Treating Inflammation in Polycystic Ovary Syndrome to Ameliorate Ovarian Dysfunction

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LIST OF ABBREVIATIONS

ACCC	Academic Computing and Communications Center
AE	Adverse Event
AUC	Area under the Curve
BBT	Basal Body Temperature
BMI	Body Mass Index
BW	Body Weight
CCT	Cream Challenge Test
CCTS	Center for Clinical and Translational Science
CITI	Collaborative Institutional Training Initiative
CL_{IV}	Clearance for an Intravenous Infusion
CL_P	Clearance of Plasma Insulin
CL_{portal}	Clearance for a Portal Infusion
CRC	Clinical Research Center
DEXA	Dual Energy X-ray Absorptiometry
DSMB	Data and Safety Monitoring Board
EMSA	Electrophoretic Mobility Shift Assay
FDA	Food and Drug Administration
FE_L	Hepatic Fractional Extraction
HBSS	Hank's Buffered Saline Solution
HIPAA	Health Insurance Portability and Accountability Act
HCG	Human Chorionic Gonadotropin
HCG-ST	Human Chorionic Gonadotropin Stimulation Test
HPF	Hepatic Plasma Flow
ICD	Informed Consent Document
IDS	Investigational Drug Service
IND	Investigational New Drug
ICL	Integrative Physiology Laboratory
IR	Insulin Resistance
ISR	Insulin Secretion Rate
IRB	Institutional Review Board
IUD	Intrauterine Device
K_m	Hepatic Insulin Delivery Rate
LH	Luteinizing Hormone
MNC	Mononuclear Cells
MRI	Magnetic Resonance Imaging
NFκB	Nuclear Factor κB
OCC	Outpatient Care Center
OGTT	Oral Glucose Tolerance Test
17OHP	17-Hydroxyprogesterone
PHI	Protected Health Information
PI	Principal Investigator
PCOS	Polycystic Ovary Syndrome
ROS	Reactive Oxygen Species
RT-PCR	Real-Time Polymerase Chain Reaction
S_I	Fractional Glucose Disappearance/Insulin Concentration Unit
UIC	University of Illinois at Chicago
UI Health	University of Illinois Hospital and Health System
V_{max}	Maximal Hepatic Degradation Rate
V_P	Extrahepatic Distribution Volume

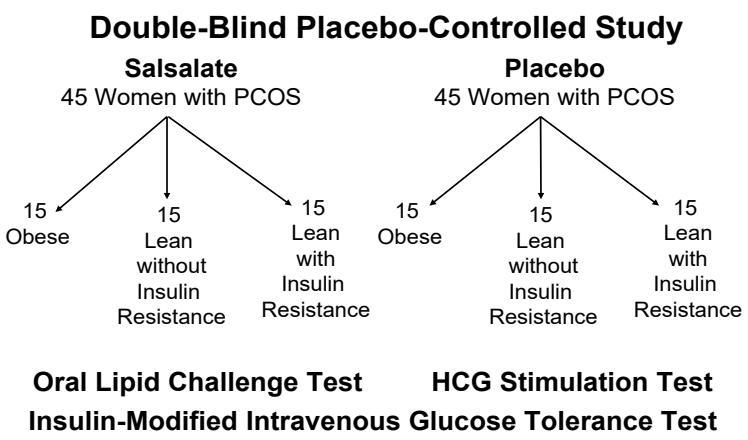
1.0 Project Summary/Abstract

Polycystic Ovary Syndrome (PCOS) is characterized by hyperandrogenism, ovulatory dysfunction and polycystic ovaries. Insulin resistance (IR) is a common feature of PCOS, and the resultant hyperinsulinemia has been theorized to promote hyperandrogenism in the disorder. However, 30-50% of women with PCOS who are lean do not have insulin resistance. Women with PCOS also exhibit chronic low-grade inflammation. We have shown that in PCOS, glucose ingestion activates nuclear factor κB (NFκB), the cardinal signal of inflammation culminating in upregulation of the inflammation pathway within mononuclear cells (MNC). This phenomenon is independent of excess adiposity and is highly correlated with circulating androgens. In addition, *in vitro* exposure to proinflammatory stimuli is capable of directly stimulating ovarian theca cell androgen production. Salicylate administration has been shown to suppress NFκB activation, and the nonacetylated form of salicylate is well tolerated in humans.

The proposed research is a randomized double-blind placebo-controlled study of 90 women with PCOS. Forty-five subjects with PCOS (15 lean without IR), 15 lean with IR and 15 obese) receiving salsalate, a nonacetylated salicylate, at an oral dose of 3-4 gm daily for 12 weeks will be compared with 45 age- and body-composition-matched control women with PCOS receiving placebo (Fig. 1). The *overarching hypothesis* is that inflammation contributes to ovarian dysfunction, independent of excess adiposity or IR.

The *specific aims* are, **I**: To examine the effect of salsalate administration on the ovarian capacity to secrete androgen on and insulin sensitivity in PCOS. **II**: To examine the effect of salsalate administration on the inflammatory response of mononuclear cells induced by lipid ingestion and glucose infusion in PCOS. The *approach* involves evaluation of ovarian androgen secretion in response to human chorionic gonadotropin administration and insulin sensitivity during the insulin-modified frequently sampled intravenous glucose tolerance test (FS-IVGTT) along with ovulation monitoring before and after salsalate administration. The inflammatory response of MNC to lipid ingestion will also be evaluated during treatment by measuring reactive oxygen species, the mRNA and protein content of inflammation markers, NFκB activation and cytokine release in culture.

Figure 1



It is our *expectation* that women with PCOS receiving salsalate will exhibit decreased ovarian androgen secretion and reduced inflammation regardless of the degree of adiposity or IR status. These results will be significant because they will show a causal contribution of inflammation to ovarian dysfunction in PCOS, thereby improving our understanding of the pathogenesis of PCOS; open previously unexplored therapeutic avenues that are not necessarily dependent on improving IR; and guide the design of future studies aimed at determining what dietary modifications, medical regimen or both will optimally attenuate inflammation in PCOS to reduce medical disease and enhance fertility.

2.0 Background/Scientific Rationale

PCOS is characterized by hyperandrogenism, ovarian dysfunction and polycystic ovarian morphology.[1] Obesity and IR are common features of PCOS.[2,3] Under the current model of pathophysiology of PCOS, the compensatory hyperinsulinemia of IR is the primary driver of hyperandrogenism. This concept was born from the cross-sectional observation that insulin is positively correlated with androgens in obese women with PCOS,[2] and is supported by reports of increased androgen production from theca cells obtained from obese women with PCOS following insulin exposure *in vitro*[4] and increases in circulating androgens in women with PCOS following insulin infusion *in vivo*.[5-7] However, these *in vitro* - *in vivo* responses were elicited with supraphysiological insulin concentrations.[4-7]

Physiological insulin infusion on the other hand does not augment androgen levels in PCOS.[6] The current model also does not explain the cause of hyperandrogenism and ovarian dysfunction in the 30-50% of women with PCOS who are lean and lack IR. Thus, some other factor contributes to these abnormalities in PCOS.

We have shown that ingestion of glucose and saturated fat elicits an inflammatory response from circulating MNC in lean women with PCOS who lack excess abdominal adiposity. The hallmark of this response is increased activation of NFκB, the cardinal signal of inflammation (Fig. 2).[8-11] These findings illustrate the separate and discrete role of MNC in manifesting inflammation in PCOS and that MNC are an excellent model to assess systemic inflammation in PCOS.

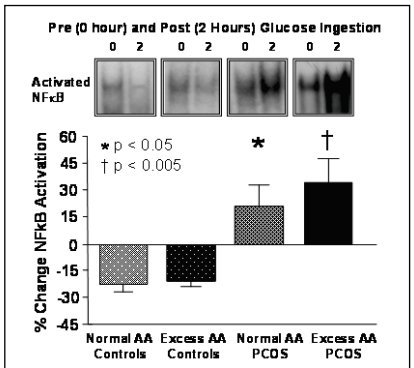


Fig.2. Gel shifts depicting the activation of MNC-derived NFκB, the cardinal signal of inflammation, following glucose ingestion in lean women with PCOS with and without excess abdominal adiposity and body composition-matched controls. Results are expressed as change

We have also shown that in PCOS, there is a link between molecular markers of inflammation from MNC and circulating androgens.[8-14] Chronic suppression of ovarian androgen production does not ameliorate inflammation in lean women with PCOS.[15] However, *in vitro* exposure of ovarian theca cells to proinflammatory stimuli upregulates CYP17, the androgen producing enzyme and increases testosterone.[16,17]

Salsalate is an inexpensive, safe, well-tolerated, well-understood anti-inflammatory agent that inhibits NFκB activation when used at higher doses.[18,19] The salsalate dose required to achieve a salicylate level in the upper therapeutic range is dependent on body mass.[20] This is achieved in lean individuals using 3.0 gm/day as the maximum dose recommended in the salsalate package insert. Individuals across the obese range (30-40 kg/m^2) require >3.0 gm/day to achieve the same objective.[21,22] Salsalate and other salicylates have also been shown to decrease IR.[19,23,24] However, the ability of salsalate to decrease IR would not be necessary if the beneficial anti-inflammatory effect of salsalate to reduce hyperandrogenism is on the ovaries. In fact, we have shown that in lean insulin-sensitive women with PCOS, salsalate reduces human chorionic gonadotropin (HCG)-stimulated ovarian androgen secretion by 44% and normalizes basal testosterone levels.[25] Our studies in MNC also confirm the ability of salsalate to suppress NFκB activation.[25] Together these observations validate the use of these measurements as endpoints to assess the effects of salsalate to probe the pathophysiology of PCOS.

Salsalate raises circulating insulin due to its ability to decreased insulin clearance from the liver which confounds the assessment of insulin sensitivity from post-treatment hyperinsulinemic-euglycemic clamp studies.[19,23,26] Performance of a novel minimal model-based analysis from an insulin-modified FS-IVGTT is able to address this confounding factor. With this approach, hepatic and extrahepatic insulin clearance can be estimated before and after salsalate treatment to obtain measures of insulin sensitivity that take into account the salsalate-induced alteration in insulin clearance. The FS-IVGTT using the minimal model approach has been used for nearly four decades as a metabolic research tool and has been widely published.[27-32] A co-investigator (Quinn) is experienced in the performance of the minimal model FS-IVGTT.[33,34]

In this context, the *rationale* for the proposed study revolves around the concept that in PCOS, inflammation contributes to ovarian dysfunction independent of excess adiposity or IR, and may also improve insulin sensitivity when IR is present. We propose to undertake a 12-week randomized, double-blind, placebo-controlled trial to test the link between inflammation and ovarian androgen secretion in PCOS unrelated to IR. If this study of pathophysiology demonstrates beneficial effects, this will pave the way for developing novel therapies for ovarian dysfunction in PCOS.

3.0 Objectives / Aims

The *main objective of this proposal* is to evaluate the ability of salsalate to reduce ovarian androgen secretion, induce ovulation and decrease lipid- and glucose-stimulated inflammation independent of body composition and IR in women with PCOS; and to also improve insulin sensitivity in IR women with PCOS. Effects of salsalate will be assessed based on the following aims:

Specific Aim 1. ***To examine the effects of salsalate administration on the ovarian capacity to secrete androgens, menstrual function, and insulin sensitivity in PCOS.***

The *hypothesis* for this aim is that salsalate treatment will decrease HCG-stimulated ovarian androgen secretion and induce ovulation in women with PCOS regardless of body composition or IR status; and may also improve insulin sensitivity in IR women with PCOS. We will test this hypothesis in a randomized double-blind placebo-controlled study. The ovarian androgen response to HCG administration will be evaluated in women with PCOS (15 lean with IR, 15 lean without IR and 15 obese) before and after administration of a therapeutic salsalate dose for ~12 weeks compared with women with PCOS receiving placebo for ~12 weeks (15 lean with IR, 15 lean without IR and 15 obese). Ovulation monitoring will occur in all subjects using timed progesterone measurements and serial follicular ultrasonography as indicated. Fractional glucose disappearance per insulin concentration unit adjusted for insulin clearance will be determined as a measure of insulin sensitivity (S_I) during a FS-IVGTT before and after salsalate or placebo administration. We anticipate that salsalate will reduce the exaggerated ovarian androgen response to HCG administration and lead to hormonal and sonographic evidence of ovulation regardless of body composition or IR status when compared with placebo. We also anticipate that salsalate will increase S_I in IR women with PCOS compared with placebo.

Specific Aim 2. ***To examine the effect of salsalate administration on the inflammatory response of mononuclear cells induced by lipid ingestion and glucose infusion in PCOS.***

The *hypothesis* for this aim is that salsalate administration will down-regulate inflammatory signal transduction and cytokine production within MNC following lipid ingestion and glucose

infusion in women with PCOS regardless of body composition or IR status. We will test this hypothesis using the study design described in Aim 1. The inflammatory response of MNC to a cream challenge test will be evaluated in women with PCOS before and after salsalate treatment with the following measurements: a) reactive oxygen species (ROS) generation; b) NFκB activation; c) mRNA and protein proinflammatory markers; d) cytokine release from cultured MNC culture. It is anticipated that lipid- and glucose-induced inflammation will decrease with salsalate use regardless of body composition or IR status when compared with placebo.

4.0 Eligibility

The protocol will prospectively study healthy reproductive-age women with PCOS recruited from clinical practice or various local advertisements (see section 5.0). Volunteers interested in study participation will respond by telephone or secure e-mail (see section 5.0). They will subsequently be provided with a detailed study description and undergo an initial pre-screening using questions from an eligibility assessment form (attached). During the prescreening, volunteers will be asked to provide their name, contact information, age/date of birth, smoking status, and information about their current medical conditions, general and gynecological health status and medication use. All of the answers obtained will be documented in the eligibility assessment form which will be kept in each volunteer's study booklet. The PI will determine subject eligibility based upon the following inclusion / exclusion criteria.

4.1 General Inclusion Criteria:

- Diagnosis of PCOS based on the presence of hyperandrogenism (skin manifestations of androgen excess such as hirsutism, acne or temporal balding – or –elevation of at least one serum androgen [i.e. total testosterone, free testosterone, androstenedione or DHEA-S] using predetermined local laboratory cutoffs), oligo/amenorrhea and evidence of withdrawal bleeding after progestin administration.
- 18-40 years of age.
- Good health as evidenced by medical history, physical examination and gynecologic examination within 30 days prior to starting the study.
- Willingness to provide informed consent according to the guidelines of the University of Illinois at Chicago (UIC) Institutional Review Board (IRB).
- Willingness to use double-barrier contraception such as condoms and topical spermicide (foam, cream or gel), condom and diaphragm, diaphragm and topical spermicide or sponge with topical spermicide if sexually active. Use of a non-hormonal intrauterine device (IUD), or permanent sterilization of the subject or her partner (i.e. tubal ligation or vasectomy) is also acceptable in all instances.

4.2 General Exclusion Criteria:

- Hyperprolactinemia.
- Uncontrolled thyroid disease.
- Evidence of Cushing's syndrome, nonclassic congenital adrenal hyperplasia or a hormone producing tumor based on physical findings and serum androgen levels on initial screening.
- Known or suspected pregnancy.
- Regular vigorous physical activity during previous 6 months.
- Use of any medications known to affect carbohydrate or sex hormone metabolism such as oral contraceptives, progestins, glucocorticoids or insulin sensitizing agents within 30 days of beginning the study.

- Acute or chronic inflammatory illnesses (e.g. URI, asthma, rheumatoid arthritis or systemic lupus erythematosus).
- Type 1 or type 2 diabetes mellitus defined as having a fasting glucose >126 mg/dl and/or a 2-hour postprandial glucose >200 mg/dl.
- Regular smoking defined as more than 2 cigarettes a month, or any smoking within 30 days of beginning the study.
- History of any illness exacerbated by salicylate use (e.g. peptic ulcer hepatic or renal disease, anemia, thrombosis, coagulopathy, congestive heart failure, hypertension or gout).
- Allergy to salicylate or dairy products.
- Medication use interacting with salicylates such as anti-platelet drugs (e.g. cilostazol, clopidogrel), anticoagulants (e.g. enoxaparin, heparin, warfarin), corticosteroids (e.g., prednisone), certain diabetes drugs (e.g. sulfonylureas such as glyburide), certain anti-seizure drugs (e.g. phenytoin, valproic acid), cidofovir, cyclosporine, drugs for gout (e.g. probenecid, sulfinpyrazone), anti-hypertensives (e.g. ACE inhibitors such as captopril, angiotensin II receptor antagonists such as losartan, and beta blockers such as metoprolol), drugs that affect the acidity of urine (e.g. ammonium chloride, acetazolamide), lithium, methotrexate, oral bisphosphonates (e.g. alendronate), pemetrexed, SSRI antidepressants (e.g. fluoxetine, sertraline), tenofovir, and diuretics (furosemide, hydrochlorothiazide, spironolactone).

4.3 Excluded or Vulnerable Populations

- Although not the primary source of recruitment, eligible UIC students and employees are welcomed to participate with every effort taken to avoid any semblance of coercion (see section 5.0).
- Eligible volunteers from all racial or ethnic backgrounds are also welcomed to participate.
- Minors and pregnant women will be excluded by virtue of the scientific design. Moreover, the study question is not directed towards minors or pregnant women.

5.0 Subject Enrollment

Volunteers with PCOS will primarily be recruited from the principal investigator's (PI) Reproductive Endocrine referral practice located in the University of Illinois Hospital and Health System (UI Health) Outpatient Care Center (OCC), Suite 4A. The prevalence of PCOS in the general female population is ~15% but is as high as 80% in the referral based patient population cared for by the PI. Volunteers with PCOS will also be recruited through physician referral and from advertisements on UIC bulletin boards, electronic bulletin boards, websites such as Google.com, local newspapers and on social media. The advertisements will include a phone number and e-mail address to contact the PI or the Ob/Gyn Research Nurse Study Coordinator who will explain the study and administer a screening questionnaire using an approved eligibility checklist administered either over the phone, or via email correspondence using a secure UIC e-mail server containing a link to a confidential web-based screening questionnaire (Screening Questionnaire – Treating Inflammation in Polycystic Ovary Syndrome to Ameliorate Ovarian Dysfunction) through UIC's Qualitrics survey tool (http://accc.uic.edu/service/surveys). Advertisements, that correspond with IRB approved flyers or images, may be used on Facebook, Twitter, Instagram or the study website (http://www.doyouhavepcos.org). Prospective volunteers who view the images will be directed to contact the study phone or email address for more information. No direct contact with prospective volunteers will be made on these platforms. No data will be collected from these platforms. In addition, other care providers who become aware of the study may refer potential subjects for participation.

HealthMatch https://healthmatch.io/ and CenterWatch https://www.centerwatch.com/ are two online platforms that will also be used to assist with study recruitment. Prospective volunteers access these platforms to learn about clinical trials with open enrollment from publicly available information gathered by these platforms (e.g. study protocols descriptions listed on ClinicalTrials.gov). If a prospective volunteer expresses interest in study participation, the platform sends the investigator an email notification, but only provides the prospective volunteer's contact information at the investigator's request. The study team can subsequently use an appropriate IRB-approved recruitment tool to confirm interest in study participation and study eligibility such as email correspondence containing a link to our confidential web-based screening questionnaire as described above. No direct contact with prospective volunteers will be made on these platforms. No data will be collected from these platforms.

A targeted recruitment campaign will also be launched with the assistance of PCOS Challenge, the largest nonprofit support organization globally for women with PCOS serving over 45,000 members (2,542 in Illinois). PCOS Challenge membership grows at over 20% per year, and 92% of PCOS Challenge members have been diagnosed with PCOS. This will be a statewide recruitment campaign that includes: 1) One month of bi-weekly emails to members in Illinois sent by the PCOS Challenge Marketing and Public Relations Department; thus, the research team will not have direct access to any email addresses; 2) A study promotional page on the PCOS Challenge website (http://www.pcoschallenge.org/) with additional information about the study (i.e. title, description, goals, eligibility criteria, exclusions, etc.); 3) A 120mm x 240mm banner ad promoting the study on every page of PCOSChallenge.com (>40,000 pages). The email message to members, the promotional page PowerPoint presentation and the 120mm x 240mm banner ad are included for review.

The CCTS will further assist in identifying potential study participants using the CRDW–UIC CIRCLE as the data source. The data extracted through REDCAP will include age, height, weight, laboratory test results, diagnoses associated with PCOS, and contact information (see Data Request Authorization Form). The extracted data will subsequently be transferred to an Excel spreadsheet that will be stored by the PI in his password protected office computer under lock and key. A letter of introduction to the study will be sent to potentially eligible reproductive-age women whose data meet the profile for having PCOS, but only after obtaining permission from the UIC physicians who care for these women. The letter will indicate that the communication was sent with permission from their physician, and will contain study contact information to inquire about study participation or to request no further contact. The data from a given individual will initially be identified by medical record number (MRN), and then coded upon study enrollment when these individuals are assigned a study number. The link between an individual and the coded data will only be known to the PI. The coded data set without direct identifiers will be stored by the PI in his password protected office computer under lock and key. Only the PI and Co-investigators will have access to the coded data set.

Each volunteer will meet the PI (or his designee) in the UIC Center for Clinical and Translational Science's (CCTS) Clinical Research Center (CRC) to explain the scientific rationale, procedures and potential risks of the study, and the rights of the study participant. Informed consent will be obtained from each volunteer prior to participation in the study using a consent form approved by the UIC IRB. An electronic note will be written in each participant's medical record regarding the consent, and a copy of the consent form will be given to the participant. The original consent form will be kept with the investigator's study records.

Eligible volunteers will be informed that participation in the study is entirely voluntary, and that they may withdraw their consent at any time. They will also be assured that withdrawal from

the study will not affect their on-going care or their relationship with their physician in any way. Volunteers who fail the screening process will be promptly notified by telephone that they are not eligible to participate in the study. Data from screen failures will not be included in the study.

An honest and open recruitment process is employed to protect against the possibility of coercion or undue influence. Volunteers are encouraged at all stages to discuss their potential participation with trusted advisors including friends, family members, or medical personnel not connected with the study. The aim is to remain objective in the recruitment process to the point that volunteers have been excluded in the past on the basis of a perceived failure to reach a fully informed state regarding the study. There will not be any threat of harm or adverse consequences if the subject does not agree to participate in the study. For students or employees in particular, they will be reassured that their decision to participate or not to participate will not affect their grades (students) or standing in the University or Hospital. Furthermore, the information provided during the consent process will be presented in a balanced way with equal emphasis on all elements of consent (e.g. there will not be over-emphasis of benefits and under-emphasis of risks).

6.0 Study Design and Procedures

This research will involve the prospective study of 90 reproductive age women (18-40 years) with PCOS using a randomized double-blind placebo-controlled study design. The randomization schedule will be stratified by body mass index (BMI) (lean: BMI ≥18 but ≤25 kg/m2; obese: BMI ≥30 but ≤40 kg/m2) and IR status study groups to ensure a balance of patients in the two study arms. IR will be defined by the insulin area under the curve from 0-120 min (insulin $AUC_{0\text{-}120}$) of an oral glucose tolerance test (OGTT) >7,000 which is highly predictive and thus, recommended for population assessment of IR in PCOS.[35] Forty five (45) of the women with PCOS will receive salsalate of which 30 will be lean (15 without IR; 15 with IR) and 15 will be obese. The other 45 women with PCOS with a similar frequency distribution of BMI and IR status will receive placebo. Thus, the study will be comprised of the following 6 groups:

1. Salsalate-treated lean PCOS without IR (n=15)
   – BMI ≤25 kg/m^2 –and– 2-hour oral glucose tolerance test (OGTT) insulin area under the curve (AUC) ≤7,000
2. Placebo-treated lean PCOS without IR (n=15)
   – BMI ≤25 kg/m^2 – and– 2-hour OGTT insulin AUC ≤7,000
3. Salsalate-treated lean PCOS with IR (n=15)
   – BMI ≤25 kg/m^2 –and– 2-hour OGTT insulin AUC >7,000
4. Placebo-treated lean PCOS with IR (n=15)
   – BMI ≤25 kg/m^2 –and– 2-hour OGTT insulin AUC >7,000
5. Salsalate-treated Obese PCOS – BMI≥30 kg/m^2–≤40 kg/m^2 (n=15)
6. Placebo-treated Obese PCOS – BMI≥30 kg/m^2–≤40 kg/m^2 (n=15)

After the initial pre-screening, volunteers will be asked to come to the CCTS CRC at 912 S. Wood Street for a screening visit at ~ 8:00 AM. The screening visit will occur within 1 to 4 weeks of beginning the study protocol and will last ~4 hours. At the start of the screening visit, volunteers will undergo informed consent followed by a complete history and physical, vaginal sonography, and blood draws for a hormonal evaluation if not performed previously, a complete blood count (CBC), a comprehensive metabolic panel (CMP) and an OGTT. Based on the screening results, volunteers deemed eligible for study participation will subsequently enter the study protocol beginning between days 3 and 13 following the onset of either a spontaneous or progestin-induced withdrawal bleed, or without regard to the onset of menses if vaginal

sonography within 21 days of study start reveals no evidence of follicular development in the face of a negative pregnancy test.

Visits 1 through 5 will occur during the first week of the study protocol. During Visits 1 through 4, subjects will undergo an HCG stimulation test (HCG-ST) in the CRC consisting of a single IM HCG injection during Visit 1 after documenting a negative urine pregnancy test, along with a single blood draw during all 4 visits at 0, 24, 48 and 96 hours after HCG administration to measure serum androgens and 17-hydroxyprogesterone. Visits 1 through 3 will be short lasting ~30 minutes. After one of these short visits, subjects will also undergo a body composition assessment consisting of a whole-body dual-emission X-ray absorptiometry (DEXA) scan in the Integrative Physiology Laboratory (IPL) at 1640 W. Roosevelt Road, Suite 187, and a magnetic resonance image of the abdomen in the Center for Magnetic Resonance Research at 2242 W. Harrison Street, Suite 103. Visit 4 will last ~8 hours during which a cream challenge test (CCT) will also be performed in the CRC consisting of dairy cream ingestion and serial blood draws at 0, 2, 3 and 5 hours after cream ingestion to measure inflammation markers. Visit 5 will last ~7 hours and occur 2 days after the CCT during which subjects will undergo a FS-IVGTT in the CRC to assess insulin sensitivity and glucose-stimulated inflammation.

Table 1. PRE-STUDY SCREENING

Entry H&P & vaginal sonography for ovarian morphology; Prolactin, TSH, LH, FSH, total / free T, DHEA-S & 17OHP (only if not obtained in previous 6 months); CBC, CMP & OGTT.

STUDY PROTOCOL WEEKS →	**0**	**4**	**8**	**12**
Salicylate Level	X	X	X	X
Sex Hormone Binding Globulin	X			X
Body Composition: MRI & DEXA	X			
HCG Stimulation Test: T, A & 17OHP @ 0, 24, 48 & 96 hours.	X			X
Cream Challenge Test: MNC-derived ROS, NFκB, RNA & protein inflammat'n markers @ 0, 2, 3 & 5 hours.	X			X
Frequently Sampled Intravenous Glucose Tolerance Test: glucose, insulin, C-peptide & FFA q 1-30 minutes x 34 samples from -20-240 minutes.	X			X
Post-Study Safety Tests: CBC & CMP.				X

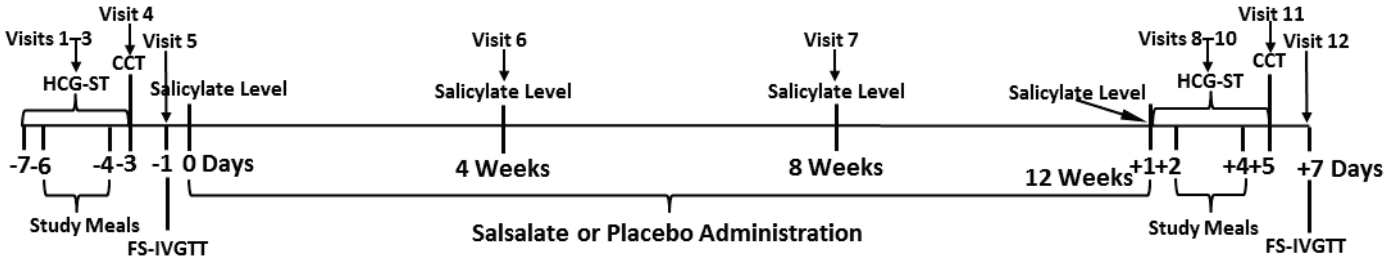
Ovulation Monitoring: BBT from 0-12 weeks; P on 7th day of BBT rise; If P >2 ng/dl documented, vaginal sonography on day of & day after LH surge.

A predefined computer-generated randomization schedule set up by a statistician will have centralized allocation and codes secured by a research pharmacist. This schedule will be used to assign recruited subjects to receive salsalate (Amneal Pharmaceuticals, Bridgewater, NJ) 3 grams/day (lean subjects) or 4 grams/day (obese subjects) in 2 divided doses or identical placebo for ~12 weeks. Study medication will actually be administered over a range of 11 to 13 weeks to accommodate scheduling glitches without impacting any anticipated treatment effects. Subjects and study personnel will be blinded to the treatment assignment. Serum salicylate levels will be measured to assess compliance from a fasting blood sample drawn at baseline (Visit 1) and after 4 weeks (Visit 6), 8 weeks (Visit 7) and 12 weeks (Visit 8) of treatment. Compliance will be defined as salicylate levels being in the therapeutic range for rheumatology practice (10-30 mg/dl) during treatment. A Data and Safety Monitoring Board (DSMB) will keep track of salicylate levels to maintain blinding (see Section 6.0). A fasting lipid profile will also be drawn at baseline (Visit 1) and after 12 weeks (Visit 8) of treatment.

During treatment, all subjects will undergo ovulation monitoring consisting of serum progesterone measurements timed by basal body temperatures along with serial follicular ultrasonography as indicated. After the ~12 weeks of treatment, subjects will undergo repeat testing consisting of an HCG-ST during Visit 8 through 11, a CCT during Visit 11 and a FS-

IVGTT during Visit 12. Visits 8 through 12 will occur during the last week of the study protocol. See Table 1 for an overview of the study protocol and Fig. 2 for the study timeline.

Figure 2



Although it is anticipated that the majority of subjects will undergo the three main test procedures (i.e. HCG-ST, CCT and FS-IVGTT) in the order described, the order in which they will be scheduled may need to be changed to accommodate scheduling glitches, since they will be performed as outpatient procedures in accordance with the CCTS CRC operating hours. The change in order will not impact the integrity of the data.

6.1 Subject-Related Procedures

- *Screening* of volunteers to confirm the diagnosis of PCOS will typically be based on previously measured hormone levels. It is anticipated that most subjects with PCOS will be diagnosed by the PI just prior to study entry, and will likely involve performance of a hormonal evaluation within 6 months of beginning the study. However, the timing of previous hormone measurements used to diagnose PCOS will be left to the discretion of the PI. If the relevant tests for this purpose have not been performed previously, the necessary hormone levels will be obtained from a screening blood draw which will also be used to measure a CBC and CMP (maximum 22 ml). Assays to measure prolactin, TSH, LH, FSH, total testosterone, free testosterone, androstenedione, DHEA-S and 17-hydroxyprogesterone (17OHP) along with the CBC and CMP will be performed by the UI Health Clinical Pathology Laboratory. Pregnancy will be ruled out in all subjects immediately prior to study testing before and after treatment with salsalate or placebo using a urine test sensitive enough to detect >50 mIU/L of HCG.

- *Screening* of volunteers with an OGTT to rule out diabetes and to determine IR status will be performed in the CCTS CRC prior to study entry after an overnight fast of ~12 hours. A polyethylene intravenous catheter will be inserted in an arm vein for blood sampling at ~9:00 AM. The size of the catheter (i.e. 18-23-guage) will be of suitable caliber for the size of the vein used to gain access. A baseline blood sample (9 ml) will be drawn in the fasting state. A 75 gram glucose beverage will subsequently be ingested over 10 minutes. Blood samples (9 ml each) will again be drawn at 30, 60, 90, 120 and 180 minutes following glucose ingestion. Every attempt will be made to insert the intravenous catheter and obtain blood samples on time. However, a sample may be obtained within a fifteen minute window after the scheduled time due to any unforeseen technical difficulty. Upon completion of the test, the indwelling catheter will be removed and subjects will be fed a high carbohydrate snack. Blood glucose and plasma insulin levels will be measured by the UI Health Clinical Pathology Laboratory.

- A complete medical *History and Physical Examination* will be performed in the in the CCTS CRC. A Pap smear will be performed at the same time only if a volunteer is due for one in accordance with ACOG guidelines. Otherwise, a copy of the most recent Pap smear will be requested after obtaining a signed release of medical information from the volunteer.

- *Vaginal Sonography* to assess ovarian morphology will be performed during the initial screening in the CCTS CRC on the day of the entry history and physical examination. Polycystic ovaries will be defined by published criteria established by Dewailly et al;[36] namely, the presence of 25 or more subcapsular follicles measuring 2-9 mm in diameter or increased ovarian volume (>10 cm^3).

- *Body Composition* will be assessed at the beginning of the study using 2 modalities:

    - *Dual Energy X-ray Absorptiometry* (DEXA, Model LUNAR iDXA, General Electric Healthcare, Wood Dale, IL) will be performed in the IPL at which time anthropometric measures of height and weight will be taken using standard techniques. These measures are required for the DEXA scan. All female participants will perform a spot pregnancy test prior to DEXA scan. If pregnancy test is positive the participant will be excluded. The instrument emits photons at 2 different energy levels as a detection scanner traverses the body. The detected energy passes through the body with differential attenuation that is computer analyzed to calculate body fat. The total fat quantity, and truncal fat quantity will be measured, the latter of which is defined as the area between the dome of the diaphragm (cephalad limit) and the top of the greater trochanter (caudal limit).[37] Fat quantity will also be measured in the central abdominal area (R1 area) which is an area of 50 cm^2 around the central point of the midline between the lateral iliac crests and the lowest rib margins at the end of normal expiration (the same midline used for waist circumference).[38] Study subjects will wear light clothing and lie on their backs during the procedure which takes 10-15 minutes to complete. Subjects will be exposed to a radiation dose of ≤0.3 mrem based upon manufacturer's specifications and calculations from Standford Dosimetry, LLC RADAR Medical Procedure Radiation Dose Calculator. A Radiation Safety Committee review <u>is not required</u> for this amount of research protocol radiation exposure given the estimated effective equivalent dose is below 100 mrem (1 mSv) per year, a limit set by the Nuclear Regulatory Commission for "general public" exposure.

    - *Magnetic Resonance Imaging* (MRI, Siemens MAGNETOM Trio 3T, Malvern, PA) will be performed at the Center for Magnetic Resonance Research, 2242 W. Harrison Street, Suite 103. The MRI scanner performs an axial scan in the supine position to measure abdominal adiposity as previously described.[39] A gradient echo pulse sequence with water suppression to obtain a fat-only image is followed by lipid suppression to obtain a water-only image and no suppression to serve as an anatomic reference. Five 4 mm slices centered at L3 are obtained and the fat signal intensity ratio [If/(If+Iw)] is calculated from the fat-only (If) and water-only (Iw) images on a pixel-by-pixel basis to produce a map. The calibration curve obtained from a previous phantom study is used to convert this ratio to a fat percentage value that is used to determine visceral and subcutaneous fat volumes with AnalyzeDirect, Inc. software (Stilwell, KS).[39]

- *HCG Stimulation Test (HCG-ST):* The test will be performed in the CRC as described by Piltonen et al.[40] over three (3) short CRC visits. A baseline blood sample (16 ml) will be

drawn after an overnight fast of ~12 hours followed by administration of a single intramuscular injection of 5,000 IU HCG (Pregnyl; Schering-Plough, Kenilworth, NJ). Fasting blood samples (16 ml each) will again be drawn at 24, 48, and 96 hours after HCG administration (*please refer to the Risks - Venipuncture / Venous Catheter Blood Sampling subsection of Section 7.0 for the approach and alternatives to venipuncture*). Every attempt will be made to obtain blood samples on time. However, the baseline sample may be obtained within a fifteen minute window and the post-HCG samples may be obtained within a sixty minute window after the scheduled time due to any unforeseen technical difficulty. Gathering of vital signs during the single blood draw visits will be considered if there is any clinical evidence subject instability. Plasma will be isolated from the blood samples collected and stored at –80^{o} C until assayed for testosterone, androstenedione and 17OHP.

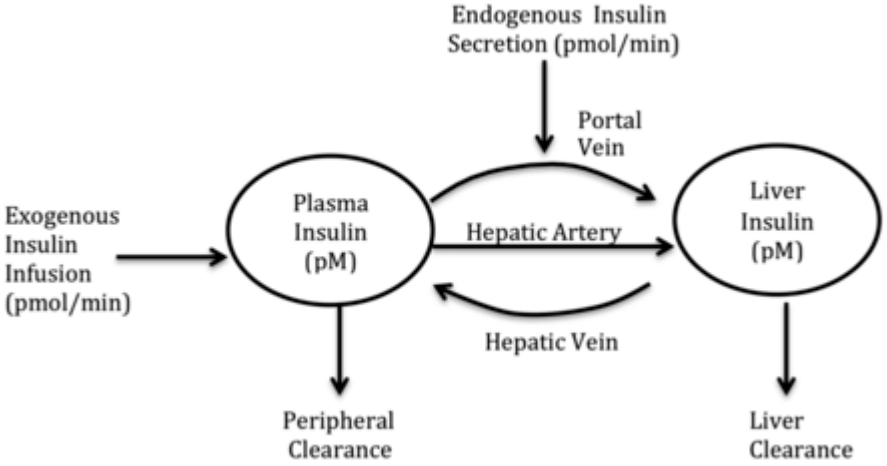
- *Cream Challenge Test (CCT):* All subjects will consume a standard weight maintenance diet consisting of 50% carbohydrate, 35% fat and 15% protein for 3 consecutive days (*days 1 – 3*) before the test through meals prepared by the CRC dietician. The test will be performed in the CRC on the day of the 96 hour HCG-ST blood draw after an overnight fast of ~12 hours as described by Deopurkar et al.[41] A polyethylene intravenous catheter will be inserted in an arm vein for blood sampling at ~8:30 AM. The size of the catheter (i.e. 18-23-guage) will be of suitable caliber for the size of the vein used to gain access (*please refer to the Risks - Venipuncture / Venous Catheter Blood Sampling subsection of Section 7.0 for the approach and alternatives to intravenous catheter placement*). A baseline blood sample (90 ml each) will be drawn in the fasting state at ~9:00 AM. One hundred (100) ml of dairy cream (gourmet heavy whipping cream; Land O Lakes Inc, Arden Hills, MN) will then be ingested over 10 minutes. This dairy cream preparation has 33 gm of fat and 1257 kJ (300 kcal)/100 ml cream. The saturated fat content of the cream preparation is 70% (28% unsaturated fat), the protein content is <2%, and the glucose content is 0%. Blood samples (80 ml each) will again be drawn at 120, 180 and 300 minutes after cream ingestion. Every attempt will be made to insert the intravenous catheter and obtain blood samples on time. However, a sample may be obtained within a fifteen minute window after the scheduled time due to any unforeseen technical difficulty. Upon completion of the test, the catheter will be removed, and subjects will be fed a study meal for lunch as described above. Subjects will also be discharged with weight maintenance study meals to consume that evening and up until they begin the FS-IVGTT. MNC will be isolated from the blood samples collected and used to measure ROS, or placed in culture, or processed for storage at –80^{o}C until used for RT-PCR, Western blotting and electrophoretic mobility shift assay (EMSA). Plasma will also be isolated from the same blood samples and stored at –80^{o}C until assayed for cytokine levels.

- *Diet Instruction and Monitoring:* All subjects will receive detailed diet instructions from a UIC Department of Medicine registered dietician on the day of the pre-treatment CCT aimed at maintaining a similar weight throughout the study. The instructions will focus on having subjects consume their habitual pre-study food intake throughout the ~12 weeks of treatment. Subjects will complete a 24 hour food record just before entering the study to serve as a guide for the diet instructions. Subjects will subsequently weigh themselves at home on a weekly basis using the same scale to alert the study team if they have lost or gained more than 4 pounds compared with their weight at study entry. The registered dietician will provide additional counseling to these subjects as needed to determine if any diet adjustments are required for weight maintenance. Subjects will also keep a 24 hour food record on a monthly basis that will be reviewed by trained staff from the Nutrition

service to assess compliance. Subjects will return the 24 hour food records to the CRC when they report for each monthly salicylate level blood draw.

- *Insulin-Modified FS-IVGTT:* The test will be performed in the CRC two (2) days after the CCT after an overnight fast of ~12 hours as described by Polidori et al.[32] An intravenous polyethylene catheter will be inserted in an forearm vein at ~7:10 AM (-50 minutes) for infusing glucose and insulin. Another intravenous catheter will be inserted in a contralateral forearm vein for blood sampling. The size of the catheter (i.e. 18-23-guage) will be of suitable caliber for the size of the vein used to gain access (*please refer to the Risks - Venipuncture / Venous Catheter Blood Sampling subsection of Section 7.0 for the approach and alternatives to intravenous catheter placement*). Baseline blood samples will be obtained at ~7:40 AM (-20 minutes), ~7:50 AM (−10 minutes), ~7:59 AM (−1 minute) and ~8 AM (0 minutes). The average of the four samples is considered the basal level. A glucose solution (0.3 gm/kg) will be administered intravenously beginning immediately after the 0 minute blood draw. Insulin (20 mU/kg Humulin; Eli Lilly, Indianapolis, IN) delivered in 1 unit/ml infused over 60 seconds starting at ~8:20 AM (20 minutes). Blood samples will be obtained at -20, −10, −1, 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 40, 50, 60, 70, 80, 90, 100, 120, 150, 180 and 240 minutes. Every attempt will be made to insert the intravenous catheters and obtain blood samples on time. However, all samples with the exception of those used to measure glucose may be obtained within a fifteen minute window after the scheduled time due to any unforeseen technical difficulty. A total of 272 ml of blood (8 ml/sample) will be collected during the entire procedure. All infusions will be stopped at 12 PM. Upon completion of the test, both catheters will be removed, and subjects will be fed a standard lunch. Plasma will be isolated from the blood samples collected and stored at –80° C until assayed for glucose, insulin, C-peptide and FFA. Preparation of glucose and insulin for infusion during the FS-IVGTT procedure will be performed by the UI Hospital Pharmacy Investigational Drug Services.

- *Mathematical Model to Assess Hepatic and Extrahepatic Insulin Clearance.* Hepatic and peripheral insulin clearances were estimated using a model for insulin kinetics during the FS-IVGTT as described in Fig. 3.



The model is based on the following main assumptions:

1. Endogenously secreted insulin enters the portal circulation where it travels to the liver before reaching the systemic circulation. The insulin secretion rate (ISR) will be calculated by deconvolution using C-peptide kinetic parameters.[42] The calculated ISR and the known insulin infusion rate will be used as inputs to the model.

2. The rate of delivery of insulin from the systemic circulation to the liver reported in pmoles/minute is equal to the plasma insulin concentration reported in pmoles/liter times the assumed hepatic plasma flow (HPF) rate (0.576 L/min/m^2).[43]
3. Insulin clearance occurs in both the liver and in extrahepatic (peripheral) tissues, which includes kidney, muscle, and adipose tissue. Extrahepatic clearance is assumed to be proportional to the plasma concentration.
4. Hepatic clearance will be modeled using either a linear or a saturable function. Both functions will be tested in each subject, and the one providing the best fit will be retained. Both functions will be considered because some previous studies have suggested linear clearance, whereas others have suggested nonlinear and/or time-dependent clearance.[30,43-45] No time-dependent changes in parameter values will be modeled.

Given these assumptions, the resulting model requires estimating either three (linear model) or four (saturable) parameters from the measured plasma insulin profiles and calculated ISR.

The following equations and parameters correspond with these main assumptions. The HPF rate used in the equations is the combined plasma flow to the liver from the portal vein and the hepatic artery.

$$\text{Insulin delivery to liver (pmol/min)}$$
$$\text{Delivery} = ISR + HPF \cdot P$$
$$\text{Hepatic insulin degradation (pmol/min)}$$
$$\text{Linear model} = FE_L \cdot \text{Delivery}$$
$$\text{Saturable model} = \frac{V_{max} \cdot \text{Delivery}}{K_m + \text{Delivery}}$$
$$\text{Extrahepatic insulin degradation (pmol/min)} = CL_P \cdot P$$

P is plasma insulin reported in pmoles/liter, ISR is reported in pmoles/minute, HPF and CL_P are reported in liters/minute, FE_L is hepatic fractional extraction (dimensionless), V_{max} is the maximal hepatic degradation rate reported in pmoles/minute, and K_m is the hepatic insulin delivery rate at which 50% of maximal degradation occurs reported in pmoles/minute.

The differential equations for the linear (Equation 1) and saturable (Equation 2) assumptions are as follows:

$$V_P \frac{dP}{dt} = \text{Infusion Rate} + (1 - FE_L) \cdot ISR - (HPF \cdot FE_L + CL_P) \cdot P$$

$$V_P \frac{dP}{dt} = \text{Infusion Rate} + ISR - CL_P \cdot P - \frac{V_{max} \cdot \text{Delivery}}{K_m + \text{Delivery}}$$

V_P is the extrahepatic distribution volume for insulin reported in liters. Model-identified parameters will be normalized by body weight (BW) for comparison across subjects. For the saturable clearance model, fractional hepatic extraction varies with changes in ISR and P. FE_L values will be calculated in the basal state when compiling parameter summaries.

To provide measures of clearance that are related to analogous measures obtained by other experimental methods, Equations 1 and 2 will be used to calculate measures that would be

estimated from steady-state insulin infusions administered either intravenously as in hyperinsulinemic-euglycemic clamps or in the portal vein as in endogenous secretion. The corresponding clearance values from the model for an intravenous infusion (CL_{IV}) or for a portal infusion (CL_{portal}) will be calculated by dividing the insulin infusion rate by the steady-state plasma insulin concentration. These linear model calculations are summarized as follows:

$$CL_{IV} = CL_P + HPF \cdot FE_L$$

$$CL_{portal} = \frac{CL_{IV}}{1 - FE_L}$$

For the saturable clearance model, the effective clearance parameters vary with the insulin infusion rate, and values will be calculated assuming an infusion rate of 240 pmol/min/m^2 as commonly used in hyperinsulinemic-euglycemic clamps. The proportion of total insulin degradation occurring in hepatic versus extrahepatic tissues will be calculated by integrating the degradation rates over the 240 minute interval. The insulin sensitivity index (S_I) will be determined from the FS-IVGTT using MINMOD Millenium version 6.02.

- *Ovulation Monitoring:* All subjects will maintain a basal body temperature (BBT) chart throughout the ~12 weeks of treatment after appropriate instructions. A blood sample (5 ml) will be drawn to measure serum progesterone to detect ovulation on the 7th day of temperature elevation of at least ¾^{o}F from baseline indicative of a biphasic BBT response. Subjects will be instructed to alert the team regarding the potential need to schedule a progesterone measurement when 3 consecutive days of temperature elevation is evident. The length of the cycle in which a luteal range progesterone (>2 ng/ml) is documented will be used to time initiation of midcycle urine luteinizing hormone (LH) monitoring with a commercially available kit. Vaginal sonography will be performed in the CRC on the day of the color change signifying the LH surge and the following day to document follicular development and collapse, respectively, as the definitive evidence of ovulation.

6.2 Specimen Collection and Storage

- *Blood specimens* will be collected by venipuncture or from a venous catheter placed by a trained medical person who will remain at the bedside to monitor the subject continuously.

- For screening, a maximum of 22 mL (4½ tsp) will be drawn to measure hormones if not performed previously, a complete blood count and a chemistry panel, and 24 mL (5 tsp) will be drawn during the screening OGTT. During the study, we will draw 64 mL (12¾ tsp) during each HCG-ST (Visits 1-4 and 8-11), 330 mL (66 tsp) during each CCT (Visits 4 and 11), and 272 mL (54 tsp) during each FS-IVGTT (Visits 5 and 12). We will also draw an additional 20 mL (4 tsp) to measure salicylate levels (Visits 1, 6, 7 and 8) and fasting lipid profiles, and an additional 5 mL (1 tsp) to measure progesterone for ovulation detection as needed only if warranted by the BBT response.

- *MNC Isolation and Processing:* Blood samples are collected in tubes containing Na-EDTA as an anticoagulant. A 12 ml sample will be layered over 12 ml of polymorphonuclear cell isolation medium (Polymorphprep™, Accurate Chemical and Scientific Corporation, Westbury, NY). Samples will be centrifuged at 450 x g for 30 minutes at 18^{o}C resulting in separation of two bands above the red blood cell pellet. MNC present in the top band will be harvested with a Pasteur pipette and washed repeatedly with Hank's buffered saline solution (HBSS) yielding greater than 95% pure MNC.[46] A fresh portion of MNC will be reconstituted

in HBSS for ROS generation or suspended in RPMI 1640 medium for culture. A second MNC portion will be suspended in RNAlater reagent (Quiagen, Germantown, MD) for 24 hours at 4°C to stabilize RNA, centrifuged at 6,000 x g for 5 minutes at 4°C to yield a pellet that will be stored at –80°C until processing for real time-PCR (RT-PCR). A third MNC portion will be suspended in phosphate buffered saline (PBS – pH 7.4), centrifuged at 13,000 x g for 2 minutes at 18°C to yield a pellet that will be stored at –80°C until processing for Western blotting. A fourth portion of MNC will be used to prepare nuclear extracts of DNA-binding protein with a method described by Andrews et al., and will subsequently be stored at –80°C until processing for electrophoretic mobility shift assays.[47]

- Plasma and/or serum will be separated from whole blood through centrifugation and aliquoted into 1.5 ml polyproplyene microtubes for storage.

- Individual aliquoted MNC, plasma or serum samples will be placed in a box labeled with the study ID and stored in a -80°C freezer.

- Samples will be labeled with a numbering system for identification of the test result. The numbering system will give each subject a unique identifier, and indicate which study visit each sample is from designated as V1, V2, V3, etc. immediately after the subject number.
- Remaining samples will be stored indefinitely future study as needed.

6.3 Study Medication Preparation and Dispensing

- A research pharmacist from the Investigational Drug Service (IDS) will prepare the study medication consisting of 500 mg capsules of salsalate along with an identical placebo. The PI currently holds an IND for salsalate administration (IND #113845).

- A research pharmacist will also prepare all of the clamp infusates.

- The study medication, HCG and clamp infusates will all be stored in the IDS and dispensed by an IDS research pharmacist when needed for use in the study.

6.4 Protected Health Information (PHI) and Other Data Collected

- PHI data will be collected at the time of study enrollment

- Subject demographics will be used for descriptive purposes.

- Anthropometric data (height and weight) and body composition data from the DEXA scan and abdominal MRI will also be used for descriptive purposes.

- Diet logs will be collected.

6.5 Data Storage and Management

- All research data will be coded using unique identifiers. Once the subject has been assigned a unique identifier, all data will carry this label. Only the PI will have the original identifier information and this information will be kept in a password-protected spreadsheet on his encrypted and password protected personal computer or secure departmental server in his locked office at UIC located in CMRB 1111.

- All subject visit data will be transcribed from a written data sheet to password protected files on an encrypted university computer and the original data sheet will be kept in a subject binder. Subject binders will be kept in a locked cabinet in the PIs locked office.

- Study personnel and the PI will have access to the study information.

- Information will be kept indefinitely.

6.6 Specimen Analyses – Laboratory Assays

- *ROS Generation Assay:* Respiratory burst activity of MNC (4 x 10^5 cells/ml) will be measured by detection of superoxide radical via chemiluminescence. A 500µl aliquot of MNC (400 cells/µl) is added to duplicate cuvettes containing a spin bar. The cuvettes are placed into a two-channel lumi-aggregometer (Chronolog Corporation, Haverton, PA). 15µl of 10mM luminol followed by 1µl of 10mM formylmethionyl leucinyl phenylalenine are added to each cuvette. Chemilumunescence is recorded in mV by computer software (Chronolog Aggrolink). The gain is adjusted to visualize the curves within range of the displayed graph. The peak responses of the duplicate samples recorded to calculate the mean peak. This method was developed by Thusu and Dandona,[48] and is similar to that published by Tosi and Hamedani.[49] In this assay system, superoxide radical release measured by chemiluminescence correlates linearly with that measured by the ferricytochrome C method. There is also a dose-dependent chemiluminescence inhibition by superoxide dismutase and catalase, and diphenylene, a specific inhibitor of NADPH oxidase, the superoxide producing enzyme. The specific inhibitory effect of diphenylene iodonium on NADPH oxidase has been established by Hancock and Jones.[50] As validated in our laboratory, the variation of ROS generation by MNC in humans using this method varies by less than 8% over a 2 week period.[12,51]

- *NADPH Oxidase p47^{phox} Subunit, p65 NFκB Subunit, IκB, TNFα, IL-6 and IL-1β Real-Time PCR (RT-PCR):* MNC are suspended in RNAlater reagent (Quiagen, Germantown, MD) for 24 hours at 4°C to stabilize RNA, centrifuged at 6,000 x g for 5 minutes at 4°C to yield a pellet that is stored at –80° C until processing for RT-PCR. Total RNA is isolated from MNC (~20 mg) using an RNAeasy kit (Quiagen, Germantown, MD). The isolated total RNA (1 µg) is treated with DNase (Life Technologies, Gaithersburg, MD), and reverse-transcribed using TaqMan reverse transcription reagents (PE Biosystems, Foster City, CA) and oligo(dT) primers according to the manufacturer's instruction. Primers and probes for the individual inflammation marker genes are selected using PRIMER EXPRESS software (PE Biosystems, Foster City, CA). The internal probes and a 28S rRNA probe are constructed as previously described.[52] Internal probes are labeled at the 5'-ends with a reporter dye (6'-carboxyfluorescein), and at the 3'-end with a non-fluorescent quencher; and subsequently phosphate-blocked at the 3'-end to prevent extension. The 5'-end of the 28S rRNA probe is labeled with VIC, a proprietary fluorescent dye (PE Biosystems, Foster City, CA). Each set of target primers and probe are co-amplified with 28S rRNA primers and probe. Fluorescence is measured at each amplification cycle in an ABI Prism 7700 Sequence Detection System (Perkin Elmer/Applied Biosystems, Foster City, CA). The signal for the 28S rRNA is used to normalize against differences in RNA isolation and RNA degradation, and in the efficiencies of the reverse transcription and PCRs. All samples are run in triplicate and quantified by normalizing the inflammation marker mRNA signal with the 28S signal. The final quantification is achieved with a relative standard curve.[52]

- *NADPH Oxidase p47^{phox} Subunit, p65 NFκB Subunit and IκB Western Blotting:* As previously described,[53] MNC cell lysates are prepared by adding 1 ml of boiling lysis buffer (1% SDS), 1mM sodium ortho-vanadate, and 10 mM Tris (pH 7.4) to MNC pellets. Total protein concentrations are determined using BCA protein assay (Pierce Chemical Co., Rockville, IL). Total cell lysate (60 mcg) are electrophoresed on 10% gels for p47^{phox} subunit and IκB. The proteins are transferred to a polyvinylidine diflouride membrane and incubated with a monoclonal antibody against p47^{phox} subunit (Transduction Laboratories Inc., San Diego, CA) or a polyclonal antibody against IκB (Rockland, Gilbertsville, PA). The membrane is washed and developed using super signal chemiluminescence reagent (Pierce Chemical Co.). Densitometry is performed using Carestream Molecular Imaging software version 5.0.2.30 (Rochester, NY).

- *NFκB Electrophoretic Mobility Shift Assay (EMSA):* As previously described,[46,47] DNA-binding protein extracts are prepared from MNC pellets, and total protein concentrations are determined using BCA protein assay (Pierce Chemical Co., Rockville, IL). The NFκB gel retardation assay is performed using an NFκB-binding protein detection kit (Life Technologies, Inc. Long Island, NY). The double-stranded oligonucleotide containing a tandem repeat of the consensus sequence for the NFκB-binding site is radiolabeled with γ-P^{32} by T4 kinase. The nuclear extract (5 µg) is mixed with incubation buffer and preincubated at 4^{o}C for 15 minutes. Labeled oligonucleotide (60,000 cpm) is added, and the mixture is incubated at room temperature for 20 minutes. Samples are electrophoresed on a 6% nondenaturing polyacrylamide gel that is dried under vacuum and exposed to x-ray film. Densitometry is performed as described above.

- *MNC Culture:* A blood sample will be drawn at 0 and 120 minutes of the CCT for MNC isolation. Whole blood will be diluted 1:2 with PBS (pH 7.4) and underlayered with histopaque-1077 for density gradient centrifugation (400g for 45 minutes) as previously described.[54] The pellet is obtained from the last wash and resuspended in RPMI 1640 culture medium (2mM L-glutamine, 100 U/ml penicillin, 100 ug/ml streptomycin, 90 mg/dl glucose) at a final concentration of 5X10^{6} cells/ml. MNC isolated from the pre-CCT fasting blood sample will be cultured in medium supplemented with palmitate bound to fatty acid-free bovine albumin (4%) at varying concentrations spanning the physiological postabsorptive to pathophysiological range (0.4, 0.8, 1.2 and 2.0 mM), in the presence and absence of 1 ng/ml of lipopolysaccharide (LPS) endotoxin (Sigma, St Louis, MO). MNC isolated from the 120 minute CCT blood sample will be cultured without FFA supplementation in the presence and absence of 1 ng/ml of LPS. After a 24 hour incubation (5% CO_2 at 37^{o} C), MNC supernatants will be collected and stored at –80^{o} C until assayed.

- *Plasma Measurements:* All assays will be performed in the Reproductive Endocrine and Inflammation Laboratory except those for salicylate and progesterone which will be measured by the Clinical Chemistry Laboratory at IU Hospital. Glucose concentrations will be measured by the glucose oxidase method (YSI, Yellow Springs, OH) while plasma insulin concentrations will be measured by direct double antibody RIA (Linco Research, St. Charles, MO). C-peptide will be measured by a 2-site immunenzymatic sandwich assay (Roche Diagnostics, Indianapolis, IN). FFA will be measured by an enzymatic colorimetric assay (Wako Diagnostics, Mountain View, CA). TNFα, IL-6 and IL-1β levels in plasma and in MNC culture supernatants will be measured by ELISA (Quantikine, R&D Systems, Minneapolis, MN). CRP levels will be measured by a high sensitivity ELISA (Alpha Diagnostics International, San Antonio, TX). Levels of LH, androstenedione, testosterone, DHEA-S, 17OH-progesterone and progesterone will be measured in duplicate by RIA (MP

Biomedicals LLC, Solon, OH). Sex Hormone Binding Globulin will be measured by electrochemiluminescence (Mayo Clinic, Rochester, MN). To reduce interassay variability, all samples from an individual subject will be measured in the same assay at the end of the study.

7.0 Expected Risks/Benefits

Risks

- **Venipuncture / Venous Catheter Blood Sampling** – For screening, we will draw a maximum of 22 mL (4½ tsp) to measure hormones if not performed previously, a complete blood count and a chemistry panel, and 24 mL (5 tsp) during the screening OGTT. During the study, we will draw 64 mL (12¾ tsp) during each HCG-ST, 330 mL (66 tsp) during each CCT, and 272 mL (54 tsp) during each FS-IVGTT. We will also draw an additional 20 mL (4 tsp) to measure salicylate levels and fasting lipid profiles, and an additional 5 mL (1 tsp) to measure progesterone for ovulation detection only if warranted by the BBT response.

  The *Study Visit Blood Sampling Plan* included as an Appendix provides a detailed accounting of the volume of blood that will be drawn from each subject during the entire study. These flow sheets provide a detailed breakdown of the tubes that will be used, the volumes that will be drawn, and the types of measurements that will be performed. The *Laboratory Flow Charts* also included as an Appendix illustrates how the collected blood will be utilized for molecular work and cell culture in the PI's laboratory to effectively generate key data to address Aims of the study.

  The total amount of blood that will be drawn on each subject during screening and the HCG-ST, CCT and the FS-IVGTT is expected to be <1,500 ml consisting of no more than 750 ml obtained ~12 weeks apart which again is a longer interval than the typical clinical practice standard for blood donations. All subjects will be screened for blood count. The decision to proceed with study participation will be reviewed on a case-by-case basis after taking into consideration the health status of the individual if the pre-study screen reveals a hemoglobin <11.6 grams; or in the case of a subject who weighs <70.8 kg, if the pre-study hemoglobin is <12.6 grams. In the latter case, blood sampling will be limited to 10.5 ml/kg within an 8 week period. If the complete blood count (CBC) performed during initial screening reveals evidence that low hemoglobin is due to iron deficiency anemia in an otherwise eligible volunteer, iron supplementation (ferrous sulphate 325 mg twice daily) will be recommended and a CBC will be performed at appropriate intervals until higher acceptable hemoglobin meeting the above criteria is documented before the volunteer enters the protocol.

  The risk of developing anemia due to blood draws during the study is uncommon but still possible in subjects with a pre-study hemoglobin at the lower limit of study eligibility (<12.0 grams, or <13.0 grams for weight <70.8 kg). Subjects in this circumstance will be advised to take iron during the study (ferrous sulphate 325 mg twice daily) as a precaution Additional iron supplementation may be recommended as appropriate if a complete blood count (CBC) performed at the end of the study reveals a low hemoglobin.

  The potential risks to the subjects associated with venipuncture needle sticks or IV catheter insertions include discomfort, possible bleeding/hematoma (~1/1000) at the site, rarely an infection, and uncommonly feeling faint from the procedure. These risks will be minimized by allowing only experienced nurses or doctors to insert intravenous lines. From

time to time, difficulty may be encountered in performing venipuncture or inserting an intravenous catheter even after several attempts. In this instance, a subject may be escorted by a physician or a research nurse from the CRC to the Imaging and Diagnostics Services Short Stay Unit located in Suite 2600 on the 2nd floor of UI Heath Hospital at 1740 W. Taylor Street immediately across the skyway adjacent to the CRC, where a member of the IV-PICC Team will perform the venipuncture or insert the intravenous catheter using ultrasound guidance at no additional risk. The subject will subsequently be escorted back to the CRC to complete the particular study visit. Use of the IV-PICC Team may extend the CRC study visit by up to 2 hours.

The studies will be performed on the CCTS CRC, which provides staff that assists the P.I. Furthermore, the P.I. has staff trained and dedicated to performing these studies. All catheters will be inserted with meticulous use of sterile technique to avoid infection and great care will be taken to minimize any discomfort or complications associated with insertion. The catheters will be kept patent with heparinized saline to prevent clotting.

- **CCT, OGTT and HCG-ST** – The major risk associated with these tests is insertion of an intravenous catheter for blood drawing as discussed above. Glucose ingestion during the OGTT may cause abdominal discomfort, nausea, increased blood pressure, flushing or sweating. Dairy cream ingestion may cause urticaria, pruritus and in rare cases anaphylaxis in individuals with an unknown dairy allergy; and much less often may cause nausea, cramps, flatulence, bloating or diarrhea in individuals with previously unknown lactose intolerance.

- **FS-IVGTT** – The major risk of this test is hypoglycemia associated with the insulin infusion along with the risk associated with insertion of intravenous catheters for the infusions and blood drawing as discussed above. The frequent glucose measurements will allow prompt detection of hypoglycemia, in which case the test will be stopped and the intravenous glucose infusion will be increased or glucose will be given orally for resolution.

- **DEXA scans** involve a very small amount of radiation. One of the risks associated with radiation exposure is cancer. The natural incidence of fatal cancer in the U.S. is about 1 chance in 5. Everyday radiation exposure from natural occurring background radiation (sun, radon exposure in the home) is approximately 100 mrem (1 mSv) per year. Each subject in this study will receive a whole body DEXA scan. The radiation dose of one whole body DEXA scan is approximately ≤0.3 mrem. This amount of radiation is very low as to make an accurate risk estimate meaningless. A negative urine pregnancy test must be documented prior to undergoing the DEXA scan.

- **HCG Administration** – HCG use can cause the following adverse effects: edema, depression, fatigue, headache, irritability, restlessness, ovarian cyst rupture, reaction or pain at the injection site, or a local or systemic hypersensitivity reaction.

  The risk of cyst rupture will be minimized by administering the HCG between days 3 and 13 following the onset of either a spontaneous or progestin-induced withdrawal bleed, or without regard to the onset of menses if vaginal sonography within 21 days of HCG administration reveals no evidence of follicle cyst development in the face of a negative pregnancy test.

- **Salsalate Treatment** – Salsalate use can cause the following adverse effects characteristic of salicylates in descending order of frequency: tinnitus, nausea, hearing impairment, rash and vertigo. These effects are transient, and resolve upon cessation of salsalate. Although cause and effect has not been established, the following less common adverse effects have also been reported: abdominal pain, abnormal hepatic function, anaphylactic shock, angioedema, bronchospasm, decreased creatinine clearance, diarrhea, gastrointestinal irritation, inflammation, bleeding or perforation, hepatitis, hypotension, nephritis, urticaria, myocardial infarction or cerebral vascular accident.

  Tinnitus is an expected side effect of salsalate treatment that occurs more frequently at doses higher than the one proposed for this study. To minimize this side effect, subjects will be advised to begin by taking one capsule (i.e. 500 mg of salsalate in subjects receiving salsalate) the first day and increase the dose in one capsule increments every 3 days thereafter until the desired dose is achieved. If tinnitus occurs at any point in the upward dose titration or later on during the treatment, an affected subject will be advised to reduce the daily study medication dose in stepped one capsule increments every 3 days. In a previous study, this approach effectively resolved symptoms enabling subjects to complete the study while still maintaining therapeutic salicylate levels. The nature of any suspicious symptoms reported by a subject will be reviewed to determine whether there is actual tinnitus before the salsalate dose is reduced in the manner described. Vital signs and potential side effects will be monitored every 4 weeks during treatment when subjects report to the CRC to have their blood draw for a salicylate level. Liver function tests, BUN and creatinine levels will be drawn within 4 weeks after study completion to be certain that no lasting untoward hepatic or renal impairment has occurred.

- **Reproductive Risk** – Pregnancy is an exclusion criterion for this study. The risks listed above would apply equally to a developing fetus as well as the primary participant. Potential risks of specific impact to a developing fetus include the small amount of radiation exposure received from the DEXA scan (point 4 above). A negative urine pregnancy test must be documented prior to undergoing the DEXA scan. Salsalate is a Category C drug for pregnancy and use of double barrier contraception will be required during the study unless either the participant or her partner have already undergone permanent sterilization.

- **Loss of confidentiality** is a risk as with all human subject-based medical research owing to disclosure of health information. These disclosures may be mandated by local or national regulatory agencies (i.e. IRB, Food and Drug Administration (FDA)) or may be unintentional. Safeguards regarding both physical and electronic copies of all subject data are in place, as dictated by the current standards of UIC and UI Health.

  Except for de-identified research data, all other information relevant to patients collected in the study will be kept confidential, including locked storage of all paperwork, and secure digital storage on a database to which only members of the research group have access. The security and integrity of this database are maintained with the direct assistance and oversight of UI Health Information Technology support teams, and the database is served centrally from a closely monitored, regularly backed up server.

- **Unforeseen Risks** – There may be risks or side effects related to the study that are unknown at this time.

Benefits

- Subjects will receive individual body composition results as well as diabetes risk information from the OGTT. Based on the results of a small pilot study that treated women with PCOS with salsalate, it is possible that subjects receiving salsalate may experience a reduction in circulating androgens and ovulation. However, because individuals respond differently to therapy, it is not known in advanced if these potential benefits will occur. There are no anticipated benefits to subjects receiving placebo.

  The knowledge to be gained from this research may be beneficial to other patients, society or science. The results will guide the design of future studies aimed at determining what dietary modifications, medical regimen or both will optimally attenuate inflammation in PCOS with the goal of reducing medical disease and enhancing fertility. In particular, the findings may help define the therapeutic molecular inflammation target to develop the most ideal anti-inflammatory agent.

8.0 Data Collection and Management Procedures

- Data will be collected by the PI, Co-I or an authorized member of the research team and coded to the study number assigned to each subject. All electronic data will be secured on the PIs encrypted and password protected computer until it can be moved to a secure UIC data server for long-term storage. Hard copy data reporting forms will be stored in a locked file cabinet until it can be scanned to electronic copy and archived onto the UIC data server. Once files are converted the original hard copy will be kept until the study is closed, at which time the documents will be destroyed. In the event that a discrepency or error is found in the source data, the PI will determine if the data can be collected again/procedure repeated in a subsequent study visit or alternative visit provided this will not disrupt the integrity of the data. Should this occur, the event will be documented and reported to the IRB. Access to the data will be limited to the PI and IRB-authorized members of his research team. The PI will examine safety and review all data on an ongoing basis. Source data will be collected in data reporting forms of both electronic and written form. The PI will review these forms and data at the completion of each subject visit to ensure both accuracy and completeness.

9.0 Data Analysis

- Data will be analyzed according to the plan for statistical analysis presented below.

10.0 Quality Control and Quality Assurance

- The PI will evaluate the data for adherence with the protocol and for accuracy in relation to source documents.
- The PI is responsible for the evaluation of data quality and will review the data in an ongoing basis.

11.0 Data and Safety Monitoring

- The IRB will be responsible for human subject protection oversight through annual periodic review of research involving human subjects. All personnel participating in research

activities will meet UIC and OHRP IRB requirements for human subjects research, including completion of an approved CITI human subjects training (or institutional equivalent) and refresher courses at required intervals.

- Each year, the PI will submit a progress report to the UIC IRB, which will review progress and re-approve the protocol. As part of these reviews, the PI will report how many subjects have been recruited, the outcomes to date and if there have or have not been any non-serious adverse reactions reported by the study participants.

- Various aspects of the study will be monitored as follows: data quality, subject recruitment, accrual and retention, adverse events, assessment of scientific reports or therapeutic development, results of related studies that impact subject safety, and procedures designed to protect the privacy of subjects.

- A **Data and Safety Monitoring Board (DSMB)** will monitor human subject safety, protocol compliance and data integrity. The DSMB will consist of three experienced physicians / nurses who as a group have a good clinical and investigative understanding of the study protocol based on their backgrounds (Dr. Uzma Syed, Assistant Professor of Clinical Medicine, Division of Endocrinology and Metabolism; Dr. William Kobak, Associate Professor of Clinical Obstetrics and Gynecology; and Dr. Eileen G. Collins, Professor of Nursing). The DSMB will be chaired by Dr. Syed who has an excellent knowledge base of endocrine and metabolic research and DSMB oversight. Dr. Syed will also be the medical safety monitor. DSMB members will not be directly involved in the study.

  Initial Meeting: The DSMB will meet prior to initiation of the study to review the protocol, resolve queries, and approve the research protocol and informed consent document.

  Interim Meetings: In open session, the DSMB will meet at least twice a year to review the ongoing recruitment process, participant withdrawal and protocol deviations. It will review any study protocol amendments and follow the continuing review process. It will review participant safety and discuss any serious adverse events that may occur, including the relationship of adverse events to study intervention. It will review the study protocol to determine if any protocol revisions are merited as suggested by interim results, adverse events, or external events such as new FDA recommendations. It will review the informed consent document and confirm consistency with risks of participation to determine if any consent document revisions are merited. It will discuss any issues raised by the clinical study team. At the conclusion of each meeting, one of the following dispositions will be recommend: continue the study without changes, continue the study with specified changes, place the study on Clinical Hold pending further discussion or investigation, or terminate the study.

  Key Features to be Assessed: Monthly salicylate levels measured at Visits 1, 6, 7 and 8 by Labcorp Clinical Laboratories will eventually be used to assess compliance after the study is unblinded upon its completion to accurately interpret the study data, but will not be used for clinical management. To avoid blinding bias, the monthly salicylate levels will not be posted in a subject's electronic medical record. Instead, DSMB members under Dr. Syed's direction will prospectively receive copies of these salicylate levels for safekeeping. Development of tinnitus during salsalate use will be used to assess salicylate toxicity clinically as in a previous large multicenter trial.[19] As an additional precaution, salsalate will be prepared as 500 mg capsules and identical placebo capsules so the blinding will not be jeopardized when changing the dose in one capsule increments to manage tinnitus.

Operating Procedures: The DSMB will convene after 5 participants have completed the study protocol or 6 months following completion of the first participant (whichever occurs first); and thereafter at least twice a year. If there is a concern about serious adverse events, more frequent meetings may be called by vote. If unexpected and/or serious adverse events occur disproportionately to what may be anticipated, the study will be placed on clinical hold and the IRB will be consulted for guidance.

Minutes will be taken by the Research Nurse Study Coordinator and forwarded to the DSMB chair for review. The final version of the minutes will be sent to all DSMB members for approval. A formal report from the chair, following approval by the DSMB members will be supplied to the IRB within six weeks of each meeting. Each report will conclude with a recommendation selecting one of the following dispositions: continue the study without changes, continue the study with specified changes, place the study on clinical hold pending further discussion or investigation, or terminate the study. In the case of clinical hold or study termination, the report will be disseminated to the research team as rapidly as possible.

Interim Reporting: The PI will provide Interim data reports to the DSMB. Types of reports for each meeting include summaries of the following: baseline clinical and demographic characteristics of study participants, the recruitment process, including numbers screened, numbers enrolled, withdrawals, adherence and deviation from the study protocol. Any adverse events, including serious, study-related adverse events, will be evaluated by the DSMB. In particular, they will be concerned with adverse events that may affect continued study participation. Specific items to be reported will include: tinnitus during study medication treatment, hypoglycemia during an OGTT or FS-IVGTT, allergic reactions to study medications or any other serious adverse events.

- Anticipated risks/adverse events noted in the consent form will be handled as follows:

Risks and/or Anticipated Adverse Events	**Assessment Measures**	**Individual <u>doing</u> Assessment**	**Assessment Intervals or Frequency**	**Interventions to Decrease or Respond to Risks**
Venipuncture DEXA scans	Patient observation	CRC Staff	During the procedure	Verbal explanation; Provide privacy; Performed by qualified, trained personnel.
Venous access and blood draws	Patient observation	CRC R.N.	During the procedure	Performed by M.D. or R.N.; Provide complete explanation and expectations before the procedure, both verbally and in the consent form.
Pain	Patient observation and communication	CRC R.N.	During the procedure	Tylenol as ordered; Explain procedure and rationale to patient.

Infection	Patient observation and vital signs	CRC R.N.	During the procedure	Blood draws performed under sterile technique; Instruct patient regarding infection signs and symptoms and importance of reporting these to the M.D.; Provide M.D. and CRC telephone number in the event of a problem.
Bleeding, bruising or anemia	Patient observation and communication	CRC R.N.	During procedure	Careful closure of stopcocks between blood draws; Meet hemoglobin IRB requirements.
Thrombosis	Observe blood flow during draws	CRC R.N.	Every blood draw to check patency of lines.	O.9% NS infusion @ 30cc/hr throughout the test.
Hypoglycemia during FS-IVGTT infusion of insulin.	Patient observation and frequent glucose measurements	CRC R.N. and Research Nurse Study Coordinator	Every 1-10 minutes during the initial 1½ hours of the FS-IVGTT starting 20 minutes before the test, then every 20-30 minutes for the next 1½ hours.	Cessation of the infusions and intravenous glucose administration if patient becomes symptomatic (i.e. headache, shakiness, visual changes and palpitations) or glucose level falls below 70 mg/dl.

- A participant will be monitored continuously during an entire CRC visit to undergo study procedures, and the PI will be present within the hospital and available by telephone, pager or e-mail throughout each visit. In the situation of an anticipated or an unanticipated Adverse Event (AE), a standard IRB Adverse Event Report Form will be sent to the CRC and IRB in a timely manner. This report will include a full description of the event, including the relationship of the AE as not related, possibly related, or definitely related to the study procedure. The PI, in consultation with the DSMB, will grade adverse events severity as follows (as recommended by the National Cancer Institute):

  0 – “No adverse event or within normal limits or not clinically significant”
  1 – “Mild AE, did not require treatment”
  2 – “Moderate AE, resolved with treatment”
  3 – “Severe AE, resulted in inability to carry on normal activities and required

professional medical attention”
4 – “Life threatening or disabling AE”
5 – “Fatal AE”

- A participant’s study will be stopped in the event she requests not to continue, or if she has a reaction to any procedure, the ingestion of the glucose beverage during the OGTT, the HCG injection, dairy cream ingestion during the CCT, difficulty obtaining a peripheral intravenous line, administration of salsalate (i.e. allergies, persistent or serious side effects, unusual anxiety). The participant’s treating physician will attend to any serious adverse events that may occur until they have resolved or refer the participant to the appropriate provider as needed. A participant will also be asked to stop the study if she is not compliant with the requirements of the study protocol. All participants will be assessed for side effects at the end of the study. Study discussions will be documented within each participant’s study binder. Follow-up with the IRB or CCTS CRC will also be noted, and copies will be kept of all correspondence.

- If a participant withdraws from the study, she will be replaced by a different participant to achieve accrual of 90 participants to complete the study. Additional reasons for withdrawal may include but are not limited to a family emergency, a new medical diagnosis, participant preference or development of any persistent study medication side effects that do not resolve with common clinical measures in a timely fashion. The IRB and CCTS CRC will be notified during an annual continuing review of subject accrual to date, and any withdrawals or dropouts.

- If a serious adverse event occurs during the study, the PI will promptly report the event to the IRB (in accordance with UIC OPRS Prompt Reporting policy) and to the study DSMB for review and possible action. In the unlikely event of any serious adverse reactions occurring during this study, the DSMB will also provide appropriate clinical expertise for advice and assistance. The study will be completely suspended in the event of death of a subject.

- The PI will keep abreast of scientific reports or therapeutic development, and results of related studies that impact subject safety; and will inform subjects immediately of new information from these reports or studies that change the risk/benefit ratio for study participation. This type of information will also be reported to the DSMB and to the IRB during an annual continuing review.

12.0 Statistical Considerations

12.1 Data Analysis

- *OGTT Dynamic Responses:* The glucose and insulin responses during the OGTT is defined as the area under the curve (AUC) created by plotting the entire sequence of glucose or insulin values and calculated using the trapezoidal rule.[55]

- *HCG-stimulated Dynamic Responses:* The testosterone, androstenedione and 17-hydroxtprogesterone responses during the HCG-ST is defined as the area under the curve (AUC) created by plotting the entire sequence of each of these hormone values, and calculated using the trapezoidal rule.[56]

- *Lipid-stimulated Responses:* Responses of the various inflammation markers from MNC or plasma will be defined as the difference between the maximum lipid-stimulated value and the baseline basal value. If baseline basal values exhibit considerable intersubject

variability, the percent (%) change will be calculated defined as follows: (maximum lipid-stimulated value – baseline basal value / baseline basal value) x 100.

- *Insulin Sensitivity:* The calculated fractional glucose disappearance per insulin concentration unit adjusted for insulin clearance will be determined during a FS-IVGTT as a measure of peripheral insulin sensitivity (S_I).

12.2 Power Analysis

We do not expect >20% dropout based on previous studies.[19,20] Thus, we do not anticipate any substantial bias in the results. Based on the high cost of adding a patient, recruitment will continue until 90 pre- and post-evaluations are completed up to a maximum of 113 patients rather than increasing the overall sample size up front.

Aim 1: The primary outcome for Aim 1 is HCG-stimulated testosterone AUC. Our salsalate treatment preliminary data sample size (n=6) is too small to calculate power for the main outcome. As there are three groups (lean non-IR, lean IR, obese), the three contrasts of interest will be comparing the mean difference in HCG-stimulated testosterone AUC between the salsalate and placebo arms within each study group (i.e. salsalate-treated lean non-IR vs placebo lean non-IR, salsalate-treated lean IR vs placebo lean IR, and salsalate-treated obese vs placebo obese). To keep the familywise type I error at 0.05, we will use a Sidak adjustment and set α=0.017 for each contrast for the primary outcome.

There are no previous studies in women with PCOS that address any of the outcome variables related to salsalate treatment. Thus, there is no pilot data of salsalate vs. placebo from which to base our power calculations. However, our preliminary baseline HCG-ST data revealed similar results in women with PCOS or controls regardless of BMI. The HCG-stimulated testosterone AUC (ng/dl x 96 hr) in women with PCOS (n=17) compared with ovulatory controls (n=16) revealed a mean (±SD) of 7370±2481 vs. 3659±959 ($p<0.0001$, effect size [ES] =1.8). Furthermore, previous data gathered in women with PCOS (n=12) evaluating HCG-stimulated testosterone AUC in response to treatment with an insulin sensitizer revealed a significant ($p<0.04$) decrease in the mean (±SD) from 4395±1294 (pre) to 3288±1259 (post).[57]

Our preliminary data presented above revealed an ES of 1.8 for the mean change in the PCOS group vs. controls. We expect that the post-salsalate treatment HCG-stimulated testosterone AUC will be similar to controls. The proposed sample size of 90 patients (15 salsalate and 15 placebo for each study group), will provide 90% power to detect an ES ≥1.4 (80% power to detect an ES ≥1.24), based on a two-sided two-sample t-test at 1.7% significance to account for multiple comparisons which will yield similar power to our proposed GLM model with a Sidak adjustment to control type I error level. We used the pre- and post-treatment HCG-stimulated testosterone AUC data above136 to obtain a reasonable estimate for common SD of change for each treatment group. A post-treatment decrease of 30% (7672 - 0.3(7672)) in the salsalate-treated group and a post-placebo decrease of 10% (7672 - 0.1(7672)) in the placebo group with common standard deviation of 990 (assuming a correlation of 0.7 between pre/post measures) will yield an ES of 1.55. This analysis is indicative of adequate power to detect a difference in our primary outcome.

The secondary outcome for Aim 1 is insulin sensitivity (S_I). Our primary comparison will be change in S_I between the salsalate and placebo groups in insulin-resistant subjects (lean IR and obese) as we do not anticipate a change in lean non-IR subjects. Previous data gathered in another insulin resistant population (n=9) evaluating insulin sensitivity in response to salicylate

treatment revealed a significant ($p<0.04$) increase in the mean (±SD) insulin sensitivity from 4.0±2.7 (pre) to 5.9±1.8 (post).[23] These data indicate that a sample size of 30 subjects per group is sufficient to achieve 80% power to detect an ES ≥1.74 for salsalate vs. placebo based on a two-sided two-sample t-test at a 0.05 alpha level to account for multiple comparisons again yielding similar power to our GLM model.

This study is not powered to compare ovulation rates among the separate groups. Thus, ovulation rate post-treatment based on detection of luteal range progesterone elevations guided by BBTs will be compared between the salsalate and placebo groups overall. Based on the inclusion criteria, patients will be anovulatory pre-treatment, thus only post-treatment rates will be compared. With a sample size of 45 in each treatment group, if we expect approximately 10% to ovulate in the placebo arm, we will have over 80% power to detect a statistically significant difference if ovulation increases by 40% in the salsalate-treated group based on Fisher's exact test with type I error set at 0.05.

Aim 2: The primary outcome for Aim 2 is lipid-stimulated NFκB EMSA density (i.e. change from baseline). The three contrasts of interest will be comparing the mean difference in lipid-stimulated NFκB EMSA density between the salsalate and placebo group within each study group (i.e. salsalate-treated lean IR vs placebo lean IR, salsalate-treated lean non IR vs placebo lean non IR, and salsalate-treated obese vs placebo obese). Our preliminary baseline CCT data revealed similar results in women with PCOS and obese controls.[11] The lipid-stimulated NFκB EMSA density in women with PCOS (n=17) compared with lean controls (n=8) revealed a mean (±SD) % change of 20.1±10.4 vs. -3.0±9.1 ($p<0.0001$, ES=1.9). The secondary outcomes for Aim 2 are glucose-stimulated NFκB EMSA density and lipid-stimulated ROS generation (i.e. change from baseline). For women with PCOS compared with lean controls, our previously published baseline OGTT data showed a mean (±SD) % change of 34.6±45.7 vs. -21.6±35.6 ($p<0.04$, ES=1.4),[13] and our baseline preliminary CCT data showed a mean (±SD) % change of 92±38 vs. -7±22 in ROS generation ($p<0.0001$, ES=3.8). For each of these outcomes, we will use a similar GLM framework as described in Aim 1 with the same three contrasts of interest. We will use a Sidak adjustment to control the type I error for each outcome. We expect post-salsalate treatment measures in PCOS will be similar to lean controls. Based on a two-sided two-sample t-test with a 0.017 α level to account for multiple comparisons which will yield similar power to our GLM model, we will have 80% power to detect an ES ≥ 1.24. Thus, our study is sufficiently powered for these outcome measures.

Based on baseline PCOS vs. control data and using the approach described to analyze the primary and secondary outcomes, there is also 80% power to detect an ES ≥ 1.24 for lipid-stimulated $p47^{phox}$ WB density (16.4±10.9 vs. -6.4±6.3 [$p<0.0001$, ES=2.6]) and lipid-stimulated MNC-derived TNFα release (8.4±11.8 vs. -7.4±9.4 [$p<0.008$, ES=1.5]). These 2 variables and those for which we lack preliminary data to estimate power; (i.e. salsalate-treated vs. placebo lipid- and glucose-stimulated p47phox, p65 NFκB and TNFα mRNA content, and plasma CRP and TNFα) are exploratory outcomes key to providing means and SDs to plan larger studies.

12.3 Statistical Analysis

Initially, a full descriptive analysis of the data will be performed. Frequency distributions will be computed for all raw data and for change from baseline responses to lipid or glucose stimulation. Summary statistics will be provided by treatment group (salsalate, placebo) overall as well as by treatment group and study group (lean non-IR, lean IR, obese).

i. Baseline data such as age, BMI, androgens, insulin sensitivity, total body fat and visceral fat will be compared between groups (salsalate: lean non-IR, lean IR, obese; placebo: lean non-IR, lean IR, obese) using analysis of variance (ANOVA) for normally distributed data or Kruskal-Wallis test for non-normal data to validate similarity of characteristics within each study group across the 2 treatment arms. If there is statistical significance, multiple comparisons between groups will be performed using a Sidak adjustment.

ii. The primary outcome for Aim 1 is a difference in HCG-stimulated testosterone AUC between salsalate vs. placebo. To test our hypothesis, we will use a GLM framework with a dependent variable of post-treatment HCG-stimulated testosterone AUC including covariates for pre-treatment HCG-stimulated testosterone AUC, treatment indicator, study group (lean non-IR, lean IR, obese) and the treatment by study group interaction. Of primary interest will be the three contrasts of difference in mean between salsalate vs. placebo for these study groups. A Sidak adjustment will be used to control the Type I error. Analysis for the secondary measure of difference in S_I between salsalate and placebo for the insulin-resistant subjects only (lean IR, obese) will be compared using the same GLM framework, although only the overall contrast of difference in means between salsalate and placebo is of interest. Assumptions and model fit will be checked for all primary and secondary outcome analyses. Data will be transformed if necessary.

Post-treatment ovulation rates based on ultrasound documentation of follicular collapse will be compared between the salsalate-treated group and the placebo group using Fisher's exact test with type I error at 0.05. Also, ovulation rates and 95% exact confidence intervals will be estimated by study group (lean with IR, lean without IR, and obese) and treatment group.

iii. Aim 2 will evaluate lipid-stimulated NFκB EMSA density (primary outcome), glucose-stimulated NFκB EMSA density and lipid-stimulated ROS generation (secondary outcomes) along with the exploratory variables described in the power analysis. Primary and secondary outcomes will be transformed (e.g. logarithmic transformation) if either measure is not normally distributed and will be analyzed using a GLM framework as described for Aim 1 to compare salsalate vs. placebo for each study group. A Sidak adjustment will be used to control type I error. Descriptive statistics will be provided for exploratory variables.

13.0 Regulatory Requirements

13.1 Informed Consent

- Written consent will be obtained in the CCTC CRC during the screening visit after the volunteer has successfully completed an initial phone screening. Each member of the research team is designated to administer the informed consent. Each research team member has been trained by the PI in administering informed consent and has completed CITI and HIPAA training as required by IRB.

- Each volunteer will receive a copy of the signed informed consent document (ICD) for his/her records. The study copy of the signed ICD will be stored in the subject's study binder in a locked file cabinet in the PIs locked office. Each member of the study team has access to these study binders. Because they are stored in the PI's office, the PI will supervise access.

13.2 Subject Confidentiality

- Subject's confidentiality will be maintained by using unique identifiers and keeping source documents with sensitive subject information in protected environments as described above. Further, all research team members are Collaborative Institutional Training Initiative (CITI) and HIPAA trained.

- All research team members have access to the study data that will be stored digitally on a secure database. Study data are kept separate from source documents containing sensitive subject information. Source documents such as initial screening forms, informed consent and other data sheets containing dates are kept in subject binders in a locked cabinet in the PI's office. Security standards will include 2 locked doors for paper records, and 2 electronic signatures for electronic data (i.e. main computer, then specific access to data files). The PI will oversee who has access to these files.

- Upon subject enrollment, the subject is assigned a unique identifier. This unique identifier will be used for all data sheets and data spreadsheet entries. Only the initial screening form, the informed consent and the identifier key will contain direct identifiers.

- The research team will request access to participant's PHI through a patient authorization document, which if signed by the subject will give the researcher permission to use PHI collected during the research study for defined purposes.

- After study completion, we plan to scan all source documents into electronic format for long term storage on an Academic Computing and Communications Center (ACCC) protected UIC server that only the PI can access. Upon verification of electronic transfer, original source documents will be shredded and disposed of into confidential file disposal bins.

- Sensitive subject information such as personal identifiers are required to determine subject eligibility and required to contact the individual for study visits. Once the subject is enrolled, the subject is assigned a unique identifier and these initial source documents are no longer needed for the successful completion of the study. The steps above describe the handling of these source documents.

- *A Certificate of Confidentiality* is not required, as the information collected if disclosed would not significantly harm or damage the participant.

13.3 Unanticipated Problems

- Based on the low level of risk during study participation, serious adverse events are unlikely to occur. Discomfort during the blood draws will be most likely problem that could occur during participation. Every effort will be made to provide comfort to participants whenever possible. Should participants experience discomfort or emotional upset from any of the research procedures, they will be reminded that they are under no obligation to participate in the study, and can withdraw consent and discontinue participation at any time without prejudicing their current or future relations with UIC or UI Health, or its physicians. Students of employees will be reminded that participation or nonparticipation in the study will not affect current or future medical care at UIC or the UI Health and/or position of employment or course of studies in these institutions.

- Additional potential problems such as breach of confidentiality and emotional upset may also occur. Every effort will be made to maintain subject confidentially using the data management procedures described above. In the event of breaches or suspected breaches of data security, including sensitive information, an unanticipated problem will be reported to the IRB using the Event Requiring Prompt Reporting form within 5 working days of becoming aware of the event. Possible data breaches include, but are not limited to, the following: lost or misplaced files/folders, improper disposal of paper containing sensitive information, loss/violation of the integrity of decryption key or process.

- A participant's study will be stopped in the event she requests not to continue, or if she has a reaction to any procedure, the ingestion of the glucose beverage during the OGTT, the HCG injection, dairy cream ingestion during the CCT, difficulty obtaining a peripheral intravenous line, administration of salsalate (i.e. allergies, persistent or serious side effects, unusual anxiety). In the unlikely event that a serious side effect occurs, the participant will immediately be referred for further evaluation and treatment. All participants will be assessed for side effects at the end of the study and any discussions will be documented within each participant's study binder. Follow-up with the IRB or CRC will also be noted, and copies will be kept of all correspondence.

- A participant will be withdrawn from the study if she fails to comply with any of the research procedure, if the study is stopped for an unforeseen reason, or in the event of physical injury or illness resulting from the research procedures. Failure to take the appropriate dose of medication not related to downward dose titration for expected symptom control can result in withdrawal of a subject from the study. The degree of noncompliance with medication use that can have a significant adverse impact on the study may vary from subject to subject and the decision to withdraw a subject for medication use noncompliance will be left to the discretion of the investigator.

- The study will be completely suspended in the extremely unlikely event of death of a subject, or at the moment it becomes apparent that any particular serious side effect is occurring at an unanticipated increased frequency.

- The PI will keep abreast of scientific reports or therapeutic development, and results of related studies that impact subject safety; and will inform subjects immediately of new information from these reports or studies that change the risk/benefit ratio that might cause them to change their mind about continued participation. This type of information will also be reported to the DSMB and to the IRB during an annual continuing review. Participants will be instructed to notify the principal investigator or his designee as soon as the desire to withdraw is apparent. The circumstances related to the request to withdraw will be reviewed to determine the necessity of performing a post-study evaluation to insure safety.

14.0 References

1. Fauser BC, Tarlatzis BC, Rebar RW, Legro RS, Balen AH, Lobo R, Carmina E, Chang RJ, Yildiz BO, Laven JS, Petraglia F, Wijeyeratne CN, Norman RJ, Dunaif A, Franks S, Wild RA, Dumesic D, Barnhart. Consensus on women's health aspects of polycystic ovary syndrome (PCOS): the Amsterdam ESHRE/ASRM-Sponsored 3rd PCOS Consensus Workshop Group. *Fertil Steril* 2012, 97:28-38.e25.
2. Burghen GA, Givens JR, Kitabachi AE. Correlation of hyperandrogenism with hyperinsulinemia in polycystic ovarian disease. *J Clin Endocrinol Metab* 1980, 50:113-116.
3. Dunaif A, Segal KR, Futterweit W, Dobrjansky A. Profound insulin resistance, independent of obesity in polycystic ovary syndrome. *Diabetes* 1989, 38:165-1174.

4. Nestler JE, Jakubowicz DJ, de Vargas AF, Brik C, Quintero N, Medina F. Insulin stimulates testosterone biosynthesis by human theca cells from women with polycystic ovary syndrome by activating its own receptor and using inositoglycan mediators as the signal transduction system. *J Clin Endocrinol Metab* 1998, 83:2001-2005.
5. Micic D, Popovic V, Nesovic M, Sumarac M, Dragasevic M, Kendereski A, Markovic D, Djordjevic P, Manojlovic D, Micic J. Androgen levels during sequential insulin euglycemic clamp studies in patients with polycystic ovary disease. *J Steroid Biochem* 1988, 31:995-999.
6. Dunaif A, Graf M. Insulin administration alters gonadal steroid metabolism independent of changes in gonadotropin secretion in insulin-resistant women with the polycystic ovary syndrome. *J Clin Invest* 1989, 83, 23-29.
7. Tosi F, Negri C, Perrone F, Dorizzi R, Castello R, Bonora E, Moghetti P. Hyperinsulinemia amplifies GnRH agonist stimulated ovarian steroid secretion in women with polycystic ovary syndrome. *J Clin Endocrinol Metab* 2012, 97:1712-1719.
8. González F, Sia CL, Shepard MK, Rote NS, Minium J. Inflammation in response to glucose ingestion is independent of excess abdominal adiposity in normal weight women with polycystic ovary syndrome. *J Clin Endocrinol Metab* 2012, 97:4071-4079.
9. González F, Sia CL, Shepard MK, Rote NS, Minium J. Hyperglycemia-induced oxidative stress is independent of excess abdominal adiposity in normal weight women with polycystic ovary syndrome. *Hum Reprod* 2012, 27:3560-3568.
10. González F, Sia CL, Shepard MK, Rote NS, Minium J. The altered mononuclear cell-derived cytokine response to glucose ingestion is not regulated by excess adiposity in polycystic ovary syndrome. *J Clin Endocrinol Metab* 2014, 99:E2244-E2251.
11. Abdelhadi OA, Shepard MK, Sia CL, Garrett TJ, González F. Activation of nuclear factor κB in response to cream ingestion is related to ovarian androgen hypersecretion in polycystic ovary syndrome. *Fertil Steril* 2013, 100 (2 Suppl):S39.
12. González F, Rote NS, Minium J, Kirwan J.P. Reactive oxygen species-induced oxidative stress in the development of insulin resistance and hyperandrogenism in polycystic ovary syndrome. *J Clin Endocrinol Metab* 2006, 91:336-340.
13. González F, Rote NS, Minium J, Kirwan JP. Increased activation of nuclear factor κB triggers inflammation and insulin resistance in polycystic ovary syndrome. *J Clin Endocrinol Metab* 2006, 91:1508-1512.
14. González F, Rote NS, Minium J, Kirwan JP. In vitro evidence that hyperglycemia stimulates tumor necrosis factor-α release in obese women with polycystic ovary syndrome. *J Endocrinol* 2006, 188:521-529.
15. González F, Sia CL, Stanczyk FZ, Blair HE, Krupa ME.. Hyperandrogenism exerts an anti-inflammatory effect in obese women with polycystic ovary syndrome. *Endocrine* 2012, 42:726-735.
16. Piotrowski PC, Rzepczynska IJ, Kwintkiewicz J, Duleba AJ. Oxidative stress induces expression of CYP11A, CYP17, STAR and 3βHSD in rat theca-interstitial cells. *J Soc Gynecol Invest* 2005, 12(2):319A.
17. Foyouzi N, Kwintkiewicz J, Duleba AJ. Modulation of Steroidogenesis of theca-interstitial cells by oxidative stress and insulin. *J Soc Gynecol Invest* 2004, 11(2):312A.
18. Goldfine AB, Silver T, Aldahi W, Cai D, Tatro E, Lee J, Shoelson SE. Use of salsalate to target inflammation in the treatment of insulin resistance and type 2 diabetes. *Clin Transl Sci* 2008, 1:36-43.
19. Goldfine AB, Conlin PR, Halperin F, Koska J, Permana P, Schwenke D, Shoelson SE, Reaven PD. A randomised trial of salsalate for insulin resistance and cardiovascular risk factors in persons with abnormal glucose tolerance. *Diabetologia* 2013, 56:714-723.

20. Greenblatt DJ, Abernethy DR, Boxenbaum HG, Matlis R, Ochs HR, Harmatz JS, Shader RI. Influence of age, gender, and obesity on salicylate kinetics following single doses of aspirin. *Arthritis Rheum* 1986, 29:971-980.
21. Goldfine AB, Fonseca V, Jablonski KA, Pyle L, Staten MA, Shoelson SE., TINSAL-T2D (Targeting Inflammation Using Salsalate in Type 2 Diabetes) Study Team. The effects of salsalate on glycemic control in patients with type 2 diabetes: a randomized trial. *Ann Intern Med* 2010, 152:346-357.
22. Goldfine AB, Fonseca V, Jablonski KA, Chen YD, Tipton L, Staten MA, Shoelson SE, Targeting Inflammation Using Salsalate in Type 2 Diabetes Study Team. Salicylate (salsalate) in patients with type 2 diabetes: a randomized trial. *Ann Intern Med* 2013, 159:1-12.
23. Hundal RS, Petersen KF, Mayerson AB, Randhawa PS, Inzucchi S, Shoelson SE, Shulman GI. Mechanism by which high-dose aspirin improves glucose metabolism in type 2 diabetes. *J Clin Invest* 2002, 109:1321-1326.
24. Flieschman A, Shoelson SE, Berner R, Goldfine AB. Salsalate improves glycemia and inflammatory parameters in obese young adults. *Diabetes Care* 2007, 31:289-294.
25. González F, Mather KJ, Considine RV, Pardue SL, Acton AJ. Suppression of nutrient-induced inflammation with a nonsteroidal anti-inflammatory agent ameliorates ovarian dysfunction in lean insulin-sensitive women with polycystic ovary syndrome. *Fertil Steril* 2015, 104 (2 Suppl):e21.
26. Koska J, Ortega E, Bunt JC, Gasser A, Impson J, Hanson RL, Forbes J, de Courten B, Krakoff J. The effect of salsalate on insulin action and glucose tolerance on obese non-diabetic patients: results of a randomized double-blind placebo-controlled study. *Diabetologia* 2009, 52:385-393.
27. Pacini G, Bergman RN. MINMOD: a computer program to calculate insulin sensitivity and pancreatic responsivity from the frequently sampled intravenous glucose tolerance test. *Comput Methods Programs Biomed* 1986, 23:113-122.
28. Toffolo G, Arduin A, De Zanche N, Avogaro A, Cobelli C. A minimal model of insulin during insulin modified IVGTT: assessment of hepatic insulin extraction. *Mod Contr Biomed Syst* 1997, 15:91-95.
29. Basu R, Breda E, Oberg AL, Powell CC, Dalla Man C, Basu A, Vittone JL, Klee GG, Arora P, Jensen MD, Toffolo G, Cobelli C, Rizza RA. Mechanisms of the age-associated deterioration in glucose tolerance: contribution of alterations in insulin secretion, action, and clearance. *Diabetes* 2003, 52:1738-1748. Erratum in: *Diabetes* 2003, 52:3014.
30. Toffolo G, Campioni M, Basu R, Rizza RA, Cobelli C. A minimal model of insulin secretion and kinetics to assess hepatic insulin extraction. *Am J Physiol Endocrinol Metab* 2006, 290:E169-E176.
31. Denti P, Bertoldo A, Vicini P, Cobelli C. IVGTT glucose minimal model covariate selection by nonlinear mixed-effects approach. *Am J Physiol Endocrinol Metab* 2010, 298:E950-960.
32. Polidori DC, Bergman RN, Chung ST, Sumner AE. Hepatic and extrahepatic insulin clearance are differentially regulated: results from a novel model-based analysis of intravenous glucose tolerance data. *Diabetes* 2016, 65:1556-1564.
33. Quinn LT. Metabolic responses during the post-exercise recovery period in non-insulin dependent diabetes mellitus. USPHS GCRC M01-RR00055 and NINR 1F31-NR06723, Ph.D. dissertation – Quinn, 1996.
34. Quinn LT. Diabetes, exercise and postprandial oxidative stress. NIH/NINR R01-NR007760-03, PI – Quinn, 2003.
35. Ciampelli M, Leoni F, Cucinelli F, Mancuso S, Panunzi S, De Gaetano A, Lanzone A. Assessment of insulin sensitivity from measurements in the fasting state and during an oral

glucose tolerance test in polycystic ovary syndrome and menopausal patients. *J Clin Endocrinol Metab* 2005, 90:1398-1406.
36. Dewailly D, Lujan ME, Carmina E, Cedars MI, Laven J, Norman RJ, Escobar Morreale HF. Definition and significance of polycystic ovarian morphology: a task force report from the Androgen Excess and Polycystic Ovary Syndrome Society. *Hum Reprod Update* 2014. 20:334-352.
37. Taylor RW, Keil D, Gold EJ, Williams SM, Goulding A. Body mass index, waist girth, and waist to hip ratio as indexes of total and regional adiposity in women: evaluation using receiver operating characteristic curves. *Am J Clin Nutr* 1998, 67:44-49.
38. Carmina E, Bucchierri S, Esposito A, Del Puente A, Mansueto P, Orio F, Di Fede G, Rini G. Abdominal fat quantity and distribution in women with polycystic ovary syndrome and extent of it relation to insulin resistance. *J Clinical Endocrinol Metab* 2007, 92:2500-2505.
39. Cotler SJ, Guzman G, Layden-Almer J, Mazzone T, Layden TJ, Zhou XJ. Measurement of liver fat content using selective saturation at 3.0 T. *J Magnetic Res Imag* 2007, 25:743-748.
40. Piltonen T, Koivunen R, Perheentupa A, Morin-Papunen L, Ruokonen A, Tapanainen JS. Ovarian age-related responsiveness to human chorionic gonadotropin in women with polycystic ovary syndrome. *J Clin Endocrinol Metab* 2004, 89:3769-3775.
41. Deopurkar R, Ghanim H, Friedman J, Abuaysheh S, Sia CL, Mohanty P, Viswanathan P, Chaudhuri A, Dandona P. Differential effects of cream, glucose, and orange juice on inflammation, endotoxin, and the expression of Toll-like receptor-4 and suppressor of cytokine signaling-3. *Diabetes Care* 2010, 33:991-997.
42. Van Cauter E, Mestrez F, Sturis J, Polonsky KS. Estimation of insulin secretion rates from C-peptide levels. Comparison of individual and standard kinetic parameters for C-peptide clearance. *Diabetes* 1992, 41:368-377.
43. Caumo A, Florea I, Luzi L. Effect of a variable hepatic insulin clearance on the postprandial insulin profile: insights from a model simulation study. *Acta Diabetol* 2007, 44:23-29.
44. Kim SP, Ellmerer M, Kirkman EL, Bergman RN. Beta-cell "rest" accompanies reduced first-pass hepatic insulin extraction in the insulin-resistant, fat-fed canine model. *Am J Physiol Endocrinol Metab* 2007, 292:E1581-E1589.
45. Meier JJ, Veldhuis JD, Butler PC. Pulsatile insulin secretion dictates systemic insulin delivery by regulating hepatic insulin extraction in humans. *Diabetes* 2005, 54:1649-1656.
46. Aljada A, Ghanim H, Dandona, P. Activation of nuclear factor-κB (NF-κB) in mononuclear cells (MNC). *Methods Mol Biol* 2002, 196:105-10.
47. Andrews NC, Faller DV. A rapid micro-preparation technique for extraction of DNA-binding proteins from limited numbers of mammalian cells. *Nucl Acids Res* 1991, 19:2499.
48. Thusu K, Abdel-Rahman E, Dandona P. Measurement of reactive oxygen species in whole blood and mononuclear cells using chemiluminescence. *Methods Mol Biol* 1998, 196:99-103.
49. Tosi MF, Hamedani A. A rapid specific assay for superoxide release from phagocytes in small volumes of whole blood. *Am J Clin Pathol* 1992, 97:566-573.
50. Hancock JT, Jones OT. The inhibition by diphenyleneiodonium and its analogues of superoxide generation by macrophages. *Biochem J* 1987, 242:103-107.
51. Dandona P, Aljada A, Mohanty P, Ghanim H, Hamouda W, Assian E, Ahmad S. Insulin inhibits intranuclear factor κB and stimulates IκB in mononuclear cells in obese subjects: evidence for an anti-inflammatory effect? *J Clin Endocrinol Metab* 2001, 86:3257-3265.
52. Balagopal P, Schimke JC, Ades PA, Adey D, Nair KS. Age effect on transcript levels and synthesis rate of muscle MHC and response to resistance exercise. *Am J Physiol Endocrinol Metab* 2001, 80:E203-E208.
53. Aljada A, Ghanim H, Dandona P. Translocation of $p47^{phox}$ and activation of NADPH oxidase in mononuclear cells. *Methods Mol Biol* 2002, 196:99-103.

54. Boyum A. Isolation of mononuclear cells and granulocytes from human blood. *Scand J Clin Lab Invest* 1968, (21 Suppl) 97:77-89.
55. Jones NH. Finding the area under the curve using jmp and a trapezoidal rule. SAS Institute 1997, http://www.jmp.com/about/newsletters/jmpercable/pdf/05_fall_1997.pdf.
56. Koivunen RM, Morin-Papunen LC, Ruokonen A, Tapanainen JS, Martikainen HK. Ovarian steroidogenic response to human chorionic gonadotrophin in obese women with polycystic ovary Syndrome. *Hum Reprod* 2001, 16:2546-2551.

APPENDICES

Telephone Eligibility Assessment Form

Electronic Eligibility Assessment Form

Clamp Infusions Preparation

Study Visit Blood Sampling Plan

González Laboratory Flowsheet

ChooseMyPlate Diet Instructions

3-Day Food Record

Basal Body Temperature Chart

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