

**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 ≥ 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- γ) 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- β). Significant association of genotype frequency with PCOS was determined by Pearson's χ^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 ≥ 35 kg/m² 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- γ); a transcription factor activated by various FA and FA metabolites (PPAR- γ agonists). The role of PPAR- γ 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- γ 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 α), 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- γ 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- γ (13).

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

1.3.1 PPAR-Gamma

The PPAR- γ 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- γ 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- γ 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- γ 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- γ 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

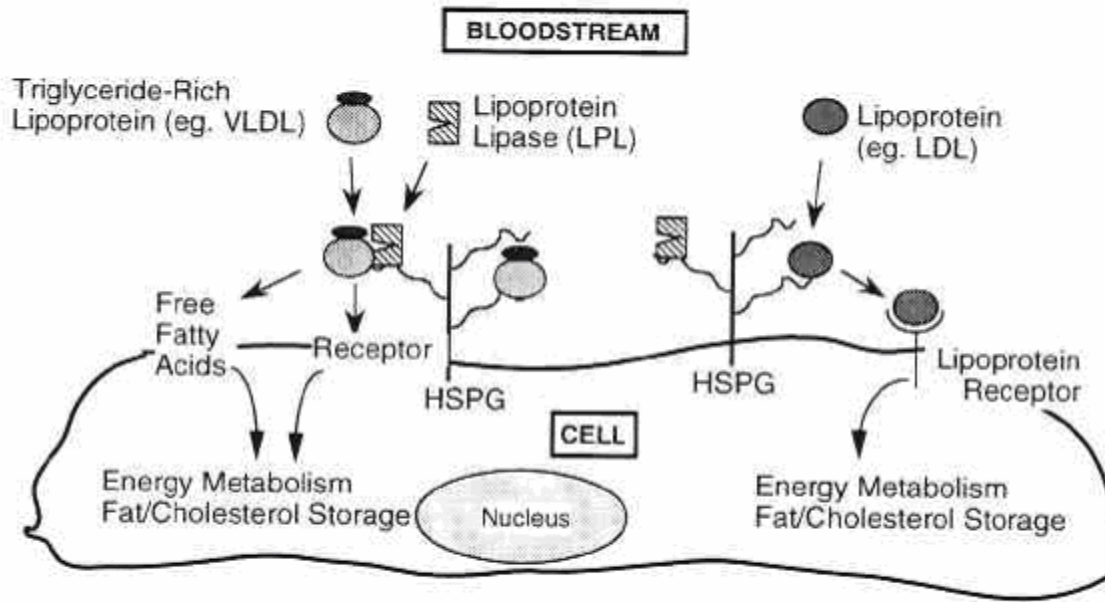


Figure 1-1. Lipoprotein lipase in the blood

(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- β) may be involved in the provision of malonyl-CoA or in the regulation of FA uptake and oxidation by mitochondria. ACC- β 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- β 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- γ . Stumvoll et al. (39) studied the gene-gene interaction between the P12A variant of PPAR- γ 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- γ 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- γ 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 ≥ 35 kg/m² 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 (± 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 ≥ 7.0 mmol/l

or ≥ 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 ≥ 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 ≥ 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 ≥ 126 mg/dl.

2.3.3 Data analyses

Baseline characteristics were compared between cases and controls by use of χ^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 ± 5.9 vs. 40.0 ± 5.2 years; $p = 0.017$), as well as heavier (BMI 31.6 ± 9.6 vs. 26.2 ± 6.0 kg/m², $p < 0.0001$; 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 ± 16 vs. 110 ± 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 ± 83 vs. 77 ± 34 mg/dl; $p = 0.0002$), lower HDL concentrations (52 ± 14 vs. 58 ± 14 mg/dl; $p = 0.003$), and higher LDL concentrations (122 ± 30 vs. 113 ± 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 μ U/ml; $p = 0.0003$), GIR (6.3 vs. 7.8 mg/dl over μ U/ml; $p = 0.003$), and HOMA-IR (3.6 vs. 2.4 mmol/l x μ 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/m²), SBP (cases: 126.6, controls: 111.0 mmHg), fasting insulin (cases: 20.0, controls: 16.5 μ 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/m²], 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 ≥ 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; ≥ 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 ≥ 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 ± 5.98 years, controls = 48.1 ± 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>Ehrmann (1999) (5)</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 2.4 ± 0.3 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>Legro (1999) (6)</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, $<27 \text{ kg/m}^2$) had 10.3% IGT and 1.5% T2DM. The prevalence of glucose intolerance was significantly higher in PCOS vs. control women ($\chi^2 = 7.0$; $P = 0.01$; odds ratio = 2.76; 95% confidence interval = 1.23-6.57).</p>
<p>Norman (2001) (7)</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>Weerakiet (2001) (8)</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

Baseline Characteristic	Cases (N = 97)	Controls (N = 95)	P- Value
Age (mean years \pm SD)	38.02 (5.89)	39.97 (5.21)	.017
Body mass index (mean \pm SD)	31.56 (9.55)	26.22 (6.00)	<.0001
Waist:Hip ratio	.82 (.10)	.75 (.06)	<.0001
Race (n)	-----	-----	.0006
African-American	16	2	-----
Caucasian	81	93	-----
SBP (mean mmHg \pm SD)	115.40 (15.75)	109.74 (12.57)	.009
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)	.0002
HDL (mean mg/dl \pm SD)	51.57 (13.93)	57.53 (14.10)	.003
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 μ U/ml \pm SD)	17.09 (11.73)	11.61 (4.08)	.0003
Glucose:Insulin ratio (mean \pm SD)	6.26 (2.66)	7.75 (2.20)	.0003
HOMA-IR (mean \pm SD)	3.62 (3.14)	2.37 (.92)	.002

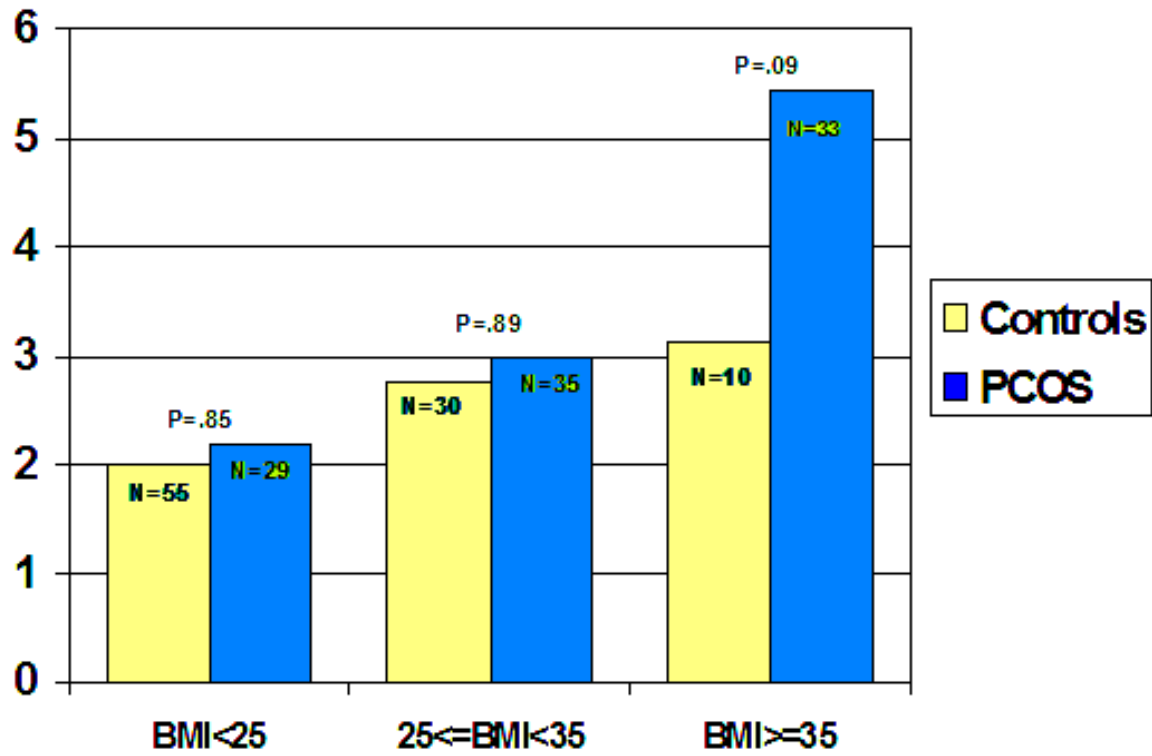


Figure 2-1. Average HOMA Scores Among PCOS Cases and Controls By BMI Strata

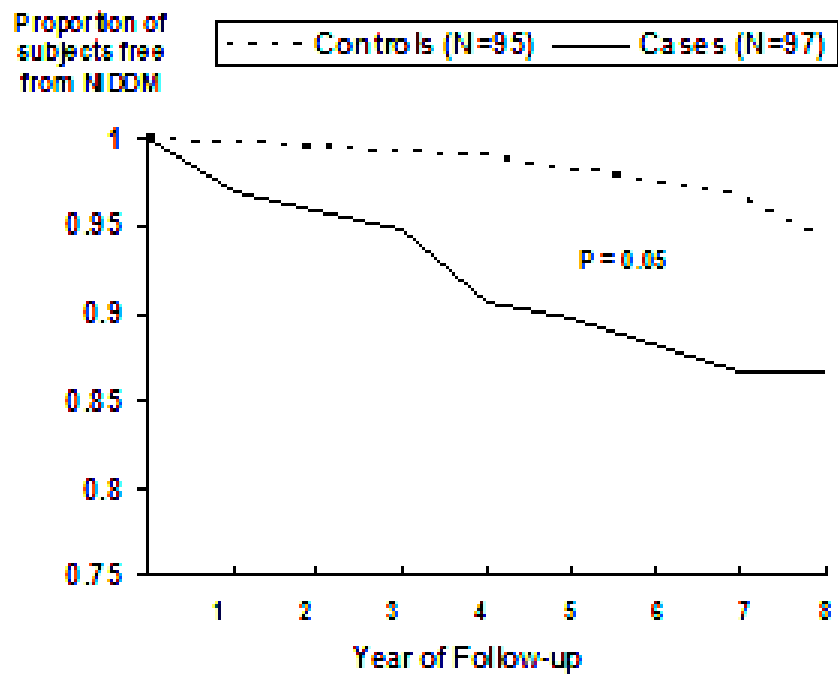
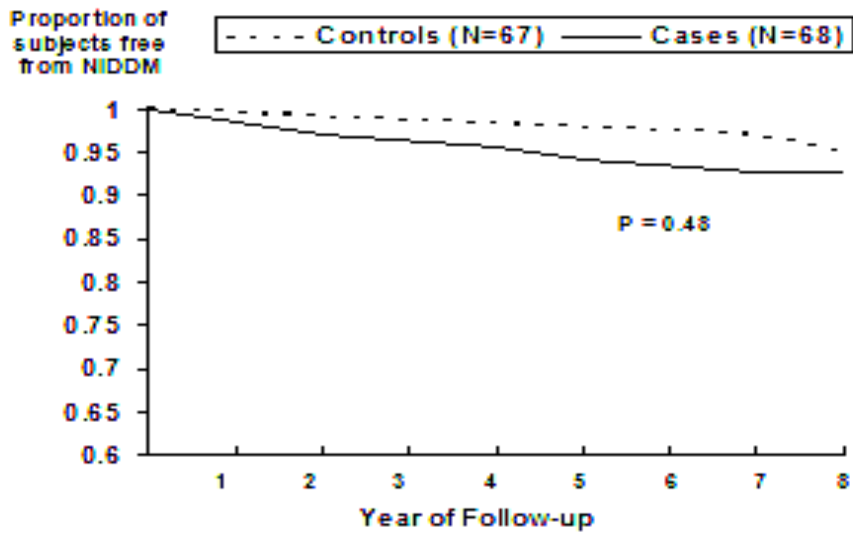


Figure 2-2. Proportion of PCOS Cases and Controls Free of T2DM By Year of Follow-up

BASELINE GLUCOSE < 85



BASELINE GLUCOSE > 85

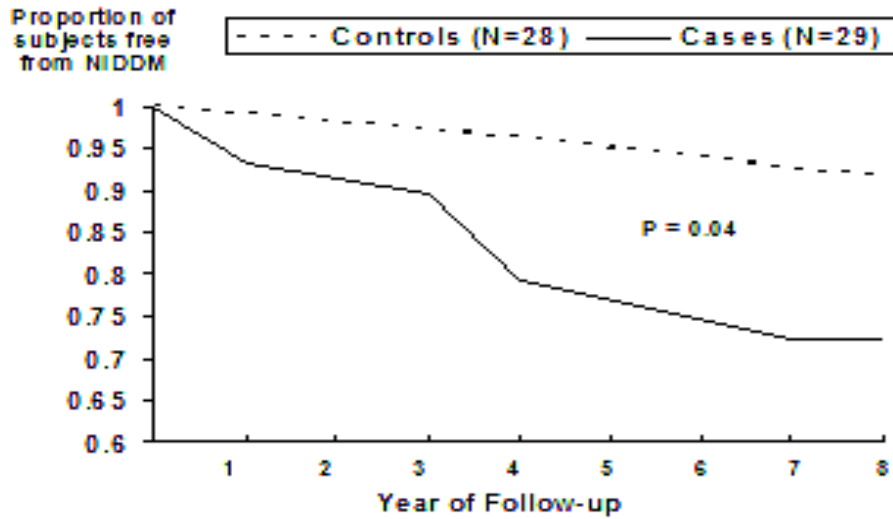


Figure 2-3. Proportion of PCOS Cases and Controls Free of T2DM By Year of Follow-up Stratified by Baseline Glucose

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 ^a	0.67 – 5.99	0.22
Baseline glucose < 85						
Controls	67	4.8%	1.0	-----	-----	-----
Cases	68	7.3%	1.67	1.35 ^a	0.30 – 6.15	0.70
Baseline glucose ≥ 85						
Controls	28	8.4%	1.0	-----	-----	-----
Cases	29	27.6%	4.29	2.38 ^a	0.44 – 12.91	0.31
PCOS/BMI Interaction						
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 ^b	0.37 - 4.56	0.68
Cases, BMI ≥ 35	33	24.2%	4.67	4.61 ^b	1.50 – 14.15	0.008
All Controls	95	5.8%	1.0	-----	-----	-----
Cases, BMI < 35	64	6.0%	1.49	1.45 ^b	0.41 – 5.08	0.56
Cases, BMI ≥ 35	33	21.3%	5.20	5.14 ^b	1.67 – 15.78	0.004

^aAdjusted for age, body mass index

^bAdjusted 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- γ , the G972R variant of insulin receptor substrate-1, three single nucleotide polymorphisms (SNPs) of acetyl-coA carboxylase beta (ACC- β), 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- β gene were computed and compared by use of χ^2 tests. Linkage disequilibrium within the ACC- β locus was calculated for all pairs of SNPs. A non-parametric T5 statistic was used to test for significant ACC- β 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- β allele frequency was significantly different among AA cases and controls. Linkage disequilibrium was significant between two ACC- β SNPs, T204540C and G194216A in both Caucasian and AA subjects. When comparing PCOS cases to same race controls, ACC- β haplotype frequencies were similarly distributed. However, not surprisingly, the distribution of ACC- β 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- β 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- β haplotype or P12A/G972R combined genotypes with PCOS. Future studies may be necessary to validate the results of this study, especially regarding ACC- β 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- γ) 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- γ 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- γ 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- γ 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- γ 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- γ 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- γ 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- β) 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- β 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- β gene may be associated with PCOS. Furthermore, it has been postulated that insulin resistance may be related to gene transcription downstream of PPAR- γ (25), such as the LPL and ACC- β genotypes selected for this analysis.

The specific aims of this analysis are 1) To test the association of P12A of PPAR- γ , G972R of IRS-1, ACC- β 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- β 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 (± 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°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- γ variant P12A**: Molecular genetic analysis of PPAR- γ 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- β** : Three SNPs were identified from the ACC- β 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). Thermocycling conditions for all ACC- β 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 *Eae*I, *Afe*I, and *Nco*I, respectively (New England Biolabs, Inc., Beverly, MA). PCR thermocycling of ACC- β variant rs2268403 created a 474-bp product in which an *Eae*I

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 χ^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- β were estimated using the PHASE software program (29, 30) and tested for significant difference between same-race cases and controls using χ^2 tests. PHASE uses the expectation-maximization algorithm to obtain maximum-likelihood estimates of haplotype frequencies. The association of pairwise comparisons of ACC- β 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- β 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 χ^2 distribution with df equal to number of haplotypes tested. StatXact was used to test for significance between ACC- β 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- β 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- β 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- β 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- β 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- β 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- β 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- β haplotype or combinations of ACC- β 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- γ 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- γ 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- α (65, 66). These results can be supported by the demonstrated effects of TZDs on PPAR- γ 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- β 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- β was not seen in this population. This was the first study to associate ACC- β 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- β haplotype had no association with PCOS demonstrating an overall lack of association. Results from this population regarding the ACC- β 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- β whose effects on PCOS merit further study.

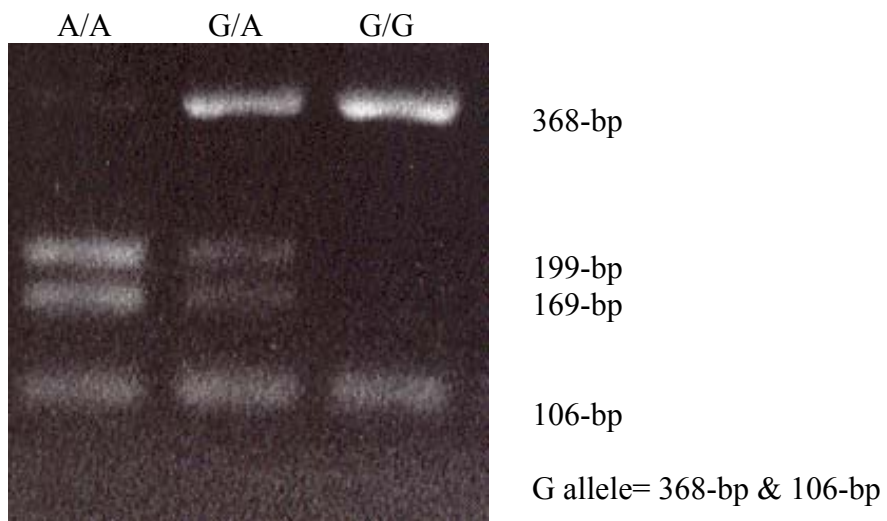


Figure 3-1. Gel electrophoresis of ACC-Beta SNP G194216A

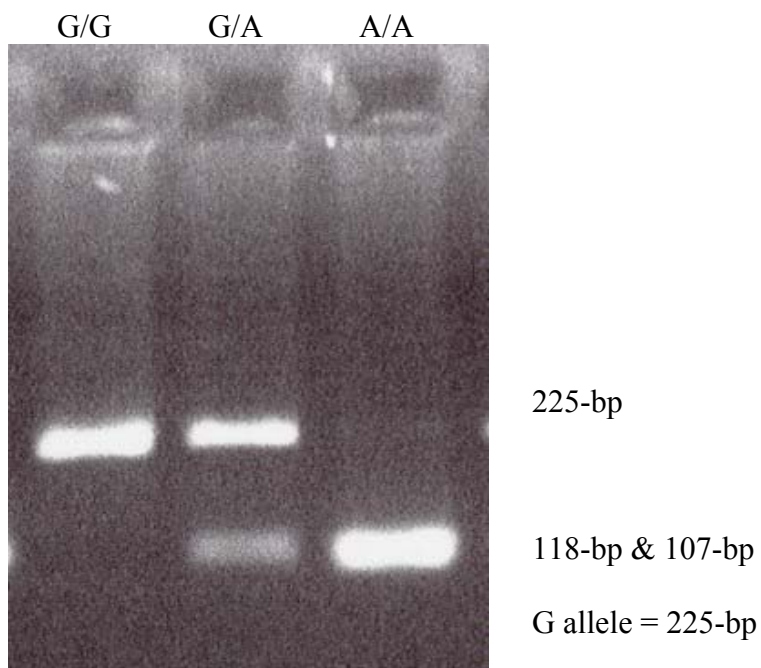


Figure 3-2. Gel electrophoresis ACC-Beta SNP T204540C

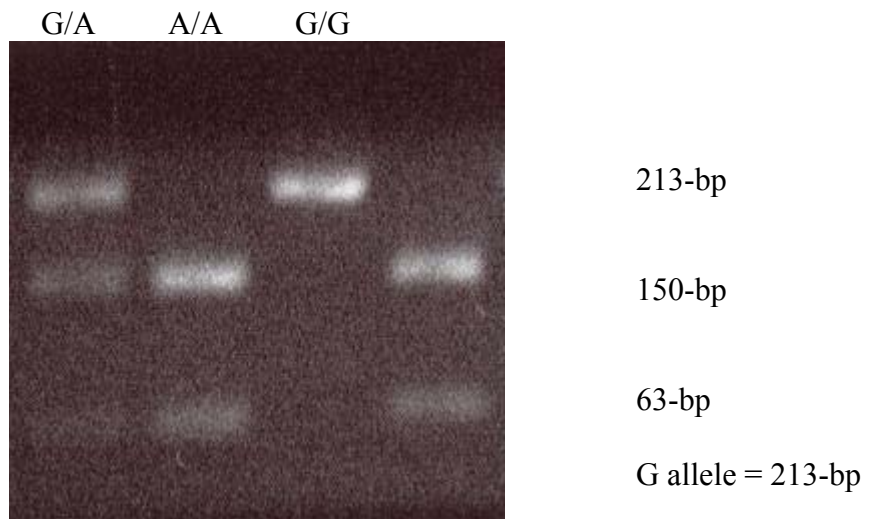


Figure 3-3. Gel electrophoresis of ACC-Beta SNP G263491A

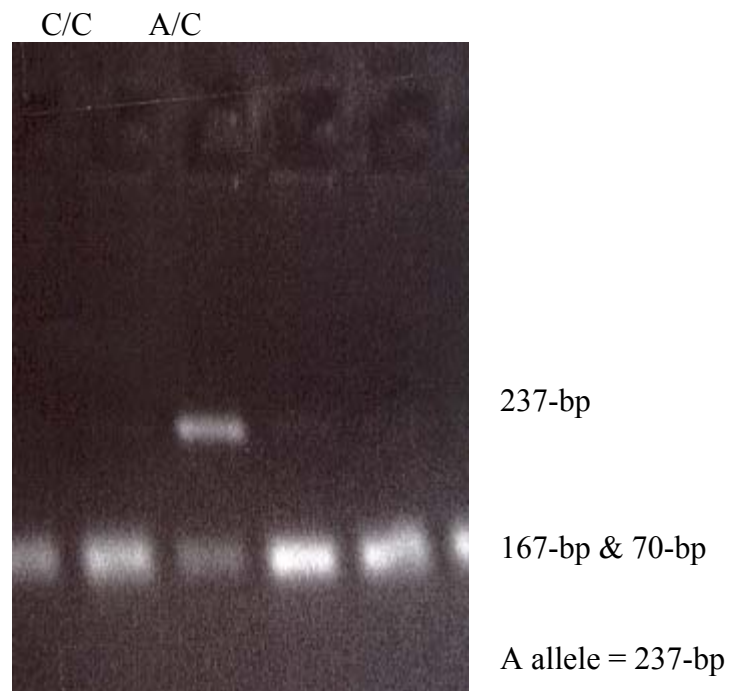


Figure 3-4. Gel electrophoresis of LPL SNP A7634966C

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

Allele 1 Frequency (N) In Caucasian subjects^b				
SNP	Cases (n = 244)	Controls (n = 260)	$\chi^{2(a)}$	P-value
PPAR- γ (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- β (G263491A)	0.68 (166)	0.67 (173)	0.64	0.73
ACC- β (G194216A)	0.83 (202)	0.82 (213)	0.77	0.69
ACC- β (T204540C)	0.55 (134)	0.54 (141)	0.07	0.98
LPL (A7634966C)	0.91 (221)	0.90 (234)	1.65	0.56
Allele 1 Frequency (No./Total) In African American subjects^b				
SNP	Cases (n = 52)	Controls (n = 54)	$\chi^{2(a)}$	P-value
PPAR- γ (P12A)	0.96 (50)	0.98 (53)	0.39 ^a	0.61
IRS-1 (G972R)	0.94 (49)	0.87 (47)	1.52	0.58
ACC- β (G263491A)	0.81 (42)	0.78 (42)	0.5	0.88
ACC- β (G194216A)	1.00 (52)	0.91 (49)	5.32 ^a	0.05
ACC- β (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 χ^2 used instead of Fisher's exact for χ^2 value only
- b. Allele 1 defined as most common allele - The ACC- β 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	<0.0001	<0.0001	0.969		-----	0.36	0.998

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- β 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

Haplotypes (G194216A, T204540C, G263491A)		Cases (n=122)	Controls (n=130)	OR^a	Exact 95% C.I.
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
African American subjects					
Haplotypes (G194216A, T204540C, G263491A)		Cases (n=26)	Controls (n=27)	OR^a	Exact 95% C.I.
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	-----	-----

^aThe 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

Association in Caucasians				
Status	N	Ln(L)	χ^2	P-value
PCOS cases	122	-299.57	39.3	0.5
Controls	130	-322.83	47.49	
PCOS + controls	252	-625.4	80.94	
Association in African Americans				
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

Genotype Combination	Caucasian Cases (n=122)	Caucasian Controls (n=130)	OR^a (95% CI)
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	-----
Genotype Combination	AA Cases (n = 26)	AA Controls (n=27)	OR^a (95% CI)
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- γ , G972R of IRS-1, ACC- β , 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- γ), the G972R variant of insulin receptor substrate-1 (IRS-1), three single nucleotide polymorphisms of acetyl-CoA carboxylase beta (ACC- β), 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- γ 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- γ (5). The genotypes in this analysis are either downstream and under the transcriptional control of PPAR- γ (i.e., ACC- β and LPL) or have been demonstrated to interact with PPAR- γ (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- γ 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- γ 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- γ '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- β) may be involved in the provision of malonyl-CoA or in the regulation of fatty acid uptake and oxidation by mitochondria. ACC- β is relevant to this study due to its critical role in fatty acid oxidation (31). The potential roles of LPL and ACC- β 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- γ , G972R of IRS-1, ACC- β 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 (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 (± 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 m^2) and by waist-to-hip ratio (waist in cm/hip in cm). A BMI <25 kg/m^2 was normal weight, <35 kg/m^2 was defined as overweight, and ≥ 35 kg/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° 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- γ 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- β*** : Three single nucleotide polymorphisms were identified along the ACC- β 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). Thermocycling conditions for ACC- β 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 χ^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- β 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- β haplotype of "100" for the ACC- β 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- β haplotype groups. Multivariable generalized linear regression modeling was used to test the significance of genotype, the P12A/G972R genotype combination and ACC- β 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 ± 8.8 vs. 28.1 ± 6.7 kg/m², $p < 0.0001$; WHR 0.82 ± 0.08 vs. 0.79 ± 0.08 , $p = 0.01$) than controls and presented with lower HDL concentrations (54.2 ± 15.5 vs. 58.1 ± 14.9 mg/dl; $p = 0.01$). Both diastolic (75.9 ± 9.6 vs. 74.2 ± 9.1 mmHg; $p = 0.10$) and systolic (116.5 ± 15.4 vs. 114.8 ± 14.3 mmHg; $p = 0.25$) blood pressures were similar between cases and controls. Caucasian cases also had higher CRP concentrations (3.8 ± 5.3 vs. 2.6 ± 3.0 mg/L; $p = 0.004$). Insulin levels were significantly elevated among PCOS cases compared to controls (19.5 ± 13.1 vs. 15.0 ± 9.8 mg/dl, $p = 0.0001$) and insulin sensitivity was significantly lower among PCOS cases (HOMA-IR 5.2 ± 5.3 vs. 3.8 ± 4.7 mU · mmol/L², $p = 0.0003$). 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- β 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 ± 8.4 and 4.2 ± 4.2 mU · mmol/L², 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 ± 7.9 and 3.0 ± 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 ± 3.2 vs 2.6 ± 3.0 mg/L). However, CRP concentrations varied between case subjects carrying G/R+R/R and G/G genotypes (7.1 ± 10.6 vs 3.3 ± 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 themG972RxPCOS 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- β SNPs (G263491A, T204540C, and G194216A) or ACC- β 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- β 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- β -/- mutant mice. The ACC- β SNPs chosen for these analyses were selected for their distribution across the ACC- β 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- β 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- γ), 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/m² 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- γ , the G972R variant of IRS-1, the three single nucleotide polymorphisms of ACC- β (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- β gene.

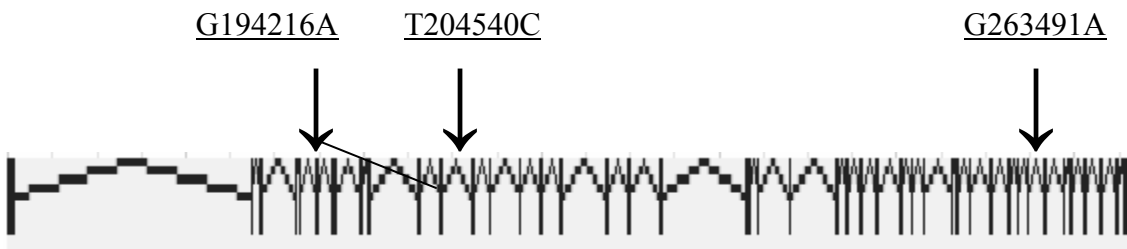


Figure 4-2. The ACC-Beta gene

Each vertical line along the ACC- β 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.

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 ² \pm SD)	31.8 \pm 8.8	28.1 \pm 6.7	<0.001
Waist:Hip ratio (mean \pm SD)	0.82 \pm 0.08	0.79 \pm 0.08	0.01
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	0.004
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	0.01
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 ^a
Taking OC (%; N)	10.9 (16/147)	13.4 (21/157)	0.51 ^a
Taking HRT (%; N)	11.6 (17/147)	14.0 (22/157)	0.52 ^a
First degree relative with PCOS (%; N)	14.3 (21/147)	3.2 (5/157)	0.002^a
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	<0.001
HOMA-IR (mean mU \cdot mmol/L ² \pm SD)	5.2 \pm 5.3	3.8 \pm 4.7	<0.001
Abnormal glucose status (%; N) ^b	17.2 (25/145)	5.9 (9/155)	0.002^a

- 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

Caucasian subjects				
Genotypes^a	Cases	Controls	χ^2	P-value^b
LPL SNP				
A7634966C – C/C (vs. X/A)	0.81 (98/121)	0.81 (106/130)	0.007	0.91
ACC-β SNPs				
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
PPAR-γ SNP				
P12A – Pro/Pro (vs. X/Ala)	0.64 (77/121)	0.63 (82/130)	0.008	0.93
IRS-1 SNP				
G972R – Gly/Gly (vs. X/Arg)	0.86 (104/121)	0.86 (112/130)	0.002	0.96
African American subjects				
LPL SNP				
A7634966C – C/C (vs. X/A)	0.81 (21/26)	0.81 (22/27)	0.004	0.95
ACC-β SNPs				
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	0.05^c
PPAR-γ SNP				
P12A – Pro/Pro (vs. X/Ala)	0.92 (24/26)	0.96 (26/27)	0.39	0.53
IRS-1 SNP				
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 χ^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	
	β	P-value	β	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	
	β	P-value	β	P-value
Age (in years)	-0.003	0.94	-0.0009	0.98
BMI	0.19	<0.0001	0.18	<0.0001
Race	1.65	0.009	1.85	0.003
G972R of IRS-1			-0.47	0.60
PCOS			0.07	0.89
G972R of IRS-1 x PCOS			3.68	0.005

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 ± 5.98 years, controls = 48.1 ± 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- γ , the G972R isoform of IRS-1, three ACC- β 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- β haplotype or combinations of ACC- β 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- γ plays a central role in adipogenesis (32-34) based on two main processes. Firstly, the insulin sensitizing effects of activated PPAR- γ 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- α (39, 40). Among diabetic populations, TZDs effect PPAR- γ 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- γ pathway and, considering PPAR- γ '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- γ 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- β impacts FA utilization through its direct positive control of malonyl-CoA production and its indirect relationship with PPAR- γ , affected through CPT1's up-regulation by PPAR- γ , making it highly feasible as a candidate gene affecting expression of the PCOS phenotype. A mutation in the ACC- β 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- β activity. Even though an effect of ACC- β 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- β 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- γ 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- β 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- β -/- mutant mice. The ACC- β SNPs chosen for these analyses were selected for their distribution across the ACC- β gene to allow a “genotype” analysis (i.e., haplotype analysis). However, in these analyses, neither LPL SNP A7634966C nor the ACC- β SNPs (G263491A, T204540C, and G194216A) nor the ACC- β 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- β 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

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 IR and T2DM.

In attempting to determine if specific genotypes impacted the PCOS phenotype, we concluded that there was no association of genotype with PCOS. The P12A variant of PPAR- γ , the G972R variant of IRS-1, the three single nucleotide polymorphisms of ACC- β (G263491A, T204540C, and G194216A), and the A7634966C SNP of lipoprotein lipase (LPL) were not significantly associated with PCOS. However, when assessing if genotype impacted insulin sensitivity or inflammation among PCOS-affected women, there was a novel association of CRP with IRS-1 variant G972R by PCOS interaction over and above age, BMI and race. Even though CRP has been studied in relation to inflammatory genetic markers, namely the CRP gene (78-80) and the interleukin-6 gene (81, 82), and CRP gene activity is under the transcriptional control of nuclear transcription factors, such as PPAR- γ , CRP activity has not been studied in association with insulin sensitivity-related genetic polymorphisms before now. This finding of significantly elevated CRP levels in association with the interaction of the G972R variant of IRS-1 and PCOS introduces a previously unexplored avenue for future research into the relationship between IR and inflammatory factors. It also provides a basis upon which the G972R variant of IRS-1 may be associated with development of T2DM through subclinical factors. By touching upon the interrelationships between IR, obesity, and inflammation, these analyses may be a new starting point for future analyses among high-risk populations.

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APPENDIX A

EVIDENCE OF A GENETIC BASIS OF PCOS: PEDIGREE STUDIES

Since 1979, studies have been attempting to elucidate a mode of inheritance for PCOS. Since the premise of this analysis is that it is a genetically-based disorder, this appendix includes a review of historically-relevant genetic family studies providing evidence of this genetic basis for PCOS. Strengths that these studies shared include recruitment and enrollment of first degree family members and a general trend of improving upon the efforts of past studies with the end result being very well-designed studies. However, in researching this literature, what stood out the most was that the research effort is still in its infancy. Over time, the definition of PCOS has changed almost with every new study, potential confounders of study results have changed with the disorder definition, and the lack of standardized methods for measuring heritability while accounting for these confounders have hindered these studies from getting any conclusive or reproducible results. With the introduction of biochemical evidence by the 1990 National Institutes of Health Consensus Criteria as a defining characteristic of a PCOS diagnosis in the US, the thought was to somehow make results more comparable and, hopefully, more conclusive. Yet, this has not happened. PCOS is a much more complex disorder than first thought and we are still left wondering if we simply have not well defined the true nature of the disorder. PCOS is most likely phenotypically and genetically heterogeneous and is, thus, difficult to study as so many phenotypic subgroups exist.

Major limitations of previous family genetic studies include small numbers of participants, lack of hormonal data, inclusion of men with premature baldness without knowing that a genetic basis for its inclusion as the “male phenotype” exists, and a lack of controlling for such confounders as medications, other diseases that present with the same phenotype, race, or BMI. This study, while not capable of clearing up questions from the past, is the first study to investigate the role of PPAR- γ in women with PCOS from a “pathway perspective” by examining genes influenced or otherwise controlled by PPAR- γ as if following a pathway of insulin signaling. Positive results from this study will open up a new avenue of thought regarding the definition of PCOS while negative results will allow research to move forward onto other paths of research.

<p>Ferriman and Purdie (1979)</p> <p><u>Title:</u> The inheritance of polycystic ovarian disease and a possible relationship to premature balding</p> <p><u>Objective:</u> To examine the etiology, definition and pathogenesis of polycystic ovarian disease and to find a link to baldness</p>	<p><u>Population</u></p> <p><u>Inclusion criteria:</u></p> <ul style="list-style-type: none"> * 825 1° female relatives of 284 hirsute women and 274 1° male relatives of 136 hirsute women * 179 non-hirsute controls aged 20 to 40 years from the same outpatient clinics used to identify hirsute women, but without hirsutism (HIR), oligomenorrhea (OM), or infertility (provided reference values) * All subjects of European descent * Subject medication use not investigated <p>* <u>Exclusion criteria:</u> Premature menopause, ovarian dysgenesis, anorexia nervosa, amenorrhea-galactorrhea, GnRH deficiency, and organic thyroid, adrenal, pituitary or hypothalamic disorders</p>	<p><u>Methods</u></p> <p><u>Cases:</u></p> <ul style="list-style-type: none"> * Ovarian size in probands determined by gynaecography * Family history elicited for all family members from probands regarding the relations with affected status, i.e., female with HIR, OM or infertility or male with premature balding (in their 20's or 30's) (PMPB) * A HIR or baldness score assessed for all subjects <p><u>Controls:</u></p> <ul style="list-style-type: none"> * Women recruited from 2 ante-natal clinics and one general medical clinic * Aged 20 to 40 years * Asked the same family history questions as the cases * They were used for reference values only 	<p><u>Results</u></p> <ul style="list-style-type: none"> * There is the existence of a genetically-based disorder consisting of OM, infertility, and, commonly, enlarged ovaries * Relative prevalence from mothers and sisters suggest a modified dominant form of inheritance * Neither the prevalence of HIR among 1° female relatives nor of premature balding among 1° male relatives differed from controls 	<p><u>Strengths</u></p> <ul style="list-style-type: none"> * Inclusion of all male and female 1° family members in analysis * Large number of participants * Acknowledgement of possible etiological differences between women with and without HIR and stratified analysis of each <p><u>Limitations</u></p> <ul style="list-style-type: none"> * Proband diagnosis based on ovarian morphology only * All data of family members from self-reported questionnaire of proband * Mode of inheritance determination based only on 1° relatives * Data collected differently on men with baldness (proband self-report) than on their controls (interview) * Prolactin assays rarely performed during screening which allowed subjects with hyperprolactinemia into the study
<p>Mandel et al. (1983)</p>	<p><u>Population</u></p> <ul style="list-style-type: none"> * Four families in whom at least two siblings had 	<p><u>Methods</u></p> <p><u>Cases and controls:</u></p> <ul style="list-style-type: none"> * Fasting blood drawn 	<p><u>Results</u></p> <ul style="list-style-type: none"> * Elevated concentrations of DHEA-S indicated 	<p><u>Strengths</u></p> <ul style="list-style-type: none"> * Fasting blood draw on all participants

<p><u>Title:</u> HLA genotyping in family members and patients with familial polycystic ovarian disease</p> <p><u>Objective:</u> To determine whether the familial occurrence of PCO is related to the major histocompatibility complex (HLA)</p>	<p>clinical evidence of PCO (N=8)</p> <ul style="list-style-type: none"> * 15 PCO subjects without affected siblings * 10 normal cycling women without evidence of endocrine disorder and not taking medication (provided reference values) * Subject ethnicities not mentioned * Confounding medications not mentioned except in controls * <u>Affected status definition:</u> PCOS cases and sisters had to have HIR with OM or amenorrhea from menarche * <u>Exclusion criteria:</u> 21-hydroxylase deficiency 	<p>between 8 and 9 AM before and 60 min post-ACTH administration to exclude 21-hydroxylase deficiency</p> <ul style="list-style-type: none"> * Blood also drawn to: <ul style="list-style-type: none"> - Obtain HLA-A, -B, -C, and -DR genotypes for analysis - Measure serum levels of luteinizing hormone (LH), follicle stimulating hormone (FHS), testosterone (T), androstenedione (A), dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEA-S), cortisol (F), progesterone (P), and 17-hydroxyprogesterone (17-OHP) 	<p>excess adrenal androgen secretion</p> <ul style="list-style-type: none"> * HLA genotyping in these families demonstrated that PCO does not exhibit linkage to the HLA system 	<ul style="list-style-type: none"> * Familial and non-familial testing of HLA for a more complete assessment of the relationship between HLA and PCO <p><u>Limitations</u></p> <ul style="list-style-type: none"> * Small number of subjects * Blood drawn at random times of the female menstrual cycle in females (probands and sisters). This causes more variation in circulating androgens between individuals than there really might be * Controlling for spontaneous ovulation (easily tested by serum P levels) by either exclusion or separate analysis was not addressed. * Medication use that may have affected androgen levels not checked or excluded in any subjects
<p>Hague et al. (1988)</p> <p><u>Title:</u> Familial PCO: A genetic disease?</p>	<p><u>Population</u></p> <ul style="list-style-type: none"> * 50 PCOS cases (17 with congenital hyperplasia) * 137 reproductive aged females in families of probands * 158 volunteer women 	<p><u>Methods</u></p> <ul style="list-style-type: none"> * Transabdominal pelvic scans where ovaries were identified and measured in 3 planes, where possible * Detailed pedigrees obtained by proband 	<p><u>Results</u></p> <ul style="list-style-type: none"> * Mode of inheritance is not autosomal dominant or X-linked dominant * Other mechanisms were considered to explain this (i.e., meiotic drive due to 	<p><u>Strengths</u></p> <ul style="list-style-type: none"> * Defined affected status very clearly defined (PCO) * Large number of family members and controls

<p><u>Objective:</u> To determine the heredity of PCO</p>	<p>with normal ovaries used for reference values * All subjects of European descent * Medications not mentioned * <u>Affected status definition:</u> PCO based on ultrasound * <u>Exclusion criteria:</u> None mentioned</p>	<p>interview Measured symptoms of PCOS (i.e., HIR, OM, amenorrhea, infertility, and obesity) in all subjects</p>	<p>genetic segregation distortion, vertical transport of infective agent, environmental factors)</p>	<p><u>Limitations</u> * Inclusion of women with PCO due to congenital adrenal hyperplasia (i.e., classical and nonclassical 21-hydroxylase deficiency and 11-hydroxylase deficiency) * Ultrasonographer not blinded to subject affection status * Medication use was not addressed, but may have affected androgen levels</p>
<p>Lunde et al. (1989)</p> <p><u>Title:</u> Familial clustering in the polycystic ovarian syndrome</p> <p><u>Objective:</u> To assess the degree of familial clustering and mode of inheritance of PCOS</p>	<p><u>Population</u> * 1° and 2° relatives of 132 Norwegian PCOS patients aged 19 to 37 years on whom ovarian wedge resection had been performed between 1970 and 1980 (Group I) * 71 controls aged 17 to 39 years (Group II) * All subjects of Norwegian descent * Medications not mentioned * <u>Affected status definition:</u> <u>All women:</u> Multicystic ovaries on gross examination <u>and</u> at least 2 of the following: menstrual irregularity,</p>	<p><u>Methods</u> * Completed questionnaire on presence of infertility and HIR in patient and control parents, siblings, uncles, aunts, and grandparents * For female relatives, a question on menstrual irregularities was included; for male relatives, a question on premature balding was asked. * Age was recorded for all relatives * The questionnaire was then discussed with entire Group II and 89 of Group I. * To evaluate proband</p>	<p><u>Results</u> * A significantly higher proportion of relatives of PCOS cases were reported to have PCOS-related signs and symptoms when compared to controls * There was evidence of familial clustering * An X-linked mode of inheritance is not supported by these data * An autosomal dominant mode of inheritance was supported by these data</p>	<p><u>Strengths</u> * Large number of participants * Inclusion of 1° and 2° male and female relatives * Proband questionnaire information reliability verified by parent and sibling examination of 12 male and 28 female relatives of 15 PCOS cases selected for geographical convenience</p> <p><u>Limitations</u> * Diagnosis of male and female relative HIR largely based on interviews with probands * There was no blood drawn for hormonal</p>

	<p>obesity, HIR, and infertility</p> <p><u>All men:</u> PMPB prior to 30 years old</p> <p>* <u>Exclusion criteria:</u> None mentioned</p>	<p>interview reliability about 1° relatives, parents and siblings of 15 probands were examined</p> <p>* Menarche was recorded and classified in both groups</p> <p>* Body hair growth evaluated and HIR scored in 89 probands, all controls, and parents and siblings of 15 probands</p>		<p>assessment. PCOS proband diagnosis based only on ovarian morphology</p> <p>* All data of family members from self-reported questionnaire by PCOS women</p> <p>* The reliability of questionnaire information was high for 1° relatives only, so suggested mode of inheritance was based on this group only</p> <p>* Mode of inheritance included the male “PCOS phenotype” of premature balding. This unclear phenotype definition could have confounded analyses.</p>
<p>Carey et al. (1993)</p> <p><u>Title:</u> Evidence for a single gene effect causing PCO and male pattern baldness</p> <p><u>Objective:</u> To determine the mode of inheritance of PCO and PMPB, within the families of affected individuals by classic segregation</p>	<p><u>Population</u></p> <p>* 14 PCOS cases with PCO having at least 1 sister with or without PCO, of which 10 had families of sufficient size to examine an extended pedigree</p> <p>* 62 1° relatives of the 10 probands</p> <p>* 107 extended family members also contacted and interviewed, including data on 24 deceased family members</p>	<p><u>Methods</u></p> <p>* Transabdominal pelvic ultrasound of all women</p> <p>* Nine of the 14 probands had irregular menses and were interviewed with a full history taken (regarding menstrual disturbance)</p> <p>* Subjective assessment by subject of degree of acne and HIR</p> <p>* Measured in a general exam was height, weight, blood pressure</p>	<p><u>Results</u></p> <p>* The authors proposed PCOS and PMPB are caused by alleles of one gene, which affects androgen production</p> <p>* Different PCOS/PMPB frequencies are caused by different thresholds of phenotypic expression</p> <p>* A variable phenotype due to modification of other genes</p> <p>* Data consistent with an autosomal dominant</p>	<p><u>Strengths</u></p> <p>* Blood drawn for hormonal and biochemical assessment and women whose blood was drawn mid-cycle were excluded</p> <p>* 75-g oral glucose tolerance test (OGTT) was used to assess insulin sensitivity and resistance in obese probands, rather than relying on self-report</p> <p>* 10 families were completely screened yielding 58 kindreds plus</p>

analysis	<p>* No reference values used</p> <p>* Ethnicities of the 14 families: 10 Caucasian, 2 Iranian, 1 Afro-Caribbean, 1 Asian</p> <p>* Women on oral contraceptives (OCs) analyzed separately</p> <p>* <u>Affected status definition:</u> <u>Probands:</u> PCO by pelvic ultrasound <u>Female relatives:</u> PCO by pelvic ultrasound or history of menstrual irregularity and/or unwanted hair with or without acne <u>Men:</u> PMPB as fronto-parietal hair loss before age 40 years <u>Exclusion criteria:</u> Blood obtained from women at mid-cycle were excluded from analysis, hyperprolactinemia, late onset 21-hydroxylase deficiency, and type A insulin resistance</p>	<p>measurements, degree of HIR and acne, history of miscarriage and infertility</p> <p>* Blood was drawn to assess LH, FSH, T, prolactin and 17alpha-hydroxyprogesterone</p> <p>* Family members were assessed for acanthosis nigricans as a measure of hyperinsulinemia (HI) and insulin resistance (IR)</p>	disorder	<p>probands (~6 subjects/family)</p> <p>* Each person was screened and interviewed separately</p> <p>1° and 2° family members were included for mode of inheritance analyses</p> <p>* Included analysis of which PCOS symptoms (ovarian morphology, irregular menses, HIR) were most highly correlated with PCO</p> <p><u>Limitations</u></p> <p>* Blood obtained from all women at random cycle times</p> <p>* Hyperinsulinemia (HI) and insulin resistance (IR) measured by presence of acanthosis nigricans alone in all but obese family members</p> <p>* Men were included in this study as affected if they had PMPB. Conclusions on mode of inheritance, then, are based on the inclusion of these men and may be inaccurate due to that.</p>
Jahanfar et al. (1995)	<p><u>Population</u></p> <p>* 19 pairs of MZ twins</p> <p>* 15 pairs of DZ twins</p>	<p><u>Methods</u></p> <p>* Subject interviewed for zygosity, menstrual</p>	<p><u>Results</u></p> <p>* Eleven pairs of twins (5 MZ and 6 DZ pairs) were</p>	<p><u>Strengths</u></p> <p>* The authors adjusted analysis for BMI for</p>

<p><u>Title:</u> A twin study of polycystic ovary syndrome</p> <p><u>Objective:</u> Used the classic twin model to investigate the etiology of PCO by comparing monozygotic (MZ) and dizygotic (DZ) twin pairs to elucidate the contribution of genetic and environmental factors in PCO</p>	<p>* 20 control subjects with regular cycle lengths and no evidence of HIR or acne ($19 < \text{BMI} < 27 \text{ kg/m}^2$) used to construct reference values for biochemical parameters</p> <p>* All subjects were Australian</p> <p>* OCs were stopped at least 3 months before blood was drawn</p> <p>* <u>Affected status definition:</u> PCO on ultrasound</p> <p>* <u>Exclusion criteria:</u> Late onset 21-hydroxylase deficiency</p>	<p>history, height, weight, waist and hip circumferences, and presences of HIR and acne</p> <p>* Transabdominal ultrasound by blinded technician</p> <p>* Blood draw after overnight fast to measure LH, FSH, SHBG, total T and DHEA-S, insulin, glucose, prolactin, androstenediol gluconoride, insulin like growth factor binding protein-1 (IGFBP-1) and – 3 (IGFBP-3)</p>	<p>scan discordant (one twin with PCO, the other not on ultrasound)</p> <p>* Model fitting analysis suggested fasting insulin levels, androstenediol gluconoride, and BMI were significantly influenced by genetics</p> <p>* Results suggest that PCO is unlikely to be inherited via a single autosomal genetic defect and may be the result of combined genetic and environmental factors or a possible sex-linked disorder associated with nonrandom X-chromosome inactivation</p>	<p>variable traits (i.e., SHBG and insulin)</p> <p>* They did not use clinical evidence of OM or HA to test models of PCO as one twin with hair or acne had cosmetic procedures to help the presence of either or both</p> <p>* Blood was drawn in the follicular phase for all unaffected women</p> <p>* The ultrasound technician blinded to clinical or biochemical results</p> <p>* A test of precision was performed of ultrasound results and interpretation</p> <p><u>Limitations</u></p> <p>* Small number of subjects</p> <p>* Variable analyses other than that for SHBG and insulin were not BMI adjusted and BMI was not assessed between cases and controls for significant differences. However, the ranges of BMI between the controls ($19 < \text{BMI} < 27 \text{ kg/m}^2$) and cases ($16 < \text{BMI} < 37 \text{ kg/m}^2$) were clearly not similar</p>
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				and should have been made more similar between cases and controls for more accurate reference values
<p>Norman et al. (1996)</p> <p><u>Title:</u> Hyperinsulinemia is common in family members of women with polycystic ovary syndrome</p> <p><u>Objective:</u> To determine if a disorder of insulin secretion was common in male and female family members of PCOS probands</p>	<p><u>Population</u></p> <ul style="list-style-type: none"> * Five families * 5 probands; 33 family members (older than 12 years) * Control subjects for PCOS, baldness, insulin and lipids were used for reference values * All subjects Australian * Women stopped OCs at least 6 months prior to blood draw * <u>Affected status definition:</u> <u>All women:</u> T> 1ng/mL or A> 2ng/mL plus SHBG< 20mmol/L plus ovarian morphology on ultrasound (i.e., 8 or more cysts with increased stromal echo on 1 or 2 ovaries) <u>All men:</u> exam, photo or clear history (i.e., from individual or family) of baldness before age 40 years * <u>Exclusion criteria:</u> Smoking 	<p><u>Methods</u></p> <ul style="list-style-type: none"> * Female members had ovarian scanning (vaginal or transabdominal (where subject refused or was too young for vaginal)) * Males assessed for premature baldness * All subjects had fasting blood obtained for androgens, SHBG, lipids, and insulin * 75-g OGTT performed every 30 minutes for 2 hours * All subjects were nonsmokers to avoid the confounding effect of smoking on insulin resistance (a relationship known since at least 1986) 	<p><u>Results</u></p> <ul style="list-style-type: none"> * Hyperinsulinemia (HI) was considered a potential metabolic and genetic marker for people who may be carriers of a family tendency for PCO * HI was common in siblings regardless of obesity * PCO and premature male pattern baldness (PMPB) common in PCOS families * Insufficient subjects to establish mode of inheritance 	<p><u>Strengths</u></p> <ul style="list-style-type: none"> * All subjects had OGTT to test insulin and glucose levels * Smokers excluded to avoid confounding of IR * Fasting blood drawn on all subjects * All ultrasound scans performed in the follicular phase * Reference values were adjusted for BMI (i.e., insulin, triglycerides, LDL, HDL) and for sex (i.e., androgens) * Clear definitions of reference values used, overweight status, and control selection <p><u>Limitations</u></p> <ul style="list-style-type: none"> * Small number of participants affecting two main parts of this study: insufficient to establish form of inheritance and insufficient to examine the relationship between HI and oligmenorrhea * Blood was drawn at

				<p>random cycle times in women</p> <ul style="list-style-type: none"> * PMPB used as male phenotype while it has not been established it has the same genetic basis as PCOS
<p>Waterworth et al. (1997)</p> <p><u>Title:</u> Linkage and association of insulin gene VNTR regulatory polymorphism with polycystic ovary syndrome</p> <p><u>Objective:</u> To study INS VNTR as a candidate genetic locus for susceptibility to PCOS</p>	<p><u>Population</u></p> <ul style="list-style-type: none"> * 147 individuals from 17 families with several PCOS cases * A case-control study of two additional clinic populations: one from the Samaritan and St. Mary's Hospitals, London presenting with anovulation and/or HIR and one from the Middlesex Hospital, London presenting with anovulation and PCO * <u>Ethnicities of families:</u> 14 European descent, 2 Asian, and 1 Iranian * <u>Ethnicity of Case-Control and Middlesex subjects:</u> All European * Medications not mentioned * <u>Affected status definition for family study:</u> <u>All women:</u> PCO on ultrasound <u>All men:</u> Male-pattern 	<p><u>Methods</u></p> <ul style="list-style-type: none"> * Blood drawn for assessment of 8 markers ranging from the tyrosine hydroxylase site to 2 markers in the insulin growth factor 2 region and spanning INS (i.e., the insulin gene locus) 	<p><u>Results</u></p> <ul style="list-style-type: none"> * The authors found positive evidence for linkage of PCOS to the INS VNTR locus on chromosome 11p15.5 * The INS VNTR III/III genotype was associated with increased risk of PCOS in two independent case-control studies * Multilocus linkage disequilibrium mapping may suggest VNTR is the predisposing locus * The authors concluded PCOS is partly due to an inherited alteration in insulin production 	<p><u>Strengths</u></p> <ul style="list-style-type: none"> * The insulin gene locus was studied from several angles at once: linkage with families, association with unrelated individuals, and association with family members <p><u>Limitations</u></p> <ul style="list-style-type: none"> * PMPB was used as the male phenotype to increase the number of affected individuals in each family, but at the expense of a clear phenotype * Small number of subjects for linkage analysis (~8 people per family)

	<p>balding with onset before age 30</p> <p>* <u>Affected status definition for Case-Control study:</u> Anovulation and/or HIR and PCO on ultrasound</p> <p>* <u>Affected status for Middlesex study:</u> Anovulatory cycles (anovulatory = OM = intermenstrual interval of more than 6 weeks) with PCO on ultrasound</p> <p>* <u>Exclusion criteria:</u> “...other causes of anovulation and HIR such as late onset congenital adrenal hyperplasia”</p>			
<p>Legro et al. (1998)</p> <p><u>Title:</u> Evidence for a genetic basis for hyperandrogenemia in polycystic ovary syndrome</p> <p><u>Objective:</u> To determine the genetic basis of endocrinological</p>	<p><u>Population</u></p> <ul style="list-style-type: none"> * 80 PCOS cases * 115 sisters of PCOS cases (used to establish prevalence of PCOS related phenotype due to genetics) * 70 age-, ethnicity-, and weight- comparable controls without history of hypertension or diabetes mellitus, either personally or in 1° relatives (used for reference values) 	<p><u>Methods</u></p> <ul style="list-style-type: none"> * All control women and probands examined by a study investigator * One fasting blood draw done on all participants between 8 and 10 AM. * Out of town sisters (N=39) had blood drawn at an outside site and were not examined by a study investigator * Height, weight, and blood pressure assessed for all subjects (not 	<p><u>Results</u></p> <ul style="list-style-type: none"> * Familial aggregation of hyperandrogenemia (HA) in PCOS kindreds suggest a genetic trait * The authors proposed that HA be used as the defining characteristic of PCOS 	<p><u>Strengths</u></p> <ul style="list-style-type: none"> * Women not fitting all criteria were analyzed and addressed separately (i.e. on OCs, insulin sensitizers, unable to get blood, OM without HA) * Reference values from controls of similar age, ethnicity, and weight * Controls tested for lack of IR by 75-g 2 hour OGTT

<p>abnormalities in families with PCOS</p>	<p>* <u>Ethnicities for probands:</u> 75 non-Hispanic white, 4 Hispanic, 1 African American * <u>Ethnicities for controls:</u> 61 non-Hispanic white, 7 Hispanic, 2 African American * Probands and controls only (not sisters) required to stop OCs at least 3 months before blood draw. Sisters on meds were considered “unknown” status * <u>Affected status definition:</u> Elevated total or free T plus oligomenorhea (less than or equal to 6 menses per year) or amonorrhea (e.g., NIH 1990 Consensus criteria) * <u>Exclusion criteria:</u> Nonclassical 21-hydroxylase deficiency, hyperprolactinemia, or androgen secreting tumors</p>	<p>mentioned how in out of town sisters) * Blood was assayed for total and free T, DHEA-S, LH, and FSH</p>		<p><u>Limitations</u> * Blood drawn at random times to monthly cycle for probands and sisters without testing for spontaneous ovulation * Did not include familial males in this study</p>
<p>Govind et al. (1999) <u>Title:</u> PCO are inherited as an</p>	<p><u>Population</u> * 29 probands * 134 1° proband family members * 10 controls * 44 1° control family members * Reference values from a</p>	<p><u>Methods</u> * Screening interview of all probands and controls * Transvaginal ultrasound used to determine ovarian morphology and volume and performed by one observer (precision of</p>	<p><u>Results</u> * PCOS/PMPB are of autosomal dominant inheritance * Sisters of women with PCOS are more likely to have endocrine abnormalities</p>	<p><u>Strengths</u> * Blood drawn in follicular phase in women with at least somewhat regular cycles and randomly in women with oligo/amonorrhea * Women on hormones</p>

<p>autosomal dominant trait: Analysis of 29 polycystic ovary syndrome and 10 control families</p> <p><u>Objective:</u> To obtain evidence of the genetic basis of PCOS and PMPB</p>	<p>group of 218 normally cycling women sampled within 7 days of menses</p> <p>* <u>Ethnicities for probands:</u> 27 Caucasian and 2 Asians</p> <p>* <u>Ethnicities for controls:</u> 10 Caucasian</p> <p>Women could continue hormones, but those were analyzed separately for LH and testosterone</p> <p>* <u>Affected status definition:</u> <u>Women:</u> PCO by pelvic ultrasound <u>5 mothers and one sister had undergone a hysterectomy and bilateral salpingo oophorectomy:</u> status defined according to history <u>Men:</u> PMPB defined by significant frontoparietal hair loss (type IV of Hamilton) before 30 years</p> <p>* <u>Exclusion criteria:</u> Late onset 21-hydroxylase deficiency Recent ovulation excluded in PCOS by progesterone measurement</p>	<p>which was checked by independent observers)</p> <p>* Single fasting blood draw to assess LH, FSH, progesterone, DHEA-S, SHBG, and testosterone</p>	<p>* PMPB men have higher testosterone than non-PMPB men</p>	<p>analyzed separately for LH and testosterone</p> <p>* Blinded verification of PCO</p> <p><u>Limitations</u></p> <p>* PMPB in men used as phenotype without any proof it is genetically related to PCOS</p> <p>* Cases and controls not race matched</p> <p>* Spontaneous ovulation in PCOS cases not tested for and excluded in analysis</p> <p>* Did not adjust for weight, which the authors identified as a possible mediator of peripheral insulin resistance</p>
<p>Urbanek et al. (1999)</p>	<p><u>Population</u></p> <p>* 150 probands with available nuclear families</p> <p>* 134 sisters of probands</p>	<p><u>Methods</u></p> <p>* Blood drawn for screening of all women for exclusion criteria and</p>	<p><u>Results</u></p> <p>* The strongest evidence for linkage was with the follistatin gene, even after</p>	<p><u>Strengths</u></p> <p>* Large number of subjects</p> <p>* Only PCOS was</p>

<p><u>Title:</u> Thirty-seven candidate genes for polycystic ovary syndrome: strongest evidence for linkage is with follistatin</p> <p><u>Objective:</u> To test 37 candidate genes for linkage and association with PCOS or hyperandrogenemia</p>	<p>* 163 trios (proband and both parents) for transmission disequilibrium testing (TDT) * <u>Ethnicities of families:</u> 148 European, 2 Caribbean * <u>Confounding medications:</u> OCs and insulin sensitizing agents * <u>Affected status definition:</u> <u>For probands:</u> <= 6 menses per year plus evidence of HA (i.e., total or free T more than 2SD above the control mean) <u>For sisters:</u> HA with or without OM * Women considered unaffected if they had normal androgen levels, were not taking any confounding medications, and had regular menstrual cycles (i.e. menses every 27-35 days) * Women not of reproductive age or not considered affected or unaffected were considered unknown <u>For men:</u> considered unaffected * <u>Exclusion criteria:</u></p>	<p>affected status * Blood drawn from all subjects for genetic testing of 37 candidate genes</p>	<p>correction for multiple testing * Linkage results for CYP11A were nominally significant before, but not after correction * The strongest effect in the TDT test was observed in the INSR region, also not significant after correction * They concluded a systematic screen of candidate genes can provide strong evidence for genetic linkage in complex diseases and can identify genes for future research</p>	<p>considered an affected phenotype * Well-designed with a focus on studying each candidate gene from a family approach as well as a case-control approach (i.e., affected sib-pair analysis to test linkage and transmission disequilibrium test analysis to test association)</p> <p><u>Limitations</u> * By using only siblings to test linkage, they lost power in their analysis</p>
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	Nonclassical 21-hydroxylase deficiency, hyperprolactinemia, androgen secreting tumors			
<p>Urbanek et al. (2000)</p> <p><u>Title:</u> Allelic variants of the follistatin gene in polycystic ovary syndrome</p> <p><u>Objective:</u> To detect variation in the follistatin gene and to assess its relevance to PCOS via pedigree and case-control studies</p>	<p><u>Population</u></p> <ul style="list-style-type: none"> * Screened the follistatin gene for DNA sequence variants in 19 families with multiple affected daughters (N=85 affected members) and in 31 unrelated women with PCOS and 15 control women * Tested a common variant in the follistatin gene for association with PCOS in 249 PCOS families (N=324 affected members) * Examined follistatin messenger RNA expression levels in cultured fibroblast cells from 18 PCOS cases and 13 control women * <u>Ethnicities of families:</u> 90 European descent, 5 Caribbean or Mexican, 2 African American, 1 Asian Indian, 1 unknown * <u>Confounding medications:</u> OCs or insulin sensitizing drugs * <u>Affected status definition:</u> 	<p><u>Methods</u></p> <ul style="list-style-type: none"> * Blood drawn for screening of all women for exclusion criteria and affected status * Blood drawn from all subjects for genetic testing of follistatin variants 	<p><u>Results</u></p> <ul style="list-style-type: none"> * Most of the follistatin gene variants are rare (i.e., 13 of 20 variants occurred at a frequency of less than 5% of parental chromosomes) * Only one sequence polymorphism was detected in the coding region and the intron/exon boundaries of the follistatin gene of 31 PCOS and 15 control women * No significant differences were found in the number of transmissions of markers in the follistatin gene among the 249 PCOS families with 324 affected members * No substantial difference was found in follistatin expression between the 18 PCOS cases and 13 controls * Contributions, if any, from the follistatin gene to the etiology of PCOS are likely to be small 	<p><u>Strengths</u></p> <ul style="list-style-type: none"> * Various methods employed to test follistatin variants, including family and case-control studies * Males excluded from analysis <p><u>Limitations</u></p> <ul style="list-style-type: none"> * Only 284 of 324 “affected” women had PCOS, the rest had HA only. While this does introduce more variance into analysis, which increase the chances of an inconclusive result, the fact that only 40 of 324 women (~12% of the total population) had HA only would probably not have changed study results if they had been removed from analysis.

	<p><u>Probands</u>: OM (≤ 6 menses/year) and HA (i.e., total or free T more than 2 SD above the control mean)</p> <p><u>Sisters</u>: HA with or without OM</p> <p>* <u>Exclusion criteria</u>:</p> <p><u>Probands</u>: Hyperprolactinemia and nonclassical congenital adrenal hyperplasia</p> <p><u>Sisters</u>: normal androgens but irregular cycles, on confounding medications, not of reproductive age</p> <p>* All brothers</p>			
<p>Kahsar-Miller et al. (2001)</p> <p><u>Title</u>: Prevalence of polycystic ovary syndrome (PCOS) in first-degree relatives of patients with PCOS</p> <p><u>Objective</u>: To determine the rate of clinically evident PCOS in 1° female relatives of PCOS probands</p>	<p><u>Population</u></p> <p>* 195 PCOS cases (Caucasian and African American) + female relatives (i.e., 78 mothers and 50 sisters of 93 probands)</p> <p>* 119 controls (aged 18 to 50 years) recruited for determination of normal androgen level ranges</p> <p>* <u>Ethnicities of probands</u>: 166 non-Hispanic white, 29 black</p> <p>* <u>Ethnicities of controls</u>: not mentioned</p> <p>* <u>Confounding medications</u>: controls excluded for OCs; no</p>	<p><u>Methods</u></p> <p><u>Cases</u>:</p> <p>* Probands interviewed about family prevalence of HIR and menstrual irregularities and to get permission to contact their mothers and sisters</p> <p>* Clinical evaluations conducted on all consenting family members to assess personal menstrual history as well as HIR and acne, height and weight</p> <p>* Serum obtained for measurement of total and free T, SHBG</p> <p>* Thyroid stimulating</p>	<p><u>Results</u></p> <p>* High degree of familial aggregation of PCOS (5- to 6-fold increased incidence among 1° female relatives)</p> <p>* Unclear mode of inheritance</p> <p>* 35% of mothers and 40% of sisters of probands will be affected by PCOS</p>	<p><u>Strengths</u></p> <p>* Authors acknowledge family bias toward treatment if a family member also has PCOS and changed the affected status to include those family members</p> <p>* A strict definition of “affected” status was used for probands and their family members</p> <p>* Analysis for differences in participating and nonparticipating family members of probands was done to ascertain self-selection bias</p> <p>* They addressed possible</p>

	<p>androgens assessed for family members on OCs</p> <p>* <u>Affected status definition:</u> <u>For probands:</u> 1990 NIH Consensus criteria (i.e., evidence of ovulatory dysfunction with HIR and/or HA) <u>For relatives:</u> modified to address previous treatment of HIR or acne</p> <p>* <u>Exclusion criteria:</u> Nonclassical adrenal hyperplasia, Cushing syndrome, hyperprolactinemia, hypothyroidism</p>	<p>hormone, prolactin, and 17-OHP to test for exclusion criteria</p> <p><u>Controls:</u> Controls underwent a cursory physical examination, HIR scoring, and blood sampling</p>		<p>differences in the results between this study and others as due to ethnicity or environment (e.g., diet)</p> <p><u>Limitations</u> * The small number of sisters recruited prevent an analysis of mode of inheritance</p>
<p>Mao et al. (2001)</p> <p><u>Title:</u> Study on the mode of inheritance for familial polycystic ovary syndrome</p> <p><u>Objective:</u> To investigate the mode of inheritance of PCOS</p>	<p><u>Population</u> * 139 PCOS cases * 1° family relatives * <u>Ethnicity:</u> All subjects Chinese * <u>Confounding medication:</u> OCs stopped for at least 3 months * <u>Affected status definition of cases:</u> Irregular menses (cycle <21 days or >35 days or bleeding for ≥10 days) <u>and</u> blood hormones (LH/FSH ≥2, T ≥2.2nmol/L, A >9nmol/L) <u>and</u> PCO by ultrasound OR males with PMPB</p>	<p><u>Methods</u> * Outcome measures were irregular menses in women and PMPB in men * Questionnaires from probands about family members * Information verified by family member interview</p>	<p><u>Results</u> * Prevalence rates of irregular cycles among mothers and sisters were 37.4% and 33.1%, respectively * Prevalence rates of PMPB among fathers and brothers were 19.4% and 6.5%, respectively * Simple segregation analysis indicated that the segregation ratio of PCOS trait in siblings was 0.3023 * Complex segregation analysis indicated that PCOS fit in with the</p>	<p><u>Strengths</u> * Large number of families enrolled</p> <p><u>Limitations</u> * PMPB was included as an affected phenotype in this study of PCOS mode of inheritance. There is no evidence of a similar or shared genetic basis for PCOS and PMPB.</p>

	* <u>Exclusion criteria</u> : Other diseases related to androgen excess, like Cushing's syndrome, etc.		inheritance model of co-dominant disorder with full penetrance and sporadic cases	
<p>Colilla et al. (2001)</p> <p><u>Title</u>: Heritability of insulin secretion and insulin action in women with polycystic ovary syndrome and their first degree relatives</p> <p><u>Objective</u>: To examine the extent of heritability of defects in both insulin action and insulin secretion among PCOS families</p>	<p><u>Population</u></p> <ul style="list-style-type: none"> * 33 PCOS cases * 48 non-diabetic 1° relatives <p>* <u>Ethnicities of relatives</u>: 31 Caucasian, 12 African American, 4 Asian, 1 Hispanic</p> <p>* <u>Confounding medications</u>: All steroid preparations (including OCs) and insulin altering medications stopped \geq 2 months before screening and enrollment</p> <p>* <u>Affected status definition</u>: Historical, physical examination, and hormonal evidence of androgen excess and NIH consensus criteria (i.e., history of OM, infertility, HIR, acne or androgenetic alopecia and HA measured by free T\geq 34.7 pmol/L)</p> <p>* <u>Exclusion criteria</u>: Nonclassical 21-hydroxylase deficiency, congenital adrenal hyperplasia, Cushing's</p>	<p><u>Methods</u></p> <ul style="list-style-type: none"> * All subjects had a fasting intravenous glucose tolerance testing (IVGTT) where 2 IVs were placed, one in each arm (one to administer glucose and tolbutamide, one to draw blood for insulin and glucose levels). Blood was drawn 34 times from -20 to 240 minutes. From IVGTT, they measured 1) first phase insulin secretion, 2) insulin sensitivity index, and 3) insulin secretion X insulin sensitivity to assess beta cell secretory function adjusted for insulin resistance * Blood drawn was assayed for insulin, glucose, testosterone, SHBG 	<p><u>Results</u></p> <ul style="list-style-type: none"> * Significant heritability was found for insulin secretion among siblings * The parameter quantifying insulin secretion in relation to insulin sensitivity was significant among siblings * The authors concluded that there is an heritable component to beta cell dysfunction in families of women with PCOS 	<p><u>Strengths</u></p> <ul style="list-style-type: none"> * The authors measured environmental influences on beta cell dysfunction (i.e. spousal correlations vs. parent-offspring and sibling correlations) * IVGTT is a very thorough method of measuring insulin and glucose levels * Age, sex and race adjusted for BMI in all analyses <p><u>Limitations</u></p> <ul style="list-style-type: none"> * Small number of subjects studied * Many of the subjects were both obese and profoundly insulin resistant, limiting variability of this measure * The authors did not address ethnic differences in insulin sensitivities that have been shown to exist. They could have addressed this by doing stratified analyses using ethnicity.

	syndrome, diabetes mellitus, hyperprolactinemia			
<p>Legro et al. (2002)</p> <p><u>Title:</u> Insulin resistance in the sisters of women with polycystic ovary syndrome: Association with hyperandrogenemia rather than menstrual irregularity</p> <p><u>Objective:</u> To determine if sisters of PCOS probands have evidence of PCOS</p> <p><u>Hypothesis:</u> IR produced anovulation in sisters with PCOS</p>	<p><u>Population</u></p> <ul style="list-style-type: none"> * 336 PCOS probands * 307 sisters of 219 probands * 47 controls (for reference values) * <u>Ethnicities for sisters and controls:</u> non-Hispanic white * <u>Confounding medications:</u> subjects taking OCs, hypertension, and insulin sensitizing medications were considered “phenotype unknown” * <u>Affected status definition:</u> Total T > 58 ng/dL or free T > 15ng/dL and OM * <u>Exclusion criteria:</u> Nonclassical 21-hydroxylase deficiency, hyperprolactinemia, androgen secreting tumors 	<p><u>Methods</u></p> <p><u>Cases:</u></p> <ul style="list-style-type: none"> * Sisters were evaluated onsite at one of 3 study centers (N=122) or offsite in a local hospital or clinical laboratory (N=185) * Fasting blood draws on all subjects * Those seen on site also had blood pressure assessment, waist and hip girth measurements at the study center * Offsite subjects had self-reported height and weight (N=166) measurements <p><u>Controls:</u></p> <ul style="list-style-type: none"> * Examined by a study investigator and had no HIR * Tested for glucose tolerance by 75-g 2 hour OGTT * Assays determined from single fasting sample for glucose, insulin, LH, FSH, total and free T, and DHEA-S 	<p><u>Results</u></p> <ul style="list-style-type: none"> * hyperandrogenism (HA) identifies sisters at risk for insulin resistance (IR) while menstrual irregularity does not * IR clusters in families with PCOS 	<p><u>Strengths</u></p> <ul style="list-style-type: none"> * The authors controlled for ethnic differences in insulin sensitivity in PCOS probands by recruiting only non-Hispanic white women * Analysis of continuous response variables were adjusted for age and BMI * Controls had no history of non-insulin dependent diabetes mellitus (NIDDM) or hypertension personally or in their 1° relatives * Fasting blood was drawn from each subject for biochemical assessment <p><u>Limitations</u></p> <ul style="list-style-type: none"> * Controls were age, weight and ethnicity comparable to PCOS probands, but not their sisters * Blood drawn at random times in all women

APPENDIX B

CELLULAR ASPECTS OF ADIPOSITY AND INSULIN RESISTANCE

An analysis of the Third National Health Examination Survey data determined a prevalence of diabetes 2.9 times higher in persons who are overweight rather than non-overweight (1). Prospective studies, like those involving Pima Indians, have supported this association (2). Body weight/fat is associated with diabetes via the presence of insulin resistance (3), which is itself a predisposing factor for type 2 diabetes (T2D) (4-6). Evidence from obesity research confirms skeletal (7), adipose, and liver (8) tissue have insulin resistance in states of overweight. When obese subjects develop T2D, glucose, insulin, and free fatty acids (FFAs) simultaneously increase, yet the defining insulin resistance, as measured by decreased total body glucose disposal, is thought to occur predominantly in skeletal tissue. There are several factors associated with both increased body fatness and insulin resistance, including systemic inflammation (CRP, PAI-1, TNF- α), endothelial dysfunction, dyslipidemia, hyperinsulinemia, and altered coagulation and fibrinolytic factors (Figure B-1).

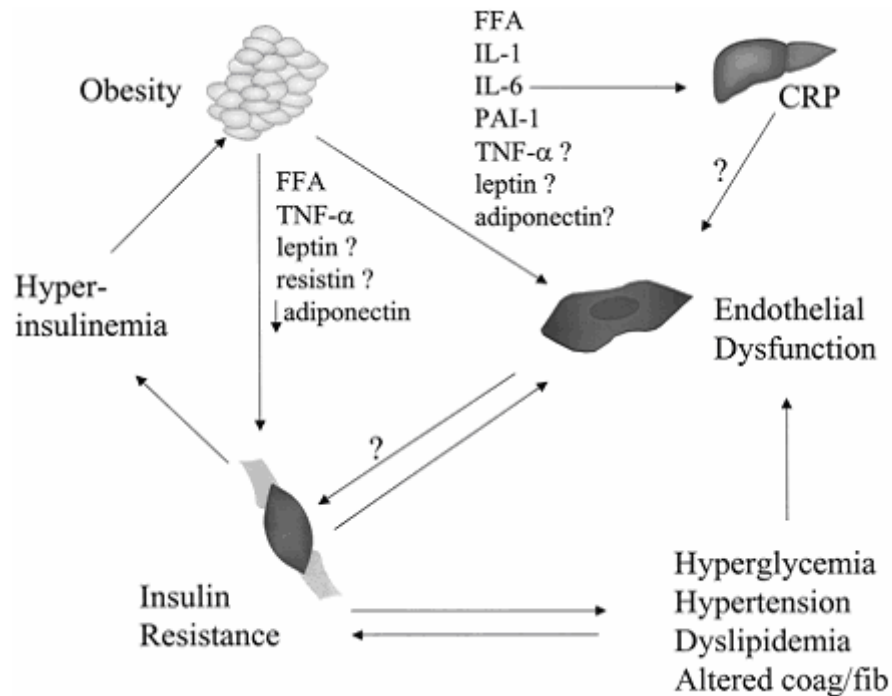


Figure B-1. Obesity and Insulin Resistance

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Several theories exist to explain the relationship between body fatness and insulin resistance. One long-standing theory, the portal hypothesis, states that visceral fat accumulation almost primarily accounts for complications due to obesity by contributing to an increase in portal vein plasma FFA concentrations (9). One problem with the portal hypothesis is that it does not address the effect of overall body fatness or subcutaneous truncal fat in insulin resistance. Recent research has found in obese women that both body fatness, as measured by whole body magnetic resonance imaging (MRI) (10), and truncal subcutaneous fat mass, as measured by the sum of skinfolds (11), were significantly related to insulin resistance. Subcutaneous truncal fat has also been associated with insulin resistance in both obese nondiabetic (12, 13) and T2D.(14-16) men.

Two alternative hypotheses exist to explain the relationship between subcutaneous truncal fat and/or overall body fatness on insulin sensitivity– the ectopic fat storage syndrome hypothesis and the adipocyte as an endocrinologically-active organ. In this dissertation, the selection of candidate genes as well as the theories of how obesity and insulin resistance relate are most supported by the ectopic fat storage syndrome hypothesis.

Ectopic fat storage, or intramuscular lipid storage, as a cause of insulin resistance, is supported by the finding of increased skeletal muscle triglyceride content in subjects with obesity or T2D. Muscle TG levels are predictive of insulin resistance in both humans and animals, as measured by muscle biopsy, CT and spectral magnetic resonance (sNMR) (17). In an extensive review of the relationship between muscle triglyceride and insulin resistance, Kelley et al. (15) addressed several paths of thought concluding that inflexibility of muscle lipid utilization as fuel in obese and type 2 diabetic subjects was the main defect by which all study results could be explained. The main premise of this concept was that insulin resistance entails disturbances of both glucose and fatty acid metabolism. In normal skeletal muscle, there is flexibility in type of fuel usage dependent upon the environment of the muscle (i.e., pre- or post-absorptive) with the ability to seamlessly switch between fuel types (i.e., glucose or fatty acids). These impairments seem to be indirectly centered on the ability of mitochondria to oxidize fatty acids, perhaps through mediation of lipid metabolite levels, such as ceramide or diacylglycerol, both of which are known to attenuate insulin signaling (18). Various methods exist to image intramyocellular lipid, including noninvasive methods, such as magnetic resonance spectroscopy or computer tomography, and invasive methods, such as muscle biopsy. Magnetic resonance spectroscopy is able to distinguish between intramyocyte and extramyocyte lipid, where it is possible to identify peaks corresponding with the methylene carbon of triglyceride (19). Extramyocyte triglyceride is contained within adipocytes within muscle and intramyocyte TG (IMTG) is found within muscle fibers. IMTG is the form increased in obesity and is correlated with severity of IR (20). From increases of IMTG found in non-obese, insulin resistant, first-degree relatives of type 2 diabetics (21), it is suggested that among insulin resistant populations, regional deposition of fat within skeletal muscle may be an early body composition abnormality, rather than arising later as a consequence of excess adiposity. This further supports the idea that disturbed lipid metabolism by skeletal muscle may have a pivotal role in development of obesity and type 2 diabetes.

In the hypothesis that the adipocyte is an active endocrine organ, the adipocyte responds to various stimuli to integrate metabolic, hormonal, and neural stimuli by releasing hormones (22-44). This view is quickly gaining popularity as several adipocyte-secreted factors (i.e., adipokines), including interleukin-6 (45), leptin (23), and tumor necrosis factor- α (23) are significantly associated with obesity, insulin resistance, and type 2 diabetes. These two hypotheses are not mutually exclusive and highlight the complexity of determining causes of insulin resistance among individuals.

B.1 BASIC CELLULAR MECHANISMS OF THE INSULIN PATHWAY

B.1.1 Insulin receptor

Insulin is the most potent anabolic hormone known and is essential for normal tissue development, growth, and maintenance of whole-body glucose homeostasis. Insulin is secreted by the beta cell of the pancreatic islets of Langerhans in response to elevated circulating glucose levels and amino acids after eating. Insulin regulates glucose homeostasis at many sites, thereby reducing hepatic glucose output (via decreased gluconeogenesis and glycogenolysis) and increasing the rate of glucose uptake. Insulin resistance primarily occurs when normal levels of circulating insulin are not sufficient to regulate these processes. Thus, insulin resistance is a defect in signal transduction.

The insulin receptor is comprised of two cysteine-rich extracellular alpha subunits and two membrane-spanning beta subunits linked by disulfide bonds to form a beta-alpha-alpha-beta heterotetramer. This receptor is a large glycoprotein weighing 300-400 kiloDaltons (kDa) mostly concentrated on adipocyte and hepatocyte cells (200,000 – 300,000 per cell). The tyrosine kinase activity of the insulin receptor was first recognized in 1981 (46) and is defined as the enzymatic (kinase) activity enabling the insulin receptor to transfer a phosphate moiety from adenosine triphosphate (ATP) to a specific amino acid residue (tyrosine). When phosphorylated, this tyrosine is part of a recognition motif for several insulin substrates. A large body of evidence suggests that the receptor tyrosine kinase activity and multisite autophosphorylation is required for biological activity and action of the insulin and insulin-like growth factor (IGF)-1 receptors (47-49). The autophosphorylation sites in the insulin receptor beta-subunit include Tyr1146, Tyr1150, Tyr1151 (50). These residues correspond to major autophosphorylation sites in the kinase domain of the prototype tyrosine kinase pp60c-src. Insulin receptor autophosphorylation, which directly regulates the receptor's kinase activity, seems to occur through a transmechanism, whereby insulin binding to the alpha subunit of one alpha-beta dimer stimulates autophosphorylation of the adjacent covalently-linked beta subunits. The insulin receptor, though, is regulated by more than insulin binding and tyrosyl-autophosphorylation. Prior to stimulation by insulin, the receptor is basally phosphorylated on serine and threonine residues. Signaling systems that stimulate serine or threonine kinases, including the insulin receptor itself, increase the Ser/Thr phosphorylation of the beta-subunit (46, 51), which has been associated with decreased tyrosine kinase activity of the insulin receptor. Chronically elevated insulin levels, such as those found in states of insulin resistance, may stimulate serine kinases perhaps through the IGF-1 receptor, which itself can be

stimulated by high insulin levels. This interaction could provide a mechanism for insulin-induced insulin resistance. Studies of women with PCOS have indicated that associated IR may be most likely due to post-receptor binding dysfunction rather than substrate-receptor binding {Dunaif, 1992 #503}.

B.1.2 Insulin Receptor Activity (Signaling)

Once the receptor is bound, the insulin signal is transmitted through a cellular network of protein kinase cascades based on both tyrosine and serine/threonine kinases. Many of insulin's actions are the result of dephosphorylation reactions, including: 1) the activation of glycogen synthase, pyruvate kinase and pyruvate dehydrogenase and 2) the inhibition of triacylglycerol lipase, phosphorylase and its kinase (52).

Insulin action begins when insulin binds to its receptor. The tyrosine kinase of the insulin receptor is initially stimulated by insulin binding and is subsequently augmented by insulin-stimulated receptor autophosphorylation. The discovery of tyrosine kinase activity of the insulin receptor allowed identification of down-stream elements in insulin action. Two mechanisms that have emerged as the foundation for insulin signal transmission are: 1) the substrate hypothesis and 2) the association hypothesis. The substrate hypothesis involves the tyrosine phosphorylation of cellular proteins by the activated insulin receptor kinase. The association hypothesis is based on the idea that autophosphorylation of the beta subunit mediates non-covalent, stable interactions between the receptor and cellular proteins and is not dependent on phosphorylation. Common insulin receptor substrates include the insulin receptor substrate-1 (IRS-1), which may function as a central molecule in insulin signal transmission by binding to src homology-2 (SH-2) domain-containing proteins during insulin stimulation and regulating associated catalytic activities that mediate the insulin response. These SH-2 domains are phosphotyrosine binding sites located in a variety of proteins that control the activation of tyrosine kinases in the insulin and other tyrosine-based receptors. IRS-1 binds strongly to the phosphatidylinositol 3'-kinase (PI3K) enzyme (53). PI3K is believed to be an important mediator of cellular growth and metabolism (54) and its activity increases several-fold after stimulation by insulin, suggesting IRS-1 and/or the insulin receptor somehow activate PI3K in vivo (55).

B.1.3 Insulin synthesis

Beta cells are the only body cells capable of synthesizing insulin, a process begun with proinsulin mRNA, by uniquely being able to initiate transcription of the preproinsulin gene. In the beta cell nucleus, the preproinsulin mRNA is matured by the sequential addition of a 5'-methylguanine cap, a poly-A tail, and

the excision of noncoding introns. Mature mRNA is translocated to the cytoplasm for translation of preproinsulin on membrane-bound ribosomes. Beta cell cytoplasm contains large amounts of preproinsulin mRNA (~10% of total mRNA) that are dormant in glucose concentrations > 3.3 mM. The initiation of translation ultimately leads to insulin biosynthesis where translocation of glucose to the ribosomes increases within minutes when glucose concentrations are greater than 3.3mM (56). After translation of approximately 50 residues, the nascent chain emerging from the ribosomal complex binds to the signal recognition sequence of a ribonucleoprotein complex and elongation is stopped as the translation complex binds to the endoplasmic reticulum, or ER. Within the ER, translation of preproinsulin mRNA is completed and the conversion to proinsulin occurs – a process taking about 30 to 60 seconds.

Proinsulin is then transported along the microtubule network in transport vesicles to the cis part of the Golgi apparatus – a process that is guanine triphosphate- and calcium-dependent (57). In the trans network of the Golgi apparatus, proinsulin is converted to insulin and the inactive by-product C-peptide by prohormone-converting endopeptidases PC3 and PC2 and exopeptidase carboxypeptidase H. Insulin is then packaged into secretory vesicles in cisternae of the Golgi apparatus to be ready for export to the plasma membrane. Insulin “grains” accumulate in the cisternae forming clathrin-coated vesicles. From the trans-Golgi network, the vesicles are carried via microtubules that form the beta cell cytoskeleton.

B.1.4 Beta Cell Dysfunction

The beta cell cytoskeleton is important for insulin secretion as disruption of its function inhibits post-translational processing and mobilization of insulin to the plasma membrane. This network consists of polymerized structures of actin filaments and microtubules and it bridges the ER, the Golgi apparatus and the plasma membrane. Furthermore, the application of glucose to these cells has been shown to increase amounts of polymerized tubulin in the beta cell (58). Polymerization of tubulin and mobilization of vesicles is regulated by microtubule-associated proteins that are bound to tubulin. These proteins are believed to be phosphorylated by cAMP-responsive protein kinases (59, 60). Likewise, the amount of polymerized actin in islet cells increases from 40% to 70% upon glucose stimulated insulin secretion (59, 61).

Insulin secretion via secretory vesicles requires recruitment from the cytosolic pool, translocation to the plasma membrane, and physical association with the plasma membrane (i.e., vesicles dock and fuse with the plasma membrane and their content spill into extracellular space, a process also known as

exocytosis). Phosphorylation of microtubulins and filaments aid in navigation of vesicles toward the cell membrane. Kinesin, the force-generating microtubule-associated adenosine triphosphatase (ATPase), has been identified as important in mobilization of insulin secretory vesicles. In the resting beta cell, kinesin is phosphorylated by casein kinase 2 and is rapidly desphosphorylated by calcineurin (62) with increased calcium levels. A small pool of insulin vesicles are contained within the beta cell for “ready release” and fusion of these vesicles with the plasmalemma allows insulin release (63). Soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptors (SNAREs) are important for directing specificity of vesicles to the membrane (60). The vesicle-SNARE (v-SNARE) is recognized by the target-SNARE (t-SNARE) on the plasma membrane. Docking of the vesicles with the plasma membrane involves formation of a core complex linking the syntaxin and synaptosomal-associated protein 25 (SNAP-25), the t-SNARE, with vesicle-associated protein 2 (VAMP-2)/synaptobrevin-2, the v-SNARE (64, 65).

Beta cells are responsible for maintaining a narrow range of blood glucose levels, working through a feedback loop, as follows: 1) hyperglycemia signals beta cells to produce insulin and suppress glucagon; 2) suppressed glucagon switches off glucose production from liver and increases glucose uptake in muscle, fat and liver; 3) hypoglycemia signals beta cells to decrease insulin secretion and increase glucagon, thus stimulating glucose production in the liver. When functioning properly, the rate of hepatic glucose production equals the rate of glucose disposal and fasting glucose levels are maintained between 80 and 115 mg/dl. In type 2 diabetes and other states of inadequate insulin, hepatic glucose production exceeds glucose disposal resulting in fasting hyperglycemia (66).

In the normal beta cell, insulin secretion is initiated when glucose enters the cell, accelerates metabolism, and closes adenosine triphosphate (ATP)-sensitive potassium channels (67). Potassium channels have high-affinity sulfonylurea receptors which are sensitive to sulfonylureas and other secretagogues (which close channels) and diazide (which opens channels). Closure of potassium channels depolarize the cell membrane and set up oscillating fluxes of calcium, which then trigger oscillating releases of insulin granules from the cell (67-69). Evidence exists that Ca⁺⁺-dependent protein phosphorylation aids in the initiation of exocytosis.

Early in type 2 diabetes, there is a lack of responsiveness of the beta cells to glucose, which later turns to a reduction in beta cell mass (70). The initial lesion may be comprised of an abnormality in glucose transport, secretagogue pathways, ion channels, or other processes involved in insulin synthesis, processing, storage and release (66). Genetically increased apoptosis may be responsible for beta cell loss (71, 72) with cofactors such as cell exhaustion from long-term hypersecretion and deposition of amyloidlike material in pancreatic islets. Further contributing factors probably include glucotoxicity and/or lipotoxicity (70, 72, 73).

Regardless of the cause, the first indication of beta cell dysfunction is a delay in the acute insulin response to glucose. In the normal response to insulin, a first phase secretion response starts immediately, peaks in 10 minutes, and ends in 20 minutes and is followed by a second secretion phase, which begins at 15-20 minutes and peaks over the next 20 to 40 minutes (74). In type 2 diabetics, the first phase response to glucose is lost and must be gone approximately 5 years before fasting hyperglycemia appears (72). The result is an excessive rise in postprandial glucose and, in response to that, a hypersecretion of second-phase insulin (72, 75).

B.2 THE INSULIN PATHWAY AND LIPID METABOLISM

B.2.1 Fat cell regulation of insulin sensitivity

Adipose tissue has an important role in insulin sensitivity and the relationship can be outlined as follows: circulating free fatty acids that have been derived from adipose tissue are elevated in many states of insulin resistance and may contribute to insulin resistance by (i) inhibiting glucose uptake, glycogen synthesis, and glucose oxidation and (ii) increasing hepatic glucose output (76).

Cross-talk between tissues in regulation of glucose metabolism involves the following steps:

- 1) Insulin is secreted from pancreatic beta cells as a result of elevated circulating glucose levels.
- 2) Insulin decreases glucose production from the liver, and increases glucose storage, uptake, and utilization in adipose and muscle tissue.
- 3) The fat cell regulates metabolism by releasing FFAs that reduce glucose uptake in muscle, insulin secretion from the beta cell, and increase glucose production from the liver.
- 4) The fat cell can also secrete adipokines, like leptin, adiponectin, and TNF, which regulate food intake, energy expenditure, and insulin sensitivity.

Plasma glucose is kept in tight regulation despite periods of feeding and fasting. This balance is governed by: 1) glucose absorption from the intestine, 2) production by the liver, and 3) uptake and metabolism by peripheral tissues. Insulin increases glucose uptake in muscle and fat, by stimulating GLUT4 translocation from intracellular sites to the cell surface, and inhibits hepatic glucose production in order to regulate blood glucose concentrations. Moreover, insulin has other non-metabolically centered functions, including cell growth and differentiation, as well as promoting the storage of substrates in fat, liver and muscle by stimulating lipogenesis, glycogen and protein synthesis and by inhibiting lipolysis, glycogenolysis and protein breakdown. Insulin resistance results in profound dysregulation of these processes, producing elevations in fasting and postprandial glucose and lipid levels. Even though up to 75% of insulin-dependent glucose disposal occurs in skeletal muscle (77), only mice with a knockout of the insulin receptor in adipose tissue have impaired glucose tolerance when compared to skeletal muscle knockout mice. These results may suggest that skeletal tissue IR is more due to insulin receptor dysfunction in adipose tissue than in skeletal tissue. Thus, adipose tissue is crucial in regulating both lipid and glucose metabolism.

Elevated FFAs are associated with insulin-resistant states and have been implicated in contributing to diabetes and obesity by inhibiting glucose uptake, glycogen synthesis and glucose oxidation, and by

increasing hepatic glucose output (76). Furthermore, they are also associated with reduced insulin-stimulated IRS-1 phosphorylation and IRS-1-associated PI3K activity (78). The link between increased circulating FFAs and insulin resistance may involve accumulation of triglycerides and fatty-acid derived metabolites (diacylglycerol, fatty acyl-CoA, and ceramides) in liver and muscle.

B.2.2 Lipid Metabolism

Lipids are digested and absorbed in the gastrointestinal tract and are transported via plasma to various organs for energy use or storage. The liver, adipose tissue and the mammary gland all use exogenous lipids to synthesize triglycerides. The liver also produces endogenous lipids to synthesize cholesterol. Overall, free fatty acids, although very important for fuel, form a small part of blood lipids. They are transported in plasma bound non-covalently to albumin. Major classes of lipids in plasma include: triglycerides, phospholipids, cholesterol and its esters, and free or non-esterified fatty acids (Figure B-2). Triglycerides (TGs), phospholipids, and cholesterol and its ester are transported in the blood as lipoproteins. Lipoproteins have a hydrophobic core comprised of TG and cholesterol ester, which is surrounded by a monolayer of apoproteins, cholesterol and phospholipids. The monolayer allows this macromolecule to be miscible in plasma for transport of endogenous and exogenous lipids. While in this complex, lipids and proteins associate and are stabilized by non-covalent forces. The protein components of lipoproteins, called apoproteins, form the lipoprotein structure providing recognition sites for cell surface receptors and can act as regulators of certain enzymes.

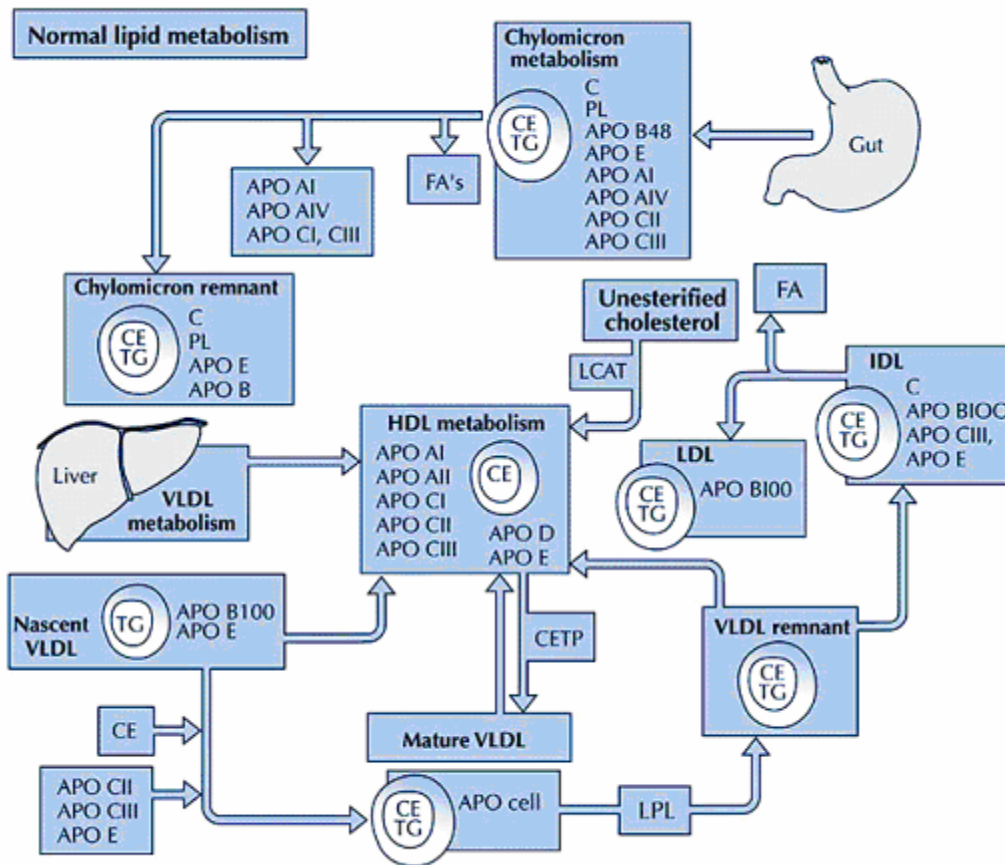


Figure B-2. Normal lipid metabolism

Bold terms are the main products at each step in the pathway. Apo – apolipoprotein; C – cholesterol; CE – cholesterol esters; CETP – cholesterol ester transfer protein; FA – fatty acid; HDL – high density lipoprotein; IDL – intermediate density lipoprotein; LCAT – lecithin acyltransferase; LDL – low density lipoprotein; LPL – lipoprotein lipase; PL – phospholipids; TG – triglycerides; VLDL – very low density lipoprotein. (Reprinted, with permission, from Current Hypertension Reports, Volume 1 © 1999 by Current Science, Inc. www.biomedcentral.com/currdiabetesrep from Nicholas (1999) Curr Hyperten Rep 1: 131-36.)

B.2.3 Lipid digestion

The products of lipid digestion in the jejunum are free fatty acids, cholesterol and 2-monoacylglycerol. When mixed with bile salts, they create mixed micelles, which are soluble in an aqueous environment.

Fatty acids are converted into their CoA esters in the mucosal cell and are esterified by the addition of various acyl transferases 2-monoacylglycerol to form triglycerides (triacylglycerol). Cholesterol is esterified into cholesterol esters and lysophospholipids are converted to phospholipids. Intestinal mucosal cells synthesize apolipoprotein B (apoB). The lipids are then repackaged to form chylomicrons, containing the core of triacylglycerol and cholesterol esters, now with lipid soluble vitamins, carotene, and others. Chylomicrons are then secreted by enterocytes into the intestinal lacteals, where intestinal lymph can enter the blood stream through the thoracic duct. During transport into the blood, these chylomicrons acquire apoE (to be recognized by specific hepatic receptors) and apoCII (an activator of lipoprotein lipase). During their transport in the vascular system, chylomicrons are hydrolyzed by lipoprotein lipase (LPL). LPL is present in capillary beds of most organs and tissues where it is bound to glycosaminoglycans on the luminal surface of the endothelial cells. ApoCII interacts with both LPL and the chylomicron to orient the catalytic site with its lipid substrate. The LPL found in the liver is different from that found in the vascular endothelium in that liver LPL is not heparin sensitive. Also, only hepatic LPL is involved in the TG hydrolysis of chylomicron remnants. LPL in different tissues is influenced by nutritional and hormonal status. It is insulin sensitive and, in the fed state, LPL activity in adipose tissue increases. Thus, in the fed state when blood insulin levels are high, dietary FA are transported to the adipocyte for storage as TG.

B.2.4 Transport of endogenous lipids

After a carbohydrate-rich meal, glucose in the liver is converted to fatty acid via acetyl CoA. At this time, the fatty acids in the liver are esterified to form TG. In the normal healthy condition, TG does not accumulate in the liver and is removed from the liver through secretion of very-low-density-lipoproteins (VLDL). This nascent VLDL is converted to mature active VLDL by acquiring apoE and apoCII from high-density-lipoprotein (HDL). The triglyceride-rich core of VLDL is transformed by being hydrolyzed to low-density-lipoprotein (LDL). As TGs are removed, VLDL becomes smaller and denser. ApoE and apoCII, which originally came from HDL, are returned to HDL. Cholesterol esters are transferred from HDL to VLDL, while TG and phospholipids transfer concurrently from VLDL to HDL. As a result of intravascular hydrolysis and protein and lipid exchanges, VLDL is converted to LDL. Thus, through the metabolism of VLDL, fatty acids synthesized endogenously are transported from liver to peripheral tissues, like muscle and adipose tissue.

The lipoproteins principally involved in cholesterol transport are LDL and HDL. LDL is the product of VLDL metabolism. It retains most of the apoB found in VLDL as well as cholesterol esters, but has

lost most of the TG found in VLDL. Since LDL particles are relatively small, they enter the extracellular space and are in direct contact with the plasma membrane of various cells. LDL's primary function is to supply peripheral tissues with cholesterol, the structural component of cells plasma membranes. Cells obtain most of their cholesterol from circulating LDL, conserving energy in the process. To expedite the process of delivering cholesterol to cells while preventing cholesterol accumulation, the cell surface has specific LDL receptors that bind and internalize LDL via endocytotic vesicles. These vesicles fuse with lysosomes which are then hydrolyzed by lysosomal enzyme. Unesterified cholesterol released from the lysosome into the cell sap suppresses the synthesis of b-hydroxy-b-methyl glutaryl (HMG) CoA reductase causing reduced cholesterol synthesis. The LDL receptor is also under feedback control. In a low cholesterol state, HMG CoA reductase and LDL receptor synthesis are increased to augment cellular cholesterol. Concurrently, esterification of cholesterol is reduced.

B.2.5 Metabolism of HDL

HDLs are synthesized in the liver and released by exocytosis. They are comprised of protein and phospholipids. Nascent HDL is converted to HDL through acquisition of cholesterol. HDL easily accepts unesterified cholesterol – either circulating or on the cell surface membrane. During metabolism of chylomicrons and VLDL, their TGs are removed by LPL leaving excess free cholesterol, which is then removed via HDL. Many studies have recognized the correlation between HDL levels and coronary heart disease (CHD). HDL is protective against the formation of CHD by channeling cholesterol toward the liver for excretion instead of toward adipose tissue for storage.

B.3 FATTY ACID OXIDATION IN SKELETAL MUSCLE

Insulin normally inhibits lipolysis (79) which lowers plasma FFA concentrations depriving cells of the primary source of circulating lipid fuel. Insulin also forces glucose into cells, stimulating glucose storage and oxidation. This increased intracellular glucose metabolism results in malonyl-CoA synthesis and generates elevated glycerol-3-phosphate, a compound used to esterify intracellular LCFA-CoA in cells, but not MCFA. Thus, in a hyperinsulinemic state, availability of FFAs to muscle is drastically reduced, which further reduces LCFA oxidation and makes available more glycerol-3-phosphate for LCFA

esterification into triglycerides. In cases of starvation or exercise, the opposite process occurs, permitting increased LCFA flux into the mitochondria. In the case of exercise, reduced activity of acetyl-CoA carboxylase- β decreases malonyl-CoA concentrations, thus relieving residual carnitine palmitoyl transferase-1 (CPT-1) activity and increasing fatty acid oxidation. Intracellular events can effect changes in the extracellular FFA on fuel oxidation. Rasmussen et al. (80) comment on how inhibition of LCFA oxidation by increased malonyl-CoA might stimulate IMTG synthesis in the face of adequate extracellular FFA (81). Even though fatty acids are easily available during exercise (82), it is possible that the ongoing hydrolysis of IMTG continues even when plasma FFA levels are suppressed. If this is accurate, then the regulation of CPT-1 by malonyl-CoA would safeguard muscle mitochondria from excess LCFA entry in states of adequate glucose concentrations. Currently, the function of IMTG remains unclear, but is potentially important for its association with insulin resistance (83). In doing research involving the functioning of CPT-1 activity, the measurement of LCFA and MCFA oxidation is an elegant approach (84).

B.3.1 FFA Oxidation and the PPAR-Gamma/RXR Complex

Once inside the muscle cell, FFAs can follow one of two pathways, via incorporation into lipids by esterification for storage and structural purposes or by beta-oxidation in mitochondria and peroxisomes. After the production of fatty acyl-CoA derivatives (FACoA) by acetyl-CoA synthase (ACS), entry of FACoA into mitochondria is mediated by CPT-1, a crucial point in regulation of lipid and glucose metabolism (85). Control of gene expression involved in fatty acid metabolism occurs through members of the PPAR- subfamily of nuclear receptors, acting as heterodimeric partners with the retinoid X receptor (RXR) (86).

B.3.2 The PPAR Gene Subfamily

The PPAR gene subfamily of nuclear receptors has three main domains: 1) the amino-terminal A/B domain, which includes a ligand-independent transactivation function and contains putative phosphorylation sites; 2) the DNA-binding domain, which includes two zinc finger motifs; and 3) the carboxyl-terminal ligand-binding domain, which involves a ligand-binding domain. The PPARs form heterodimers with the retinoid-X-receptor (RXR) (87). This PPAR/RXR complex binds to a DNA consensus sequence, or the peroxisome proliferators response element (PPRE). Several PPAR-sensitive genes encode for proteins involved in lipid metabolism. These proteins include those directly involved in

inter-organ lipid transport (apolipoproteins A-I, A-II, C-III), in lipid uptake (lipoprotein lipase, fatty acid translocase, fatty acid transport protein), and in fatty acid metabolism (acyl-CoA oxidase, acyl-CoA synthetase, CPT-1, long-chain acyl-CoA dehydrogenase). Other PPAR responsive genes are the mitochondrial uncoupling proteins, malonyl-CoA decarboxylase, and the pyruvate dehydrogenase (PDH) kinase isoform PDK4 (88-90). These same genes may also be involved in regulation of both the flux through and the cross-talk between glucose and fatty acid metabolic pathways.

Phosphorylation of PPARs affect their trans-activating capacity and this process may be influenced by ligand-binding (91, 92). PPAR- γ is phosphorylated by mitogen-activated protein kinases (MAPKs) (93) through binding to serine residues in the N-terminal region of PPAR-. The multilevel control of PPAR- activity ensures fine-tuning and adjustment of lipid metabolism to changes in energy demand.

B.4 LIPOTOXICITY AND INSULIN RESISTANCE

Obesity is currently considered the number one preventable cause of disease in humans (NIH, March 2004). Complications associated with obesity include dyslipidemia, insulin resistance, type 2 diabetes, and heart disease, as well as the metabolic syndrome X. In animal research, genetically obese Zucker diabetic fatty rats with the same symptoms of metabolic syndrome are considered to have “lipotoxicity” (94-98). Lipotoxicity in these animals has been attributed to products of excessive non-beta-oxidative metabolism of FA excess in skeletal muscle, pancreatic islets, and myocardium (95-98). High levels of these products are thought to cause lipotoxic-associated complications by disrupting cell function and ultimately by promoting programmed cell death (“lipoapoptosis”) (96, 97) (Figure B-3).

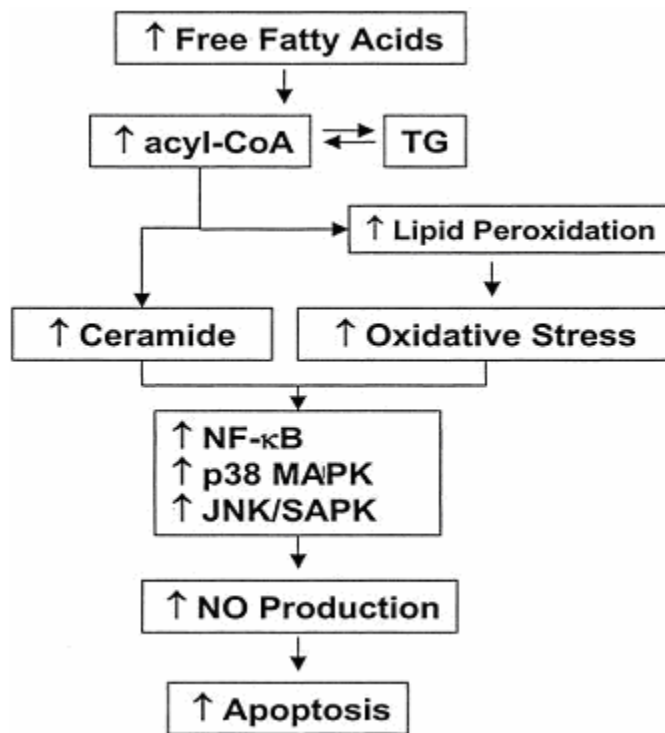


Figure B-3. Lipotoxicity and Insulin Resistance

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B.5 THE INSULIN PATHWAY AND SYSTEMIC HOMEOSTASIS

B.5.1 Glucose Homeostasis

Insulin resistance is defined as a dysfunction in the ability of insulin to maintain whole-body glucose homeostasis. The three events that are coordinated to maintain normal glucose homeostasis are the secretion of insulin by pancreatic beta cells, the suppression of hepatic glucose production, and the stimulation of glucose uptake by liver and muscle (99). In a study by Shulman (100), glycogen synthesis represented the main pathway for non-oxidative glucose disposal in normal subjects and the rate glycogen formation was 60% reduced in diabetic subjects, providing evidence that glycogen synthesis was profoundly disturbed in individuals with type 2 diabetes to the point of being the major intracellular metabolic defect accounting for type 2 diabetes. To further investigate this finding, investigators assessed potential rate-limiting steps for insulin-stimulated muscle glucose metabolism, namely glycogen synthase, hexokinase, and GLUT4, as each of these has been found defective in patients with type 2 diabetes (101). If glycogen synthase is the major defect in glycogen synthesis, then it would be expected that the concentration of glucose-6-phosphate (G6P) would be higher in type 2 diabetics. However, Rothman et al. (102) found G6P levels in type 2 diabetics (n=6) to be lower than those found in normal subjects (n=6) ($p < 0.01$). These findings indicate reduced activity of either muscle glucose transport and/or hexokinase activity to be most likely for development of insulin resistance. To distinguish between potential defects in hexokinase and glucose transport, Cline et al. (101) used a novel carbon-13 and phosphorous-31 nuclear magnetic resonance approach to measure glucose, G6P, and glycogen concentrations. In diabetic patients, they found ~80% lower rates of whole-body glucose metabolism, muscle glycogen synthesis, and G6P concentrations. They concluded that glucose transport was the rate-controlling step in insulin-stimulated muscle glycogen synthesis in diabetic subjects. Due to this, the current focus of research is on mechanisms responsible for a defect in insulin-stimulated GLUT4 transporter activity.

B.5.2 Glucose Homeostasis and FFAs

Over 50% of the current US population is overweight (103), putting a higher percentage of individuals at risk for insulin resistance and type 2 diabetes (104) than ever before. Even though it's not clear how obesity produces insulin resistance, 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 (4, 105) and 2) both chronic and acute (106-109) plasma FFA elevations produce acute insulin resistance.

FFAs have effects on total body rates of glucose uptake, glycogen synthesis, and glycolysis. In fact, elevated FFAs have been shown to inhibit all three pathways equally and to the same degree (108). Thus, the FFA-induced defect is localized at the glucose transport and/or phosphorylation level. The finding that acutely increased intramyocellular triglyceride levels develop concurrently with insulin resistance, while establishing an association, does not prove a cause-effect relationship. One hypothesis to explain the relationship between FFAs and insulin resistance is that an insulin resistance causing signal is generated during the synthesis or breakdown of intramyocellular triglyceride (110-113). Results of studies have suggested that FFA may produce insulin resistance by PKC 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. FFAs have also been shown to cause hepatic insulin resistance by suppressing hepatic glucose production (114-118).

B.5.3 Glucose Homeostasis and PPAR-Gamma

The general theory by which PPAR- γ activation improves sensitivity of glucose metabolism to insulin is as follows:

- 1) PPAR- γ increases adipose tissue remodeling and fat mass accretion brought about by enhanced adipocyte differentiation through induction of target genes mainly involved in lipid metabolism;
- 2) Fatty acids derived from hydrolysis of triglyceride-rich lipoproteins are redirected towards adipose tissue rather than skeletal muscle, which increases glucose metabolism in muscle;
- 3) There is a concomitant increase in gene expression regarding glucose uptake and insulin signaling in both adipose tissue and muscle, and a modulation of fat-derived signaling molecules that could affect peripheral glucose processing.

Furthermore, PPAR- γ seems to protect beta cells against intracellular triglyceride accumulation often associated with type 2 diabetes, hence improving beta cell function (119) (Figure B-4) .

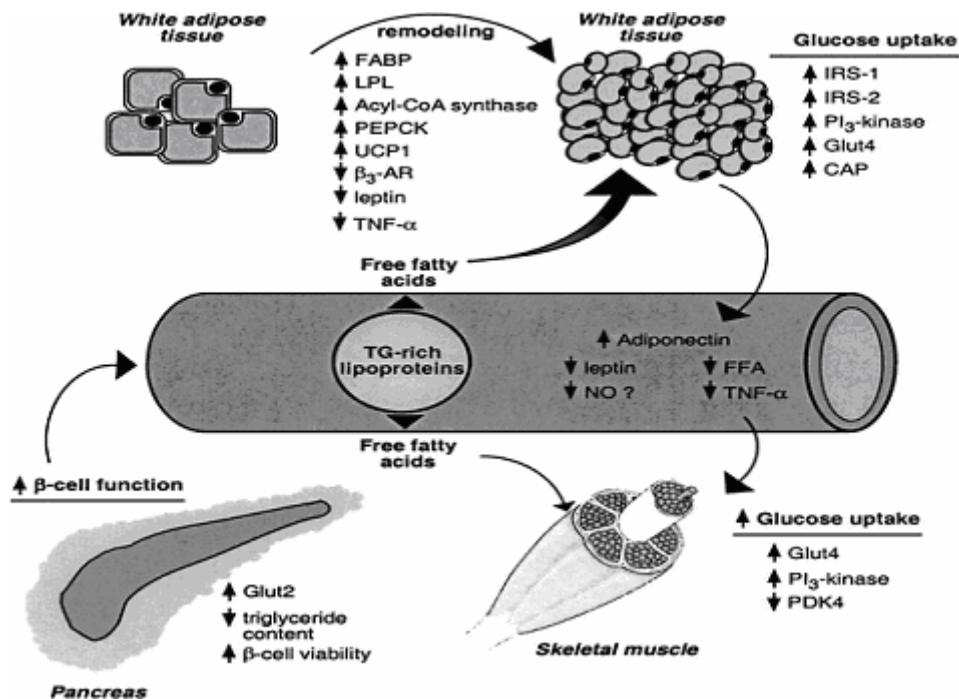


Figure B-4. Effect of PPAR-gamma activation on insulin sensitivity

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B.5.4 Fatty Acid Homeostasis

Usually, FA delivery to nonadipose tissue is tightly controlled by the need for fuel. Plasma FFA levels rise during exercise and fasting leaving practically no unoxidized FA in these cells. During chronic overnutrition, FA influx into tissues may exceed FA usage and compensatory up-regulation of FA oxidation is required to maintain intracellular FA homeostasis. Excess FAs probably provide signals for metabolic adjustments by being PPAR- ligands (120-123). PPAR- γ is a transcription factor that up-regulates CPT-1 and acyl-CoA oxidase (ACO) (122, 123). In most normal nonadipose tissue, PPAR- γ and fatty acid synthesis enzymes are expressed at low levels compared to adipocytes (124).

B.5.5 Abnormal FA homeostasis

The normal liporegulatory system exists only in tissues of normally leptinized organisms. When leptin resistance is present, FA-mediated up-regulation of oxidative enzymes does not occur (125). Surplus FAs

may then enter lipogenesis and nonoxidative metabolism pathways (95, 97), such as lipid peroxidation and ceramide-mediated apoptosis. The main cause of lipid accumulation in nonadipocytic tissue is the high rate of lipogenesis derived from elevated FFA levels and triacylglycerol levels in addition to intracellular FA synthesis (95). PPAR- γ , a lipogenic transcription factor, functions as an up-regulator for lipogenic enzymes (121, 122, 126), acetyl-CoA carboxylase and fatty acid synthase, both of which catalyze FA synthesis, and glycerol-phosphate acyl transferase, which catalyzes FA esterification. High levels of PPAR- γ in nonadipocytic tissue, such as pancreatic islets, in Zucker diabetic fatty rats is accompanied by increased expression of the adipocyte determination and differentiation factor 1 (ADD-1)/sterol regulatory element binding protein (SREBP)-1 (127, 128). SREBP-1 is a candidate for the proximal transcription factor that increases lipogenic capacity, because it can be up-regulated by diet-induced hyperinsulinemia (129, 130).

There are currently various products of non-beta-oxidative FA metabolism capable of injuring cells, including triacylglycerol (TG) excess (steatosis) (131), ceramide excess (96, 132, 133), and products of lipid peroxidation (134). TG excess in nonadipocytic tissue could potentially directly interfere with specific cell functions, including muscular contraction, and may also cause fibrosis in this tissue (131). Ceramide accumulation in beta cells has been long implicated in the apoptotic pathway of autoimmune destruction of beta cells and has been known to attribute to increased sphingomyelin breakdown (135). Ceramide itself can be formed directly by de novo synthesis from FAs (96). This pathway appears to be central to FA-induced apoptosis (96, 97). Furthermore, ceramide increases expression of inducible nitric oxide synthase (iNOS) through nuclear factor kappa B activation (136), thereby increasing the production of nitric oxide (NO) (137). NO consequently forms potent oxidants that cause apoptosis (138, 139). This pathway is shared between obesity-related type 2 diabetes and autoimmune type 1 diabetes. Lipotoxicity and lipoapoptosis can be blocked at several sites, including the restoration of leptin action, the reduction of lipid excess, blockade of ceramide formation, and inhibition of iNOS formation (96, 97, 136).

B.6 THE INSULIN PATHWAY AND OXIDATIVE STRESS

A large body of evidence suggests that hyperglycemia causes the generation of reactive oxygen species (ROS) and this ultimately leads to increased oxidative stress in numerous tissues. Without the necessary compensatory response from the endogenous antioxidant network, the system becomes overwhelmed and goes into redox imbalance, which then leads to activation of stress-sensitive intracellular signaling

pathways. One main consequence of this is the production of gene products that cause cell damage and are largely responsible for late complications of diabetes. Other consequences of the same or similar signaling pathways include the mediation of insulin resistance and impaired insulin secretion. The presence of antioxidant protection against effects of both hyperglycemia and free fatty acids supports a causal relationship between oxidative stress and worsening of these metabolic abnormalities.

Insulin resistance often precedes the development of the more severe type 2 diabetes by many years, is highly prevalent in the general population (~10 - 25%), and is multifactorial (140, 141). It is caused by both genetic and acquired factors. Hyperglycemia produces oxidative stress via increased production of mitochondrial ROS (142), nonenzymatic glycation of protein (143, 144), and glucose autoxidation (145, 146). Elevated FFA can cause oxidative stress via increased mitochondrial uncoupling (147, 148) and beta-oxidation (149, 150), leading to increased ROS. Hyperglycemia- and FFA-induced oxidative stress has been shown to lead to activation of stress-sensitive signaling pathways. This, then, worsens insulin secretion and action, leading to type 2 diabetes. Hyperglycemia has been shown to cause major complications of diabetes including nephropathy, retinopathy, neuropathy, and macro- and micro-vascular damage (140, 142, 151, 152).

Oxidative stress has been associated not only with type 2 diabetes, but also with insulin resistance in vivo (153-157). Oxidative stress leads to activation of multiple serine kinase cascades (158-160). Many potential targets of these kinases are in the insulin signaling pathway, including the insulin receptor and IRS proteins. In 3T3-L1 adipocytes, induction of oxidative stress with H₂O₂ inhibits insulin-stimulated glucose transport (161-163). The effect is selective for insulin-stimulated signaling compared with platelet-derived growth factor-stimulated signaling (164) and was reversed by pre-incubation with the antioxidant LA (162). In L6 muscle cells, activation of p38 MAPK by oxidative stress (H₂O₂) is linked to H₂O₂-mediated inhibition of insulin-stimulated glucose transport (165). Inhibition of insulin signaling was reversed by a specific p38 MAPK inhibitor (165).

B.6.1 Oxidative Stress and Beta cell Dysfunction

The beta cell is particularly sensitive to damages from oxidative stress. Through the effects of GLUT2 (the high K_m glucose transporter) (166-169), glucokinase (the glucose sensor) (170-173), and glucose metabolism, beta cells are responsible for sensing and secreting insulin in response to circulating glucose (174). Mitochondrial metabolism is necessary for linking stimulus to secretion (174-177). Therefore, ROS have the ability to damage mitochondria and thus to attenuate insulin secretion (178). Several studies have found that beta cell dysfunction is the result of: 1) chronic exposure to elevated glucose

levels, 2) chronic exposure to FFA, and 3) the combined effects of elevated glucose and FFAs. These effects appear to be dependent upon the oxidative stress induction of the NF-kappaB and additional stress-sensitive targets (179-181). There is evidence suggesting chronic hyperglycemia found in type 2 diabetics contributes to impaired beta cell function (182, 183).

B.7 THE INSULIN PATHWAY AND THE ENDOTHELIUM

The endothelium is a dynamic autocrine/paracrine organ that regulates vascular tone and interaction of the vessel wall with substances in circulation. In the endothelium, vasodilators and vasoconstrictors are produced and their balance is maintained in the normal system. Of the 4 major vasodilators, nitric oxide (NO) is the main one and, has multiple vascular-protective actions. Among these actions is the inhibition of: 1) vascular smooth muscle cell growth and migration, 2) platelet aggregation and thrombosis, 3) monocyte adhesion, 4) inflammation, and 5) oxidation (184). Vasoconstrictors, the main one being angiotensin II, promote vascular damage and inflammation.

B.7.1 The Endothelium and Type 2 Diabetes

It has been demonstrated that endothelial dysfunction is an early step in the atherosclerotic process leading to coronary artery disease (CAD) (185). While obesity itself attributes a risk of CAD 2 to 3 times higher than that found in the general population, increased adiposity with T2D has a risk estimate 3 to 4 times higher than that found in obesity alone (186). Several in vivo and in vitro studies have demonstrated endothelial dysfunction (ED) in subjects with type 2 diabetes (187, 188). It has been hypothesized that ED in type 2 diabetes could be a result of either decreased synthesis of nitric oxide (NO), increased inactivation of NO, or decreased responsiveness to NO.

While the exact pathogenesis of ED in diabetes is unknown, multiple mechanisms are likely to be involved, including abnormalities in adipokine production, especially TNF- α . TNF- α may be associated with cardiovascular disease (CVD) (189-192). In one study, plasma levels of TNF- α were elevated in subjects with premature CVD, independent of insulin sensitivity (192). Conversely, TNF- α levels have been found to decrease after weight reduction and in parallel with improvement of endothelial function (193). The effects of TNF- α on vascular endothelium may be direct as well as indirect through promoting

the release of mediators from adipocytes or other host cells. At the molecular level, TNF- α has been found to increase leukocyte adhesion to endothelium.

B.8 NUTRIENT SENSING PATHWAYS AND INSULIN RESISTANCE

Insulin resistance is considered a cardinal feature of metabolic defects found in obesity and is postulated to develop as an adaptation to increased nutrient availability. Energy balance and metabolic homeostasis are maintained by complex regulatory systems. Normally, the body senses changes in energy balance and activates appropriate responses, which include decreased food intake, increased energy expenditure, and regulation of substrate oxidation and intermediate metabolism. So, why is obesity now considered the number one preventable cause of death? Much evidence has suggested that obesity is a fuel partitioning disorder. According to Neel's hypothesis of the thrifty genotype (194), 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 (Figure B-5).

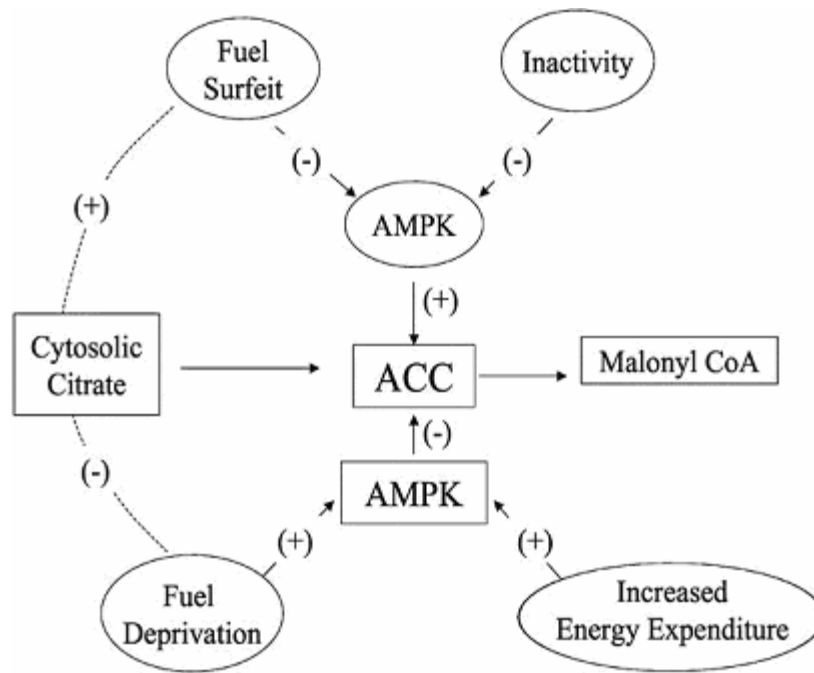


Figure B-5. ACC regulation in skeletal muscle

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To sense nutrient availability, cells must possess biochemical sensors that detect nutrient levels and initiate adaptive responses. Malonyl-CoA has been identified as a biochemical sensor (195) 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 (85), muscle (196), the pancreatic beta cell (197), and endothelium (198) and probably works similarly in the adipocyte (199) and the central nervous system. 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 allosteric activator of ACC; and AMPK, an enzyme activated by decreases in the cell's energy state as measured by increases in the AMP/ATP and creatine:creatine phosphate ratios (200-203). Current thought is that muscle contraction regulates ACC solely by activating AMPK. Activated AMPK phosphorylates ACC at Ser79 and inhibits its activation by citrate. Conversely, an abundance of

glucose increases malonyl-CoA concentration via increased cytosolic citrate levels (204) and decreased AMPK activation (112). Glucose deprivation works through the same pathway in the opposite direction.

Generally, activation of AMPK switches on catabolic pathways that generate ATP, while switching off anabolic pathways and other nonessential processes that consume ATP. It does this by direct phosphorylation of regulatory proteins and by indirect effects on gene expression. The downstream effects of AMPK activation include inhibition of fatty acid, cholesterol, and protein synthesis (205, 206). The direct targets for phosphorylation by AMPK that are responsible for the inhibition of translation remain unclear. Another effect of AMPK activation is the numerous genes regulated AMPK (i.e., IRS1, ACC- β , GLUT4). The genes that are up-regulated by AMPK in muscle are similar to those induced by endurance exercise training, including GLUT4 and mitochondrial oxidative enzymes (207). In most cases, direct AMPK target proteins responsible for effects on gene expression are not known (Figure B-6).

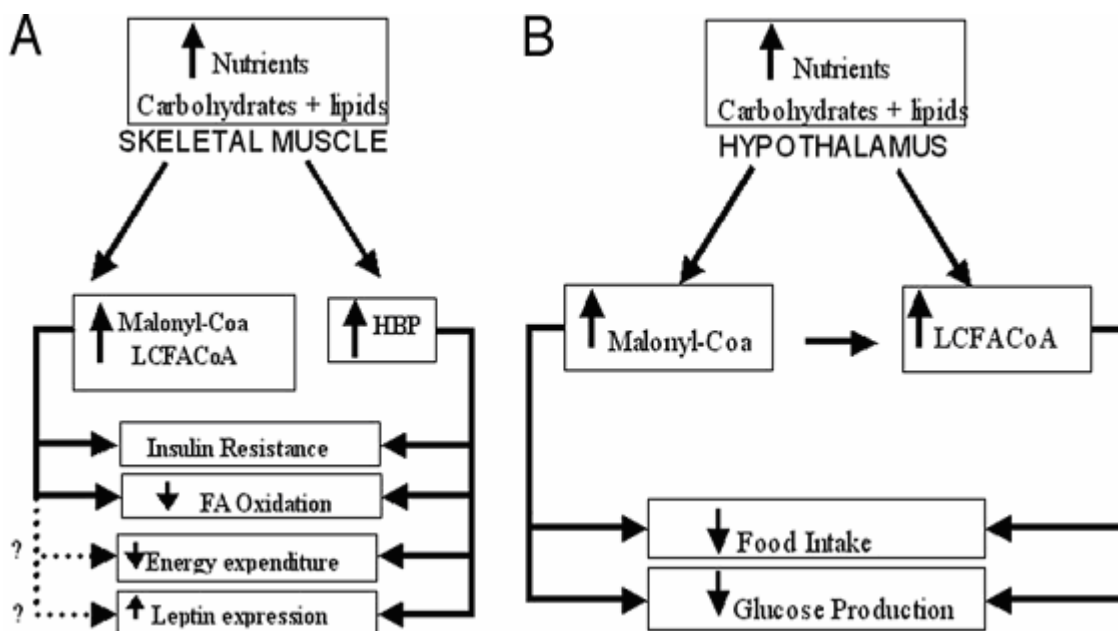


Figure B-6. Signals of nutrient abundance

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AMPK activation causes metabolic changes that would benefit subjects with type 2 diabetes and the metabolic syndrome, such as increased glucose uptake and metabolism by muscle and other tissues,

decreased glucose production by the liver, and decreased synthesis and increased fatty acid oxidation. Results from experiments with mice (208-211) suggest the AMPK system is the probable target of antidiabetic drug metformin (212-214).

Direct evidence of the relationship of malonyl-CoA and AMPK to obesity was provided by Winder et al. (215) in a study of the effects of AMPK activator, AICAR, on rats. The authors found that rats administered AICAR consumed less food than pair-fed rats and, while their mean end weights were similar, AICAR administered rats had over 30% diminished epididymal and retroperitoneal fat pads. These results are supported by Saha et al. (216) where rat fat pads were diminished by 30-40%. In the hypothalamus, malonyl-CoA and AMPK fuel-sensing and signaling mechanisms play a role in initiating signaling events that regulate food intake. AMPK activity has been found to decrease in various rodent hypothalamic nuclei as a result of 1) re-feeding after fasting (217), glucose (217) and insulin administration (218), and leptin injection (217); 2) hypothalamic concentration of malonyl-CoA diminishes with starvation (219); and 3) the central administration of C75, a FA synthase inhibitor, prevents the diminished malonyl-CoA in the hypothalamus during starvation (219). These studies suggest that factors that elevate malonyl-CoA concentrations in hypothalamic nuclei diminish food intake, just as factors that decrease concentrations in hypothalamic nuclei increase food intake.

B.8.1 The Hexosamine Biosynthesis Pathway

The hexosamine biosynthesis pathway (HBP) is a second nutrient sensing pathway by which insulin action and energy homeostasis may be modulated. The HBP is activated by increased glucose flux. The mechanisms of the HBP pathway are as follows: 1) After transport and phosphorylation of glucose to glucose-6-phosphate (G6P), G6P is used to synthesize glycogen and used in glycolysis; 2) One to 3% of incoming glucose is converted to fructose-6-phosphate (F6P) and enters the HBP; 3) the glutamine: F6P amino transferase (GFAT) catalyzes the first committed step of HBP and regulates the flux through this pathway (220-222). HBP plays an important role in insulin action modulation in both adipose and muscle tissue. Glucose-induced insulin resistance is blunted by inhibition of GFAT activity and expression (223), suggesting the damaging effects of hyperglycemia are mediated by HBP activation. HBP induces insulin resistance by inhibiting multiple sites of the insulin-signaling cascade. Several investigations have shown that insulin-dependent glucose uptake and glycogen synthesis are both down-regulated by HBP activation (224, 225).

HBP plays a prominent role in energy balance, suggested by its role in inducing leptin expression (226). Transgenic mice over-expressing GFAT in adipose and muscle tissues, have elevated plasma

leptin levels (227). HBP controls expression of leptin via transcriptional activation of the leptin promoter (228). Elevated leptin levels, in the normal system, cause decreased food intake, yet obese individuals usually have elevated leptin levels, suggesting leptin resistance. It is still not clear how nutrient-induced leptin actions fail to fully initiate counter-regulatory responses to nutrient excess in obesity-susceptible individuals.

B.9 INSULIN SIGNALING AND TYPE 2 DIABETES MELLITUS

Type 2 diabetes is caused by the combined effects of peripheral insulin resistance and defects in beta cell secretion. Murine knockout and transgenic model studies suggest that disruptions of the insulin/IGF-1 signaling mechanisms, and alterations in the functions of IRS proteins, might contribute to these defects. On a cellular level, insulin/IGF-signaling regulates multiple processes, including carbohydrate and lipid metabolism, gene transcription, DNA synthesis, anti-apoptosis, and cell proliferation. Insulin and IGF-1 and -2 bind to members of the insulin tyrosine kinase family, and while the insulin and IGF-1 receptors are relatively specific for insulin and IGF-1, respectively, IGF-2 provides cross-talk between these two receptors. Although the current view asserts that diabetic complications arise from deleterious effects of hyperglycemia, type 2 diabetes might be best understood as a global disorder of insulin/IGF-1 signal transduction that dysregulates gene expression and cell function in various tissues.

In animal studies of absent insulin receptors, mice without insulin receptors in skeletal muscle do not develop diabetes, but exhibit elevated fat mass, FFAs and serum triglycerides (229). Mice lacking hepatic insulin receptors also fail to develop diabetes, but display a complex metabolic phenotype, including glucose intolerance and decreased serum triglycerides and FFAs (230). Also, overexpression of dominant negative dysfunctional insulin receptors results in disturbed lipid metabolism and eventual glucose intolerance (231). Results of these studies suggest a multisystem interaction in insulin resistance and the development of type 2 diabetes by either exacerbating compensatory mechanisms or by directly interfering with insulin secretion. Specifically, combined insulin resistance in beta cells and hepatic/muscle might be an important component of type 2 diabetes.

B.10 GENETIC, LIPID METABOLISM, AND INSULIN RESISTANCE

B.10.1 PPAR-Gamma, Lipid Metabolism and IR

PPAR- γ belongs to a nuclear receptor subfamily, of which all members contain a ligand-dependent transactivation domain in its C-terminal region, a highly conserved DNA binding domain comprised of two zinc fingers, and a ligand-dependent activation domain in the NH₂-terminal region (232). There are three different PPAR- γ mRNAs (233, 234) – 1, 2, and 3. The P12A variant of PPAR- γ is a mutation in the PPAR- γ 2 receptor. Natural ligands for all PPAR- γ s include fatty acids and their derivatives (235). The PPAR- γ 2 isoform is expressed mostly in white adipose tissue, but is also detectable in brown adipose and skeletal muscle tissues (236). Compared with lean subjects, PPAR- γ 2 only has increased expression in obese subjects (124). Furthermore, it has been found that, compared to subcutaneous fat, PPAR- γ expression is increased in visceral fat pockets of obese subjects (237).

B.10.2 PPAR-Gamma and Adipogenesis

PPAR- γ has a central role in adipogenesis (238-240). The effects of PPAR- γ on adipogenesis are based on two main processes. First, these effects are based on increased expression of genes that promote fatty acid trapping and storage in adipocytes, such as fatty acid binding protein (240), LPL (126), and acyl-CoA synthase (241). Second, these effects are the result of repression of genes that induce lipolysis and the release of fatty acids, such as the beta3-adrenergic receptor (242) and cytokines leptin (243, 244) and TNF- α (245, 246). These results can be supported by the demonstrated effects of thiazoladinediones (TZDs) on PPAR- γ activation (247, 248). Treatment with TZDs seems to favor redistribution of white adipose tissue, with decreased visceral depots relative to subcutaneous fatty regions (247-250). This fat cell redistribution includes a shift in the cell type population resulting in more small adipocytes and fewer large, insulin insensitive adipocytes (251-253). The loss of mature, large adipocytes is thought to be caused by PPAR- γ -mediated induction of apoptosis (251, 253).

B.10.3 PPAR-Gamma2 Point Mutation P12A

This mutation is a partial loss-of-function mutation associated with decreased receptor activity (254, 255) resulting in greater insulin sensitivity, lower BMI, and an improved lipid profile (255-259). This relationship is attenuated by certain factors, including BMI, suggesting resultant insulin sensitivity is primarily due to body fat mass (255), selected population for study and ethnicity (260). Depending upon the population and ethnicity studied, the P12A mutation has been associated with inverse findings (261-263) or no significant findings at all (264-267). These conflicting findings may be explained as gene-gene or gene-environment interactions. In addition to the P12A effects on body fatness and insulin sensitivity, it has also been shown to effect insulin secretion in healthy (268) and diabetic individuals (258) in response to free fatty acids.

B.10.4 Lipoprotein lipase, Lipid Metabolism and IR

The two main roles of lipoproteins are 1) to prevent the dispersal of lipids by exchange or diffusion and 2) to deliver lipids to specific target tissues. Functions of apolipoproteins include being ligands or cell surface receptors as well as cofactors for cell surface lipases, like LPL. LPL's activity occurs in the luminal surface of capillary endothelial cells (269) and it attaches to triacylglycerol (TAG) in particles to provide non-esterified fatty acids (NEFA) and 2-monoacylglycerol to tissues (270-273). Both of these compounds are used in tissues as storage for energy as TAG and as energy for the heart, as well as the regulation of thermogenesis in brown adipose tissue (270). Lipoprotein molecules become smaller after hydrolysis by LPL and further hydrolysis causes loss of TAG and the apoE moiety, resulting in a LDL molecule (270).

Another function of LPL is as a bridge between lipoproteins and specific cell surface proteins (274, 275). It can promote the proliferation of vascular smooth muscle cells (276) and has several other important functions, including 1) inducing the expression of the TNF- γ gene (277); 2) synergizing with interferon- γ in stimulation of macrophage NO synthetase expression (278, 279); 3) activating endothelial nitrite reductase (NAD(P)H) oxidase (280); and 4) reducing secretion of apoE (281). In summary, LPL is glycosylated, exists as a dimer, and is engaged in several molecular functions, include those with lipid/water interfaces, cofactor apoC2, heparin sulphate-proteoglycans (HSPG), substrate molecules at the active site, and specific lipoprotein receptors (272, 273, 282). Several natural mutations have been identified and seem to affect the function of the LPL enzyme.

LPL expression is attenuated through hormones, notably insulin, and this directly impacts fatty acid utilization (272, 273). 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 (272, 283, 284). Cold exposure also stimulates LPL activity, but in brown adipose tissue only (285, 286). LPL expression is further regulated by disease states, notably atherosclerosis and diabetes (287-289). Transcriptional control of LPL also impacts fatty acid usage. Metabolites that induce LPL gene transcription include the PPRE in liver and adipose tissues and in macrophages in response to fibrates, some fatty acids, glucose, and TZDs (126, 288, 290). Cytokines also induce LPL gene transcription, especially TNF- α , by eliminating binding of nuclear factor-Y and Oct-1 to the LPL promoter in 3T3-L1 cells, and interferon- γ , via suppression of macrophage LPL gene transcription (291)

Decreased LPL activity has been seen in individuals with type 2 diabetes and insulin resistance (292-295). During these states, the increased production of adipokines, like TNF- α and IL-6, has been postulated to be the cause or reduced LPL expression (293, 296). Furthermore, the resultant decrease in LPL activity contributes to hypertriglyceridemia, decreased HDL levels, and increased risk of coronary heart disease (297).

B.11 ADIPOKINES IN INSULIN RESISTANCE AND TYPE 2 DIABETES MELLITUS

Cytokines are known products of both inflammatory and immune systems. Cytokines secreted from adipose tissue, or adipokines, are theorized to be responsible for initiating a proinflammatory state, also called systemic inflammation, which may affect the development of insulin resistance and endothelial dysfunction (i.e., the first stage of the development of atherosclerosis). Increased systemic inflammation has been found among individuals with obesity, insulin resistance, and T2D. The importance of fat is mediated through the relation of FFAs to adipose tissue and the production of fat factors that affect inflammation of the vessel wall. FFAs are implicated among those substances that cause postprandial endothelial dysfunction associated with a high-fat meal (298). Elevated serum levels of TG and FFA found in states of obesity and T2D have led researchers to believe that it is lipotoxicity that is responsible for the initiation and progression of hepatic and peripheral insulin resistance and pancreatic beta cell dysfunction (299).

Cytokines are currently viewed as major players in the development of atherosclerosis and diabetes (300, 301). TNF- α and IL-6 are the most investigated adipokines and are correlated with all measures of

obesity and are strongly related to insulin resistance (189, 302-308). The direct correlation between visceral adiposity and circulating adipokine levels supports the assumption that adipokines may be important in the metabolic changes that commonly occur in insulin resistance states (299). Other adipokines that have been widely studied in relation to insulin resistance include adiponectin (26, 309, 310), leptin (45), and plasminogen activator inhibitor-1 (311). Resistin may also be associated with insulin resistance, although results of studies have been controversial (312-319).

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APPENDIX C

INSULIN RESISTANCE IN POLYCYSTIC OVARY SYNDROME

In 1990, the National Institutes of Health established the new diagnostic criteria for PCOS (1). The basis of diagnosis is based on clinical evidence of hyperandrogenemia (elevation in plasma concentration of an androgen) and/or hyperandrogenism (hirsutism) and chronic oligo-anovulation (often defined as six or fewer menses in the last 12 months), with the exclusion of other causes of hyperandrogenism such as non-classical adrenal steroid 21-hydroxylase deficiency, hyperprolactinemia or androgen-secreting neoplasm. PCOS is often difficult to diagnose due to its intersecting phenotypic traits that can easily be mimicked by other endocrine disturbances (or phenocopies). These disturbances are (1) an altered LH/FSH ratio; (2) cystic ovaries; (3) hyperandrogenism; and (4) oligomenorrhea. Conditions that mimic PCOS include late-onset adrenal hyperplasia (LOAH), Cushing's syndrome, and androgen producing tumors of the ovary or adrenal glands. As yet, a clearly defined and universally agreed upon cause for PCOS does not yet exist.

PCOS is also associated with metabolic disturbances somewhat reflective of metabolic cardiovascular syndrome or "syndrome X". Burghen and colleagues (2) were the first to report women with PCOS had higher basal and glucose-stimulated insulin levels than weight-matched controls. They also found blood levels of insulin and androgen in PCOS patients correlated (3). The elevated insulin levels were initially attributed to the insulin resistance of obesity. However, insulin excess was found to occur in nonobese patients with PCOS (4), and the insulin resistance of PCOS was found to be excessive for the degree of obesity (5). Since then, several studies have demonstrated in various ethnic groups that PCOS-affected women are commonly affected by hyperinsulinemia and insulin resistance, altered first-phase insulin secretion, impaired glucose tolerance, dyslipidemia, hypertension and impaired fibrinolysis.

C.1 GLUCOSE TOLERANCE IN PCOS

In normal glucose regulation, individuals maintain normal serum glucose levels by increasing pancreatic insulin secretion. Responses to insulin include glucose uptake into peripheral organs, mostly muscle and fat cells, and suppression of gluconeogenesis and glucose secretion. When peripheral insulin resistance exists, serum glucose levels can still be maintained if the pancreas can secrete additional insulin to overcome the resistant state. Once the pancreas can no longer adequately compensate, postprandial glucose levels rise, this is defined as impaired glucose tolerance (IGT). Glucose tolerance is one metabolic parameter impacted by PCOS. Glucose tolerance has been systematically studied in women with PCOS since 1987. Dunaif et al. (6) found that affected women had significantly increased glucose levels during an oral glucose tolerance test compared with age- and weight-matched ovulatory hyperandrogenic and control women. They showed that 20% of the obese PCOS women had either IGT or NIDDM using National Diabetes Data Group criteria. There were, however, no significant differences in glucose tolerance between lean PCOS and age- and weight-matched controls. This observation suggests obesity is a greater mediating factor for IGT than PCOS. In 1999, Legro et al. (7) conducted a prospective study of the prevalence of glucose intolerance and parameters associated with risk for glucose intolerance in PCOS-affected women. Of 254 women with PCOS, aged 14 to 44 years, 31.1% had IGT and 7.5% had NIDDM. Lean PCOS-affected women ($BMI < 27 \text{ kg/m}^2$), 10.3% had IGT and 1.5% had NIDDM. Compared to control women ($N=80$) of similar weight, ethnicity, and age, the prevalence of glucose intolerance was significantly higher in women with PCOS. The variables most associated with postchallenge glucose levels were fasting glucose levels, PCOS status, waist-to-hip ratio, and body mass index. They concluded women with PCOS are at significantly elevated risk for IGT and NIDDM at all weights and at a young age (i.e., the third or fourth decades of life) (8). Dunaif et al. (8) found the prevalence of glucose intolerance is significantly higher in obese PCOS women than in their normal weight counterparts, in whom IGT occurs occasionally. This finding is consistent with the synergistic effect of obesity and PCOS in determining IGT. In a 10-year follow-up study of women with PCOS, fasting and glucose-stimulated insulin and C-peptide were significantly increased in PCOS women when compared to their baseline levels, suggesting a worsened insulin resistant state (9). Studies of insulin secretion have demonstrated the importance of beta-cell function in the development of IGT and NIDDM (10). Recently, Collila et al. (11) found that beta-cell dysfunction is heritable in families of women with PCOS and this may be a factor in future development of NIDDM.

In summary, it is accepted that PCOS is often associated with profound insulin resistance as well as defects in insulin secretion. These abnormalities, with concurrent obesity found in ~50% of PCOS women, explain the increased prevalence of glucose intolerance in PCOS.

C.2 INSULIN ACTION IN PCOS

C.2.1 Insulin action in vivo in PCOS

In normally working systems, insulin has several actions, including glucose transport, glycogen synthesis, protein synthesis, and mitogenesis. Insulin is the most potent anabolic hormone known, and promotes the synthesis and storage of carbohydrates, lipids and proteins, while inhibiting their degradation and release into the circulation. Insulin stimulates the uptake of glucose, amino acids and fatty acids into cells, and increases the expression or activity of enzymes that catalyze glycogen, lipid and protein synthesis, while inhibiting the activity or expression of those that catalyze degradation. Research on the effects of insulin on glucose metabolism is usually examined in studies of insulin resistance, which can be examined using the euglycemic glucose clamp technique. Euglycemic glucose clamp studies have demonstrated significant and substantial decreases in insulin-mediated glucose disposal in PCOS (5, 12). The decrease seen in PCOS (i.e., ~35-40%) is of similar magnitude to that seen in NIDDM (8). Obesity (as a measure of fat mass), body fat location (waist-to-hip girth ratio), and muscle mass independently contribute to overall insulin sensitivity (13-15). Alterations in these parameters could influence IR in PCOS. Women with PCOS have increased prevalence of obesity (16), (17) and women with upper (abdominal) rather than lower (hip/thigh) obesity have an increased frequency of hyperandrogenism (15). Studies of lean women with PCOS compared to normal control women of similar weight and body composition have confirmed affected women are insulin resistant independent of potentially confounding parameters (18). Research in cultured cells have confirmed the impression from in vivo studies that an intrinsic defect in insulin action is present in PCOS (19). While mechanisms for dysfunctional insulin action are unknown in PCOS, it is clear that PCOS affects insulin sensitivity over and above obesity alone, further supporting an intrinsic defect in insulin action in PCOS.

C.2.2 Insulin secretion in PCOS

Hyperinsulinemia in PCOS is primarily a result of a compensatory increase in insulin secretion secondary to substantial peripheral insulin resistance. Basal insulin secretion is increased and hepatic extraction of insulin is decreased in PCOS (20). Obesity further exacerbates IR and hepatic glucose production defects are seen in obese PCOS women (17, 21). Insulin secretion increases as insulin sensitivity decreases to maintain glucose homeostasis. This relationship is called the disposition index and is a hyperbolic function. In both nonobese and obese PCOS women, insulin secretion is inappropriately low for the degree of IR (i.e. values fall below the disposition index curve), suggesting a beta-cell dysfunction (22, 23). This suggests PCOS women may be at unusually high risk for developing glucose intolerance.

C.2.3 Insulin clearance in PCOS

Hyperinsulinemia can result from decreases in insulin clearance as well as from increased insulin secretion. Decreased insulin clearance is usually present in insulin-resistant states since insulin clearance is receptor-mediated, and acquired decreases in receptor number and/or function are often present in insulin resistance secondary to hyperinsulinemia and/or hyperglycemia (24, 25). Very few studies have examined the issue of insulin clearance in PCOS. Morin-Papunen et al. (26) recently studied the effects of metformin vs. ethinyl estradiol-cyproterone acetate in obese and nonobese women with PCOS. Metformin is believed to improve hyperinsulinemia and hyperandrogenemia by decreasing central obesity and the release of free fatty acids from adipose tissue (27, 28). In nonobese women with PCOS, euglycemic clamp research of posthepatic insulin clearance has not found abnormalities in women with PCOS (5, 29). Circulating insulin to C-peptide molar ratios are increased in PCOS, suggesting decreased hepatic extraction of insulin, but these ratios reflect insulin secretion as well as extraction and are unreliable measures of insulin clearance (30, 31). O'Meara et al. (32) designed a study of insulin clearance in women with PCOS and found decreased hepatic insulin extraction by model analysis of C-peptide levels. Hence, in PCOS, hyperinsulinemia is most probably the result of both increased basal insulin secretion and decreased hepatic insulin clearance.

C.3 MOLECULAR MECHANISMS OF INSULIN RESISTANCE IN PCOS

Insulin resistance may be due to several factors along the insulin signaling pathway. The insulin signaling pathway may be considered “dysfunctional” at the point of the insulin receptor (binding of insulin to the receptor) or at the intracellular level (post-receptor). In PCOS, the dysfunction in insulin signaling seems to be due to post-receptor defect.

The insulin dysfunction in PCOS is postulated to be the main factor elevating androgen levels. In 1921, Achard and Theirs reported on a bearded woman who was also diabetic. Kahn and colleagues (33) reported on three lean adolescent women with acanthosis nigricans. Acanthosis nigricans is the presence of hyperpigmentation and thickening in skin folds of the neck, axilla, or beneath the breasts. This type of insulin resistance soon became known as Type A syndrome or HAIR-AN (hyperandrogenism, insulin resistance, and acanthosis nigricans). This idea led to the discovery of the influence of insulin on steroidogenesis. The relationship of acanthosis nigricans to insulin resistance seems likely to be related to hyperinsulinemia, since the basis of acanthosis nigricans is epidermal hyperplasia and insulin promotes epidermal cell growth in culture (34). Insulin resistance was then found to be the best biochemical correlate of acanthosis nigricans (35). Hyperinsulinemia appears to be a major factor in the ovarian dysfunction of PCOS. Any treatment lowering insulin levels, will concurrently lower androgen levels as well, as is seen in weight loss (36). Recent research suggests insulin is capable of enhancing a variety of steroidogenic pathways in ovarian thecal cells, ovarian granulosa cells, adrenalcortical cells, and the periphery (37). Furthermore, insulin seems to be capable of exerting these effects directly (38). Thus, insulin excess could contribute to functional adrenal hyperandrogenism.

C.3.1 Constraints of insulin action studies in PCOS

General consensus in the literature is that insulin resistance is commonly found in obese women with PCOS. The pathogenesis of IR in PCOS is controversial and one suggestion is that obesity is responsible for insulin action dysfunction in these women. Many conflicting results from these studies can be explained by differing diagnostic criteria, especially those differences found between studies conducted in the United States and the United Kingdom, and by the inclusion of lean and obese women in the subject population. Studies using ovarian morphology as the basis for PCOS diagnosis have found that only anovulatory women with PCO morphology are insulin resistant. Hence, studies that have defined PCOS by PCO morphology could have included women that were not insulin resistant. Also, studies including

ovulatory women with hyperandrogenism would bias the study with non-IR subjects. However, regardless of study locale, there is strong evidence supporting the association of anovulation with IR.

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