CI)</b>
11/11	21/5	20/7	1.47 (0.33 – 6.87)
11/12	3/23	5/22	0.57 (0.08 – 3.40)
11/22	0/26	1/26	-----
12/11	2/24	1/26	2.17 (0.10 – 132.66)
12/12	0/26	0/27	-----
22/11	0/26	0/27	-----
22/12	0/26	0/27	-----

a. calculated using StatXact

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#### **4.0 THE ASSOCIATION OF GENETIC VARIANTS OF PPAR-GAMMA, INSULIN RECEPTOR SUBSTRATE-1, LIPOPROTEIN LIPASE, AND ACETYL-COA CARBOXYLASE-BETA WITH INSULIN SENSITIVITY AND SYSTEMIC INFLAMMATION AMONG WOMEN WITH POLYCYSTIC OVARY SYNDROME AND CONTROL SUBJECTS**

##### **4.1 ABSTRACT**

Background: Polycystic ovary syndrome (PCOS) is a heterogeneous condition associated with obesity and insulin resistance (IR). Previous studies of obese and type 2 diabetic populations have found that intramuscular fat storage is strongly correlated with IR, suggesting a shared or related genetic component. Genotypes associated with fatty acid metabolism may elucidate how fat storage may impact insulin sensitivity among PCOS cases.

Study Design: Retrospective case-control

Specific Aim: To evaluate the association of the P12A variant of PPAR- $\gamma$ , G972R of IRS-1, ACC- $\beta$ , one LPL SNP, and the P12A/G972R variant combinations with insulin sensitivity (HOMA-IR) and C-reactive protein (CRP) concentrations among PCOS case and control subjects.

Methods: DNA was obtained from 304 Caucasian and African American (AA) PCOS case and control subjects (147 PCOS, 157 controls). Fasting blood lipids, insulin, glucose, obesity and CRP were evaluated for association with genotype. One-way analysis of variance tested for each genotype on HOMA-IR and CRP with PCOS status. Multivariate generalized linear regression modeling was used to test the significance of genotype on HOMA-IR and CRP while controlling for relevant covariates.

Results: PCOS cases had significantly higher CRP concentrations ( $p=0.004$ ) and HOMA-IR scores ( $p=0.0003$ ) than controls. Univariate modeling indicated that the IRS-1 variant G972R significantly impacted CRP levels and HOMA-IR. Generalized linear modeling determined that

BMI and triglyceride levels attenuated the association of G972R with HOMA-IR, removing the statistical significance of all other covariates (age, PCOS, G972R, and G972RxPCOS interaction). Generalized linear modeling of CRP included age, BMI, race, G972R, PCOS, and the G972RxPCOS interaction. In this final model, BMI ( $p < 0.001$ ) and race ( $p = 0.003$ ) were significant predictors of CRP concentrations. More importantly, the G972RxPCOS interaction was a significant predictor of CRP ( $p = 0.005$ ). The final model accounted for 22% of variability seen in CRP concentrations.

Conclusions: Using multivariable linear regression modeling, neither the IRS-1 variant G972R nor PCOS were significantly predictors of CRP. However, the interaction of G972R and PCOS significantly predicted CRP concentrations where the R972 allele was associated with higher CRP concentrations among PCOS cases.

## 4.2 INTRODUCTION

The molecular mechanisms responsible for insulin resistance (IR) are poorly characterized. One potential mechanism involves storage of fat in aberrant locations, such as muscle and liver. Previous studies of individuals with IR, obesity and type 2 diabetes (T2DM) suggest that a genetic predisposition leads to ectopic fat deposition rather than this process being solely due to acquired adiposity (1, 2). In this analysis, genotypes selected for their association with fatty acid (FA) metabolism are the P12A variant of peroxisome proliferator-activated receptor gamma (PPAR- $\gamma$ ), the G972R variant of insulin receptor substrate-1 (IRS-1), three single nucleotide polymorphisms of acetyl-CoA carboxylase beta (ACC- $\beta$ ), and one SNP of lipoprotein lipase (LPL). These genotypes are biologically relevant due to their potential role in the pathogenesis of type 2 diabetes and the expression of PCOS.

PPAR- $\gamma$  is a nuclear transcription factor activated by thiazolidinediones (TZDs) and specific fatty acids (3). It appears to be the major regulator of adipogenesis and, therefore, has biological relevance to FA metabolism and insulin resistance/hyperinsulinemia (HI/IR) (4). Research has indicated that PCOS-related insulin resistance may be associated with gene transcription downstream of PPAR- $\gamma$  (5). The genotypes in this analysis are either downstream and under the transcriptional control of PPAR- $\gamma$  (i.e., ACC- $\beta$  and LPL) or have been demonstrated to interact with PPAR- $\gamma$  (i.e., IRS-1). Inconsistent findings have been reported regarding the association of the P12A polymorphism of this gene with insulin sensitivity (6-11), obesity (11-16), and blood lipid concentrations (17, 18). Furthermore, the P12A polymorphism has been shown to have both a positive association (19, 20) as well as a lack of association (21, 22) with PCOS.

The IRS-1 gene was selected for its independent association with PCOS. The IRS-1 gene functions immediately downstream of the insulin receptor. A common mild loss-of-function mutation (G972R) that has been associated with decreased insulin sensitivity (23), T2DM (24) and PCOS (23, 25-27). Stumvoll et al. (28) studied the gene-gene interaction between the P12A variant of PPAR- $\gamma$  and the G972R variant of IRS-1 and found significantly increased insulin sensitivity among the X/Ala carriers compared to Pro/Pro genotyped individuals within the X972Arg background that was not present either in the whole population or against the

Gly972Gly background. The authors concluded that the X/Ala + X/Arg genotype combination was particularly advantageous in the face of the nonprotective Arg972 allele.

Lipoprotein lipase (LPL) is a serine esterase expressed in adipocytes and striated muscle. LPL gene activity is selectively induced by PPAR- $\gamma$  in adipose tissue and its main function is the hydrolysis of triglycerides in triglycerides-rich lipoproteins, such as chylomicrons and very low density lipoproteins (29). The FFAs released by triglyceride hydrolysis are oxidized to generate ATP in muscle. In adipose tissue, FFA are re-esterified and stored in adipose tissue. Hence, LPL is pivotal in lipoprotein and energy metabolism.

ACC is a complex multifunctional enzyme system that is indirectly affected by PPAR- $\gamma$ 's control of carnitine palmitoyl transferase-1 (CPT-1) synthesis. ACC is a biotin-containing enzyme that catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, the rate-limiting step in fatty acid synthesis, which then directly affects CPT-1 production (30). The beta form (ACC- $\beta$ ) may be involved in the provision of malonyl-CoA or in the regulation of fatty acid uptake and oxidation by mitochondria. ACC- $\beta$  is relevant to this study due to its critical role in fatty acid oxidation (31). The potential roles of LPL and ACC- $\beta$  have not previously been examined in women with PCOS.

The specific aims of this study were to evaluate the association of the P12A variant of PPAR- $\gamma$ , G972R of IRS-1, ACC- $\beta$  SNPs (G263491A, T204540C, and G194216A) and haplotypes, LPL SNP A7634966C, and the P12A/G972R variant combinations with insulin sensitivity and systemic inflammation (as measured by C-reactive protein) among Caucasian and AA control and PCOS case subjects.

## **4.3 METHODS**

### **4.3.1 Subjects**

The present analysis was conducted using women recruited for the Cardiovascular Health and Risk Measurement Study (CHARM). The CHARM study was established in 1992 to investigate

the effect of polycystic ovary syndrome on cardiovascular risk factors and associated disease (i.e., CVD) in women. Women with PCOS, the population of women recruited for CHARM may be considered at high-risk for developing CVD. Women diagnosed with PCOS between 1970 and 1993 who were at least 30 years of age at the time of recruitment were identified from the records of an academic reproductive endocrine practice located at Magee-Womens Hospital, Pittsburgh, PA. The clinical diagnosis of PCOS was made if there was (1) a history of chronic anovulation in association with either (A) clinical evidence of androgen excess (hirsutism) or biochemical evidence of an elevated total testosterone concentration ( $>57.64$  ng/dl ( $2\text{nmol/l}$ )) or (B) a ratio of luteinizing to follicle stimulating hormone  $> 2.0$ . Eligible women were contacted by phone between 1992 and 1994 for a telephone interview and for further recruitment for a clinical visit. During that time, age ( $\pm 5$  years)- and race-matched neighborhood control subjects were selected using a combination of voter's registration tapes for 1992 from the Greater Pittsburgh area and Cole's Cross Reference Directory of Households and were similarly recruited. After initial phone contact, 244 PCOS-affected women and 244 controls completed a clinical visit where they received weight and height measurement, a fasting blood draw, waist and hip measurements, standard blood pressure assessment and a questionnaire-based interview. In 1996-1999, the same population of women was re-contacted for a second clinical visit also which included weight and height assessment, a fasting blood draw, waist and hip measurements, standard blood pressure assessment and a questionnaire-based interview, including questions on age at visit and self-reported race (Caucasian, AA, Asian, or other). Of the original 488 women seen between 1992 and 1994, 335 were enrolled for a second clinical visit. At this second visit, 329 women consented to a blood draw for DNA analysis. After genomic DNA extraction, 24 samples were devoid of leukocytes and were unusable for further analyses. One subject was excluded on the basis of an insulin score well over 3 SD from the mean, which significantly affected insulin-based outcome measures. For this analysis, obesity was defined by body mass index (BMI) (weight in kg/height in  $\text{m}^2$ ) and by waist-to-hip ratio (waist in cm/hip in cm). A BMI  $<25$   $\text{kg}/\text{m}^2$  was normal weight,  $<35$   $\text{kg}/\text{m}^2$  was defined as overweight, and  $\geq 35$   $\text{kg}/\text{m}^2$  was defined as obesity. The present analysis is comprised of the remaining 304 follow-up visit women (147 cases and 157 controls), of which 251 were Caucasian (121 cases and 130 controls) and 53 were AA (26 cases and 27 controls). All participants gave written, informed consent as approved by the Institutional Review Board of the University of Pittsburgh.

## **4.3.2 Laboratory analyses**

### **4.3.2.1 Blood lipids**

All blood lipid assessments (mg/dl) and fasting glucose (mg/dl) were measured at the Heinz Nutrition Laboratory under the direction of Dr. Rhobert Evans. The laboratory is carefully monitored and participates in the Centers for Disease Control standardization programs. High density (HDL) and low density (LDL) lipoproteins were determined after selective precipitation by heparin/manganese chloride and removal by centrifugation of very low density (VLDL) (32). Duplicate samples, standards and control sera were included in each run. The coefficient of variation between runs was 2.1%. Triglycerides were determined enzymatically using the procedure of Bucolo et al. (33). Duplicate samples, standards and control sera were included in each triglyceride run. Coefficient of variation between runs was 1.7%.

### **4.3.2.2 Insulin and glucose measurement**

Serum insulin levels (mU/L) were assessed using RIA (Linco, Research, Inc., St. Charles, MO). Cross-reactivity of the antibody with pro-insulin was less than 0.2%. The interassay coefficient of variation was  $2.6 \pm 0.7\%$ . Standards, blanks, and quality controls were run concurrently with all samples. Glucose (mg/dl) was quantitatively determined by an enzymatic determination read at 340/380 nm with a procedure utilizing the coupled enzyme reactions catalyzed by hexokinase and glucose-6-phosphate dehydrogenase (34). The coefficient of variation between runs was 1.8%. Fasting glucose and insulin were used to assess homeostasis assessment model (HOMA-IR), a measure of insulin resistance. In HOMA-IR, values were calculated from the fasting concentrations of insulin and glucose using the following formula: (fasting serum insulin (mU/L) x fasting plasma glucose (mmol/L))/22.5 (35). HOMA-IR ( $\text{mU} \cdot \text{mmol/L}^2$ ) has been shown to be significantly correlated with clamp IR in a large number of subjects with both normal and impaired glucose tolerance (6, 9) and with the index of sensitivity obtained from the fasting intravenous glucose tolerance testing among normal and insulin resistant volunteers, as well as diabetics (36). Abnormal glucose status (AGS) for this analysis was defined as a glucose level  $\geq$  110 mg/dl.

#### 4.3.2.3 C-reactive protein

C-reactive protein (CRP; mg/L) was measured by ultrasensitive competitive immunoassay based on purified protein and polyclonal anti-CRP antibodies (Calbiochem, La Jolla, CA). The CRP assay had a sensitivity of 0.08 µg/ml and an average interassay coefficient of variation of 8.0%. This assay is sensitive to values within the normal range and CRP levels obtained at one point in time have been shown to be both reproducible and representative over extended periods of time (37).

#### 4.3.2.4 Genotype Analyses

Genomic DNA was assessed for blood samples drawn from 147 PCOS case and 157 control subjects (Caucasian and AA) seen at the second visit between 1996 and 1999. Buffy coats were collected from 20 cc whole blood from each CHARM individual seen at the second visit and immediately frozen at  $-80^{\circ}$  C at the University of Pittsburgh, Graduate School of Public Health, Heinz Nutrition Laboratory. Genomic DNA was subsequently extracted in 2004 using established methods (38). Ambiguous samples were analyzed a second time. ***P12A***: Molecular genetic analysis of P12A was performed using the polymerase chain reaction (PCR) primers, sense (5'-GGCCAATTCAAGCCCAGTC-3') and anti-sense (5'-GATATGTTTGCAGACAGTGTATCAGTGAAGGAATCGCTTTCCG-3'), producing a 270-base pair (bp) PCR product. Carrier status of the P12A variant of the PPAR- $\gamma$  gene was determined by restriction fragment length polymorphism (RFLP) analysis (12). ***G972R***: The G972R polymorphism in IRS-1 involved *Bst*NI restriction enzyme digestion of a 262-bp PCR product amplified by PCR primers sense (5'-CTTCTGTCAGGTGTCCATCC-3') and anti-sense (5'-TGGCGAGGTGTCCA-CGTAGC-3'). Carrier status of the G972R variant of the IRS-1 gene was determined by restriction fragment length polymorphism (RFLP) analysis (39). ***ACC- $\beta$*** : Three single nucleotide polymorphisms were identified along the ACC- $\beta$  gene located on chromosome 12 (**Figures 4-1 and 4-2**). PCR primers for rs2268403 (A/G) (sense – AGGGAAGAGGCCATTCGTTGGTA-3' and anti-sense – 5'-GGGTTCTTGGCTGTGAACCAAACA-3'), rs2268393 (C/T) (sense – 5'-TGCCAGTTGCACAGAATTCCAACC-3' and anti-sense 5'-ACAATGGGAACAGCTACACCACC-T-3'), and rs3742023 (A/G) (sense – 5'-ATTACCTTGCTCGTCCTGTACCA-3' and anti-sense – 5'-TATGAGGTTAAAGCCAGGCTGTCC) were identified and created using the Primer Quest

primer creation program on Integrated DNA Technologies website ([www.idtdna.com](http://www.idtdna.com)). Thermocycling conditions for ACC- $\beta$  SNPs were 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute, finalized by a 7 minute soak at 72°C. Restriction enzymes used for each SNP were *EarI*, *AfeI*, and *NcoI*, respectively (New England Biolabs, Inc., Beverly, MA). PCR cycling of rs2268403 created a 474-bp product in which an *EarI* restriction site presented concurrently with the G  $\rightarrow$  A change at nucleotide 194216 to generate the G194216A mutation (rs2268403). After *EarI* digestion at 37°C for 2 hours and 3% agarose gel electrophoresis, the expected product sizes were 368 and 106 bp for the G194216 variant; 368, 199, 169, and 106 bp for the heterozygote; and 199, 169, and 106 for G194216A. After thermocycling of rs2268393, the expected product was 225-bp and the sequence contained an *AfeI* restriction site introduced by the T  $\rightarrow$  C variant at nucleotide 204540 to generate the T204540C mutation. Digestion by *AfeI* (37°C for 2 hours) produced the expected lengths of 225 bp for the T204540; 225, 118, and 107 bp for heterozygotes; and 118 and 107 bp for the T204540C mutation. The rs3742023 SNP, after PCR thermocycling, was contained within a 213-bp fragment itself containing an *NcoI* restriction site introduced with the G  $\rightarrow$  A nucleotide 263491 alteration. Expected product sizes were 213 for the G263491 homozygous; 213, 150, and 63 for the heterozygote; and 150 and 63 for the G263491A mutation. ***LPL***: One SNP was identified along the lipoprotein lipase gene located on chromosome 8 (**Figure 4-3**). Single nucleotide polymorphism rs3735964 was assessed by PCR thermocycling with Primer Quest primers identified and created by Integrated DNA Technologies (sense (5'-TGCAATGAGCCAGATGGAGTACCA-3') and anti-sense (5'-TGCTGAAGGACAACACACATGCAG-3')). PCR cycling of rs3735964 created a 237-bp product in which an *EarI* restriction site presented concurrently with the A  $\rightarrow$  C change at nucleotide 7634966 to generate the A7634966C mutation. After *EarI* digestion at 37°C for 2 hours and 3% agarose gel electrophoresis, the expected product sizes were 237 bp for the A7634966 variant; 237, 167, and 70 bp for the heterozygote; and 167 and 70 bp for A7634966C (**Figure 4-4**).

### **4.3.3 Data analyses**

#### **4.3.3.1 Genotype frequencies**

Genotype frequencies for each SNP were computed by gene counting and compared between cases and controls by use of Pearson's  $\chi^2$  tests. Genotype conformation to Hardy-Weinberg equilibrium proportions were tested using Fisher's exact test. All single nucleotide polymorphisms in this study were in Hardy-Weinberg equilibrium.

#### **4.3.3.2 Haplotype estimation**

The haplotype frequencies for ACC- $\beta$  were estimated using the PHASE software program (40, 41). PHASE uses the expectation-maximization algorithm to obtain maximum-likelihood estimates of haplotype frequencies. Haplotypes were described here by a three-digit code, where the first digit indicated the allele present in T204540C, the second indicated G194216A, and the last referred to G263491A. A "0" in T204540C meant the estimated allele present is a "T" and a "1" represented a "C". In G194216A and G263491A, a "0" represented a "G", and a "1" indicated an "A" allele. For example, an ACC- $\beta$  haplotype of "100" for the ACC- $\beta$  gene meant that the subject had the "C" allele for T204540C, a "G" allele for G194216A, and a "G" allele for G263491A.

#### **4.3.3.3 Association analyses**

One-way analysis of variance was used to test for significant differences in mean HOMA-IR and CRP concentrations within case status among the each genotype variant and the ACC- $\beta$  haplotype groups. Multivariable generalized linear regression modeling was used to test the significance of genotype, the P12A/G972R genotype combination and ACC- $\beta$  haplotype differences on HOMA-IR and CRP while controlling for BMI, race, current smoking, family health history of selected chronic diseases including PCOS, triglycerides, and HDL cholesterol. Statistical significance defined as a p-value <0.05. Analysis packages used in this manuscript were SAS, version 8 (SAS Institute, Inc., Cary, NC) and PHASE, version 2.1.

## 4.4 RESULTS

### 4.4.1 Demographic Characteristics among Caucasian and AA Subjects

In **Table 4-1**, PCOS cases were significantly more obese (BMI  $31.8 \pm 8.8$  vs.  $28.1 \pm 6.7$  kg/m<sup>2</sup>,  $p < 0.0001$ ; WHR  $0.82 \pm 0.08$  vs.  $0.79 \pm 0.08$ ,  $p = 0.01$ ) than controls and presented with lower HDL concentrations ( $54.2 \pm 15.5$  vs.  $58.1 \pm 14.9$  mg/dl;  $p = 0.01$ ). Both diastolic ( $75.9 \pm 9.6$  vs.  $74.2 \pm 9.1$  mmHg;  $p = 0.10$ ) and systolic ( $116.5 \pm 15.4$  vs.  $114.8 \pm 14.3$  mmHg;  $p = 0.25$ ) blood pressures were similar between cases and controls. Caucasian cases also had higher CRP concentrations ( $3.8 \pm 5.3$  vs.  $2.6 \pm 3.0$  mg/L;  $p = 0.004$ ). Insulin levels were significantly elevated among PCOS cases compared to controls ( $19.5 \pm 13.1$  vs.  $15.0 \pm 9.8$  mg/dl,  $p = 0.0001$ ) and insulin sensitivity was significantly lower among PCOS cases (HOMA-IR  $5.2 \pm 5.3$  vs.  $3.8 \pm 4.7$  mU · mmol/L<sup>2</sup>,  $p = 0.0003$ ). PCOS cases were significantly more likely to have first-degree family members with PCOS (14.3% vs. 3.2%;  $p = 0.002$ ) as well as being more likely to present with abnormal glucose status (17.2% vs. 5.9%;  $p = 0.002$ ). Women with PCOS and controls were similar in their rates of smoking, oral contraceptive use and hormone replacement therapy use (NS).

### 4.4.2 Estimated Allelic Frequencies among Caucasian and AA Subjects

Among Caucasian participants, similar genotype frequencies existed between cases and control subjects for all genotypes and no significance was found between case and control allele frequencies (**Table 4-2**). Genotype frequencies of AA case and control subjects, while not statistically significant, had larger differences than Caucasian subjects. AA cases had a borderline statistically significantly higher rate of the G/G allele in the G194216A SNP of ACC- $\beta$  than same race controls (100% vs. 81%;  $p = 0.05$ ).

### 4.4.3 Genotype Associations with Outcome Variables

All genotypes were assessed for potential impact on HOMA-IR and CRP (Data not shown) and only the G972R variant of IRS-1 was significantly associated. The main effect of IRS-1 variant G972R was assessed by comparing mean HOMA-IR and CRP levels between G/G genotype carriers and G/R+R/R genotype carriers (**Table 4-3**). The mean HOMA-IR score was significantly higher among G/R+R/R carriers than G/G carriers ( $6.3 \pm 8.4$  and  $4.2 \pm 4.2$  mU · mmol/L<sup>2</sup>, respectively;  $p=0.01$ ). Comparing genotypes within case status, the G/R+R/R variant was uniformly associated with higher HOMA-IR scores than the G/G variant within both cases and controls. Due to this, the IRS-1 variant G972R by PCOS interaction was not found to be significant ( $p=0.61$ ). CRP concentrations were similarly assessed by comparing mean differences across genotype. The G/R+R/R variant was associated with significantly higher CRP levels than the G/G isoform ( $4.8 \pm 7.9$  and  $3.0 \pm 3.4$  mg/L, respectively;  $p=0.01$ ). CRP levels were also found to be significantly higher among PCOS case than control subjects ( $p=0.004$ ). The G972RxPCOS combined effect determined a differential effect of genotype on controls and cases. Similar CRP levels were observed between controls carrying the G/R+R/R and G/G genotypes ( $2.8 \pm 3.2$  vs  $2.6 \pm 3.0$  mg/L). However, CRP concentrations varied between case subjects carrying G/R+R/R and G/G genotypes ( $7.1 \pm 10.6$  vs  $3.3 \pm 2.6$  mg/L). The G972R x PCOS interaction was statistically significant in this analysis ( $p=0.01$ ).

Predictors of HOMA-IR were assessed to determine the basis for significance in varying HOMA-IR scores observed between cases and controls (**Table 4-4**). In the first model (Model 1) evaluating main effects only, age was not predictor of HOMA-IR ( $p = 0.41$ ), however, BMI ( $\beta = 0.18$ ;  $p < 0.0001$ ) and triglycerides ( $\beta = 0.02$ ;  $p < 0.0001$ ) were both highly significant predictors of HOMA-IR. In Model 2, main effects of IRS-1 variant G972R and PCOS and the G972RxPCOS interaction were added to Model 1. In this model, neither G972R ( $p=0.14$ ), PCOS ( $p = 0.58$ ), nor the G972RxPCOS interaction ( $p = 0.96$ ) were significant predictors of HOMA-IR scores. Overall, this final model accounted for 25% of variability in HOMA-IR ( $R^2 = 0.247$ ).

**Table 4-5** explored the main effects and interaction effects of IRS-1 variant G972R and significant covariates on CRP. As in HOMA-IR, age was not a significant predictor of CRP ( $p = 0.94$ ), yet BMI ( $\beta = 0.18$ ;  $p < 0.0001$ ) and race ( $\beta = 1.85$ ;  $p = 0.009$ ) were significant main

effects predictors. When genotype, PCOS, and the G972R x PCOS interaction term were added to the model, neither G972R nor PCOS were significant predictors. However, the G972RxPCOS interaction term ( $\beta = 3.68$ ;  $p = 0.005$ ) was statistically significant, indicating that PCOS cases with the R972 allele had statistically significantly higher CRP concentrations than all other comparison groups. The final model, including all main effects and the G972R x PCOS interaction effect accounted for 22% of the variability seen in CRP levels ( $R^2 = 0.223$ ).

#### 4.5 DISCUSSION

The P12A variant of PPAR-gamma has been found in several studies to significantly associate with obesity (11-16). In the current analyses, BMI was strongly correlated with the PCOS phenotype, so much so as to potentially obscure the relationship between subclinical causal mechanisms of diabetic or CVD outcomes and PCOS, such as HOMA-IR or CRP.

LPL SNP A7634966C, the ACC- $\beta$  SNPs (G263491A, T204540C, and G194216A) or ACC- $\beta$  haplotype were evaluated for significant association with HOMA-IR and CRP. Studies of variants among the LPL gene have indicated an association with insulin sensitivity among type 2 diabetics (43) and Mexican Americans with atherogenic profiles (44), as well as adverse lipid profiles among atherogenic men and women from Geneva (45). The ACC- $\beta$  gene has been less extensively studied in relation to metabolic phenotypes among humans, but has been found to relate to fat storage (46), obesity(47), and T2DM (47) among ACC- $\beta$   $-/-$  mutant mice. The ACC- $\beta$  SNPs chosen for these analyses were selected for their distribution across the ACC- $\beta$  gene to allow a “genotype” analysis (i.e., haplotype analysis). However, these analyses did not reveal any significant associations of LPL SNP A7634966C or the ACC- $\beta$  gene with PCOS or associated outcomes (i.e., HOMA-IR or CRP). The smaller sample size could have limited the power of this analysis and results should be verified among a larger population.

The G972R isoform of IRS-1 has previously been significantly associated with obesity (42) and, in these analyses, the G972R genotype seemed to significantly interact with PCOS to affect CRP concentrations. Even after adjustment for age, BMI, race, and triglycerides, G972R and the

PCOS/G972R interaction remained significant with CRP levels. The conclusion being that G972R interacted with case status to affect CRP levels so that women with PCOS who are carriers of the R972 allele may be at increased risk of having elevated CRP concentrations. This intriguing data is consistent with the emerging relationships between obesity, inflammation, and insulin resistance.

Even though C-reactive protein has been genetically studied in relation to inflammatory genetic markers, namely the CRP gene (48-50) and the interleukin-6 gene (51, 52), and CRP gene activity is under the transcriptional control of nuclear transcription factors (i.e., PPAR- $\gamma$ ), CRP activity has not been studied in association with insulin resistance-related genetic polymorphisms before now. Furthermore, this study includes genotypes that, since their discovery by Haga et al. in 2002 (53) by genome wide scans among 24 unrelated Japanese women, have not been studied for their affect on insulin sensitivity or systemic inflammation. We suggest that future studies based upon these results might elucidate connective mechanisms not previously explored.

The main limitation of this study is its lack of power to detect significant differences among PCOS-affected AA subjects and their controls. Compared to Caucasian case and controls subjects, AA subjects did not significantly differ by case status in measurement of any outcome variable. Given the smaller sample size of AA subjects (26 cases and 27 controls), this analysis had 39% power to detect a 4.5 kg/m<sup>2</sup> difference in BMI, the variable closest to statistical significance (p=0.08), and would have required approximately 120 case and control subjects (1:1) to have 70% power to detect this same difference. Caucasian cases and controls had 88% power to detect a 2% mean difference in BMI, indicating sufficient power within this subgroup.

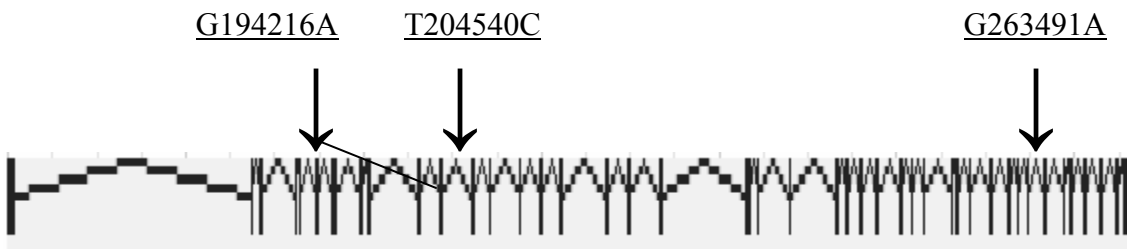
In summary, the P12A variant of PPAR- $\gamma$ , the G972R variant of IRS-1, the three single nucleotide polymorphisms of ACC- $\beta$  (G263491A, T204540C, and G194216A), and the A7634966C SNP of LPL did exhibit a potential for significantly impacting on the expression of HOMA-IR and CRP. Overall, the main finding of this study is the novel association of CRP concentrations with the interaction of IRS-1 variant G972R and PCOS over and above age, BMI and race. These results indicate that among women with PCOS, carriers of the R972 allele of IRS-1 variant G972R have significantly increased risk of presenting with elevated CRP concentrations compared to any other G972RxPCOS interaction classification. These analyses

introduce a previously unexplored avenue for future research into the relationship between insulin resistance and inflammatory factors.



**Figure 4-1. The human chromosome 12**

The red arrow indicates the location of the ACC- $\beta$  gene.



**Figure 4-2. The ACC-Beta gene**

Each vertical line along the ACC- $\beta$  gene (running 5' to 3') indicates a known allelic variant. The black arrows indicate the location of the three SNPs in this analysis.



**Figure 4-3. The human chromosome 8**

The red arrow indicates the location of the LPL gene.



All figures obtained from [www.ncbi.nih.gov](http://www.ncbi.nih.gov).

**Figure 4-4. The LPL gene**

Each vertical line along the LPL gene (running from 5' to 3') figure denotes a known allelic variant. The black arrow indicates the location of SNP A7634966C.

**Table 4-1. Prevalence of Demographic Characteristics Among Caucasian and AA Subjects**

Outcome Variable	Cases (N = 147)	Controls (N = 157)	P-value
Age (mean years $\pm$ SD)	41.5 $\pm$ 7.2	42.8 $\pm$ 7.1	0.12
Body mass index (mean kg/m <sup>2</sup> $\pm$ SD)	31.8 $\pm$ 8.8	28.1 $\pm$ 6.7	<b>&lt;0.001</b>
Waist:Hip ratio (mean $\pm$ SD)	0.82 $\pm$ 0.08	0.79 $\pm$ 0.08	<b>0.01</b>
SBP (mean mmHg $\pm$ SD)	116.5 $\pm$ 15.4	114.8 $\pm$ 14.3	0.25
DBP (mean mmHg $\pm$ SD)	75.9 $\pm$ 9.6	74.2 $\pm$ 9.1	0.10
Race			0.91
Caucasian	121	130	-----
African American	26	27	-----
C-reactive protein (mean mg/L $\pm$ SD)	3.8 $\pm$ 5.3	2.6 $\pm$ 3.0	<b>0.004</b>
Cholesterol (mean mg/dl $\pm$ SD)	209.4 $\pm$ 36.8	202.8 $\pm$ 33.8	0.13
Triglycerides (mean mg/dl $\pm$ SD)	139.9 $\pm$ 83.1	122.0 $\pm$ 65.9	0.07
HDL (mean mg/dl $\pm$ SD)	54.2 $\pm$ 15.5	58.1 $\pm$ 14.9	<b>0.01</b>
LDL (mean mg/dl $\pm$ SD)	127.6 $\pm$ 34.3	119.8 $\pm$ 31.3	0.07
HDL2 (mean mg/dl $\pm$ SD)	14.8 $\pm$ 9.7	17.1 $\pm$ 12.4	0.09
Smoking (%; N)	31.5 (29/147)	27.6 (29/157)	0.83 <sup>a</sup>
Taking OC (%; N)	10.9 (16/147)	13.4 (21/157)	0.51 <sup>a</sup>
Taking HRT (%; N)	11.6 (17/147)	14.0 (22/157)	0.52 <sup>a</sup>
First degree relative with PCOS (%; N)	14.3 (21/147)	3.2 (5/157)	<b>0.002<sup>a</sup></b>
Fasting glucose (mean mg/dl $\pm$ SD)	98.9 $\pm$ 28.0	95.0 $\pm$ 28.9	0.20
Fasting insulin (mean mlU/ml $\pm$ SD)	19.5 $\pm$ 13.1	15.0 $\pm$ 9.8	<b>&lt;0.001</b>
HOMA-IR (mean mU $\cdot$ mmol/L <sup>2</sup> $\pm$ SD)	5.2 $\pm$ 5.3	3.8 $\pm$ 4.7	<b>&lt;0.001</b>
Abnormal glucose status (%; N) <sup>b</sup>	17.2 (25/145)	5.9 (9/155)	<b>0.002<sup>a</sup></b>

- a. P-value calculated using StatXact Fisher's exact test for two independent binomials
- b. Abnormal glucose status defined as fasting glucose  $\geq$  110 mg/dL

**Table 4-2. Estimated Genotype Frequencies Among Caucasian and AA Subjects**

<b>Caucasian subjects</b>				
<b>Genotypes<sup>a</sup></b>	<b>Cases</b>	<b>Controls</b>	<b><math>\chi^2</math></b>	<b>P-value<sup>b</sup></b>
<b>LPL SNP</b>				
A7634966C – C/C (vs. X/A)	0.81 (98/121)	0.81 (106/130)	0.007	0.91
<b>ACC-<math>\beta</math> SNPs</b>				
G263491A – G/G (vs. X/A)	0.42 (51/121)	0.42 (55/130)	0.0006	0.98
T204540C – X/C (vs. T/T)	0.81 (98/121)	0.80 (104/130)	0.04	0.84
G194216A – G/G (vs. X/A)	0.68 (82/121)	0.65 (84/130)	0.28	0.60
<b>PPAR-<math>\gamma</math> SNP</b>				
P12A – Pro/Pro (vs. X/Ala)	0.64 (77/121)	0.63 (82/130)	0.008	0.93
<b>IRS-1 SNP</b>				
G972R – Gly/Gly (vs. X/Arg)	0.86 (104/121)	0.86 (112/130)	0.002	0.96
<b>African American subjects</b>				
<b>LPL SNP</b>				
A7634966C – C/C (vs. X/A)	0.81 (21/26)	0.81 (22/27)	0.004	0.95
<b>ACC-<math>\beta</math> SNPs</b>				
G263491A – G/G (vs. X/A)	0.65 (17/26)	0.59 (16/27)	0.21	0.65
T204540C – X/C (vs. T/T)	0.85 (22/26)	0.78 (21/27)	0.40	0.52
G194216A – G/G (vs. X/A)	1.00 (26/26)	0.81 (22/27)	5.32	<b>0.05<sup>c</sup></b>
<b>PPAR-<math>\gamma</math> SNP</b>				
P12A – Pro/Pro (vs. X/Ala)	0.92 (24/26)	0.96 (26/27)	0.39	0.53
<b>IRS-1 SNP</b>				
G972R – Gly/Gly (vs. X/Arg)	0.88 (23/26)	0.78 (21/27)	0.07	0.30

- a. Genotype represents most common genotype
- b. Pearson  $\chi^2$  test used
- c. P-value calculated using StatXact Fisher's exact test for two independent binomials

**Table 4-3. The Effect of the G972R variant of IRS-1 on HOMA-IR and CRP Among PCOS Case and Control Subjects**

IRS-1 Variant G972R	PCOS Status	HOMA-IR		CRP	
		Mean ( $\pm$ SD)	P-value	Mean ( $\pm$ SD)	P-value
G/G		4.2 ( $\pm$ 4.2)	Main effect	3.0 ( $\pm$ 3.4)	Main effect
G/R + R/R		6.3 ( $\pm$ 8.4)	(0.01)	4.8 ( $\pm$ 7.9)	(0.01)
	Control	3.8 ( $\pm$ 4.7)	Main effect	2.6 ( $\pm$ 3.0)	Main effect
	Case	5.2 ( $\pm$ 5.3)	(0.0003)	3.8 ( $\pm$ 5.3)	(0.004)
G/G	Control	3.6 ( $\pm$ 3.9)	Genotype x	2.6 ( $\pm$ 3.0)	Genotype x
G/R + R/R	Control	5.3 ( $\pm$ 7.6)	PCOS	2.8 ( $\pm$ 3.2)	PCOS
G/G	Case	4.8 ( $\pm$ 4.3)	Interaction	3.3 ( $\pm$ 2.6)	Interaction
G/R + R/R	Case	7.4 ( $\pm$ 9.3)	(0.61)	7.1 ( $\pm$ 10.6)	(0.01)

Generalized linear modeling used.

**Table 4-4. Predictors of HOMA-IR Among PCOS Case and Control Subjects**

Predictor	Model 1		Model 2	
	$\beta$	P-value	$\beta$	P-value
Age (in years)	-0.03	0.41	-0.03	0.41
BMI	0.19	<0.0001	0.18	<0.0001
Triglycerides	0.02	<0.0001	0.02	<0.0001
G972R of IRS-1			1.65	0.10
PCOS			0.31	0.58

Generalized linear modeling used.

**Table 4-5 Predictors of CRP Among PCOS Case and Control Subjects**

Predictor	Model 1		Model 2	
	$\beta$	P-value	$\beta$	P-value
Age (in years)	-0.003	0.94	-0.0009	0.98
BMI	0.19	<b>&lt;0.0001</b>	0.18	<b>&lt;0.0001</b>
Race	1.65	<b>0.009</b>	1.85	<b>0.003</b>
G972R of IRS-1			-0.47	0.60
PCOS			0.07	0.89
G972R of IRS-1 x PCOS			3.68	<b>0.005</b>

Generalized linear modeling used.

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## 5.0 SUMMARY AND CONCLUSIONS

The research addressed in this dissertation has been segmented into three complementary topics as follows:

1. the increased risk of T2DM conferred to women by a diagnosis of PCOS;
2. the potential association of novel lipogenic genotypes with PCOS; and
3. the association of these novel lipogenic genotypes with subclinical measures of insulin resistance and systemic inflammation among PCOS-affected women, by which an increased risk of T2DM might be explained.

### 5.1 ASSOCIATION OF PCOS WITH T2DM

It has been recognized that women with PCOS have increased risk of developing T2DM. Incidence rates of T2DM in two prior studies were 9% (1) and 16% (2) among women with PCOS at baseline, regardless of basal glucose tolerance. Even though these studies had small cohort sizes, younger age groups, and shorter follow-up periods, the risks of their populations developing T2DM starting from either IGT or normal glucose tolerance were similar to the 13.4% rate of progression found in our population. Our population allowed insight into the natural development of T2DM in women with PCOS, mainly due to older age at first visit (38.0 years for cases and 40.0 years for controls) and length of follow-up time (8 years) (i.e., age at follow-up: cases =  $46.6 \pm 5.98$  years, controls =  $48.1 \pm 5.36$  years;  $p = 0.08$ ).

BMI was found in these analyses to be a contributing factor toward development of T2DM; a fact supported by previous studies (1, 2). However, unlike previous investigations where only women with PCOS were included, these analyses were capable of investigating how BMI

interacted with PCOS through determination of its effect on risk of T2DM in control subjects. We found that BMI was both a confounder and an effect modifier of PCOS on development of T2DM. Specifically, BMI was not the only contributing factor to the development of T2DM. Compared to controls (HR=1.0), PCOS conferred ~1.5 times the risk of developing T2DM and a much higher ~5.1-fold risk was observed in morbidly obese cases.

One explanation for the increased incidence of T2DM found in PCOS cases was the association of IR with PCOS, which is found in approximately 50% to 70% of affected women (3). Compared to the prevalence of IR found in the general US population, PCOS confers a 2- to 4-fold higher risk of developing IR (4). Further, IR alone is a risk factor for the development of T2DM and, given that PCOS affects ~5% of the reproductive-aged female population in the US, the increased risk of developing T2DM attributed to PCOS could affect up to 3.5% of the US female population.

Other factors which may contribute to increased risk of T2DM are the hyperinsulinemia and hyperandrogenemia that co-exists with IR in PCOS-affected women. One major hypothesis of how insulin sensitivity is related to the PCOS phenotype is based on the insulin-glucose-androgen pathway. In this hypothesis, it is postulated that primary peripheral IR may increase circulating insulin levels to compensate for decreased insulin sensitivity or dysfunctional glucose metabolism. In an effort to subdue rising glucose concentrations at the periphery, secondary hyperinsulinemia may produce HA by over-stimulating insulin-sensitive, androgen-secreting tissues (i.e., the ovarian theca cell). The resulting hyperandrogenemia may then directly or indirectly suppress ovulation at the level of the ovary (5) causing infertility. Androgen levels in women with PCOS have been positively correlated with measures of hyperinsulinemia in several studies (6-10) and, thus, may be associated with development of T2DM.

## **5.2 ASSOCIATION OF GENOTYPE WITH PCOS**

The strong genetic basis of PCOS has been well demonstrated by family studies of first degree relatives of affected women (11-16), as well as by the presence of IR among lean women with

PCOS (17-20). In this study, allele frequencies for the P12A variant of PPAR- $\gamma$ , the G972R isoform of IRS-1, three ACC- $\beta$  SNPs (T204540C, G194216A, and G263491A) and their haplotypes, LPL SNP A7634966C, and the P12A-G972R combined genotypes were assessed for association with PCOS. No genotype frequencies were significantly different between controls and PCOS-affected women. There were also no significant associations of the ACC- $\beta$  haplotype or combinations of ACC- $\beta$  haplotypes with PCOS. Furthermore, the genotype frequencies for the P12A + G972R combined genotypes did not seem to interact with case/control status. Significant results in previous studies of P12A (21, 22) could have been, at least partially, due to differing diagnostic criteria used for PCOS (i.e., diagnosis based upon polycystic-appearing ovaries rather than clinical and hormonal measures).

Women with PCOS are generally more overweight than age- and race-matched control subjects putting them at increased risk for IR and, though it is not clear how obesity and IR are interrelated, evidence suggests that elevated FFA may be mediators in this association. This interrelationship can be evidenced in findings that most obese people have elevated FFA plasma levels (23, 24) and that both chronic and acute elevation of plasma FFA produce acute IR (25-28). The potential in this study for association of PCOS with the selected candidate genes was based upon significant findings of similar studies among diabetic populations (29, 30) and upon knowledge of cellular mechanisms by which IR may occur. Two main theories of obesity-related IR were addressed in this analysis and determined the selected candidate genotypes. The first hypothesis explored was the ectopic fat storage hypothesis, which establishes that IR is the result of a skeletal muscle composition disorder where lipid storage inside muscle tissue causes metabolic dysfunction. The second hypothesis that may be considered mutual and complementary was Neel's hypothesis of the thrifty genotype (31), which purports that the ability to store excess energy was advantageous in ancestral societies subjected to periods of starvation, but is disadvantageous during periods of energy excess, as is commonly found in modern society. Both of these hypotheses imply the genetic basis of metabolic dysfunction as a causal mechanism of certain disease like obesity, T2DM, and PCOS.

PPAR- $\gamma$  plays a central role in adipogenesis (32-34) based on two main processes. Firstly, the insulin sensitizing effects of activated PPAR- $\gamma$  are based on increased expression of target genes that promote FA trapping and storage in adipocytes, such as LPL (35). Secondly, these effects are the result of repression of genes that induce lipolysis and the release of FAs (36),

leptin (37, 38) and TNF- $\alpha$  (39, 40). Among diabetic populations, TZDs effect PPAR- $\gamma$  activation (41, 42) to increase insulin sensitivity via these processes. TZDs function via redistribution of white adipose tissue resulting in decreased visceral depots relative to subcutaneous fatty regions (41-44). This fat cell redistribution includes a shift in the cell type population resulting in more small adipocytes and fewer large, insulin insensitive adipocytes (45-47). The insulin sensitizing effects of TZDs on PCOS-affected women through these mechanisms also seem to increase fertility and ovulation. The PCOS phenotype seems to be intricately bound to FA metabolism through the PPAR- $\gamma$  pathway and, considering PPAR- $\gamma$ 's apparent master gene status, divergent results between studies are not necessarily an indication of anomaly so much as an indication of the complexity of PCOS. Its phenotypic diversity is probably a reflection of its genotypic heterogeneity and the lack of significance of the P12A variant in this analysis was most probably due to the use of unrelated cases and controls, thereby increasing the genetic heterogeneity within this population.

LPL is a candidate gene whose expression is partially regulated by PPAR- $\gamma$  and is included in this study due to its direct impact on FA metabolism. FA utilization is impacted by expression and transcription of LPL. The expression of LPL is attenuated by insulin, directly impacting FA utilization (48, 49), and by diseases such as atherosclerosis and diabetes (50-52). Metabolites that induce LPL gene transcription include the PPARs in liver and adipose tissue and in macrophages in response to fibrates, some FAs, glucose, and TZDs (35, 51, 53). Decreased LPL activity has been seen in individuals with T2DM and IR (54-57). Furthermore, the resultant decrease in LPL activity contributes to hypertriglyceridemia, decreased HDL levels, and increased risk of CHD (58). Since LPL is regulated by insulin resistance disorders, a mutation affecting the activity of the LPL gene would not be surprising among PCOS-affected women. This analysis did not find such an effect, but it cannot be ruled out as only one SNP was used to test our hypothesis. Testing multiple SNPs may give more power to test for a gene effect on disease.

According to Neel's thrifty genotype theory (31), multiple cellular mechanisms are present to sense increased availability of food and to trigger biological responses designed to most efficiently store energy. Malonyl-CoA has been identified as a biochemical sensor used to trigger a switch from FA to glucose oxidation for fuel usage (59) via deactivation of carnitine palmitoyl transferase-1 (CPT-1). During states of high glucose or insulin concentrations,

malonyl-CoA accumulation inhibits CPT-1 levels, increases glucose oxidation and reduces lipid oxidation, preferring lipid storage as triglyceride. The regulation of malonyl-CoA in muscle is controlled by specific central players, including acetyl-CoA carboxylase (ACC), the rate-limiting enzyme in malonyl-CoA synthesis; cytosolic citrate, an activator of ACC; and AMPK, an enzyme activated by decreases in the cell's energy state (60-63). Currently it is postulated that muscle contraction (i.e., glucose depletion) regulates ACC inhibition solely by activating AMPK, which phosphorylates ACC and decreases malonyl-CoA levels. Conversely, an abundance of glucose increases malonyl-CoA concentration via increased cytosolic citrate levels (64) and decreased AMPK activation (65), thereby increasing the conversion of FA into triglyceride resulting in obesity. ACC- $\beta$  impacts FA utilization through its direct positive control of malonyl-CoA production and its indirect relationship with PPAR- $\gamma$ , affected through CPT1's up-regulation by PPAR- $\gamma$ , making it highly feasible as a candidate gene affecting expression of the PCOS phenotype. A mutation in the ACC- $\beta$  gene could affect energy homeostasis by upsetting the normal balance between glucose and FA homeostasis, proffering an increased risk profile for CHD to carriers of a mutation increasing ACC- $\beta$  activity. Even though an effect of ACC- $\beta$  was not seen in this population, it does not rule out this gene as one of interest in future studies among populations with more ethnic diversity. This is the first study to attempt to associate ACC- $\beta$  with PCOS and is important in addressing first impressions of the potential contribution this gene could make among affected women. The current sample size of Caucasian subjects, allowed 67% power to detect a 15% allele frequency difference between cases and controls and 37% power to detect a 10% difference in allele frequencies. The significant 9% difference in allele frequency between AA cases and controls had a 10% power to detect, suggesting this result may need to be repeated in a larger population for validation.

### **5.3 ASSOCIATION OF GENOTYPE WITH SUBCLINICAL MEASURES OF CHD**

The P12A variant of PPAR- $\gamma$  has been significantly associated with obesity (66-71). In the current analyses, it is clear that BMI is intimately associated with the PCOS phenotype, so much

so as to potentially obscure the relationship between subclinical causal mechanisms of diabetic or CVD outcomes and PCOS. Results of multivariate analyses performed in this study supported the fact that P12A is a genotype very strongly associated with BMI. The removal of significance of every other factor that, prior to inclusion of BMI was significantly associated with CRP, may indicate that the adverse effect of the P12A genotype may be through its action on body composition.

Studies of variants among the LPL gene have indicated an association with insulin sensitivity among Type 2 diabetics (72) and Mexican Americans with atherogenic profiles (73), as well as adverse lipid profiles among atherogenic men and women from Geneva (74). The ACC- $\beta$  gene has been less extensively studied in relation to metabolic phenotypes among humans, but has been found to relate to fat storage (75), obesity(76), and type 2 diabetes (76) among ACC- $\beta$  -/- mutant mice. The ACC- $\beta$  SNPs chosen for these analyses were selected for their distribution across the ACC- $\beta$  gene to allow a “genotype” analysis (i.e., haplotype analysis). However, in these analyses, neither LPL SNP A7634966C nor the ACC- $\beta$  SNPs (G263491A, T204540C, and G194216A) nor the ACC- $\beta$  haplotype were significantly associated with HOMA-IR or CRP in modeling of PCOS adjusted for genotype.

The G972R isoform of IRS-1 has also been significantly associated with obesity (77). Using multivariable modeling, IRS-1 variant G972R seemed to significantly impact CRP among PCOS cases compared to controls. Even after adjusting for body mass index and race, G972R remained concurrently significant with the PCOS/G972R interaction, implying that not only may G972R impact expression of CRP levels, but that G972R interacted with case status to affect CRP levels. The possibility that an insulin pathway specific genotype could independently effect systemic inflammation, over and above obesity and race, has not previously been demonstrated and is the most compelling result of these analyses.

## **5.4 OVERALL STUDY STRENGTHS**

These analyses had three main strengths. Firstly, the length of follow-up for assessment of health outcomes was a strength mainly due to the fact that other populations of women with PCOS who have been assessed for development of T2DM were not followed for as long a time. During our follow-up, progression of disease was consistently tracked and reported through repeated clinic visits. Repeated clinic visits also provided a built-in verification of the presence of a diagnosed disease. Secondly, the use of controls followed concurrently with the cases provided an excellent backdrop for determining increased rate of disease progression, disease development, or subclinical measures due to PCOS. Not only could cases be compared to themselves at a previous visit, but to a control subject about whom the same level of medical history was known. Thirdly, consistency in clinical assessment and disease reporting was upheld for all subjects at all visits, which after the considerable length of follow-up, is significant for assessing effects of PCOS on health. Additionally, this study included genotypes that, since their discovery by Haga et al. in 2002 (83) by genome wide scans among 24 unrelated Japanese women, have not been studied for their affect on insulin sensitivity or systemic inflammation and, thus, provides a first impression of the potential for inclusion in future research.

## **5.5 OVERALL STUDY LIMITATIONS**

Sample size was a major limitation of this analysis. When attempting to determine both outcomes and genotype frequency differences for significance between PCOS cases compared to controls, a true difference could really have been present, but could not be established using the relatively small subcohort sizes necessary to carry out the appropriate analyses. Future analysis of ACC- $\beta$  and LPL genotype frequencies may be of interest among PCOS populations of races other than Caucasian to more accurately determine if there is an effect of genotype among these populations toward development of PCOS.

A second major limitation of this study was the reliance of patient self-report to determine the diabetic status of subjects, rather than medical chart review to get an accurate assessment both of actual physician diagnosis and date of onset. Using clinic-recorded current medication in combination with patient self-report of medical diagnosis strengthened the accuracy of presence of T2DM. Date of diagnosis of T2DM was also based upon patient self-report, however, the fact that women seen in this study were administered similar questionnaires with the same questions about diagnosis of diabetes and date of diagnosis over three points in time increased the accuracy of this estimate. There were five women assessed who had to be assigned a date of diagnosis through linear interpolation using the midpoint of their last clinic visit and the last year of follow-up. Since women could have been diagnosed with T2DM at either the second or third visit and did not need to be followed a full eight years once they were considered affected, they may have been diagnosed between the first and second visit and not have remembered their year of diagnosis. However, the time span between 1992-1994 and 1996-1999 visits for any individual woman had the possibility to be relatively accurate. Since, in practice, women recruited first for the 1992-1994 visit were generally recruited first in each follow-up visit, the average follow-up time for this population when seen in 1996-1999 was ~3.5 years. Linear interpolation of this data may be a reliable estimate of year of onset.

Another potential limitation of this analysis was the inclusion of women taking hormones (OC/HRT) in assessment of development of T2DM and subclinical measures. Since similar rates of hormone use was recorded between cases and controls, the effect of use was not considered to be a major methodological limitation.

## **5.6 OVERALL SUMMARY AND CONCLUSIONS**

Women with PCOS had significantly greater risk of developing T2DM compared to age-adjusted control women. Not surprisingly, risk of future development of T2DM in PCOS-affected women seemed to be greatly modified by obesity. Future studies of incidence of T2DM related to polycystic ovary syndrome should focus on larger groups of older women followed through



























































































































































































































































