# Cover page

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| Official Title on ClinicalTrials.gov: | PCOS, Sleep Apnea and Metabolic Risk in Women |
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## A. SPECIFIC AIMS

Polycystic ovary syndrome (PCOS) affects between 5 and 8% of women making it one of the most common endocrinopathies in women (3, 4). The disorder typically has its onset at puberty with evidence of excessive androgen production, obesity, and insulin resistance. Women with PCOS are more insulin resistant than weight-matched controls (5) and have an exceptionally high prevalence (6) of early-onset impaired glucose tolerance (30 - 40 percent), and type 2 diabetes (up to 10 percent) (7, 8). In addition, the rate of deterioration in glucose tolerance is accelerated in PCOS (7, 9). Vascular disorders are also common in this population due, at least in part, to disordered lipid metabolism. Hypertriglyceridemia, increased levels of very-low-density lipoprotein (VLDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and decreased levels of high-density lipoprotein (HDL) cholesterol are typical (10) (11). While insulin resistance contributes to glucose intolerance and dyslipidemia, it has become increasingly clear that other factors must be involved in the pathogenesis of the metabolic derangements so commonly seen in PCOS.

Over the past several years, chronic decreases in sleep duration and/or quality have been identified as a risk for the development of a number of metabolic derangements (12-14) that are strikingly similar to those seen in PCOS (3, 10, 15). Specifically, decreased sleep quality due to obstructive sleep apnea (OSA) has been causally linked to insulin resistance, glucose intolerance, dyslipidemia and hypertension independent of body mass index (BMI) (12-14). Until recently however, it had not been recognized that **OSA is present in a disproportionate number of women with PCOS: the risk for OSA is at least 5-fold higher, and perhaps as much as thirty-fold higher (16), compared to the risk in similarly obese women without PCOS.**

It was initially thought that hyperandrogenemia or obesity are the key determinants of the high prevalence of OSA in PCOS. However, both published data (16) as well as results of our Preliminary Studies are not consistent with this hypothesis. We have found that plasma concentrations of free testosterone are virtually identical in PCOS women with and without OSA. In addition, measures of OSA severity, such as the apnea-hypopnea index (AHI), do not correlate with either the degree of androgen elevation or with body mass index (BMI) among PCOS women with OSA.

Both progesterone and estrogen reportedly "protect" against the development of OSA (17, 18) (19-24) (25), and levels of these sex steroids are typically low in PCOS relative to those in normal cycling women. **Thus, we will test the hypothesis that circulating progesterone and estrogen levels are significant determinants in the development of OSA among hyperandrogenic women with PCOS. Using a novel paradigm that permits us to independently modulate sex steroid concentrations, we will be able to address the specific and independent effects of progesterone and estrogen in the pathogenesis of OSA among women with PCOS.**

OSA appears to be an underrecognized, yet significant factor in the pathogenesis of metabolic derangements in PCOS. In a recent study, we showed that measures of insulin resistance and glucose tolerance in PCOS women are strongly predicted by the severity of OSA (15). These intriguing findings suggest that there may be two "subtypes" of women with PCOS, i.e. those with and those without OSA, and that these two subtypes may be associated with distinct metabolic and endocrine alterations. PCOS women with OSA may be at much higher risk for diabetes and cardiovascular disease than PCOS women without OSA and may benefit from therapeutic interventions targeted to decrease the severity of OSA. **Thus, a major goal of the present project is to contrast the metabolic and hormonal features of these two subtypes of PCOS, explore causative mechanisms for the high prevalence of OSA in PCOS, and test the hypothesis that continuous positive airway pressure (CPAP) treatment may decrease the risk of diabetes and other cardiovascular and metabolic abnormalities in PCOS women with OSA.**

**Specific Aim 1. To determine if women with PCOS who have OSA differ from those without OSA as a consequence of differences in circulating concentrations of the sex-steroids estrogen and progesterone.** We hypothesize that in comparison to PCOS women without OSA, those who have OSA will have lower circulating levels of estradiol, progesterone, or both estradiol and progesterone at baseline. We will further test our hypothesis by comparing stimulated steroid levels in these two groups. Specifically,

we will compare the ovarian steroidogenic response to a single dose of the GnRH agonist leuprolide in PCOS women with and those without OSA. Developed by our group, the GnRH agonist test has become a novel method to assess ovarian steroidogenic function.

**Specific Aim 2. To determine if OSA improves in women with PCOS treated with estrogen or progesterone.** For these studies, we will enroll women with PCOS who have OSA. Subjects will have a detailed baseline metabolic and metabolomic profile together with a baseline polysomnogram. Subjects will then receive a single dose of depot-leuprolide in order to suppress ovarian production of estrogen, progestin, and androgen over a period of 3 months. Six weeks after administration of depot-leuprolide, subjects will have repeated assessment of metabolic, metabolomic, and sleep measures to determine if these outcomes are altered by the combined suppression of ovarian sex steroids. Subsequently, subjects will be randomized in a double-blind placebo controlled fashion to one of two treatment arms for a period of six weeks. Treatment arms are: estrogen plus placebo *or* progesterone plus placebo. At the end of this second six weeks, subjects will have metabolic, metabolomic, and sleep measures repeated. Primary outcome measures will be compared both within and between treatment arms.

**Specific Aim 3. To determine if metabolic disturbances present in PCOS women with OSA are ameliorated by the treatment of OSA with continuous positive airway pressure (CPAP).** The presence of OSA in women with PCOS has a significant negative effect upon metabolic measures. In particular, the presence of OSA in PCOS is associated with hyperinsulinemia, elevations in plasma glucose, and lipid abnormalities that include higher levels of triglycerides and LDL-cholesterol. OSA in PCOS is also associated with hypercortisolemia that persists throughout both day and night. Results of our preliminary studies indicate that CPAP treatment of OSA results in attenuation of cortisol levels not only during sleep, but throughout waking hours as well. This aim will serve to test the hypothesis that correction of OSA in PCOS will lead to improved metabolic function which can be attributed, at least in part, to a reduction in circulating levels of cortisol.

**Relationship of the present Project to Projects 1, 3, 4, and the Metabolomics Laboratory in this SCOR Proposal**

The studies planned in this Project will integrate with and complement those of Projects 1, 3, and 4 as outlined below. Metabolomics profiling is integrated into all SCOR Projects; specific interactions with the current Project are detailed below.

1. Insights into the basis for gender differences in normal sleep and pathologic sleep (OSA) will be facilitated by interactions between the present Project and Project 1 (Van Cauter, PI). In Project 1, metabolic measures, slow wave sleep (SWS), rapid-eye movement (REM) sleep, and non-REM sleep will be characterized in obese men and obese women, with and without OSA. Those with OSA will be treated with CPAP and reevaluated for sleep and metabolic outcomes. In the present Project, similarly obese women with PCOS, with or without OSA, will be characterized as above. Those with OSA will then be randomized to CPAP treatment or to receive medication designed to modify the sex steroid milieu. This integrated study design will permit us to compare normal sleep to sleep disrupted by OSA in obese men and women and obese women with PCOS. The present project will benefit from the ability to distinguish the independent effects of gender (men vs women) from circulating sex steroid concentrations (PCOS vs non-PCOS). The effects of CPAP treatment of OSA on sleep and metabolic outcomes can also be compared between groups studied in Projects 1 and 2.
2. Elevation in triglyceride (TG) concentration is a common feature of the metabolic abnormalities associated with obesity and the increase in cardiovascular risk resulting from a given rise in plasma TG concentration is approximately 2-fold greater in women than in men. Project 3 (Mittendorfer, PI) and the present Project are linked by a shared hypothesis, i.e., that the hyperandrogenemia and relative progesterone deficiency are important factors in the pathogenesis of TG elevation (Project 3) as well as OSA (present Project 2) in women with PCOS. Detailed studies of TG kinetics will be undertaken before and after modulation of plasma progesterone or testosterone concentrations in women.

3. In Project 4 (Brady, PI) primary human adipocytes will be prepared from fat biopsies obtained from PCOS women with and without OSA as well as from PCOS women with OSA before and after treatment with either CPAP or hormonal modulation. In vitro assessment of adipocyte insulin sensitivity will be determined by phospho-specific immunoblotting in conjunction with glucose uptake and anti-lipolysis assays. In parallel, adipocytes from these subjects will be cultured for 1-5 days prior to metabolic assays, to ascertain if removal of adipocytes from circulating factors will improve insulin signaling.

4. This Project, along will all others in this SCOR proposal, will provide samples of blood and urine to the Metabolomics Laboratory at Duke University (C. Newgard, Director) for highly sensitive and specific assays, as described in the overall Introduction to this proposal. This collaboration presents a unique opportunity for this Project to determine if metabolomic measures in women with PCOS and OSA differ from those of PCOS women without OSA. In addition, it will be possible to determine if interventions with CPAP or hormonal modulation are associated with changes in these same measures. This methodology provides a powerful tool to examine traditional hormones (e.g., insulin, adiponectin, ghrelin, resistin) and inflammatory markers (e.g., CRP, IL-6, TNFalpha), as well as less traditional, but potentially significant, measures such as free fatty acids and acylcarnitines (a complete list of analytes is provided in the overall introduction to this application). The sophisticated methodologies used by the Metabolomics Laboratory have already elucidated a previously unsuspected metabolic pathway in the regulation of glucose stimulated insulin secretion in the pancreas, a lipid-derived metabolite involved in the mediation of insulin resistance in muscle, and products of branch-chained amino acid metabolism that appear to provide a "signature" of human obesity. Finally, the Laboratory has found that there is heritability in the metabolic profiles in families with a high incidence of early-onset cardiovascular disease. Thus, the collaborative interaction between this Project and the Metabolomics Laboratory offers both real and potential opportunities to define metabolic pathways involved in the pathogenesis of PCOS and OSA.

***A summary of abbreviations used throughout this application is provided below.***

| | |
|---|---|
| **A1C** | Glycohemoglobin |
| **ACTH** | Adrenocorticotropin |
| **AHI** | Apnea-Hypopnea index |
| **BMI** | Body mass index |
| **CI** | Confidence interval |
| **CPAP** | Continuous positive airway pressure |
| **CRP** | C-reactive protein |
| **DHAS** | Dehydroepiandrosterone sulfate |
| **fsIVGTT** | Frequently-sampled intravenous glucose tolerance test |
| **FSH** | Follicle stimulating hormone |
| **FTI** | Free thyroxine index |
| **GnRH** | Gonadotropin releasing hormone |
| **HDL** | High density lipoprotein |
| **HOMA** | Homeostasis model assessment |
| **HPA** | Hypothalamic-pituitary-adrenal |
| **HSD** | Hydroxysteroid dehydrogenase |
| **IL** | Interleukin |
| **LDL** | Low density lipoprotein |
| **LH** | Luteinizing hormone |
| **OSA** | Obstructive sleep apnea |
| **PCOS** | Polycystic ovary syndrome |
| **REM** | Rapid eye movement |
| **SHBG** | Sex hormone binding globulin |
| **SWA** | Slow wave activity |
| **SWS** | Slow wave sleep |
| **T** | Testosterone |
| **TG** | Triglyceride |
| **TNF** | Tumor necrosis factor |
| **TT4** | Total thyroxine |
| **VLDL** | Very low density lipoprotein |

## B. BACKGROUND AND SIGNIFICANCE

PCOS affects approximately 5-8% of women in the United States and typically manifests at the time of puberty with menstrual irregularity, hirsutism, and obesity (4). The ability to diagnose PCOS at an early age has important implications, since those affected have a substantial risk for subsequent development of a number of metabolic (5, 26) and cardiovascular (11, 27, 28) disorders. Specifically, women with PCOS have among the highest reported rates of early-onset impaired glucose tolerance and type 2 diabetes (7, 8) as well as a substantial increase in risk for hypertension (29), dyslipidemia (30, 31), coronary (30) and other vascular disorders (32-34). An important addition to this list of health risks is obstructive sleep apnea (OSA) which is present in a disproportionate number of women with PCOS. The risk for OSA is at least 5-fold higher (see Preliminary Studies), and perhaps as much as thirty-fold higher in PCOS (16), than in similarly obese women. Results of our Preliminary Studies suggest that there may in fact be two "subtypes" of women with PCOS -- those with OSA and those without OSA -- and that these two subtypes may be associated with distinct metabolic and endocrine alterations. PCOS women with OSA may be at much higher risk for diabetes and cardiovascular disease than PCOS women without OSA and may benefit from therapeutic interventions targeted to decrease the severity of OSA. Most importantly, nearly all published studies that have characterized metabolic and cardiovascular abnormalities in PCOS have not controlled for the potential impact of OSA and chronic sleep loss.

*The Background that follows is intended to provide an overview of the prevalence of chronic sleep loss and OSA and the roles played by sex steroids and body fat in the pathogenesis of these conditions. This is followed by a brief review of the metabolic consequences of OSA, a description of the key metabolic derangements in PCOS and finally, by a review of the current state of knowledge regarding causes and consequences of OSA in PCOS.*

### B1. Chronic sleep loss and obstructive sleep apnea: role of sex steroids and adiposity

Over the past several decades the average duration of sleep has declined for most Americans. During the 1960's, the mean sleep duration was between 7 and 8 hours per night; today, the percentage of both men and women who sleep less than 6 hours per night has increased dramatically (1) **(Figure B1-1)**. Chronic sleep loss imposes a significant negative impact upon individual health as well as an enormous economic cost to society (35, 36). A number of studies have reported that shortened sleep duration is associated with increased mortality (37, 38). In the Nurses Health Study, it was found that sleeping less than 6 hours per night was associated with an increased risk of death, even after adjusting for age, smoking, alcohol, exercise, depression, snoring, obesity, and history of cancer and cardiovascular disease (38). Reduced sleep time has also been reported as a risk factor for the development of obesity as for type 2 diabetes (39, 40) (11, 12). Results of the Sleep Heart Health Study showed that subjects sleeping 5 hours or less per night had an adjusted odds ratios for diabetes of 2.51 (95% CI, 1.57-4.02) when compared to those who slept 7 to 8 hours per night (40). This trend in shorter sleep duration mirrors the progressive rise in overweight and obesity in the United States (41) **(Figure B1-1)** and evidence continues to emerge to support a causal link between these two conditions. Should either or both trends continue along their current trajectory, the metabolic and cardiovascular health consequences as well as economic costs will be staggering.

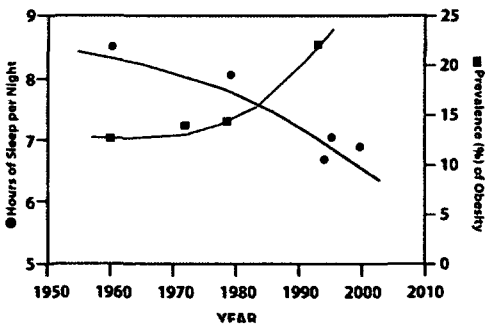



Figure B1-1. **Rising prevalence of obesity mirrors the trend toward shorter sleep duration in Americans. Drawn from composite data in (1, 2).**

Obstructive sleep apnea (OSA) is one of the major causes of chronic sleep disruption. It is characterized by episodic partial or complete upper airway obstruction during sleep leading to intermittent hypoxia, sleep fragmentation and a reduction in the quantity of deep non-rapid eye movement (NREM) sleep (stages 3 and 4, commonly referred to as slow wave sleep [SWS]). Sleep disruption resulting in reduced SWS has been associated with a rise in plasma cortisol levels and interpreted to indicate that

SWS has a "restraining" effect on the hypothalamic-pituitary-adrenal axis (42). Consistent with this is the finding that pharmacologic augmentation of SWS leads to a significant decline in salivary free cortisol levels (43).

Current estimates of OSA prevalence in the United States (2, 44) are likely to underestimate the true prevalence of the disorder since 82% of men, and an even greater (93%) proportion of women with moderate to severe OSA have not been clinically diagnosed (45). It has been consistently noted that men have a higher prevalence of OSA compared to women (45). In community based studies, the male:female ratio is usually between 2:1 and 3:1 (46) in contrast to a ratio of 8:1 in clinic-based studies (47).

OSA has been independently associated with glucose intolerance and insulin resistance even after adjustments for obesity and age (48-52). Treatment of OSA with CPAP can improve insulin sensitivity (53) and is associated with a reduction in postprandial glucose and glycohemoglobin levels in individuals with type 2 diabetes (54).

Differences in concentrations of circulating sex steroids – estrogens, progestins, and androgens – appear to play an important role in the differences between men and women, both in normal sleep as well as OSA. However, women tend to be underrepresented in most studies of OSA (**Table B1-1**).

**Table B1-1.**

| Authors | Reference # | Sample Size | Women in Sample (%) |
|---|---|---|---|
| Punjabi, et. al. | (49) | 150 | 0 |
| Ip, et. al. | (48) | 270 | 27 |
| Meslier, et. al. | (50) | 595 | 0 |
| Punjabi, et. al. | (51) | 2,656 | 54 |
| Tassone, et. al. | (52) | 30 | 30 |
| Harsch, et. al. (CPAP treatment) | (53) | 40 | 15 |
| Babu, et. al. (CPAP treatment) | (54) | 25 | 36 |

Role of Estrogen and Progesterone. Estrogens and progestins have been generally characterized as protective against the development of OSA in women. Much of the evidence to support this view is derived from studies in which sleep was evaluated in relation to pregnancy status (17, 18), age and phase of the menstrual cycle (19, 20), menopausal status (21), or in response to hormone replacement therapy (21). Lower estradiol levels have been reported in association with poor sleep quality among women aged 45 – 49 yr (22) and with a higher frequency of apneic events in women across a broader age spectrum of 24 to 72 yr (20). Among post-menopausal women, there was a modest, but statistically significant, decrease in the occurrence and frequency of sleep apnea in those randomly assigned to receive estrogen replacement rather than placebo (21).

Progesterone is the key hormone thought to underlie the differences in sleep measures that exist across the normal menstrual cycle. Progesterone levels are low during the follicular (pre-ovulatory) phase; post ovulation (luteal phase), when progesterone is synthesized by the corpus luteum, plasma levels rise by up to two log orders. When sleep measures are obtained and compared between follicular (low progesterone) and luteal (high progesterone) phases, it is apparent that upper airway resistance is lower during the luteal phase (19).

The expected rise in progesterone with pregnancy is thought to attenuate the severity of preexisting OSA as well as to "protect" from its development in women without OSA pre-conception (18). These effects have been ascribed to levels of progesterone that would normally counterbalance the increase in OSA risk imparted by pregnancy-associated weight gain. Progesterone is thought to promote its effects through direct stimulation of respiratory drive via an increased ventilatory response to both hypercapnea and hypoxia (23, 24). Progesterone may also act to enhance upper airway dilator muscle activity (25) and reduce airway resistance.

Role of Androgens. Androgens are thought to play a significant role in the sexual dimorphism in sleep architecture and in the pathogenesis of OSA (55, 56). O'Connor, et al (57) analyzed records of 830 patients with OSA to determine whether there were differences in polysomnographic features between men and women, particularly with respect to the distribution of respiratory events during REM and non-REM sleep. Although the apnea-hypopnea index (AHI) during total sleep time was significantly higher in men compared

to women (31.8 ± 1.0 vs 20.2 ± 1.5; P<0.001), the number of respiratory events occurring in REM sleep was greater in women as reflected by the so-called REM difference (i.e., the difference in the AHI in REM and AHI in non-REM sleep) in women and men. The REM difference was greater in women than men (28.1 ± 1.5 vs. 10.3 ± 1.1; P<0.001) at all levels of severity of sleep apnea. These findings were consistent and remained significant even after adjustment for the effects of covariates including weight, age, and duration of apnea. Thus, women with obstructive sleep apnea appear to have a higher proportion of respiratory events in REM compared to men, and to have a higher prevalence of apnea occurring mostly during REM.

Several studies have also shown that testosterone influences both neural control of breathing (58) and upper airway mechanics (59). Zhou et al (60) examined the effect of testosterone on apneic threshold in women during sleep. Eight normal, healthy, pre-menopausal women were studied before and after treatment with transdermal testostosterone (5 mg/day) administered in the follicular phase of the menstrual cycle. The authors concluded that testosterone increases apneic threshold in premenopausal women, thus leading to breathing instability during sleep.

Role of Body Fat and its Distribution. The risk of OSA is increased as a function of both total body fat mass as well as body fat distribution. Visceral fat appears to be more metabolically active and the quantity of visceral fat has been shown to highly correlate with OSA risk (61-63). The relative proportion of visceral fat to total body fat is higher in obese men compared to obese women. This difference is thought to contribute to the higher prevalence of OSA in men than women. Factors responsible for gender differences in body fat distribution include sex steroid concentrations, especially androgens. These factors are particularly relevant to the pathogenesis of OSA in women with PCOS.

**B2. Metabolic Consequences of OSA**

As previously noted, OSA is characterized by the combination of episodic sleep disruption and hypoxemia, each of which can trigger at least three major hormonal responses: activation of the hypothalamic-pituitary-adrenal (HPA) axis with increased cortisol production/secretion, increased catecholamine output from sympathetic nervous system stimulation, and increased release of adipokines from adipose tissue. These responses appear to contribute to the metabolic abnormalities associated with OSA, particularly to the decline in insulin sensitivity and glucose tolerance.

Hypothalamic-pituitary-adrenal axis. The onset of sleep is normally characterized by a modest inhibition of cortisol secretion that is concurrent with slow wave sleep (SWS) and lasts between 60 and 120 min (64). Nocturnal awakenings are consistently followed by a pulse in cortisol secretion (65) whereas the final morning awakening (the awakening response) is associated with a rapid rise in cortisol lasting approximately 60 min (64). Work from our laboratories (66) has shown that partial or total sleep deprivation results in increases in plasma cortisol levels by 37% and 45%, respectively. Most notably, this elevation is evidenced on the day following sleep loss and during the time when the HPA axis is usually quiescent.

Profiles of cortisol secretion in patients with OSA have been variably reported as normal in some studies and abnormal in others (67). In one report, 8 of 28 OSA patients demonstrated a disruption in the circadian rhythm with cortisol levels that were higher late in the day than in the early morning. This so-called "inverted" cortisol profile was associated in all cases with abnormal blood pressure regulation. When compared to obese subjects without OSA, obese subjects with OSA had an exaggerated ACTH response to the administration of CRH although cortisol responses did not differ between groups (68). Whether alterations in cortisol metabolism are a cause, consequence, or both in OSA remains unresolved.

**B3. Metabolic Abnormalities Associated with PCOS.**

Both lipid and non-lipid criteria identify individuals at increased risk for coronary heart disease and type 2 diabetes (69-74). Because women with PCOS have high rates of impaired glucose tolerance and type 2 diabetes (7, 8) as well as a substantial number of risk factors for cardiovascular disease (75), it has been generally assumed that many are also likely to meet criteria for the "metabolic syndrome". We recently reported that fully one-third of non-diabetic women with PCOS have developed the metabolic syndrome well before the end of their fourth decade, and usually prior to the end of their third decade of life. This prevalence is four times higher than that observed in women between the ages of 20 and 30 years and twice that of women between ages 30 and 40 years (76). Indeed, the metabolic syndrome prevalence was

similar to that in women between the ages of 50 and 60 years (76). We have also found that the prevalence of the metabolic syndrome is similar across ethnic/racial backgrounds.

Insulin resistance and hyperinsulinemia in PCOS. Even though the molecular basis for insulin resistance in PCOS remains incompletely understood, it is well documented that the compensatory hyperinsulinemia contributes both directly and indirectly (77-79) to the increase in plasma androgen concentrations that characterize PCOS. Insulin acts directly by binding to its cognate receptor on the ovarian thecal cell to stimulate testosterone synthesis (80). Insulin can also act indirectly to raise the serum concentration of free testosterone, the level of which does not appear to be tightly regulated in the female, by lowering the serum concentration of sex hormone binding globulin (SHBG) (79).

Insulin resistance is a central factor in the pathogenesis of the metabolic syndrome in both men and women and there is ample evidence to support a causal link between hyperinsulinemia and the characteristic features of PCOS. A reduction of serum insulin levels in women with PCOS results in a decrease in ovarian androgen biosynthesis, an increased SHBG concentration, and a resultant decrease in free testosterone concentrations (81, 82). Insulin also plays a key role in the impaired glucose tolerance/diabetes (81, 82) of PCOS and attenuation of hyperinsulinemia, whether through weight reduction (83) or administration of either metformin (84, 85) or a thiazolidinedione (86-88) substantially attenuates the metabolic perturbations of PCOS.

Insulin resistance and impaired glucose tolerance/type 2 diabetes. While obesity is a major factor in the development of insulin resistance in PCOS, it is now established that a component of insulin resistance in PCOS is independent of body weight (82, 89). Both lean and obese women with PCOS are more insulin resistant than their non-PCOS counterparts matched for total and fat-free body mass as documented using the hyperinsulinemic-euglycemic clamp (82, 89), frequently sampled IVGTT (26, 87, 90-92) and protocols using a graded glucose infusion (87, 90).

In long-term follow-up studies of women with PCOS there is an increased prevalence of type 2 diabetes when compared to appropriate controls (29). Two large, prospective studies in PCOS place the prevalence of IGT between 30-40% and type 2 diabetes between 5-10% (7, 8). These prevalences approach those in Pima Indians, a population with one of the highest rates of development of type 2 diabetes (93). More recently, we (7) and others (9) have found that the conversion rates from normal glucose tolerance to IGT or type 2 diabetes in PCOS are substantially elevated.

ß-Cell Dysfunction in PCOS. Because glucose intolerance results only when defects in insulin secretion and insulin action co-exist (94), we postulated that insulin secretory defects could play an important role in the propensity to develop diabetes in PCOS. Initial evidence for ß-cell dysfunction in PCOS was derived from analyses of basal and postprandial insulin secretory responses in women with PCOS relative to weight-matched controls with normal androgen levels (95). The incremental insulin secretory response to meals was markedly reduced in women with PCOS, resulting from a reduction in the relative amplitude of meal-related secretory pulses rather than from a reduction in the number of pulses present. This pattern, which resembled that of type 2 diabetes more than that of simple obesity (96), was striking in that it was evident in nondiabetic women with PCOS.

Insulin secretion is most appropriately expressed in relation to the magnitude of ambient insulin resistance. The product of these measures can be quantified (97) (the so-called "disposition index") and related as a percentile to the hyperbolic relationship for these measures established in normal subjects (97, 98). When first-phase insulin secretion is analyzed in relation to the degree of insulin resistance, women with PCOS exhibit a significant impairment in ß-cell function (86, 91). We have additionally quantified ß-cell function in PCOS by examining the insulin secretory response to a graded increase in plasma glucose and by the ability of the ß-cell to adjust and respond to induced oscillations in the plasma glucose level (26). Results from both provocative stimuli were consistent: when expressed in relation to the degree of insulin resistance, insulin secretion was impaired in PCOS subjects.

Dyslipidemia in PCOS. Women with PCOS are frequently characterized as having elevated triglyceride (TG) levels, increased levels of VLDL and LDL, and a lower HDL cholesterol (99), a lipid pattern similar to that seen in patients with type 2 diabetes. The mechanisms responsible for the adverse effects of PCOS on plasma TG homeostasis are not known. Insulin resistance has been postulated to play a key role in

causing hypertriglyceridemia in PCOS. However, we found that treatment with the insulin sensitizing agent troglitazone markedly improved insulin sensitivity in PCOS women but had little, if any, effect on plasma TG concentration (100). In addition, lean women with PCOS are found to have normal plasma TG concentrations despite being hyperinsulinemic (30). We therefore propose that increased plasma TG concentrations in obese women with PCOS are due, at least in part, to hyperandrogenemia and relative progesterone deficiency, and further that the presence of OSA has a modulating effect upon triglyceride metabolism, as will be formally tested in SCOR Project 3 (Mittendorfer, PI).

**B4. Obstructive Sleep Apnea in Women with PCOS**

Women with PCOS have been documented to develop OSA at rates that equal and may even exceed those in men. The high prevalence of OSA has been thought to be a function of both elevated levels of testosterone (a defining feature of PCOS) as well as the obesity that commonly accompanies the disorder. However, it appears that the high prevalence of OSA in PCOS cannot be fully accounted for on the basis of these two factors alone. In two studies (16, 101), the severity of sleep apnea did not correlate with BMI and in a third (102), even after controlling for BMI, PCOS women were 30 times more likely to have sleep disordered breathing and 9 times more likely than controls to have daytime sleepiness. Insulin resistance was found to be a stronger predictor of sleep disordered breathing than was age, BMI, or circulating testosterone concentrations (16). It also appeared that women with PCOS taking oral contraceptives were less likely to have sleep disordered breathing (16), consistent with recent results from the Sleep Heart Health Study Research Group in which hormone replacement therapy was associated with a lower likelihood of sleep disordered breathing among postmenopausal women (103). Finally, women with PCOS had a significantly higher mean apnea-hypopnea index compared to weight-matched controls (22.5 ± 6.0 vs. 6.7 ± 1.7; $P<0.01$), with the difference being most pronounced in REM sleep (41.3 ± 7.5 vs. 13.5 ± 3.3; $P<0.01$) (102). Because the risk imparted by obesity does not appear to be sufficient to fully account for the high prevalence of sleep disordered breathing in PCOS, additional factors have been invoked including the hyperandrogenemia (44, 55-57) that is characteristic of PCOS, as discussed below.

Androgen levels in PCOS. In response to stimulation by LH, the ovarian theca cell synthesizes androstenedione and testosterone. Androstenedione is converted by 17ß-hydroxysteroid-dehydrogenase (17ß-HSD) to form testosterone or aromatized by the aromatase enzyme (cytochrome P450arom) to form estrone. Results of studies both in vivo and in vitro (using cultured theca cells) are consistent and suggest that theca cells from PCOS ovaries are more efficient at converting androgenic precursors to testosterone than are normal theca cells (30). Clinically, this has been documented using a single, diagnostic dose of a GnRH agonist such as nafarelin or leuprolide (104). Our studies (104), as well as those of others (105), have shown that the ovarian steroidogenic response of women with PCOS is more robust than that of normally cycling women and qualitatively similar to the response seen in normal men. This response has been used as a diagnostic tool as well as a probe to define the pathogenesis of steroidogenic dysfunction in PCOS, as is proposed in this Project.

Insulin plays both direct and indirect roles in the pathogenesis of hyperandrogenemia in PCOS. Insulin acts synergistically with LH to enhance theca cell androgen production. Insulin also inhibits hepatic synthesis of sex hormone binding globulin (SHBG), the key circulating protein that binds to testosterone, and thus increases the proportion of testosterone that circulates in the unbound, biologically available or "free", state.

Results of our Preliminary Studies do not support a major role for hyperandrogenemia in the pathogenesis of OSA in PCOS. Consequently, it is important to examine alternate hypotheses. We have proposed that the relative reduction in circulating progesterone concentrations as well as estrogen concentrations, as discussed below, may contribute to the apparent excess of OSA in PCOS.

Progesterone and estrogen levels in PCOS. In normally cycling women, the luteal phase of the menstrual cycle is characterized by an increase in progesterone production from the corpus luteum and consequent slowing of GnRH, and thus LH, pulsatility. In the presence of chronic oligo- or anovulation, as in PCOS, the normal post-ovulatory rise in progesterone does not occur and the restraint on the GnRH pulse generator is thus absent (106). Thus, on average, circulating progesterone levels in PCOS women are lower than those in normally cycling women (107, 108).

Underproduction of ovarian estrogen results from low intraovarian aromatase expression and a consequent reduction in the production of the estrogens, estrone and estradiol, from their respective precursor androgens, androstenedione and testosterone. While estrone is also synthesized from peripheral aromatization (especially in adipose tissue), levels of this steroid are normal or even slightly elevated in PCOS. However, estrone is a weak estrogen with approximately $1/10^{th}$ the potency of estradiol (109). In sum, estrogen levels are subnormal in PCOS (110, 111).

## C. PRELIMINARY STUDIES

In this section, data will be presented both from unpublished as well as recently published studies from our group that support the following:

**C1.** Overweight and (visceral) obesity are exceptionally common in women with PCOS.
**C2.** Impaired glucose tolerance and type 2 diabetes are present at an early age and in a disproportionate number of women with PCOS.
**C3.** Hypertriglyceridemia is a common lipid abnormality in PCOS and predicts the presence of additional metabolic defects.
**C4.** There is a 5 - 6 fold increase in risk for OSA in PCOS.
**C5.** Neither the degree of androgen elevation nor BMI are significant predictors of OSA severity in PCOS.
**C6.** Androgen levels are significantly reduced over 3 months in PCOS in response to a single dose of the GnRH agonist leuprolide.
**C7.** Insulin sensitivity and glucose tolerance decline in response to suppression of slow wave sleep
**C8.** Treatment of OSA with CPAP in PCOS results in significant reductions in 24 hr secretory profiles for both cortisol and norepinephrine.

**C1. Overweight and obesity are exceptionally common in women with PCOS.**

The University of Chicago Center for PCOS database currently numbers 1062 subjects, 829 of whom are women with PCOS; the remaining 223 subjects are comprised of their first-degree relatives. Among the women with PCOS, all have been uniformly evaluated and diagnosed by the P.I. of this Project since the inception of the database in 1989. These women have all been seen in the Endocrinology clinics at the University of Chicago and are either physician- or self-referred. The overwhelming majority of these women (see **Figure C1-1**) are obese (81%) and a substantial proportion (35.5%) are profoundly obese with a BMI that exceeds 40.0 kg/m$^2$. These prevalences are consistent with those reported elsewhere in the U.S. (8).

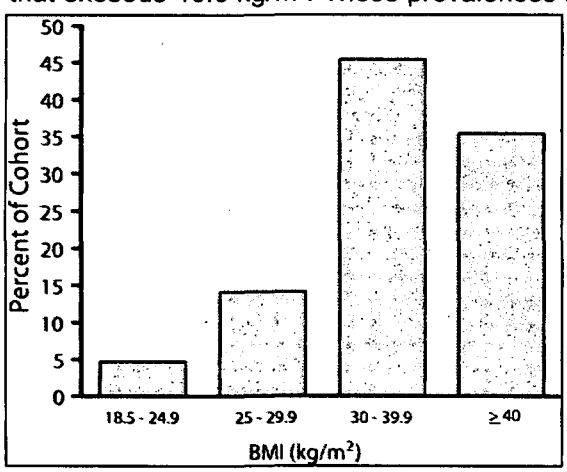



**Figure C1-1. Distribution of BMI among 829 PCOS women at the University of Chicago. BMI exceeds 30 kg/m$^2$ in over 80% of PCOS subjects.**

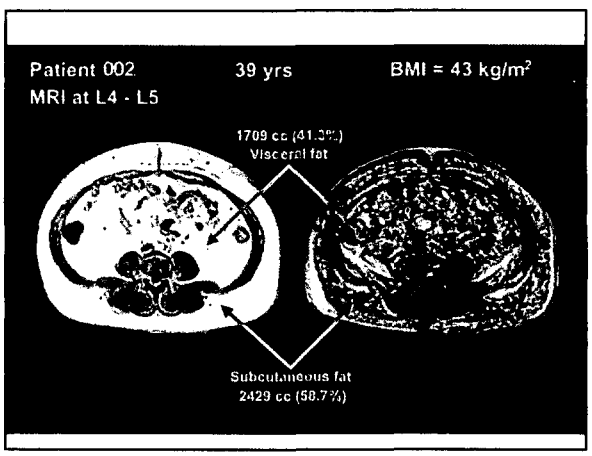



**Figure C1-2. MRI image at L4-L5 in a representative woman with PCOS. This technique provides the ability to quantify the relative proportion of visceral fat.**

As reviewed in the Background, the quantity of visceral fat appears to be more highly correlated with metabolic (insulin resistance) and cardiovascular (dyslipidemia) abnormalities than does either total or

subcutaneous fat mass. We have quantified visceral and subcutaneous adiposity in 12 women with PCOS. The images from the L4 – L5 level are evaluated with a computer algorithm that employs volume averaging. A representative example is shown in **Figure C1-2**. The relative proportion of visceral adiposity (41.3%) is substantially higher than that reported in normal weight and obese women (112).

**C2. Prevalence of impaired glucose tolerance and type 2 diabetes in PCOS**

We initially reported (7) that among women with PCOS, glucose tolerance was normal in 55%, while 35% of women had impaired glucose tolerance, and 10% had type 2 diabetes. Our sample of subjects who has had an OGTT has more than tripled since that time, but these prevalences remain nearly identical: 55% have normal glucose tolerance while 39% and 6% have IGT and type 2 diabetes, respectively. Our current analyses of oral glucose tolerance in 402 women with PCOS are summarized below.

**Table C2-1. Oral Glucose Tolerance and Androgen Levels in 402 Women with PCOS**

| | Normal Glucose Tolerance | Impaired Glucose Tolerance | Diabetes |
|---|---|---|---|
| **N (%)** | 222 (55) | 156 (39) | 24 (6) |
| **Age (yr)** | 28.0 ± 0.5 | 30.9 ± 0.7 * | 30.5 ± 1.4 |
| **BMI (kg/m2)** | 36.2 ± 0.6 | 38.4 ± 0.6 * | 39.1 ± 1.2 |
| **Total T (ng/dl)** | 72.5 ± 2.3 | 86.4 ± 4.6 * | 94.3 ± 19.2 |
| **Free T (pg/ml)** | 21.1 ± 0.6 | 27.0 ± 1.6 * | 27.2 ± 4.4 |
| **SHBG (nM)** | 16.7 ± 0.7 | 14.1 ± 0.9 | 11.4 ± 2.3 |
| **DHAS (mcg/dl)** | 160.7 ± 7.4 | 176.5 ± 11.5 | 166.4 ± 31.7 |

**Data are mean ± SEM. *P<0.05 (ANOVA with Tukey post-hoc correction for multiple comparisons) Normal Glucose Tolerance vs IGT Groups. T=testosterone; SHBG=sex hormone binding globulin; DHAS=dehydroepiandrosterone sulfate.**

These results are consistent with earlier reports substantiating the exceptionally high prevalence of impaired glucose tolerance ("prediabetes") and frank type 2 diabetes in women with PCOS. The high prevalence of obesity (see **C1**, above) in our population is a likely contributor to this finding as well as to the risk of additional metabolic abnormalities and obstructive sleep apnea (see below).

**C3. Hypertriglyceridemia is common and predictive of additional metabolic defects in PCOS.**

Women with PCOS have a substantial number of risk factors for cardiovascular disease in addition to the high rates of impaired glucose tolerance and type 2 diabetes as noted in **Section C2** above. The metabolic syndrome is defined by both lipid and non-lipid criteria that identify individuals at increased risk for coronary heart disease and type 2 diabetes. Thus, it has been generally assumed that most women with PCOS are likely to meet criteria for the metabolic syndrome.

We sought to identify factors that serve as predictors for the metabolic syndrome using data derived from a cohort of women of diverse geographic and racial/ethnic backgrounds who have been diagnosed with PCOS by uniform criteria (88). Each individual component of the metabolic syndrome was assessed to determine its ability to predict the presence (positive predictive value) or absence (negative predictive value) of the requisite number of remaining components needed to establish the diagnosis of the metabolic syndrome **(Table C3-1)**. The presence of a fasting plasma glucose of 110 mg/dl or greater had the highest positive predictive value (84%) for the presence of the metabolic syndrome. However, only 19 subjects met this criterion. Elevated triglycerides, a much more common finding, also had a high positive predictive value for the presence of the metabolic syndrome: 98 (83%) of the 118 women with a triglyceride level ≥ 150 mg/dl had at least two additional components of the metabolic syndrome.

**Table C3-1. Positive and Negative Predictive Values of Components of the Metabolic Syndrome**

| | # with criterion | # with criterion and ≥ 3 total criteria | % with criterion with MS **(Pos. Predictive Value)** | # without criterion | # without criterion and ≤ 3 total criteria | % without criterion without MS **(Neg. Predictive Value)** |
|---|---|---|---|---|---|---|
| **Waist Circumference** > 88 cm | 287 | 119 | **41%** | 74 | 71 | **96%** |
| **HDL Cholesterol** < 50 mg/dl | 240 | 115 | **48%** | 125 | 118 | **94%** |
| **Triglycerides** ≥ 150 mg/dl | 118 | 98 | **83%** | 247 | 223 | **90%** |
| **Hypertension** ≥ 130 mmHg systolic **or** ≥ 85 mmHg diastolic | 78 | 61 | **78%** | 290 | 228 | **79%** |
| **Fasting Glucose** ≥ 110 mg/dl | 19 | 16 | **84%** | 347 | 242 | **70%** |

Our analyses were also notable for the finding that fully one-third of non-diabetic women with PCOS have developed the metabolic syndrome well before the end of their fourth decade, and usually prior to the end of their third decade of life. This prevalence is markedly higher than the 6.7% prevalence of metabolic syndrome reported in women between the ages of 20 and 30 years and the 15% prevalence reported in women between ages 30 and 40 years from the Third National Health and Nutrition Examination Survey (NHANES III) (76). Indeed, the prevalence of the metabolic syndrome observed in the study population approached that seen in women who are usually between the ages of 50 and 60 years (76). Obesity appeared to have an independent effect on risk for the metabolic syndrome: women in the highest quartile of BMI had nearly a 14 fold increased chance of having the metabolic syndrome compared to women in the lowest quartile of BMI and none of the 52 women whose BMI was less than 27.0 kg/m$^2$ met criteria for the metabolic syndrome.

**C4. There is a 5-6 fold increase in risk for OSA in PCOS**

We have obtained a polysomnogram and metabolic measures in 49 women with PCOS and 22 women without PCOS (Controls). Among the 22 Controls, 4 (18%) met criteria for OSA (using the most common AHI criterion of ≥ 5 events/hr of sleep). In contrast, 27 (55%) of 49 women with PCOS had OSA. **The unadjusted odds ratio of 5.5 for OSA in PCOS is highly significant (p=0.006; 95% CI 1.6 – 18.7). Even after adjustment for age and BMI, the odds ratio remains highly significant at 5.5 (p=0.009; 95% CI 1.5 – 20.6).** Within group comparisons are shown in **Tables C4-1** and **C4-2.** The magnitude of metabolic abnormalities is greater in the presence of OSA as well. Control women with OSA had significantly higher diastolic blood pressure levels; 2hr glucose levels were also higher, but significance was lost after correction for multiple comparisons. PCOS women with OSA were significantly higher in age relative to those without OSA, but it is important to note that these women are only in their second and third decades of life. In addition, BMI and waist circumference were significantly higher in PCOS women with compared to those without OSA. As was the case in controls, 2hr glucose levels were also significantly higher, but significance was lost after correction for multiple comparisons. Finally, those with OSA had higher fasting insulin concentrations as well as higher levels of fasting triglycerides. Thus, in women with or without PCOS, the presence of OSA is associated with more profound metabolic abnormalities.

**Table C4-1.**

| | Controls Without OSA | Controls With OSA | P-value* |
|---|---|---|---|
| n | 18 (82%) | 4 (18%) | |
| Age (yr) | 29.9 ± 1.2 | 34.4 ± 1.9 | 0.123 |
| BMI (kg/m$^2$) | 35.3 ± 1.5 | 40.7 ± 4.3 | 0.160 |
| Waist Circ (cm) | 97.1 ± 3.6 | 110.4 ± 8.6 | 0.123 |
| Systolic BP (mmHg) | 114.1 ± 3.3 | 134.0 ± 6.2 | 0.012 |
| Diastolic BP (mmHg) | 67.6 ± 1.9 | 80.3 ± 3.8 | 0.007* |
| Fasting glucose (mg/dl) | 88.3 ± 2.2 | 94.9 ± 5.2 | 0.228 |
| 2hr glucose (mg/dl) | 111.6 ± 6.5 | 151.6 ± 29.1 | 0.046 |
| Fasting Insulin (pmol/L) | 11.6 ± 1.7 | 11.8 ± 3.2 | 0.972 |
| HOMA | 0.4 ± 0.1 | 0.5 ± 0.1 | 0.877 |
| A1C (%) | 5.4 ± 0.1 | 5.8 ± 0.3 | 0.067 |
| Cholesterol (mg/dl) | 163.5 ± 6.4 | 172.0 ± 8.6 | 0.553 |
| HDL (mg/dl) | 54.8 ± 3.2 | 49.0 ± 5.3 | 0.417 |
| Triglycerides (mg/dl) | 76.2 ± 13.0 | 86.5 ± 9.2 | 0.712 |
| LDL (mg/dl) | 93.7 ± 6.5 | 105.5 ± 9.3 | 0.416 |
| Total T (ng/dl) | 39.8 ± 2.7 | 28.8 ± 3.4 | 0.082 |
| Free T (pg/ml) | 9.3 ± 0.7 | 8.5 ± 1.3 | 0.614 |

**Table C4-2.**

| | PCOS Without OSA | PCOS With OSA | P-value* |
|---|---|---|---|
| n | 22 (45%) | 27 (55%) | |
| Age (yr) | 27.6 ± 0.7 | 31.8 ± 1.0 | 0.002* |
| BMI (kg/m$^2$) | 35.2 ± 1.5 | 42.2 ± 1.1 | 0.001* |
| Waist Circ (cm) | 101.9 ± 2.5 | 119.4 ± 3.0 | 0.001* |
| Systolic BP (mmHg) | 121.0 ± 2.8 | 125.8 ± 3.0 | 0.254 |
| Diastolic BP (mmHg) | 70.6 ± 1.7 | 75.1 ± 1.6 | 0.056 |
| Fasting glucose (mg/dl) | 93.1 ± 1.6 | 99.0 ± 2.4 | 0.046 |
| 2hr glucose (mg/dl) | 126.1 ± 6.9 | 144.4 ± 5.2 | 0.040 |
| Fasting Insulin (pmol/L) | 16.2 ± 2.1 | 23.8 ± 1.6 | 0.005* |
| HOMA | 0.6 ± 0.1 | 0.9 ± 0.1 | 0.014 |
| A1C (%) | 5.5 ± 0.1 | 5.6 ± 0.1 | 0.366 |
| Cholesterol (mg/dl) | 184.3 ± 5.3 | 186.5 ± 5.6 | 0.774 |
| HDL (mg/dl) | 52.5 ± 3.0 | 46.1 ± 1.7 | 0.069 |
| Triglycerides (mg/dl) | 101.2 ± 5.9 | 135.2 ± 10.3 | 0.007* |
| LDL (mg/dl) | 111.6 ± 5.2 | 113.4 ± 5.0 | 0.806 |
| Total T (ng/dl) | 72.3 ± 6.7 | 70.8 ± 4.3 | 0.856 |
| Free T (pg/ml) | 20.6 ± 1.5 | 21.3 ± 1.4 | 0.736 |

**Data are mean ± SEM. *Asterisk indicates significance after correction for multiple comparisons (Bonferroni correction).**

## C5. Neither the degree of androgen elevation nor BMI are correlated with OSA severity in PCOS.

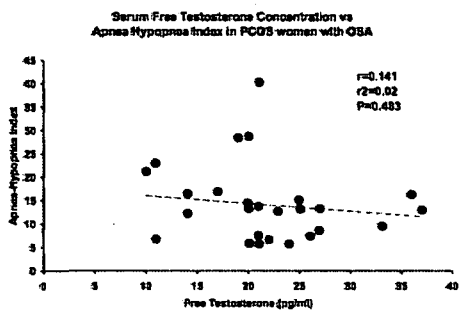



Figure C5-1

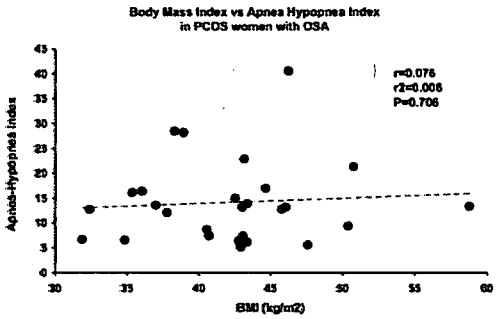



Figure C5-2

To explore potential relationships between serum free testosterone concentration and BMI with severity of OSA, we related these measures to the AHI in the 27 PCOS women with OSA discussed under **C4**, above. Scatterplots relating AHI to serum free testosterone (**Figure C5-1**) and AHI to BMI (**Figure C5-2**) are notable for the poor correlation between these measures and OSA severity. These results underscore the need to explore the effects of alternate sex hormones (i.e., progesterone and estrogen) as contributing to OSA in PCOS.

## C6. Androgen levels are significantly reduced without improvement in OSA over after significant reduction in testosterone concentrations in a subject with PCOS.

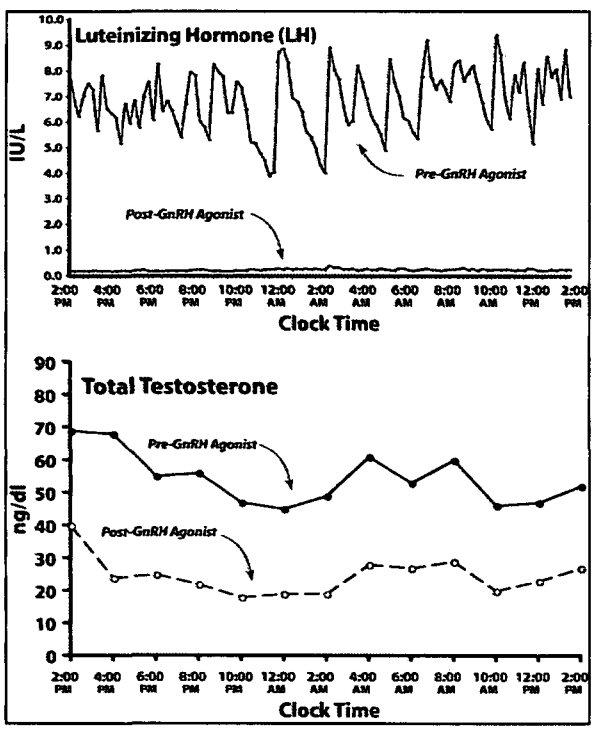



**Figure C6-1 (top panel)** depicts serum LH levels measured every 20 min over 24 hr before (Pre) and again 3 months after (Post) administration of a single 11.25 mg dose of depot leuprolide in a women with PCOS and OSA. The LH levels at 3 months are at the lower limit of detection of the LH assay over the entire 24 hr sampling interval.
**Figure C6-1 (lower panel)** depicts total testosterone concentrations measured every 2 hr over 24 hr before (Pre) and after (Post) administration of a single 11.25 mg dose of depot leuprolide in the same subject.

In light of the complete suppression of LH, circulating testosterone represents the normal contribution by the adrenal gland. Although the total testosterone levels decreased by approximately 50% and were well within the normal range, the magnitude of obstructive sleep apnea did not improve in this subject. The AHI was 14.2 events per hour sleep prior to treatment and 13.1 events per hour of sleep at 3 months. These results represent the response of a single individual. Studies planned under Specific Aim 2 are therefore designed to explore these findings in depth.

## C7. Insulin sensitivity and glucose tolerance decline in response to suppression of slow wave sleep

We have investigated the effects of all night SWS suppression via induction of microarousals without decrease in total sleep time on glucose metabolism in healthy young adults. Six young healthy lean adults

(age 20 – 31 yr; BMI 19 – 24 kg/m$^2$) were studied under two conditions (baseline, SWS suppression) in a randomized order separated at least by 4 weeks. Mean BMI did not change over the study period. Sleep disorders were ruled out by polysomnography. Undisturbed sleep was recorded on two consecutive baseline nights (B1, B2). Sleep was continuously monitored and acoustic stimuli (1000-2000Hz, 40-110dB) were administered during NREM sleep to induce microarousals and suppress SWS for three consecutive nights (S1, S2, S3). At the end of each condition, subjects had an fsIVGTT after an overnight fast. Insulin sensitivity (SI) was derived from minimal model analysis. Glucose tolerance (Kg) was calculated as the linear slope of the natural log of plasma glucose between the 5th and 19th minutes after the glucose injection. Sleep fragmentation decreased the amount of SWS (min; 74.8±7.0 on baseline vs 9.0±2.3 on S1, 13.5±3.5 on S2, 11.8±1.3 on S3; p=0.0001) with maintenance of total sleep time between study conditions (min; 465.6±8.6 on baseline vs 457.6±6.3 on S1, 467.2±3.0 on S2, 457.0±4.6 on S3; p=0.56). SI was 24% lower (7.3±0.9 vs 5.5± 0.6, p= 0.046) and Kg was decreased by 25% (2.0±0.3 vs 1.5±0.2, p=0.028) with SWS suppression compared to baseline. These data suggest that sleep disruption, whether selective for SWS or the result of OSA, lead to deterioration in insulin sensitivity. Studies proposed in this SCOR application will follow-up on these findings with a particular focus on the role of counterregulatory hormones (cortisol, norepinephrine) in mediating the decline in insulin sensitivity related to OSA.

**C8. Treatment of OSA in PCOS results in significant reductions in 24 hr secretory profiles of cortisol and norepinephrine.**

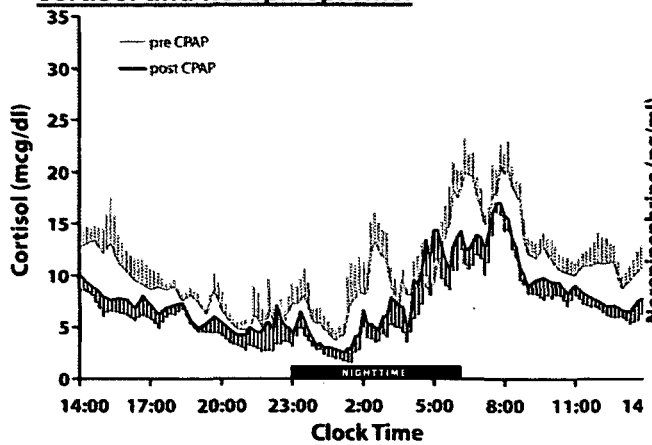



Figure C8-1

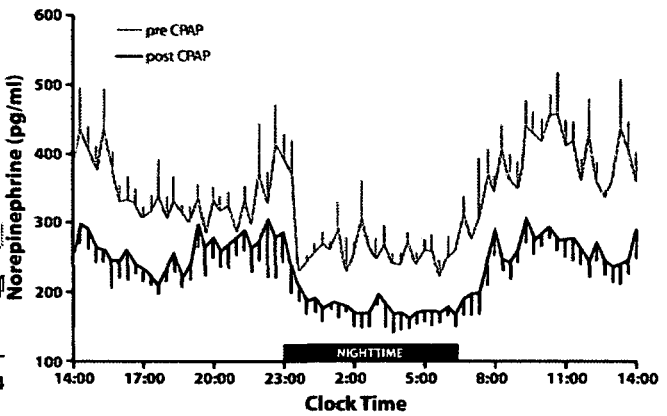



Figure C8-2

We tested the hypothesis that treatment of OSA with CPAP would ameliorate metabolic and hormonal dysfunction in PCOS. Results obtained in 5 non-diabetic women with PCOS and OSA are shown. All subjects had a polysomnogram, and 24-hr cortisol profile at baseline and after CPAP treatment for 8 weeks. Compliance was confirmed by built-in CPAP monitors and by subject contact every 2 weeks. For each individual 24-hr cortisol profile, the nadir was defined as the minimum of a regression curve calculated using a robust locally weighted procedure with a 2 hr window. For each individual 24 hr catecholamine (NE) profile, mean levels were calculated using the raw data. Data are mean SEM. Cortisol=mcg/dL; NE=pg/ml.

Subjects were 33.0 ± 2.5 yr old; mean BMI was 51.0 ± 9.0 kg/m$^2$ and did not change over the study period. Mean AHI was 17.4 ± 2.2 consistent with moderate OSA. CPAP treatment led to a significant (p=0.005) reduction in mean cortisol levels over 24-hr (7.7 ± 0.9 vs 10.2 ± 1.0) **(Figure C8-1)**. Significant reductions were seen in both daytime (7.9 ± 0.9 vs 10.3 ± 0.9, p= 0.003) and nighttime (7.5 ± 0.9 vs 10.1 ± 1.5, p= 0.02) cortisol levels after CPAP treatment. The cortisol nadir decreased by 40% (2.0 ± 0.4 vs 3.3 ± 0.8, p=0.03). The reduction in cortisol nadir was significantly correlated with AHI (r=1.0, p=0.006). Mean 24 hr NE levels decreased by 29% after CPAP (233.7 ± 18.4 vs 330.6 ± 11.6, p= 0.001) **(Figure C8-2)**. Daytime (260.0 ± 19.2 vs 363.6 ± 16.0, p=0.002) and nighttime (183.7 ± 18.4 vs 267.0 ± 8.0, p= 0.006) NE levels were decreased after CPAP. OSA is likely to contribute to elevated cortisol and NE levels in women with PCOS and could play a role in the risk for adverse metabolic alterations in this patient population.

## D. RESEARCH DESIGN AND METHODS

### D1. Study subjects and overview of experimental protocol

PCOS subjects will be recruited from the Endocrinology Clinics of the University of Chicago. All will be at least 18 but less than 40 yr of age. All recruited subjects will be obese (BMI of at least 30 kg/m$^2$). This BMI criterion is met in 80.7% of PCOS subjects in our database, as shown in Preliminary Studies. A schematic of the overall study design for Specific Aims 1 – 3 is presented in **Figure D1-1 below**. A summary grid of all testing procedures planned is shown in **Table D1.** Sample size calculations and statistical considerations follow and are presented in **Section D3**, below.

*All subjects enrolled in this project will have a confirmed diagnosis of PCOS.* A diagnosis of PCOS will require: 1) the presence of oligo/amenorrhea; 2) hyperandrogenemia, defined by a supranormal plasma free testosterone level (> 10 pg/ml); 3) hyperandrogenism, as evidenced by infertility, hirsutism, acne, or androgenetic alopecia; and 4) exclusion of nonclassic 21-hydroxylase deficiency congenital adrenal

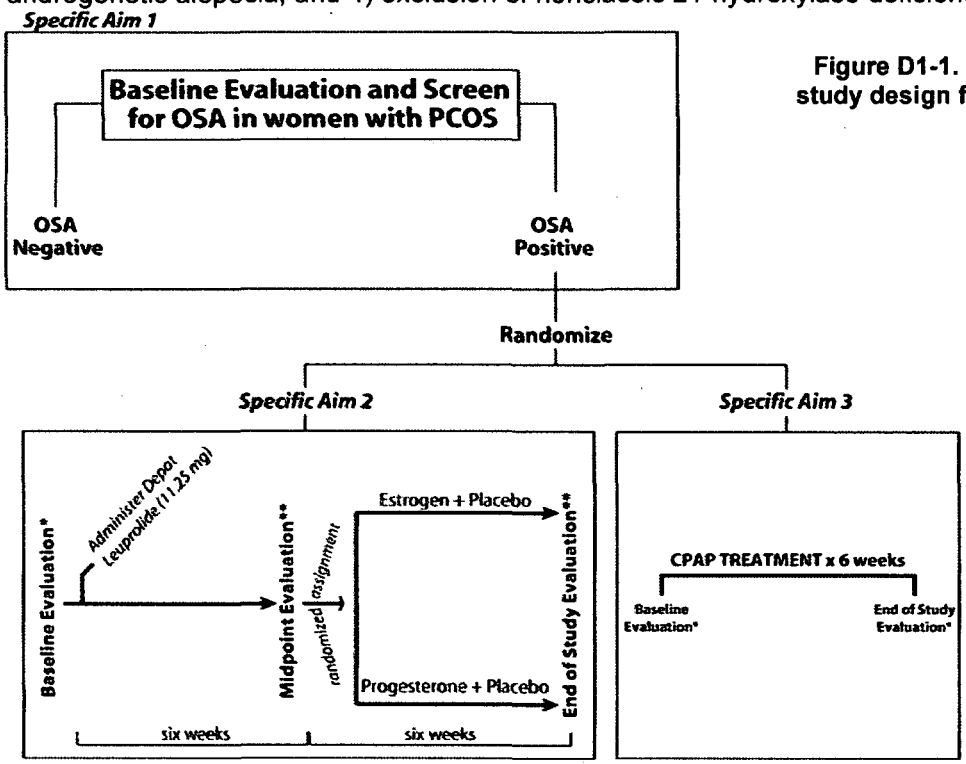



**Figure D1-1. Schematic of overall study design for Specific Aims 1 - 3.**

hyperplasia, Cushing syndrome, hypothyroidism, or significant elevations in serum prolactin. Thus, all subjects will meet the so-called NIH criteria for PCOS (113) as well as the Rotterdam criteria for PCOS (114). For at least 2 months before the study, all subjects must not take steroid preparations (including oral contraceptives), medications known to alter insulin secretion and/or action, or medications known to influence sleep. All studies will be conducted in the follicular phase of the menstrual cycle (days 1 – 7) or after 2 or months of amenorrhea. A negative pregnancy test will be required prior to all testing procedures. Subjects who are known to be diabetic will be excluded; those who prove to be diabetic on screening will not continue in subsequent protocols. Subjects with hypertension (systolic > 140 mmHg and/or diastolic > 90mmHg) will be included only if well-controlled on stable medication with either ACE inhibitors or diuretics. Subjects on beta-blockers will be excluded as these drugs affect both insulin sensitivity and sleep. Subjects must have regular life styles (no shift work, no travel across time zones within 4 weeks of study), and habitual bedtimes. Other exclusion criteria will be: habitual alcohol use of more than 2 drinks per day and excessive caffeine intake of more than 300 mg per day.

| Table D1. Summary Grid of Tests by Specific Aim.<br><br>THE PRESENCE OF THE NUMBER "1" DENOTES PRIMARY OR KEY OUTCOME FOR SPECIFIC AIM; NUMBER "2" DENOTES SECONDARY OUTCOME MEASURE. SAMPLE SIZE POWERED ON PRIMARY OUTCOME MEASURE(S).<br><br>ALL STATISTICAL ANLYSES WILL BE CORRECTED FOR MULTIPLE COMPARISONS. | SPECIFIC AIM 1 | | SPECIFIC AIM 2 | | SPECIFIC AIM 3 |
|---|---|---|---|---|---|
| | Obese PCOS OSA Negative | Obese PCOS OSA Positive | Obese PCOS OSA Positive | | Obese PCOS OSA Positive |
| | | | Depot Leuprolide | Depot Leuprolide | |
| | | | Estrogen plus Placebo | Progesterone plus Placebo | CPAP |
| **SAMPLE SIZE** | **23** | **23** | **20** | **20** | **20** |
| Oral glucose tolerance test (OGTT) | 2 | 2 | 2 | 2 | 2 |
| Frequently sampled IVGTT (fsIVGTT) | 2 | 2 | 2 | 2 | 1 |
| Androgen levels (total, free T, et al.) | 1 | 1 | 2 | 2 | 2 |
| Estradiol, Estrone, Progesterone | 1 | 1 | 2 | 2 | 2 |
| Gonadotropins (LH, FSH) | 2 | 2 | 2 | 2 | 2 |
| Metabolomics profile (blood, urine) | 2 | 2 | 2 | 2 | 2 |
| 24 hr hormonal profiles (including Cortisol and Catecholamines) | 2 | 2 | 2 | 2 | 1 |
| Plasma catecholamines | 2 | 2 | 2 | 2 | 2 |
| Abdominal MRI, Bioimpedance | 2 | 2 | 2 | 2 | 2 |
| Adipose tissue biopsy | 2 | 2 | 2 | 2 | 2 |
| Serum for adiponectin | 2 | 2 | 2 | 2 | 2 |
| Sleep and Activity Assessment | 2 | 2 | 2 | 2 | 2 |
| Sleep questionnaires | 2 | 2 | 2 | 2 | 2 |
| Wrist activity monitoring | 2 | 2 | 2 | 2 | 2 |
| Multiple sleep latency test (MSLT) | 2 | 2 | 2 | 2 | 2 |
| Sleep recording/Polysomnography | 1 | 1 | 1 | 1 | 1 |
| Wake EEG/Neurobehav. assessments | 2 | 2 | 2 | 2 | 2 |
| Daytime blood pressure recording | 2 | 2 | 2 | 2 | 2 |
| 24 hr heart rate monitoring | 2 | 2 | 2 | 2 | 2 |
| GnRH agonist test with steroid intermediates | 2 | 2 | 2 | 2 | 2 |

## D2. Screening and Specific Aim 1 Procedures

*46 subjects will need to be enrolled in Specific Aim 1 in order to ensure a sample size of 20 in the group of PCOS women without OSA; this figure of 46 subjects is based on an estimated 55% prevalence of OSA among PCOS women as shown in Preliminary Studies. This calculation accounts for the effects of expected dropouts from the study. The primary outcome measures for this Aim are the differences in progesterone and estrogen concentrations between groups.*

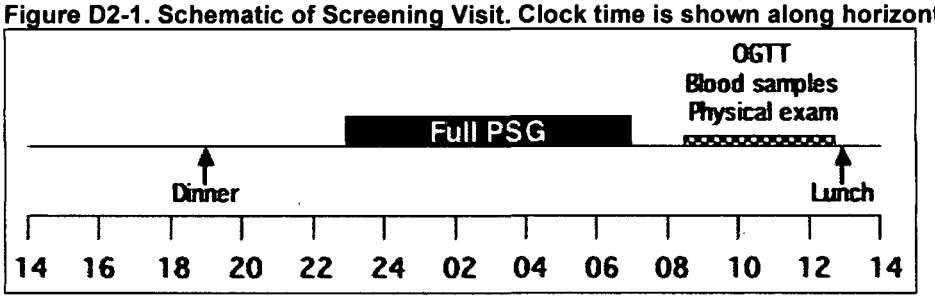
Following an initial phone screening, eligible subjects will be admitted to the GCRC in the early evening and will undergo the following screening process as described in **Figure D2-1, below**.

*N.B.* THE **SCREENING** PROTOCOL DETAILED BELOW WILL PERTAIN TO **ALL** SPECIFIC AIMS. BASED UPON THE RESULTS OF THIS SCREENING, SUBJECTS WILL BE CLASSIFIED AS EITHER **OSA NEGATIVE**, IN WHICH CASE THE

EVALUATION IS COMPLETED, OR WILL BE CLASSIFIED AS **OSA POSITIVE**. OSA POSITIVE SUBJECTS WILL THEN BE GIVEN THE OPTION TO ENTER INTO THE PROTOCOLS DETAILED IN SPECIFIC AIM 2 *OR* SPECIFIC AIM 3. SUBJECTS WILL BE INFORMED THAT IF THEY CHOOSE TO CONTINUE INTO AIM 2 OR AIM 3, THEY WILL BE RANDOMLY ASSIGNED USING A RANDOMIZATION SCHEDULE PREPARED GY THE GCRC BIOSTATISTICIAN, DR. THEODORE KARRISON. SUBJECTS RANDOMLY ASSIGNED TO SPECIFIC AIM 2 WILL ALSO BE INFORMED THAT FURTHER RANDOMIZATION WILL OCCUR TO ONE OF TWO TREATMENTS, AS DETAILED BELOW.

## Screening and Specific Aim 1 Procedures, cont'd.

**Figure D2-1. Schematic of Screening Visit. Clock time is shown along horizontal axis.**




Subjects will receive instructions on the logs/scales and practice the psychomotor vigilance test (PVT) to be used in the study. Subjects will also complete screening questionnaires including the Pittsburgh Sleep Quality Index, Berlin Questionnaire, Epworth Sleepiness Scale, Center for Epidemiologic Studies-Depression (CES-D) scale, Functional Outcome of Sleep Questionnaire (FOSQ), and the Beck Scale. An overnight full polysomnography will be performed to differentiate the subjects with from those without OSA.

**For all protocols in this SCOR application the presence of OSA will be defined by an apnea-hypopnea index (AHI) of greater than 15 events per hour sleep (AHI > 15); the absence of OSA will require an AHI of less than 5 events per hour sleep (AHI < 5). This criterion is consistent with current standards and ensures that subjects with periodic limb movement disorder (PLM arousal index > 1) are excluded from study.**

On the day following the full polysomnography, a 2 hr OGTT, a complete history and physical examination, routine laboratory tests (CBC, electrolytes, renal, liver function tests) and an EKG will be performed. This screening visit will also serve to familiarize the subject with the study environment and the experimental procedures.

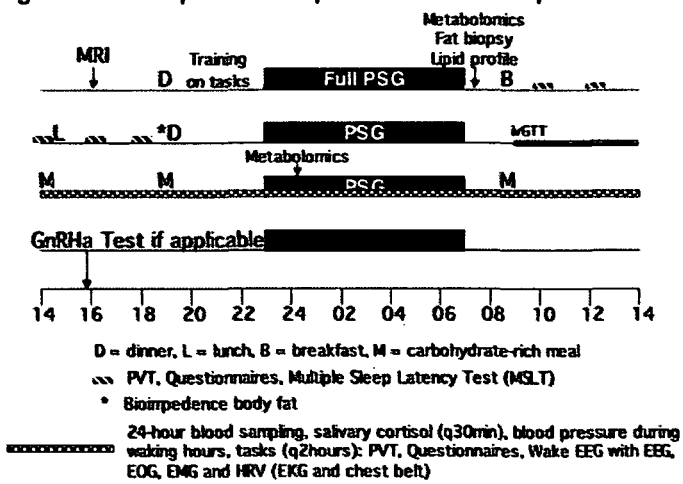
Once the Screening Visit has been completed if the subject is deemed eligible to continue, they will be instructed on completing one week of continuous wrist actigraphy at home; subjects will be asked to maintain a standardized schedule of bedtimes and mealtimes designed in accordance with their usual habits. They will be asked not to deviate from this schedule by more than 30 minutes. During this pre-study period, subjects will also be asked to fill out a daily log of sleep and meal times. Subjects who have shown poor compliance will be given the option to repeat the week of ambulatory recordings. If compliance remains poor, subjects will be excluded from further study.

After the screening visit and completion of the ambulatory recordings, the subsequent experimental procedures depicted in **Figure D2-2** and described below will occur.

The subjects will have an abdominal MRI exam at 4 pm to quantify visceral and subcutaneous fat. They will then be admitted to GCRC at 18:00 (day 1) and a standardized dinner will be provided at 19:00. The subjects will also review the PVT and questionnaires that will be used during the study. After a night of full polysomnography (day 2), 2 blood samples (12 cc for Metabolomics and 1 cc for detailed lipid profile) and a urine sample (5 cc for Metabolomics) will be obtained. This will be followed by the subcutaneous fat biopsy. The subjects will then receive a breakfast. At 9:45, 11:45, 13:45, 15:45 and 17:45, the subjects will perform the PVT and complete various questionnaires (sleepiness, vigor, mood, hunger and appetite) followed by a Multiple Sleep Latency Test (MSLT). A lunch will be provided around 14:30, depending on the MSLT duration. After the 17:45 MSLT, a bioimpedence will be obtained to measure body fat. A dinner

will be offered around 19:00 after these procedures. After a night of polysomnography (day 3), the subjects will undergo a frequently sampled intravenous glucose tolerance test (fsIVGTT) at approximately 09:00, depending on their usual bedtimes. Starting at 14:00, 24 hour blood sampling will be initiated, with intervals of every 15, 30 or 60 minutes for measurements of glucose, C-peptide, insulin, adiponectin, GH, cortisol, leptin, C-reactive protein (CRP, assayed only every 4 hours), estrogen, testosterone, progesterone, ghrelin and catecholamines (q60min) levels. Free fatty acids (FFA) will be obtained before and, 1 hour and 2 hours after each meal. FFA will also be obtained every hour during the night. Concomitant with the 24-hour blood sampling, during the waking period, saliva samples will be obtained every 30 minutes for free cortisol assay, and daytime systolic and diastolic blood pressures will be measured at 15-minute intervals.

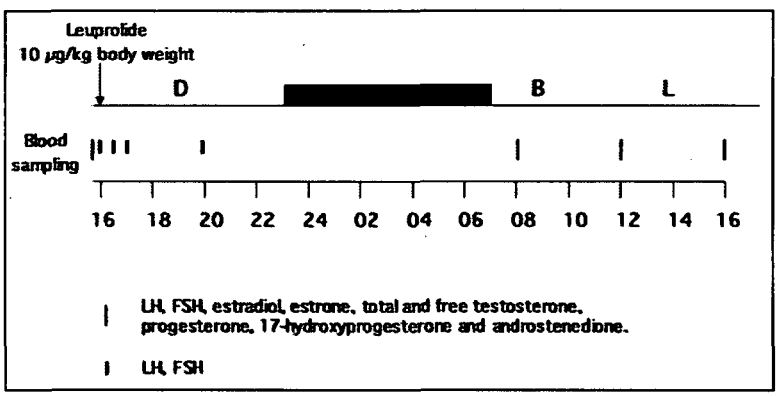
**Figure D2-2. Experimental protocol after completion of ambulatory recordings**




Every 2 hours, the subjects will perform the PVT and complete the visual analog scales. Subjects will also have a recording of wake EEG brain activity and an EKG to assess their heart rate variability (HRV). Caloric intake will be controlled with carbohydrate-rich meals served at 14:00 for lunch, 19:00 for dinner, and 9:00 for breakfast, after a night of polysomnography. The subjects will be asked to eat each meal entirely within 20 minutes. Accelerometer-based assessments of activity will be obtained continuously during the entire study (Actical). At the end of the 24-hour blood sampling period (14:00 on day 4), the subject will receive a lunch.

**At 15:50, a GnRH agonist test will be performed, as depicted in FIGURE D2-3 below.**

At 15:50, a 15cc blood sample for LH, FSH, estradiol, estrone, total and free testosterone, progesterone, 17-hydroxy-progesterone, and androstenedione will be obtained. At 15:59, a 5 cc blood sample will be taken for LH and FSH. At 16:00, leuprolide acetate 10 µg/kg will be given as a single subcutaneous injection. At 16:30, 17:00 and 20:00, 5 cc samples will be obtained for LH and FSH. A 15 cc sample will be obtained for estradiol, estrone, total and free testosterone, progesterone, 17-hydroxyprogesterone, and androstenedione after 16, 20, 24 hours GnRHa administration, i.e. at 8:00, 12:00 and 16:00.




As depicted in **Figure D1-1**, it is at this point that subjects will have completed the Screening and Specific Aim 1 Procedures. Details of procedures for subjects randomized into Specific Aim 2 or Specific Aim 3 are provided below.

### D3. Detail of methods pertaining to Specific Aims 2 and 3

*The PCOS women with OSA studied in Aim 1 plus additionally enrolled PCOS women with OSA (for a total of 60 subjects) will be randomized in a ratio of 2:1 to participate in Aim 2 (n=40) or Aim 3 (n=20). Block randomization will be performed using computer-generated random numbers. The primary outcomes measure for Aim 2 is the AHI difference pre- and post-interventions, as detail in Section D3; the primary outcomes measure for Aim 3 is the change in 24 hr cortisol concentration pre- vs post-CPAP*

Subjects randomized to Specific Aim 2 will have suppression of endogenous LH secretion, and thus ovarian sex-steroid production, by administration of a single intramuscular dose (11.25 mg) of the GnRH agonist depot leuprolide. Leuprolide is a synthetic nonapeptide analog of naturally occurring gonadotropin-releasing hormone (GnRH). The analog possesses greater potency than the natural hormone. When the depot form of leuprolide is administered at a dose of 11.25 mg, there is prolonged (3 month) suppression of pituitary gonadotropin levels and ovarian steroid levels as documented in the literature (115) and exemplified in Preliminary Studies. Six-weeks after administration, subjects will return to the GCRC for repeated evaluation, identical to that conducted at Baseline under Specific Aim 1.

After this midpoint assessment, subjects will again enter into a randomized phase of the protocol. Subjects will be assigned to receive one of two treatment regimens in a randomized, double-blind, placebo controlled fashion, for a period of 6 weeks. Subjects will receive daily oral doses of either **2 mg micronized estradiol (Estrace®)** *plus* **placebo** –OR– **200 mg micronized progesterone (Prometrium®)** *plus* **placebo**.

Rationale for preparation and dose of estrogen and progesterone. Natural estradiol is poorly absorbed in its native form and undergoes rapid metabolism in a first-pass effect through the liver. Thus, micronization (micronized estradiol) or acetylation (ethinyl estradiol) is required to achieve adequate blood hormone levels. Micronized estradiol is extremely well-absorbed and much of the compound is metabolized to estrone sulfate (65%) and estrone (15%). This "pool" of estrone and estrone sulfate then serves as the source of regenerated estradiol. At a dose of 2 mg per day, the steady-state levels of estradiol and estrone are typically 220 pmol/L and 68 pmol/L, respectively (116) and most closely recapitulate those seen in normal women in the midfollicular phase of the menstrual cycle (110). Because ethinyl estradiol is poorly converted to estrone, it will not be selected for use in these protocols. Micronized progesterone at a dose of 200 mg per day has been reported to result in attainment of serum progesterone concentrations typically seen in the late follicular/early luteal phase of the cycle (data provided by manufacturer).

Subjects randomized to Specific Aim 3 will be treated with nasal CPAP for a period of 6 weeks. Compliance will be documented based upon results provided by a microchip sensor that detects not only if the unit is powered on, but also if the mask is being worn by the subject. These data cannot be altered by the individual and there is no access to this microsensor by the user. The subjects who have OSA will undergo one night of CPAP titration study at the end of the baseline assessment. After 6 wks of home CPAP, the experimental study outline above will be repeated with CPAP use at night. Subjects whose average nightly CPAP use is > 5 hr will be considered compliant.

**D3. Statistical Considerations**

**re: Specific Aim 1**

With 20 subjects per group and assuming a two-sided alpha level of 0.05, there would be approximately 80% power to detect a 0.89 SD difference in progesterone or estrogen levels between PCOS women without and PCOS women with OSA. These values are derived from data obtained in a group of 10 PCOS women without OSA. Using the SD from the preliminary data, this study would be powered to detect a 42% difference in hormone levels between groups.

46 subjects will need to be enrolled for this Aim in order to ensure a sample size of 20 in the group of PCOS women without OSA; this figure of 46 subjects is based on an estimated 55% prevalence of OSA among PCOS women as shown in Preliminary Studies.

Progesterone and estrogen levels will be compared between the two groups using a two sample *t* test. A log transformation of the data will be performed if there is evidence of non-normality. Statistical correction will be made for multiple comparisons between groups.

**re: Specific Aims 2 and 3**

The PCOS women with OSA studied in Aim 1 plus additionally enrolled PCOS women with OSA (for a total of 60 subjects) will be randomized in a ratio of 2:1 to participate in Aim 2 (n=40) or Aim 3 (n=20). Block randomization will be performed using computer-generated random numbers.

•*Specific Aim 2*

For this Aim, there are three time points of interest: baseline (T1), six weeks after administration of depot leuprolide (T2), and at the end of study after receiving six weeks of either estrogen or progesterone

(T3). Treatment assignment to either estrogen (n=20) or progesterone (n=20) will be randomized using computer-generated random numbers.
If the variance in AHI is denoted by $\sigma^2$ and the correlation between pre- and post-treatment AHI by r, then the variance of the change in AHI is given by $2\sigma^2(1-r)$. With 20 subjects per group and assuming a two-sided alpha level of 0.05, the **Table D3-1** below lists the difference in AHI change detectable between the two treatment groups with 80% power for varying values of r.

**Table D3-1. Difference in AHI change detectable between the two treatment groups with 80% power for varying values of r.**

| r | Effect Size | Difference Detectable with $\sigma = 8$ |
|---|---|---|
| 0.25 | $1.08\sigma$ | 8.6 |
| 0.50 | $0.89\sigma$ | 7.1 |
| 0.75 | $0.63\sigma$ | 5.0 |
| 0.90 | $0.40\sigma$ | 3.2 |

Polysomnography results in a group of 17 PCOS women with OSA (defined by a total AHI ≥ 10) indicate that the mean AHI was 18.4 ± 8 (Mean ± SD). Using the SD from these preliminary data, for example, the present study would be powered to detect a difference of 0.63 × 8 = 5.04 if the correlation is 0.75 (see **Table D3-1**). This would be equivalent to a mean change in AHI of 9.2 (a 50% decrease from baseline assuming a baseline level of 18.4) in the progesterone treated group versus a mean change of 4.2 (23% decrease from baseline assuming a baseline level of 18.4) in the estrogen treated group. The change in AHI (T3 – T1) will be compared between the two groups using a two sample *t* test. This would amount to performing repeated measures analysis of variance on AHI and testing for group (progesterone vs. estrogen), time (pre- vs. post-treatment), and group by time interaction effects. The analysis will be performed both ways. Additionally, testosterone, estrogen, and progesterone levels at time T2 will be compared to the levels at T1 using a paired *t* test.

*•Specific Aim 3*
For this Aim the primary endpoint is the change in 24 hr cortisol concentration from baseline in response to CPAP treatment. There are two time points of interest: baseline (T1) and end of study after receiving six weeks of CPAP (T2). If the variance in cortisol is denoted by $\sigma^2$ and the correlation between pre- and post-treatment cortisol by r, then the variance of the change in cortisol is given by $2\sigma^2(1-r)$. With 20 subjects and assuming a two-sided alpha level of 0.05, the **Table D3-2** below lists the cortisol change detectable with 80% power for varying values of r.

**Table D3-2. Cortisol change detectable with 80% power for varying values of r.**

| r | Effect Size | Difference Detectable with $\sigma = 2.2$ |
|---|---|---|
| 0.25 | $0.77\sigma$ | 1.7 |
| 0.50 | $0.63\sigma$ | 1.4 |
| 0.75 | $0.44\sigma$ | 1.0 |
| 0.90 | $0.28\sigma$ | 0.6 |

Preliminary data in a small group of OSA+ PCOS subjects indicate that the mean cortisol level was 10.2±2.2 (Mean±SD). Using the SD from the preliminary data, for example, this study would be powered to detect a difference of 0.63×2.2 = 1.4 if the correlation is 0.50 (see **Table 2**).
Cortisol levels at time T2 will be compared to the levels at T1 using a paired *t* test. Additionally, the association between the changes (T2 – T1) in cortisol and in total AHI with treatment will be assessed using Pearson correlation coefficients or Spearman rank correlation coefficients, as appropriate.

**D4. Potential pitfalls and plans for their resolution**

re: Specific Aim 1. In studies conducted under Specific Aim 1, subjects will be recruited to participate in a wide variety of procedures over a number of weeks. Recruitment and retention may pose challenges, however we have longstanding experience with studies of this nature and our recruitment procedures have been successful to date; our retention rates in similar studies has been over 90%. Subjects are contacted on a regular basis by our study coordinator. Subjects also receive monetary compensation for participation in the protocols. We have successfully recruited subjects in the past through cooperation with other physicians who care for patients with PCOS and we plan to do so again in the event that recruitment goals are lagging.

re: Specific Aim 2. Studies in this Aim are anticipated to flow smoothly. Potential problems include drop-outs related to intolerance/untoward effects of study medication and lack of compliance. It is anticipated that additional subjects may need to be recruited to replace the drop-outs. Another potential pitfall is that the degree of testosterone suppression expected after depot-leuprolide administration may be insufficient (i.e., testosterone levels may remain above the normal female range). If this proves to be the case, we will use testosterone concentration as a covariate in our analyses related to metabolic changes pre- and post-leuprolide. Likewise, even though micronized estrogen and progesterone are optimized for oral absorption, if serum levels of estrogen and progesterone achieved are below the expected range observed in normal women, we will use these hormone concentrations as covariates in our statistical analyses. Although transdermal (patch or cream preparations) were an alternative to oral hormone therapy, the assessment of compliance with their use would have posed challenges. Pill counts, patient contact on a regular basis, and measurment of hormone levels in blood will be used to ensure and document use of study medication.

re: Specific Aim 3. This protocol requires high levels of commitment from study subjects who must use CPAP on a regular basis for the duration of the study. Compliance with CPAP use is automatically recorded and powering on the device can be differentiated from patient use via sensors incorporated into the CPAP mask. Thus, we will have device-generated compliance records.

**D5. Methods of procedure**

**Wrist activity monitoring**

The Actiwatch wrist activity monitor (Mini-Mitter Company, Inc., Sunriver, Oregon) is a miniature device (30 x 30 x 10 mm) that is accurate, sturdy and lightweight (< 68g including batteries). It has an extended memory capacity. Specially designed hardware and software for data downloading, graphing and analysis are available for this system. Wrist activity recordings will provide objective estimations of sleep duration for one week prior to the study.

**Polysomnography**

Sleep recordings will be performed in the laboratory using a Nihon Kohden (Neurofax EEG- 1100A) digital EEG acquisition system. Recordings will include electroencephalography (EEG), electrooculography (EOG), electromyography (EMG), electrocardiography (EKG) signals and a chest belt to evaluate respiration. Full polysomnography will also include oronasal airflow by thermocouples, respiratory effort from thoracic and abdominal piezo electric belts, and pulse oximetry. Prior to study sessions, all subjects will undergo one night of full polysomnography to detect sleep disordered breathing (AHI > 15, no OSA is defined as AHI < 5) and to exclude periodic limb movement disorder (PLM arousal index > 1). This screening night will also serve as habituation night. During the experimental protocol, three nights will be recorded. Each 30-sec epoch of recording will be visually scored as stage Wake, I, II, III, IV or REM following standard criteria by an experienced rater who will be blinded to the age and gender of the subject and the study condition. Respiratory events, periodic limb movements and microarousals will be scored according to established criteria. Spectral analysis of sleep EEG will be performed using specially designed software (PRANA, PhiTools, Strasbourg, France) in the delta, theta, alpha, sigma, beta and gamma bands.

**Sleep questionnaires**

*The Pittsburgh Sleep Quality Index (PSQI).* This index is derived from a self-rated 21 item questionnaire that assesses sleep quality and disturbances over a 1 month time interval. Seven "component" scores

ranging from 0 to 3 for each component are generated from this survey; one global score (PSQI) is derived from the sum of these seven scores. A global PSQI score greater than 5 has a diagnostic sensitivity of 89.6% and specificity of 86.5% in distinguishing good and poor sleepers. The seven components include: subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medication, and daytime dysfunction.

*The Berlin Questionnaire.* This questionnaire is used to assess risk for sleep apnea through questions regarding risk factors – snoring behavior, wake time sleepiness or fatigue, and the presence of obesity or hypertension. This instrument predicts high risk for sleep apnea (an RDI of 5 or greater) with a sensitivity of 0.86, specificity or 0.77, a positive predictive value of 0.89, and a likelihood ratio of 3.2.

*The Epworth Sleepiness Scale (ESS).* This survey was used to assess the level of chronic daytime sleepiness. A score of 10 or greater is indicative of excessive daytime sleepiness.

**Wake EEG recordings and neurobevioral assessments**

The subjects will be instructed to relax without falling asleep and to fixate a point on the wall for 3 min while breathing at a stable pace and then to close their eyes for 3 min while breathing normally. During the recording, the subjects will be under constant supervision. Spectral analysis of wake EEG will be performed using specially designed software (PRANA, PhiTools, Strasbourg, France) in the delta, theta, alpha, sigma, beta and gamma bands. After artifact removal, a spectral analysis, using a Fast Fourier Transformation, will be computed for 2-sec epochs for the two EEG derivations with a resolution of 0.5 Hz. The data will then be averaged for the first and the second 3-min periods in different frequency ranges, e.g. from low delta (0.5-2 Hz) to gamma (>30 Hz). Measures derived from the wake EEG include the alpha-slow wave index (ASI) defined as the ratio of EEG power in the alpha band over sum of power in the theta and delta bands) and the EEG power in the beta range of the wake EEG, a marker of alertness largely distinct from the ASI. Subjective ratings of sleepiness (Stanford Sleepiness Score), mood and vigor (VAS), Positive Affect Negative Affect Scale), hunger, appetite for various food categories, will be obtained daily in the mid to late morning, mid afternoon and evening, together with the wake EEG. Subjective sleep quality and quantity will be obtained using the Karolinska sleepiness scale every morning upon awakening. Neurobehavioral function will be measured using 5-min psychomotor vigilance test (PVT).

*Multiple sleep latency test*

The gold standard to evaluate physiological sleepiness is the multiple sleep latency test (MSLT), a series of five nap opportunities presented at 2-hour intervals beginning approximately 2 hours after initial (morning) awakening. Individuals undergoing an MSLT are instructed to allow themselves to fall asleep during the nap. Subjects are tested under standardized conditions in their street clothes and are not permitted to remain in bed between nap test sessions. Electrophysiological measures (EEG, EOG, EMG) needed to detect sleep onset and score sleep stages are recorded during nap opportunities. Unequivocal sleep is an epoch of stage 2, 3, or 4 or rapid eye movement sleep (REM) sleep. Sleep latency is defined as the elapsed time from the start of the test to the first 30-sec epoch scored as sleep.

**2-hour oral glucose tolerance test (OGTT)**

The 2-hour OGTT will be used as a screening test for assessment of glucose tolerance status (normal, impaired glucose tolerance and diabetes) as well as an outcome measure. After an overnight 12-hour fast, an intravenous catheter will be placed into an antecubital vein for blood drawing. Baseline samples of glucose, insulin and C-peptide will be taken at –15 and 0 minutes. At time 0, 75 g of glucose is administered orally, and blood samples are collected for the measurement of glucose, insulin, and C-peptide concentrations at 30, 60, 90 and 120 min. Diagnostic measures include baseline and 2-hour glucose concentrations. Normal glucose tolerance, impaired glucose tolerance and type 2 diabetes will be defined based upon the plasma glucose concentration at 2 h using the criteria of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus of the American Diabetes Association. Specifically, a diagnosis of normal glucose tolerance, impaired glucose tolerance, or diabetes is assigned if the glucose level at 2 h is less than 140 mg/dl, between 140 – 200 mg/dl, or 200 mg/dl or more, respectively.

**Frequently sampled intravenous glucose tolerance test (fsIVGTT)**
The fsIVGTT will be used to estimate insulin sensitivity by the Bergman minimal model as well as the adequacy of the first phase insulin secretory response to an intravenous glucose bolus. Starting at 09:00 after an overnight fast, blood samples (1 ml) will be drawn every 5 min for 10 min (3 baseline samples), at which time 0.3 g/kg glucose will be administered as an intravenous bolus. Blood samples will be taken at 2, 3, 4, 5, 6, 8, 10, 12, 15, 19, 21, 22, 24, 26, 28, 30, 40, 50, 60, 70, 90, 100, 120, 140, 180, 210 and 240 min. At time 20 min, intravenous insulin (0.02 U/kg) will be administered. The following summary measures will be derived: baseline insulin concentration, first phase area under the insulin curve from 0 min to 19 min; acute insulin response to glucose (AIRg) from 0 min to 10 min; insulin response expressed as a percentage of baseline insulin level; glucose tolerance index (Kg), insulin sensitivity index (SI;), and glucose effectiveness at the basal insulin level (SG). The so-called disposition index (DI), i.e., AIRg x SI, is calculated to express beta-cell function in relation to the degree of insulin resistance.

**Blood sampling and hormonal assays**
24-hour blood sampling will be performed at 15-minute intervals for 2.5 hours after each standardized meal (served at 9:00,14:00 and 19:00) and for 2 hours after scheduled bedtime, and at 30-minute intervals at all other times for measurements of glucose, C-peptide, insulin, GH, cortisol, leptin, CRP (assayed every 4 hours), estrogen, testosterone, progesterone, acylated and non-acylated ghrelin Blood samples will be obtained every hour for catecholamines. FFA will be obtained right before each meal and 1 hour and 2 hours after. FFA will also be obtained every hour during the night. During periods of frequent blood sampling, a sterile heparin-lock catheter will be inserted in the forearm and the line will be kept patent by a slow drip (10 cc/hr) of heparinized saline (750 IU/dl). All blood samples will be collected through the lateral arm of a three-way stopcock. During waking hours blood samples will be collected at the bedside. During sleep hours the intravenous line will be extended and fed through a light-tight port in the wall, thus allowing disturbance-free blood drawing from a next-door sampling room. The total amount of blood withdrawn during each of the proposed studies will not exceed 500 ml. Blood samples will be centrifuged immediately at 4°C and plasma will be frozen and stored at -80°C until assay. For each hormonal variable, all samples obtained from the same subject will be measured in the same assay. C-peptide, Insulin, GH and cortisol leptin, CRP (assayed every 4 hours), estrogen, testosterone and progesterone will be measured by chemiluminescence assays using the Immulite Immunochemistry System (Diagnostic Products Corporation). Results with the DPC Immulite, as assessed by our previous experience, or according to College of American Pathologists surveys reporting use of the instrument in clinical lab settings, demonstrate sensitivity, accuracy and reproducibility that meet all the requirements of the proposed research. Ghrelin levels will be measured by RIA (Linco Research, Inc., St. Louis, MO) kit for acylated and total ghrelin. Sensitivity is 10 pg/mL.

**Plasma catecholamines** (q60min) will be measured using the ESA Coulechem Md 5001 instrument. In this method, the catechols are first partially purified from plasma by absorption to a silica powder. Norepinephrine (NE) and epinephrine (E) are separated upon emergence from an HPLC column and quantified in a partially purified state on the basis of a reversible oxidation reduction as each catecholamine passes across a set of electrodes. The precision (CV's) of the ESA procedure, at physiologic catechol levels (NE >100 pg/ml, E 20 - 60 pg/ml) has been found to be, 6.0 - 1.8 and 4.8 - 8.0% respectively for NE and E intra- or inter-assay. Our group has recently published 24-h profiles of catecholamine levels, showing exquisite delineation of meal responses and age effects.

**Triglycerides** will be measured using a Serum Triglyceride Determination kit (Sigma-Aldrich, Inc.).
**FFA** concentrations will be assayed with enzymatic colorimetric kits (Wako Chemicals USA, Richmond, VA).

**Hormonal assays for the GnRHa Test**
Estradiol will be assayed by a modification of a Pantex kit method after extraction of plasma with a sensitivity of 2.8 pg.ml and precision of 11%. Steroid intermediates (17-hydroxyprogesterone and

androstenedione) will be measured after extraction, purification by thin-layer chromatography, and RIA. These methods are sensitive to approximately 25 ng/dl and have a precision averaging 13%. Total testosterone will be measured by a nonchromatographic method using a Diagnostics Products kit with a sensitivity of <20 ng/ml and a precision of 11%. Free testosterone will be computed from a competitive protein binding analysis with a sensitivity of 3 pg/ml, precision of 13%.

**Body composition and body fat distribution**
Waist circumference and waist/hip ratio will be measured. Bioimpedence will be performed. Body composition and fat distribution will be assessed by abdominal MRI (112).

**Subcutaneous fat biopsy (to be performed by PI in the GCRC)**
The abdominal area adjacent to the umbilicus is prepped with betadine solution. An area approximately 12 to 15 cm in diameter is prepped and allowed to air dry. Anesthetic agent is pre-mixed by removing 5cc of 1% plain lidocaine from its bottle and replacing the volume removed with 5cc of 8.4% sodium bicarbonate solution. Lidocaine field block is performed using 10cc of the premixed lidocaine/bicarbonate mixture. The area is allowed to become anesthetized for approximately 10 min after the field block, during which time it is covered with sterile 4x4. An incision approximately 0.5cm long is made parallel to the patients' waistline in the central portion of the field-blocked area, through the dermis and into the subcutaneous adipose tissue. Pre-filled 60cc syringes containing 10cc normal saline are used to aspirate the adipose tissue using a 13 gauge 3-inch hypodermic needle. Gentle aspiration back/forth motion is used to aspirate the adipose tissue. This is subcutaneous biopsy and the proper angle is approx parallel to the skin and extending up 15 degrees from the plane of the abdomen. Up to 5 syringes are used per abdominal site, depending on the sample size needed for the study. Steri-strips are used to close the wound site and folded 4x4's are placed over the strips and secured using Elastoplast. Direct pressure is applied to the wound for 5-10 minutes immediately afterwards. Ice packs are given to the patient to apply to the area after the procedure.

**Assessments in adipocytes (performed by M. Brady, PI – Project 4)**
Adipocyte preparation: Adipose sample is weighed and placed in DMEM + 1% BSA (Type V, Sigma #A7888) + 5 mM glucose + 10 mM Hepes (pH 7.4) (DMEM*). Collagenase stock solution is prepared using 3.5 ml of DMEM*/gram of fat) and 1 mg/ml collagenase (type II, Sigma #C6885). Adipose tissue is minced with a double pair of scissors, approximately 100-150 strokes. Samples are then incubated for 5 min at 37oC. Cells are transferred to new vials containing collagenase solution and incubated for a further 60-75 min at 37oC, swirling vials every 5 min. Cells are transferred to 15 ml conical tubes containing 10 ml of DMEM*. Adipocytes are washed 3X using centrifugal centrifugation (120 rpm) using 10 mls of DMEM*/wash. After final wash, excess media is completely removed and packed cells are used for in vitro assays.

Signaling Assays: 100 ml of packed cells are transferred into 1.5 ml epindorf tubes. 100 ml of DMEM* containing 2X final concentration of insulin (0-20nM) is added to the tubes. Cells are gently vortexed and incubated at 37oC for 10 min. Cells are then washed 3X in DMEM (no BSA) and lysed by addition of 200 ml of 2X Laemli buffer. All samples are boiled for 2 min, and then spun at 10,000 rpm for 10 min, RT. Samples are separated by SDS-PAGE, transferred to nitrocellulose for immunoblotting with appropriate antibodies.

Metabolic Assays: 200 ml of packed cells are transferred in triplicate into 1.5 ml epindorf tubes. 200 ml of DMEM* containing 2 mCi 14C-glucose, -/+ 10 nM insulin is added to appropriated tubes. Reactions are incubated at 37oC for 30 min, and vortexed every 5 min. At the end of the assay, half of the cells are transferred to glass test tubes and glycogen isolated using ethanol precipitation. Glycogen pellets are dried overnight, solubilized in 1 ml of water, and 14C-glucose incorporation into glycogen is determined by liquid scintillation counting. The other half of the reaction is transferred to 7 ml scintillation vials containing 900 ul of PBS and 4 ml of Betafluor is added to each vial. Vials are capped and shaken vigorously to extract the

lipids. The next day, the top 3 ml from each vial are transferred to new vials and radioactivity incorporated is determined by liquid scintillation counting.

Adipocyte culture: Primary adipocytes are resuspended in DMEM +2% FBS in a 1:1 ratio of cells to media and allowed to recover at 37oC, 6% CO2 for 30 minutes. After recovery, cells are added to Matrigel Basement Membrane Matrix in a 2:3 ratio, and 250 ul of cell/Matrigel suspension are plated per well on a 12 well plate. Plates are then placed at 37oC for 30 minutes to allow the matrigel to polymerize, after which 500 ul of DMEM +2% FBS are added per well.

**Cardiovascular measures**
Systolic and diastolic arterial blood pressure will be measured at 15-min intervals during the daytime from the non-dominant arm using ambulatory monitoring equipment (Accutracker II, Suntech Medical Instruments). Resting EKG recordings (Nihon Kohden) using two thoracic electrodes will be used to record heart rate and HRV. Time domain measures of HRV including SDNN, NN50, pNN50, r(iNN) will be calculated using ectopy and artifact free 5-min bins. Spectral power estimated in the high frequency band (HF component; 0.15 – 0.40 Hz) will be used as a measure of parasympathetic activity. Other measures of cardiac sympathovagal balance will include spectral power in the low frequency band (LF component; 0.04 – 0.14 Hz) and the LF/HF ratio. The total power in the 0.04 – 0.40 Hz band will be used to normalize absolute LF and HF values.

**Measures of energy expenditure**
We will use accelerometer-based assessments (Actical, Mini-Mitter Co. Inc, Bend, OR) to monitor the stability of activity levels in each study condition during the entire study period. The Actical is a small lightweight device (17g; 28 x 27 x 10 mm) sensitive to omni-axial accelerations, which are sampled at 32 Hz, converted to activity counts, and integrated over 1-minute epochs. The participants will wear the Actical around the clock (except when bathing) attached to an elastic waist band above their right iliac crest.

**Metabolomics**
Blood and urine will be collected, temporarily stored at – 80 C, and sent to the Metabolomics Laboratory at Duke University. Measurement of analytes in a targeted fashion is achieved by addition of known quantities of stable isotope-labeled internal standards to an array of sample types, including extracts of cells in culture, as well as bodily fluids and tissue extracts of model organisms and human subjects. The laboratory has also recently obtained the new Waters Acquity Ultra Performance LC (UPLC)™ and LCT Premier™ LC/MS systems. The enhanced chromatographic peak capacity and sensitivity of the UPLC, coupled with the high resolution and mass accuracy of the LCT Premier, allows for "unbiased" analysis of as many as 5000 individual metabolites in a single specimen. Although the identity of most of the analytes is unknown when using the LC/MS instrument, the Waters MarkerLynx™ statistical analysis software can be used to define principal components (groups of analytes) that discriminate different samples (*e.g.*, samples taken before and after a period of drug therapy or before and after an intervention such as continuous positive airway pressure (CPAP)). Once a set of metabolites has been shown to correlate with functional states, the identity of the individual analytes can be determined by additional chemical and MS-based analyses.

## E. HUMAN SUBJECTS RESEARCH

### 1. Risks to the Subjects

a. Human Subjects Involvement and Characteristics

- Study subjects will include women with PCOS. Subjects will be between 18 and 40 years of age.
- Women > 40 years of age will not be recruited to avoid the confounding variable of age related alterations upon ovarian steroidogenesis. It is anticipated that the majority of subjects will be aged between 25 and 35 years.
- Women with nonclassic 21-hydroxylase deficiency congenital adrenal hyperplasia will be identified by and AM 17-hydroxyprogesterone blood level. In cases where there is question, subjects will be advised to undergo an adrenocorticotropin stimulation test. Women with this disorder will be excluded from further study as will those with Cushing's syndrome. There will be no exclusion criteria based upon race, but subjects with systemic illnesses, including heart, renal, liver, or malignant disease will be excluded. Individuals with known peanut allergy or allergies to medications used in this study will be excluded.
- Pregnancy status will be checked. Pregnant women will be excluded from study.
- With the exception of children, the proposed studies will *not* include special classes of subjects. Specifically, studies will *not* include fetuses, neonates, pregnant women, prisoners, institutionalized individuals.
- Individuals known to be diabetic will not be enrolled in studies; those who prove to be diabetic upon screening will discontinue participation in the protocols.
- Subjects with hypertension (systolic > 140 mmHg and/or diastolic > 90mmHg) will be included only if well-controlled on stable medication with either ACE inhibitors or diuretics. Subjects on beta-blockers will be excluded as these drugs affect both insulin sensitivity and sleep.

b. Source of Materials

- Research materials to be obtained from study subjects includes blood and urine samples for hormonal and biochemical measures. Blood samples will be collected for research purposes.
- Fat cells from the subcutaneous tissue will be obtained by biopsy procedure.
- Existing records will not be incorporated as part of the study files.

c. Potential Risks

- The clinical procedures involve a minimal discomfort associated with venipuncture. There might be slight bruising at the site that will recover in a short period of time. There is also slight risk of inflammation of the vein or infection. However, our nurses who perform these procedures are highly trained and thus these risks are minimal. The amount of blood withdrawn is minimized to prevent significant blood loss. Baseline hematocrit will be measured in all subjects to ensure that this risk is not excessive.

- There are no known direct or permanent side effects from a single injection of either diagnostic leuprolide (10mcg/kg body wt) or depot leuprolide (11.25mg). In women the changes in hormones may change the timing or pattern of the menstrual cycle during which it is given, or cause transient water retention, breast tenderness, or mood changes. Occasional patients have reported headache, GI upset, back pain, mild fever, or increased libido after a single dose of leuprolide. Swelling and tenderness at the injection site may develop. The safety of leuprolide during pregnancy and risk of treatment to an unborn fetus is unknown. Women will be instructed to use an effective non-hormonal form of contraception, such as condom with spermicidal foam or IUD.

- Insulin. Insulin administered as part of the fsIVTT will raise plasma insulin levels transiently, but hypoglycemia is rare given that the insulin is administered only after a glucose bolus has been given as part of the procedure.

- Micronized progesterone will not be given to individuals with known peanut allergy since the capsules are known to contain peanut oil. Likewise, this medication will not be given to women with undiagnosed vaginal bleeding or a history of deep vein thrombosis. Patients may experience headache, breast tenderness, bloating, or depression as a result of use of this medication. Patients will be informed of these risks and all efforts will be taken to minimize them.

- Micronized estrogen will not be given to individuals with known peanut allergy since the capsules are known to contain peanut oil. Likewise, this medication will not be given to women with undiagnosed vaginal bleeding or a history of deep vein thrombosis. Patients may experience nausea, headache, breast tenderness, or bloating as a result of use of this medication. Patients will be informed of these risks and all efforts will be taken to minimize them.

- The GCRC nurses who perform these procedures are highly trained and thus the risks directly associated with intravenous puncture and blood withdrawal described above are minimal. For those subjects randomized to take pioglitazone, monitoring of hepatic function will be performed as described above. Subjects may choose not to participate in the study, and may discontinue the study at any time point. Conventional diagnostic and therapeutic modalities will be made available to all subjects where appropriate. In the event of physical injury resulting from this research, the University of Chicago Hospitals will provide emergency care at no cost to the subject, if such care is necessary. The University of Chicago Hospitals will also provide non-emergency care, but the Medical Center assumes no responsibility to pay for such care or to provide financial compensation. Confidentiality is always respected. Specimens are logged by number according to routine practices of the institution. Data collected are included in the patients' charts and results of the studies are made available to patients, their legal guardian, and their referring physicians. Samples are not available to any investigators other than those listed herein.

**2. Adequacy of Protection Against Risks**

a. Recruitment and Informed Consent

- Efforts will be made to recruit women and minorities to participate in the proposed studies. Since the disorder being investigated affects women of all ethnicities, attempts will be made to have subjects represented from all known groups. Community screenings will be conducted in Chicago neighborhoods known to have high population densities for individuals of American Indian, Asian, and Hispanic origin. The University of Chicago has established relationships with Community- and Church-based organizations throughout the metropolitan region. These organizations will be targeted for recruitment efforts.

- Subjects will be recruited from the Outpatient Clinics at the University of Chicago, and by advertisement in appropriate newsletters or other media. The nature of the research project will be described in detail to the subjects and written summaries, in lay terms, of the research proposed will be provided for review. Written informed consent will be obtained by the P.I. from the subjects and/or parent/legal guardian where appropriate.

The protocols and consent forms have been approved by the Institutional Review Board at the University of Chicago

- Because women are the target population for the study of PCOS, the PI anticipates no difficulty in recruitment of women to participate in the proposed protocols.

- Minority populations have actively participated in the PI's research program in the past, with the distribution as noted above. As described above, outreach efforts will be intensified to ensure enrollment of underrepresented minority populations.

- Children are not specifically targeted for the present studies, nor will they be excluded. It is anticipated that a small number of children may be enrolled who are 2 years post-menarche. It is further anticipated that fewer than 5% of subjects will be less than 18 years of age.

b. Protection Against Risk

The following will be specifically instituted to protect human subjects from research risks:

- subjects with systemic illnesses, including heart, renal, liver, or malignant disease will be excluded. Pregnant women, women with known peanut allergy or allergy to any planned study medication will be excluded.

- The nature of the research project will be described in detail to the subjects and written summaries, in lay terms, of the research proposed will be provided for review. Written informed consent will be obtained by the P.I. from the subjects and/or parent/legal guardian where appropriate. The protocols and consent forms have been approved by the Institutional Review Board at the University of Chicago.

- The amount of blood withdrawn will be minimized to prevent significant blood loss.

- Baseline hematocrit will be measured in all subjects to ensure that this risk is not excessive. A cooling or heating apparatus will be available in the event that local venipuncture site swelling develops.

- Women will be instructed to use an effective non-hormonal form of contraception, such as condom with spermicidal foam or IUD for studies that will alter sex-steroid levels.

**3. Potential Benefits of the Proposed Research to the Subjects and Others**

- Subjects will gain knowledge about their ability to secrete insulin and metabolize glucose. They sill also be informed of their sleep pattern and efficiency and will benefit from treatment, if needed, for obstructive sleep apnea. Because many of the patients will be at risk for developing diabetes, they will benefit from frequent clinical monitoring, should their condition progress during the study period. Patients will benefit from accurate diagnosis which will guide proper treatment. The information gained may provide insights into the pathogenesis of type 2 diabetes and may have important therapeutic implications in the future. The risks involved in ascertaining this information (see above) are minimal in relation to the potential benefit of the information gained.

**4. Importance of the Knowledge to be Gained**

- The proposed studies hold the potential for development of new knowledge regarding the etiology and pathogenesis of PCOS, obstructive sleep apnea, and the metabolic derangements associated with these conditions. Because these disorders are extraordinarily common and because they have significant impact on the health and well-being of those affected, the proposed studies are of great potential importance. The insights gained may lead to a better understanding of ways to prevent and/or treat type 2 diabetes, obstructive sleep apnea, and PCOS.

**Inclusion of Women:**
Because women are the target population for the study of PCOS, the PI anticipates no difficulty in recruitment of women to participate in the proposed protocols. The vast majority of the proposed study population will be comprised of women with PCOS, spanning across a broad spectrum of age, race, and ethnicity. PCOS typically manifests during the late teen years or early adulthood, but can be evident as early as age 12 years. The protocols in this proposal will be conducted in women ranging in age from 18 – 40 years. Before age 18 years and after age 40 years, there are significant effects of age upon ovarian steroidogenesis which can confound analyses in this proposal.

**Inclusion of Minorities:**
Minority populations have actively participated in the PI's research program in the past, with the distribution as noted above. Our studies have included women with PCOS of all racial and ethnic backgrounds. We have made, and will continue to make, efforts to recruit women from both local and distant sites. We will advertise in neighborhood publications within the City of Chicago, as well as the surrounding suburbs, and northwest Indiana. Our recruitment of minorities is facilitated, in part, by our location within the City of Chicago, our referral base (local, Midwest, and national). Outreach efforts will be intensified to ensure enrollment of underrepresented minority populations.

**Inclusion of Children:**
Children will not be included in the proposed studies.

## F. VERTEBRATE ANIMALS

**None**

## G. SELECT AGENT RESEARCH

**Not applicable**

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## I. MULTIPLE PI LEADERSHIP PLAN

**Not applicable**

## J. CONSORTIUM/CONTRACTUAL ARRANGEMENTS

**Not applicable**

## K. RESOURCE SHARING

**No novel resources will be developed in this application.**