Smoking, Sex Hormones and Pregnancy

Study Protocol

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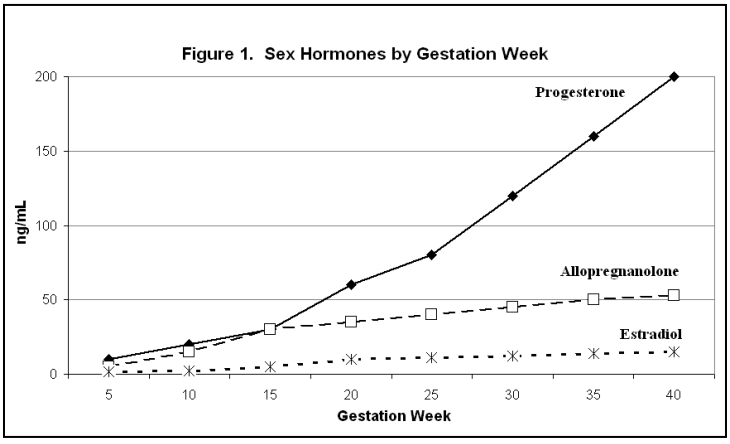
SIGNIFICANCE

Women and Smoking. Compared to men, women are at an increased risk for both smoking-related morbidity and mortality, as well as smoking relapse.[1-6] Women respond less favorably to smoking cessation treatment and, on average, quit smoking at an older age than men.[2-3] Women of childbearing age are of particular concern given the short-term and long-term risks associated with pregnancy such as placental previa, restricted fetal growth, sudden infant death syndrome, and, later in the child's life, attention-deficit disorder and overweight/obesity. [7-9] More than 90% of women who quit smoking during pregnancy relapse shortly after delivery, resulting in the continuation of smoking-related risks well after delivery as mothers are the primary source of secondhand smoke (SHS) exposure for offspring. [10] SHS contains more than 50 carcinogens, as well as other toxins. Children exposed to SHS have increased risk of respiratory and ear infections, and miss more days from school. [11] To date few, if any, interventions have addressed potential biological and/or neurobiological causal mechanisms associated with the sex differences in risk for smoking relapse. *In sum, effective smoking cessation interventions for women of childbearing age are lacking, perhaps due to a limited understanding of the causal pathways involved in smoking relapse.*

Sex Hormones & Drug Abuse Behavior. The evidence for the role of sex hormones on drug abuse behaviors is growing. The animal literature provides strong evidence indicating that progesterone is protective against drug abuse behaviors whereas estrogen promotes drug abuse behaviors.[12-13] The clinical literature is less clear but recent work provides support for this theory. For example, one of our recent studies, utilizing a randomized prospective design, observed better smoking cessation outcomes among those women who quit smoking during the luteal phase (low estradiol/high progesterone) compared to those who quit in the follicular phase (high estradiol/low progesterone). [14] Further support comes from a second study that also observed improved smoking cessation outcomes among those with a quit date in the luteal phase compared to the follicular phase while using bupropion as a smoking cessation aid. [15] However, discordant results come from two additional studies. These studies observed improved smoking cessation outcomes in the follicular phase rather than the luteal phase.[16, 17] In addition to differences in methodology, one of the primary differences between these four studies is that the first two [14, 15] did not use nicotine replacement therapy whereas the second two did. [16, 17] These data ultimately prompted a hypothesis suggesting that the timing of a quit attempt during the menstrual cycle and the pharmacotherapy used may be equally important in determining success of a quit attempt,[18] indicating nicotine response may be a factor that needs to be addressed. Relatedly, there is a growing body of animal literature to support that a metabolite of progesterone – allopregnanolone – acts as a potential protective factor. [12] Allopregnanolone is a neuroactive steroid which increases the release of dopamine and has been shown to be associated with behavioral effects of alcohol in a non-dependent sample of women, as well as withdrawal symptoms in a sample of dependent women. [19, 20] One theory is that withdrawal symptom severity, and therefore risk for smoking relapse, may be associated with changes in dopamine release. This may be one of the mechanisms involved with these observed associations between sex hormones and smoking relapse. *Increasing evidence exists indicating that the sex hormones influence addictive behaviors and sex hormones are known to change dramatically during pregnancy. However, to our knowledge, no study to date has investigated the association between sex hormones and smoking during pregnancy.*

Sex Hormones & Pregnancy. Levels of progesterone, allopregnanolone, and estradiol all change dramatically during pregnancy (**Figure 1**). Progesterone is produced mainly from the corpus luteum during early pregnancy, then by the placenta in mid- to late-pregnancy. Levels of progesterone vary widely among women but levels generally increase from approximately 10 ng/ml in the first trimester up to 200 ng/ml in the third trimester. [21] Similarly, allopregnanolone increases by ten-fold during pregnancy. [22] Estradiol, primarily from the ovaries, increases from 2 to 18 ng/ml during pregnancy. [21] Given that the literature indicates that progesterone is protective against drug abuse behaviors whereas estradiol facilitates these behaviors, the ratio between these two hormones may be of critical importance. During pregnancy, the estradiol/progesterone (E2/P) ratio can decrease from 0.20 to 0.09. [21] In *brief, while all three sex hormones increase during pregnancy,*



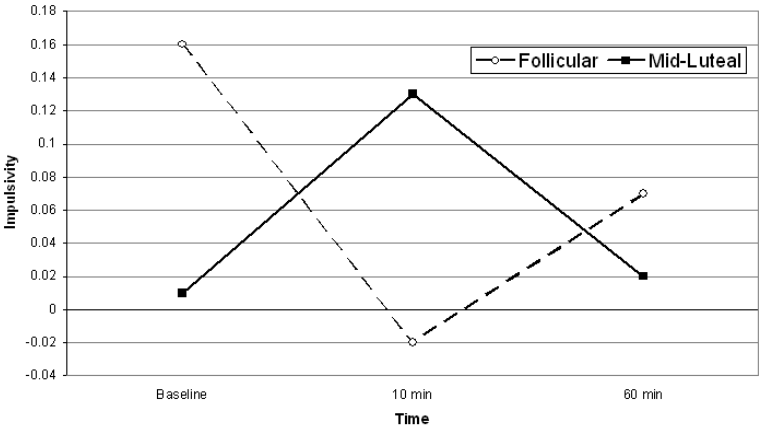
the sex hormones that may be protective against drug abuse behaviors (progesterone, allopregnanolone) increase more dramatically than the sex hormone that may facilitate drug abuse behaviors (estradiol); this results in the ratio to shift in favor of those hormones that are protective, possibly offering an overall protective effect against drug abuse behavior during pregnancy.

Formulation of Central Hypothesis. Pregnancy provides an ideal clinical model for testing our central hypothesis given the literature to-date indicates the following: (1) while pregnancy is the most common time for women to quit smoking, the majority are unable to sustain smoking abstinence after delivery, [7] (2) sex hormones increase during pregnancy, with progesterone increasing the most dramatically, [21] and (3) progesterone and allopregnanolone appear to be protective against drug abuse behaviors. [12] Therefore, *our central hypothesis is that higher levels of progesterone and allopregnanlone, along with lower estradiol/progesterone ratios, will be associated with a favorable change in smoking-related symptomatology and nicotine response.*

INNOVATION

This project incorporates at least three highly innovative aspects. First, this study will be among the first to systematically investigate the role of sex hormones on smoking-related symptomatology and nicotine response during pregnancy. This information will directly improve the state of the scientific knowledge by filling a gap in the literature and potentially offering a new direction in smoking cessation and relapse prevention interventions. For example, if progesterone proves to be associated with a reduction in smoking-related symptomatology during pregnancy, treatment of exogenous progesterone may facilitate cessation and help prevent relapse. Delivery of exogenous progesterone during the third trimester is an accepted treatment for pre-term delivery prevention;[23] therefore investigation into the delivery of exogenous progesterone as a smoking cessation treatment during times of high risk for relapse (such as immediately prior to delivery and/or in the post-partum period) would be warranted. Further, this type of treatment may extend beyond the pregnant population, to non-pregnant women, who are at an increased risk for relapse compared to their male counterparts. [2] Second, to our knowledge no studies to date have investigated the role of allopregnanolone on drug abuse behavior during pregnancy in a clinical population. Adding this neurosteroid to our project represents a new focus in our research by investigating a possible neurobiological protective factor in smoking behavior. Considering the substantial role of allopregnanolone on drug abuse behavior in the animal literature, the knowledge gained from this project has the potential to directly influence and inform new postpartum smoking relapse prevention programs given that exogenous distribution of allopregnanolone has recently been proven to be safe in non-pregnant clinical populations. [22] Third, utilizing the two clinical models (pregnancy and oral contraceptives) will allow us to better assess the independent effects of sex hormones on smoking-related symptomatology and nicotine response. While motivations for smoking may change dramatically during pregnancy for a variety of reasons (i.e. infant health, social norms), utilizing these two clinical models will allow us to better focus on the relationship between sex hormones, smoking-related symptomatology and nicotine response by comparing the associations of sex hormones and study outcomes between clinical models.

Figure 2. Changes in Impulsivity by Menstrual Phase



THE APPROACH

PRELIMINARY STUDIES

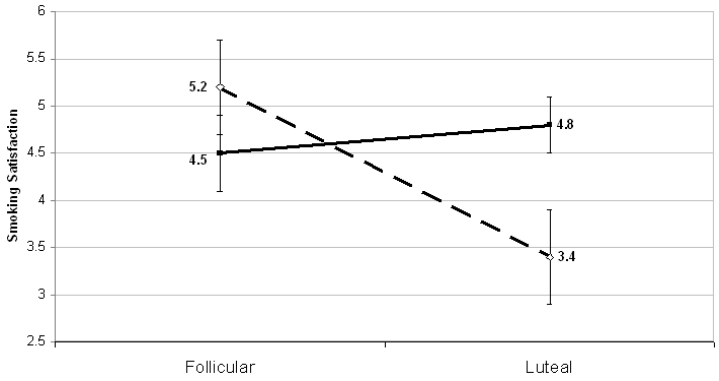
Study #1: Impulsivity Varies by Menstrual Phase after Exposure to Nicotine. Goal: This cross-over study aimed to measure differences in nicotine response between follicular (F) and mid-luteal (ML) menstrual phases in a sample of female smokers between the ages of 18 and 40 (n=20). Results: After exposure to nicotine nasal spray, participants in the F phase became significantly more impulsive on the Immediate Memory Task computer program [24] compared to participants in the ML phase (Figure 2). *Relevance: Sex hormones appear to impact the level of impulsivity, such that higher levels of progesterone are associated*

with less impulsivity after exposure to nicotine.

Study #2: Changes in Estradiol/Progesterone (E2/P) Ratio is Associated with Changes in Craving. Goal: This study aimed to assess the influence of the E2/P ratio on smoking-related symptoms of withdrawal (MNWS), [25] smoking urges (Brief-QSU), [26] and reinforcing effects of smoking (mCEQ) [27] using a within subject analysis in a sample of female smokers (n=50) with regular menstrual cycles between the ages of 18 and 40. Results: During ad libitum smoking, an increase in the E2/P ratio was associated with an increase in craving on two independent items of measurement: mCEQ (t=2.38, p=0.02) and MNWS (t=1.92, p=0.06). *Relevance: Changes in sex hormone levels are associated with changes in craving for cigarettes.*

Study #3: Smoking Satisfaction & Enjoyment Vary by Menstrual Phase during Ad Libitum Smoking. Goal: The purpose of this cross-sectional study was to investigate the differences in reinforcing effects (mCEQ) [27] of smoking by menstrual phase and depressive symptoms in a sample of female subjects (n=39) who were classified into four groups: no depressive symptoms follicular phase (NDS-F), no depressive symptoms luteal phase (NDS-L), depressive symptoms follicular phase (DS-F) and depressive symptoms luteal phase (DS-L). Results: Smoking Satisfaction varied by menstrual phase in the NDS group but not in the SDS group (F-NDS: 5.2±0.5; L-NDS: 3.4±0.5; F-SDS: 4.5±0.4; L-SDS: 4.8±0.3; p=0.016; **Figure 3**) and a similar trend was observed within the Enjoyment subscale (F-NDS: 4.4±0.6; L-NDS: 1.7±0.6; F-SDS: 3.6±0.5; L-SDS: 2.9±0.5; p=0.089). *Relevance: Among those without depressive symptoms, sex hormones appear to impact the level of reinforcing effects of smoking, such that higher levels of progesterone are associated with less smoking satisfaction and less enjoyment.*

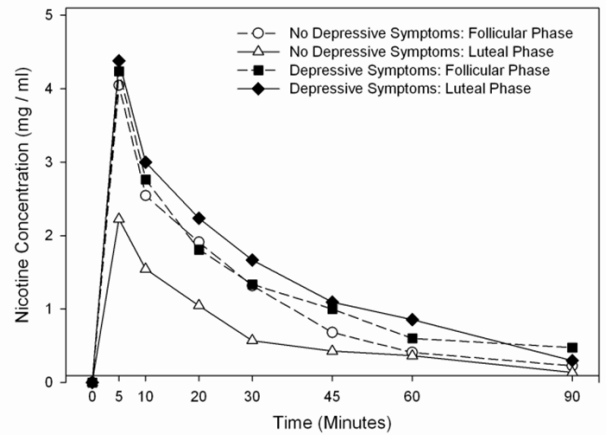
Figure 3. Smoking Satisfaction Subscale by Depressive Status and Menstrual Phase



Study #4: Hormonal Contraceptive Use is Associated with Higher Levels of a Cigarette Biomarker. Goal: This cross-sectional study aimed to measure differences in NNAL, a biomarker of carcinogen exposure to cigarette smoking, between three groups of adolescent smokers (ages 13 to 19): females on hormonal contraceptives (F-HC; n=33), females not on hormonal contraceptives (F-no HC; n=27) and males (M; n=40). Results: After controlling for cigarettes/day, F-HC had the highest levels of NNAL values compared to F-no HC and M (1.00±0.89 vs. 0.46±0.31 vs. 0.71±0.59; f value=4.90, p=0.010). *Relevance: Sex hormones appear to impact biomarker levels associated with the exposure to cigarette smoking.*

Study #5: Pharmacokinetics of Nicotine varies by Menstrual Phase in Women without Depressive Symptoms. Goal: This cross-over study aimed to measure menstrual phase differences in pharmacokinetics after exposure to nicotine nasal spray within a sample of abstinent female smokers with regular menstrual cycles who either had depressive symptoms (n=23) or did not (n=24). Results: There was a significant interaction between depressive symptoms status and menstrual phase such that those without depressive symptoms had a significant menstrual phase difference in their maximum nicotine concentration levels (p=0.020; Figure 4). Relevance: In women without depressive symptoms higher progesterone levels are associated with lower nicotine response

Figure 4. Mean Plasma Nicotine Concentrations Following Nicotine Nasal Spray Use



Study #6: Age of Menarche Predicts Onset of Smoking but Perception of Maturity does not. Goal: The goal of this retrospective study was to investigate the association of self-reported age of onset of daily smoking with self-reported age of menarche and perceived maturity level at age of menarche in a sample of female smokers (n=54) between the ages of 18 and 40. Results: Older age at menarche was associated with an older age at initiation of daily smoking (f-value=5.60, p-value=0.02). Perception of maturity was not significantly associated with smoking initiation (f-value=0.41, p=0.53). *Relevance: Menarche represents marked sex hormone fluctuations and instability; it may be that the fluctuating sex hormones impact smoking behavior more than the perception of these life changes.*

PROGRESS REPORT

For the past 20 years we have conducted research funded under NIDA grant R01-DA08075. This project's overall goal is to investigate the influence of ovarian hormones, both with and without the use of nicotine replacement therapy, on multiple outcomes: women's smoking behavior, relapse to smoking after a quit attempt, relapse-related factors including withdrawal symptoms, premenstrual symptoms, weight gain, caloric intake, negative mood, and nicotine response.

During the past funding period (May 2007 to October 2011), our findings were disseminated in eight peer-reviewed journal articles and 20 poster/oral presentations at local, national, and international scientific meetings (see Section 2.5 for Progress Report Publication List). As of February 2011, we have begun analyzing data from our completed pharmacokinetics subgroup. Based on these results we have two manuscripts in various stages of publication. These manuscripts will describe the menstrual phase differences in the changes in serum cortisol and nicotine levels after a nicotine challenge via nicotine nasal spray in a sample of 52 female smokers who have been abstinent for four days.

Menstrual Phase & Depressive Symptoms in Acute Smoking Abstinence (Continuation Grant R01-DA008075) *Project Period* reported on includes May 2007 to October 2011. This project's s*pecific aims* are: Aim 1: Determine the effect of depressive symptoms, alone and in concert with ovarian hormones (i.e. menstrual phase: follicular versus luteal) on withdrawal symptoms, nicotine craving, smoking urges, premenstrual symptoms and cortisol levels (measuring stress response) during acute smoking abstinence; Aim 2: Determine if depressive symptoms and menstrual phase moderate nicotine response following acute smoking abstinence. *Study Design*: This study utilized a randomized cross-over design to compare within subject effects of menstrual phase and between subject effects of depressive symptoms. A convenience sample is stratified by depressive symptoms, then randomized to testing order (follicular phase then luteal phase or vice versa). Each participant then completed two six-day testing sessions including two days of ad libitum smoking followed by four days of smoking abstinence. During these testing sessions, participants attended daily clinic visits to biochemically verify smoking status, collect blood samples of measurement of sex hormones and complete self-reported items assessing mood and withdrawal symptoms. Participants also completed two nicotine lab exposure sessions during alternate menstrual phases. *Summary*: As described in the Preliminary Results Section, our previous work demonstrates not only our expertise in studying the hormonal influence on smoking behavior in women, but also the importance of this work. As detailed above (Section D.1) our most recent work has provided additional evidence for a relationship between sex hormones and drug abuse behaviors. Specifically we have shown higher levels of progesterone appear to be associated with blunted levels of impulsivity, less favorable responses to nicotine, and changes in levels of craving. *New Direction*: We are proposing to continue our exploration of the possible causal mechanisms of sex hormones on smoking-related symptomatology and nicotine response in pregnant women. During pregnancy progesterone, allopregnanolone and estradiol increase dramatically. This will provide us with an ideal clinical model to further investigate the relationship between sex hormones and smoking behavior, as well as fill a gap in the current body of literature and potentially provide new information and direction to improve smoking cessation rates during pregnancy. Our proposed study is further strengthened and innovative due to the inclusion of a more controlled study of sex hormone effects via the clinical model of oral contraceptives.

RESEARCH PLAN

Overview. Using two clinical models – pregnancy (Sample 1) and oral contraceptives (OC; Sample 2) – we aim to (1) examine the association between levels of progesterone (Prog), allopregnanolone (Allo) and the estradiol/progesterone (E2/P) ratios with smoking-related symptomatology during ad libitum smoking, and (2) determine the association between Prog, Allo, and E2/P with the changes in smoking-related symptomatology and response to nicotine following overnight abstinence. Sample 1 will enroll approximately 112 pregnant women to ensure a final sample size of 84 participants to participate in a cross-sectional study at two time points: 12 weeks to 22 weeks gestation (n=42) and 32 to 37 weeks gestation (n=42). Sample 2 will enroll approximately 78 OC users to ensure a final sample size of 52 participants in a randomized cross-over, double-blind study design to complete testing periods during "low" and "high" dose levels of exogenous progesterone with a consistent dose of exogenous estrogen. All participants will complete identical data collection procedures including providing saliva (cortisol to measure stress), urine (cotinine and 3-Hydroxycotinine to measure nicotine exposure, and medication compliance), and blood (progesterone, allopregnanolone, estradiol, and nicotine) samples, as well as ecological momentary assessments (EMA) daily for seven-days. Participants will also complete a three-hour smoking lab session (with the first morning

cigarette) and a four and a half-hour nicotine exposure lab session (after at least 13 hours of overnight abstinence). These sessions will contain a nicotine challenge via nicotine nasal spray or smoking topography with timed-series of physiological, subjective and behavioral responses.

Recruitment. Our primary recruitment strategy for both study samples is via two clinical sites: (1) Obstetrics & Gynecology Clinic at Hennepin County Medical Center (HCMC), the fourth largest hospital in Minnesota and is a safety net facility for low-income, uninsured and other vulnerable populations (see Section 2.14 for letter of support) and (2) Fairview Riverside Women's Clinic at Fairview University Medical Center (FUMC; see Section 2.14 for letter of support). Among these two clinics an estimated 4,900 infants are born each year. Given an estimated 23% of women of childbearing age currently smoke about 50% quit smoking during pregnancy, [7] about 15% of pregnant women experience a pregnancy-related complication [28] and an estimated 15% are using psychotropic medications, [29] we roughly estimate approximately 407 pregnant women will meet our inclusion/exclusion criteria (details described below) for Sample 1 each year (4,900 infant births/year x 23% smoking prevalence x 50% smoking cessation x 85% no pregnancy complications x 85% no psychotropic medications). We will also recruit non-pregnant women who are on oral contraceptives from these two clinical sites. Based on preliminary results from our current study, we estimate 40% of women who smoke are oral contraceptive users and 15% are currently being treated for psychiatric problems. At each clinical site we will post flyers and business cards in the waiting and exam rooms at the Hennepin County Medical Center Obstetrics & Gynecology Clinic, and the Fairview Riverside Women's Clinic. These flyers and business cards will instruct potential participants to call study staff for more information.

In addition to recruiting at clinical sites we will also use two additional recruitment methods. The first is via the *Minnesota Academy of Family Physicians Research Network*. Approximately 80 MAFP members practice in the Minneapolis/St. Paul metro and greater surrounding area. The overall clinical populations within this network include 64% women who are White (82%), Asian (8%), Black (6%) and other/unknown (14%). Since MAFP physicians often have individual practices and are familiar with conducting clinical research, they will refer potentially eligible participants to our research study (see Section 2.14 for letter of support). The second recruitment method is *advertising in the mainstream media*. While this type of recruitment can be challenging, our team has been successful with this form of recruitment with both non-pregnant and pregnant women. For example, our current study recruiting non-pregnant women between the ages of 18-40 has had success with Facebook advertising. Specifically, over the past six months we have advertised on Facebook resulting in 4,121 clicks to our website, an estimated 130 phone calls to our clinic and an enrollment of 23 participants (~4 participants/month). Similarly, Dr. Lewis' (Co-I) ongoing study on pregnancy and depression recently sent out an email to the local newspapers listserv. This email was opened 21,700 times, resulting in 338 clicks to the study website. A total of 109 participants were enrolled into the study, the majority of which were recruited directly from this single email. In addition to these two examples, we regularly use television, radio, mailings, and other internet sources to identify study participants with similar success.

Study Samples. While all participants in Sample 1 (Pregnant Women) and Sample 2 (Oral Contraceptive Users) complete identical data collection procedures, there are some differences in inclusion/exclusion criteria, timing of the testing periods and follow-up procedures. Those differences are described here and in Table 1.

Sample 1 – Participants. To meet recruitment goals, we will aim to enroll 38 pregnant women per year (~3-4 women/month). Inclusion criteria: females 18-35 years old, in stable physical/mental health with confirmed pregnancy, self-reports smoking ≥ 5 cigarettes/day for the past year, has established prenatal care, single gestation, either 12-22 or 32-37 weeks gestation, English fluency, and able to provide informed consent. Exclusion criteria: use of psychotropic medications or illicit drugs with the exception of marijuana use ≤ 2 times per month and a willingness to abstain from marijuana during the testing period, current use of other types of tobacco or nicotine, or pregnancy complications (i.e. gestational diabetes, known congenital anomaly, fetal growth restriction, pregnancy hypertension and history of >2 miscarriages). Finally, participants will not be eligible to participate in the study at more than one time point (i.e. 12-22 and 32-37 weeks gestation) due to the potential ethical issues associated with administering nicotine nasal spray multiple times during pregnancy and/or unintended encouragement to continue smoking to be eligible to participate again.

Sample 1 - Timing of Testing Period. The testing period will occur during either 12-22 or 32-37 weeks gestation (participants complete one testing period only). These gestational weeks were selected to capitalize on the expected variability in sex hormones (e.g. 12-22 weeks gestation expected progesterone values are **19.5** to 89.4 ng/mL and 32-37 weeks gestation expected progesterone values are 48.4 to 200.0 ng/mL).[20] We will not be assessing the first trimester (due to the high risk for miscarriage and delay of initial prenatal care) nor late third trimester (due to the possibility of early delivery).

Table 1. Methodology Comparison of the Two Study Samples

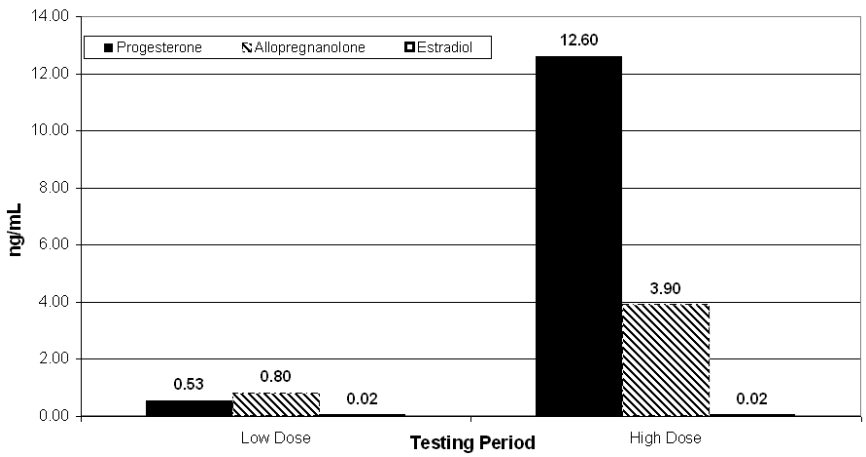
	Sample 1 *Pregnant Women (n=84)*	Sample 2 *Oral Contraceptive (OC) Users (n=52)*
Inclusion Criteria	• 18-35 years old • Stable physical/mental health • ≥ 5 cigarettes/day for ≥ 1 year • 16-20 or 32-36 weeks gestation • Established prenatal care • Single gestation • English fluency • Able to provide informed consent	• 18-35 years old • Stable physical/mental health • ≥ 5 cigarettes/day for ≥ 1 year • Current OC user willing to switch to study supplied OC • English fluency • Able to provide informed consent
Exclusion Criteria	• Use of psychotropic medication, illicit drugs or other types of tobacco/nicotine • Pregnancy complications	• Use of psychotropic medication, illicit drugs or other types of tobacco/nicotine • Current breastfeeding • Planning to become pregnant within next 3 months • Conditions or medications contraindicated to progesterone
Timing of Testing Period	One eight-day testing period completed, schedule per participant availability: • 16-20 weeks gestation, *or* • 32-36 weeks gestation	Two eight-day testing periods completed, order randomly assigned: • Low dose progesterone, *and* • High dose progesterone
Clinic Visits	• Screening Visit • 4 follow-up visits	• Screening Visit • 6 follow-up visits (4 visits during each testing session)
Follow-up	• Smoking cessation counseling offered.	• Participants return to ad lib smoking for six weeks then completing identical testing session in opposite phase (i.e. low or high dose progesterone). • Smoking cessation counseling offered.

Sample 1 - Follow-up. After completing the testing period (described below) participants will receive smoking cessation behavioral counseling, self-help materials, advice for pharmacotherapy (and a prescription, if necessary) per the discretion of the study physician (Dr. Allen, PI) and referrals to smoking cessation programs. Additional weekly phone counseling will be offered.

Sample 2 - Participants. To meet recruitment goals, we will aim to enroll 27 OC users per year (~3 women/month). Inclusion criteria: females 18-35 years old, in stable physical/mental health, self-reports currently smoking ≥ 5 cigarettes/day for at least the past year, using OC for at least the past three months without complication, willing to switch to study supplied OC, English fluency, and able to provide informed consent. *Exclusion criteria:* use of psychotropic medications or illicit drugs with the exception of marijuana use ≤ 2 times per month and a willingness to abstain from marijuana during the testing periods, current use of other types of tobacco or nicotine, current breast feeding, planning to become pregnant within the next three months, current use of finasteroid (propecia), efavirenz, red clover, ketoconazole and other drugs that are CYP3A4 inhibitors, and conditions contraindicated to progesterone treatment (including, but not limited to, thrombophlebitis, deep vein thrombosis, pulmonary embolus, clotting or bleeding disorders, heart disease, diabetes, history of stroke, allergy to peanuts, hypersensitive to progesterone and liver dysfunction).

Sample 2 - Timing of Testing Period. As in Sample 1, the timing of the testing period has been selected to maximize the variability in sex hormones by OC use. Participants will be randomly assigned to order to complete two testing periods: "low" dose progesterone versus "high" dose progesterone. To obtain these values participants will be supplied with Tri-Sprintec (Barr Laboratories Inc.; Appendix A) and generic Prometrium (Teva Pharmaceuticals USA IncAppendix A). The "low" dose progesterone is seven days of placebo Tri-sprintec + placebo generic Prometrium twice a day (7AM and 7PM). The "high" progesterone dose is seven days of placebo Tri-Sprintec + 200mg of generic Prometrium twice a day (7AM and 7PM).

Figure 5. Expected Sex Hormone Values in Oral Contraceptive Users by Testing Period



The Wednesday after their Screening Visit (described below), participants will begin taking the Tri-Sprintec medication.

Participants will be on Tri-Sprintec for three weeks prior to the first testing period. We have chosen this specific type of OC for four reasons. First, and foremost, it delivers a constant level of estrogen, allowing for an investigation of the varying levels of Prog, Allo and the E2/P ratio. Second, this type of progesterone (norgestimate, prometrium) has been shown to be converted into Allo (Dr. Richard Hauger, Personal Communication; 2/15/11). [30-31] Third, we have had success with reassigning women to this OC in our current study with little to no side effects. Finally, using an OC rather than attempting to track the natural menstrual cycle allows for ease in scheduling and related logistics.

Table 2. Study Measures

	Screening Visit	Testing Period: Baseline Visit (Day 0)	(Day 1-5)	Follow-up Visit (Day 6)	Smoking Lab Session (Day 7)	Nicotine Lab Session (Day 8)
Independent Variables						
Serum Progesterone		X		X		X
Serum Allopregnanolone		X		X		X
Serum Estradiol/ Progesterone Ratio		X		X		X
Dependent Variables (Aims 1 & 2)						
Symptomatology Forms (MNWS, [1] Brief QSU, [2] mCEQ, [3] PANAS, [4] PSS-10, [5] CES-D, [6] LTEQ [7])	X	X	X	X	X	X
Salivary Cortisol and mSSS [8] *			X			
Dependent Variables (Aim 2)						
Physiological Response (Blood Pressure, Pulse)					X	X
Subjective Response (SSS) [9]					X	X
Behavioral Response (FT, WR, 2B, IMT, DMT) [10]					X	X
Plasma Nicotine						X
Other Items						
Informed Consent	X					
Liver Function Tests [11]	X					
Demographics	X					
Medical History	X					
Smoking Behavior [12]	X					
Adverse Events (including C-SSRS)	X	X		X	X	X
Expired Carbon Monoxide	X	X		X	X	X
Urinary Cotinine and 3-Hydroxycotinine		X		X		X

These items will be collected five times during the course of Day 5 only – at waking, 30 minutes later, two hours later, 8:0 nd at bed time. [1] Minnesota Nicotine Withdrawal Scale, Hughes & Hatsukami, 1998; [2] Brief Questionnaire of Smoking U iffany & Drobes, 1991; [3] Modified Cigarette Evaluation Questionnaire, Cappelleri et al, 2007; [4] Positive and Negative cale, Watson et al, 1988; [5] Cohen Perceived Stress Scale, Chaaya et al, 2010; [6] Center for Epidemiologic Study – Depre night et al 1997; [7] Modified Subjective State Scale, al'Absi, et al., 1998, al'Absi et al, 2003; [7] Lesiure Time Ex uestionaire, Godin & Shephard, 1983; [8, 9] Subjective State Scale, al'Absi, et al., 1998 and al'Absi et al, 2003; [10] Beha asks include Fingertapping, Hindmarch, 1980, Word Recall, Phillips & Fox, 1998, 2-Back, Meyers et al, 2008, Immediat elayed Memory Task, Dougherty et al, 2002; [11] Liver function tests of ALT and AST will be conducted in Sample 2 (OC nly. [12] Includes Fagerstrom Test of Nicotine Dependence (FTND), age of smoking initiation, number of past quit atte ongest past quit attempt, motivation to quit smoking and TimeLine FollowBack (TLFB).

Given that the norgestimate dose in OC's is relatively low during both the "low" and "high" dosage testing periods participants will also be given 200mg of generic Prometrium twice a day (7AM and 7PM) during the "high" dose week and placebo pills during the "low" dose week. Participants will receive their first two doses of active or placebo Progesterone at the Baseline Visit (described below). They will be instructed to take their first dose at 7PM.This technique has been successfully used in prior research [32] and is generally well tolerated. The most common adverse effect is sedation. Less common effects include breakthrough bleeding, nausea and breast tenderness. [33-34] While it is possible that progestins may contribute to risk for thromboembolism; it is rare and we will be using natural progesterone instead of synthetic progestins. Natural progesterone is not known to be associated with thromboembolic risk. [35] Using these techniques we will expect to see stable estradiol levels with varying levels of Prog and Allo (**Figure 5**). Participants will receive additional study medication as needed at the clinic visits.

Sample 2 - Follow-up. Upon completion of the first testing period, participants in Sample 2 will be asked if they want to return to smoking. If they want to maintain their smoking abstinence, they will also receive

support for cessation. Based on observations in our current study, it is expect that up to 20% of women will want to maintain their smoking abstinence. If the participants want to return to smoking, they will be instructed to smoke at their 'regular rate' for the next three weeks and then attend an identical second testing period during the opposite testing phase (i.e. "low" or "high" dose).

Data Collection Procedures. All participants (Sample 1 and Sample 2) will complete identical testing procedures as described below and in **Table 2**.

The first visit will be a *screening visit.* At this visit informed consent will be obtained, followed by collection of demographic and smoking behavior information. Medical histories will be collected to further evaluate eligibility. The Columbia-Suicide Severity Rating Scale (C-SSRS) will be competed with each subject in order to assess suicidal ideation or behavior. Results will be reviewed by study staff and participants will be referred to physicians (S. Allen, MD) or (D. Hatzukami, PhD) if necessary. Measurements of height, weight (measurements taken with coats, shoes and other heavy items removed), blood pressure (measured after five minutes of rest), heart rate, and carbon monoxide will also be collected. A blood sample will be collected for liver function tests (AST and ALT) for Sample 2 participants only. At the end of this visit the all remaining clinic visits will be scheduled and participants will be compensated for their time.

The *baseline visit (Day 0)* will be the first day of the testing period. At this visit participants will have their weight measured and then be shown into a private room to complete self-reported items of smoking-related symptomatology and related factors (See Table 2 and Section E.5 for details). After the questionnaires are completed, blood pressure, and heart rate will be measured. Next, the participant will provide urine (for assessment of nicotine exposure) and blood (for assessment of sex hormones) samples. Remaining blood and urine samples will be saved in the biorepository. The participants will then be trained on how to use the study supplied Electronic Data Capture Device (EDCD). Upon the completion of this visit, the upcoming visit dates/times will be confirmed and participants will be compensated for their time.

During the *testing period (Days 0-7)* all participants will complete daily *Ecological Momentary Assessments (EMA)* using a study supplied Electronic Data Capture Device (EDCD). The EDCD will be programmed to "beep" to indicate an assessment is available. Given that recent research indicates that smoking-related symptomatology varies by time of day [36] participants will be prompted to complete assessments at random times (between the hours of 7AM and 11PM) up to four times per day, for an average of three times per day. Notification of an available assessment will happen via a "beep" sound. The EDCD will continue to "beep" once per every 15 minutes until the assessment is completed or after the two-hour window allotted for completion of assessment elapses. Each assessment will take an estimated 15 minutes to complete and will measure self-reported levels of smoking, withdrawal, craving, mood, stress, and physical activity (See Table 2 and Section E.5. for details). We have opted to use EMA due to several benefits versus standard paper-and-pencil format including improved quality of data, increased participant compliance, and participant preference. [37-38]

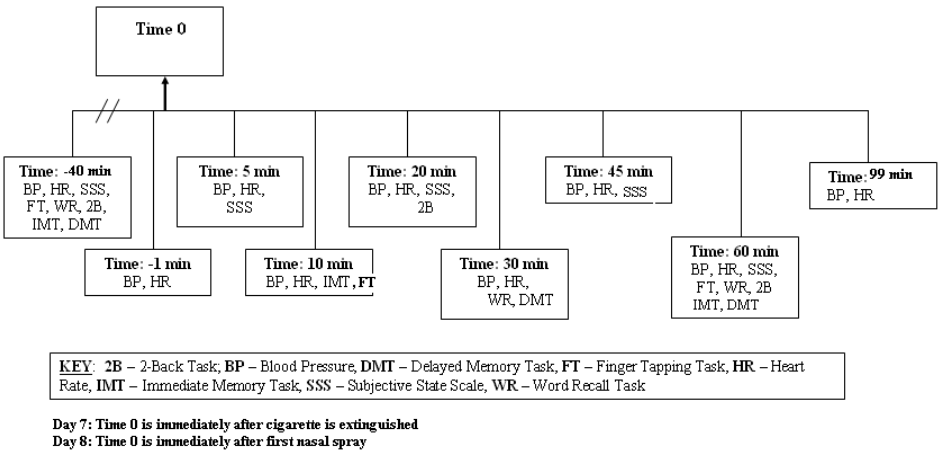
The interim clinic visit (Day 3 or Day 4), participants will return to the clinic. Upon arrival, participants will have their weight measured, followed by measurement of blood pressure, heart rate and carbon monoxide. Cigarettes smoked per day since Day 0 will be collected retrospectively from participants. Participants will also be asked about any new medications or changes to current medications. Participants will receive training on how to do the saliva samples on day 5 and receive the specific materials to do the saliva samples. Any difficulties the participant has had using the EDCD will be addressed before the participant leaves. At the end of this visit the participants will be compensated for their time.

On the day prior to the follow-up visit *(Day 5)* participants will collect salivary cortisol samples outside the clinic to measure physiological stress levels. Participants will be reminded to collect saliva samples via prompts from the EDCD. We are measuring saliva cortisol rather than blood serum levels, because samples are needed throughout the day and multiple blood draws are impractical, burdensome and would impose ethical issues in pregnancy. Saliva sample collection will occur at the following times to assess the diurnal pattern of cortisol: immediately after waking, 30 minutes later, two hours later, 8:00 pm and before going to sleep. We have successfully used this method in prior projects, [14, 39] as well as in our ongoing study. Participants are compliant with the testing procedures. Participants will be supplied with saliva sample swabs (Salivette™ tubes, Sarstedt, Rommelsdorf, Germany). Tubes will be labeled to indicate the date and time each sample should be completed. Previous research has shown that this ambulatory monitoring procedure can be adequately performed by participants, and that the integrity of the salivary cortisol is not compromised by storage at room temperature for 24 hours. [40] During each saliva sample collection, participants will complete the monitoring Subjective State Scale (mSSS) via the EDCD to monitor mood and potential confounders such

as time of last cigarette, physical activity, and caffeine use (Appendix B).

At the *follow-up clinic visit (Day 6),* participants will return to the clinic. Upon arrival, participants will have their weight measured and provide urine (nicotine exposure and medication compliance via visual inspection of urine color which will change due to riboflavin medication filler) and blood (sex hormones) samples. Remaining blood and urine will be saved in the biorepository. Next, participants will complete self-reported items of smoking-related symptomatology and related factors (See Table 2 and Section E.5 for details) followed by measurement of blood pressure and heart rate. While participants are completing the questionnaires study staff may complete a NicAlert (Appendix C) to confirm self-reported smoking status (NicAlert level of ≥ 3 indicating a cotinine value of ≥ 100 ng/mL). Participants will then practice using the nicotine nasal spray but will be prompted to refrain from smoking for one hour prior to the visit to limit nicotine exposure. They will also practice smoking through the smoking topography machine and practice using the computer tasks and other measures used in the lab sessions (See Section E.5. for details). Finally, participants will be instructed to quit smoking by midnight (Appendix D). Upon the completion of this visit, the lab session will be confirmed and participants will be compensated for their time.

Figure 6. Nicotine Lab Sessions



The *smoking lab session* will occur on *Day 7*. At this visit, participants will smoke their first morning cigarette (i.e. they will be instructed to not smoke on Day 7 until instructed to do so by study staff). Participants will attend a three hour visit beginning as close to their usual first morning cigarette as possible (starting at approximately 7am-10am, per participant preference). Puff topography, a precise measure of smoking behavior,[67,68,69] will be used to examine whether prolonged use of progesterone affects topography measures that may indicate smoking compensation.[70] Puff topography will be assessed using a handheld topography device (type pending) that provides a valid measurement of puff number, puff volume, inter-puff interval and other indices.[66] Carbon monoxide readings will be collected before and 2-5minutes after puff topography. [36] Prior to the initiation of the lab session, participants will have their weight, blood pressure, and heart rate measured, and they will complete subjective measures to assess their self-reported level of smoking-related symptomatology and related factors after overnight abstinence (See Table 2 and Section E.3.5 for details). Once the smoking lab session begins, participants will be instructed to turn off their cell phones and will not be allowed to consume caffeinated beverages or any food items. Measurements of smoking response will start 40 minutes prior to the participant smokes her cigarette and will be administered in a fixed order (**Figure 6**). Response to smoking will be quantified via seven performance domains known to be associated with nicotine response:[41] (1) physiological (blood pressure, heart rate), (2) subjective (Subjective State Scale); (3) motor speed (Finger Tapping) (4) episodic memory (Word Recall); (5) working memory (2-Back); (6) attention/impulsivity (Immediate Memory Task), and (7) delayed memory (Delayed Memory Task). (See Section E.5. for more details.) In order to access plasma nicotine levels, a blood draw via an intravenous catheter will occur at baseline (-1 minute before smoking a cigarette) and +2 minutes after the last puff of the cigarette. A total of 30cc of blood will be collected. 15 cc of blood will be analyzed for nicotine levels and hormone levels and 15 cc of blood will be added to the biorepository.

The *nicotine exposure lab session* will occur on *Day 8.* Participants will attend this four and a half-hour visit in the morning (beginning at 7am-10am depending on participant preference) to control for diurnal variation in outcome variables. While participants are completing the questionnaires study staff will download the information from the EDCDs. Procedures will be identical to Day 7 except instead of subject smoking a cigarette, they will administer nicotine nasal spray and repeat the protocol twice. Nicotine nasal spray (Nicotrol, Pharmacia & Upjohn; Appendix A) is an FDA-approved use of nicotine delivery. One spray delivers 0.5 mg of nicotine; therefore, one dose (four sprays - two in each nostril) is approximately equivalent to the

nicotine delivery from two cigarettes.[42-44] The nasal spray is the only available delivery method that closely mimics the kinetics of smoking, is easy to administer, and delivers a consistent dose.[42-43] While the nicotine nasal spray results in an adverse burning sensation, this sensation resolves on its own within minutes. In our current study we have completed over 400 nicotine nasal spray lab sessions and have never had to postpone data collection due to problem with the adverse effects of the nasal spray. We also chose the nicotine nasal spray because: (a) there are ethical and practical difficulties of instructing people to inhale measurable amounts of tobacco smoke from cigarettes, (b) it is difficult to control nicotine intake via smoke inhalation, (c) the spray isolates the effects of nicotine from that of the many other compounds found in tobacco smoke, (d) it has the fastest delivery system and best approximates the rise in nicotine concentrations as seen with cigarette smoking, and (e) it has been given a Pregnancy Category Risk of D – indicating that although nicotine has known adverse effects, the benefits of use may outweigh the risks. While there may be some initial concerns with administering nicotine to a pregnant woman, nicotine replacement products have been recommended for smoking cessation during pregnancy.[45] In a recent study by Oncken and colleagues, [46] 11 doses of nicotine nasal spray were administered over a six-hour period in a sample of pregnant smokers who abstained from smoking overnight. This study found that nicotine nasal spray reduced overall maternal nicotine exposure compared to active smoking. Compared to the 11 doses used in this study, our study will be using a single dose of nicotine nasal spray (less than 5% of the acceptable maximal dose and less than 10% of the dose used by Oncken and colleagues); therefore, there are limited ethical concerns with using nicotine nasal spray within our sample of pregnant smokers. Further this protocol was reviewed by a bioethicist at the University of Minnesota, Dr. Steven Miles, who felt there were no major ethical issues in administering this dose of nasal nicotine spray to pregnant women (see Section 2.14 for letter of support). In order to access plasma nicotine levels, a blood draw via an intravenous catheter will occur at baseline (-1 minute before the nasal spray) and at the expected maximum concentration (+5 minutes after the nasal spray; Allen et al, 2012). Blood collected at baseline will also be used to analyze sex hormones, more specifically progesterone and allogregnanalone. A total of 30cc of blood will be collected, 15 cc of blood will be analyzed for nicotine levels and hormone levels and 15 cc of blood will be added to the biorepository. Participants will also provide a urine sample to assessnicotine exposure and medication compliance via visual inspection of urine color which will change due to riboflavin medication filler. Remaining urine will be saved in the biorepository. Upon completion of the four and a half-hour lab session, any possible adverse events will be assessed for all participants. Finally, all participants will be compensated for their time and will be offered brief smoking cessation counseling and self help materials for smoking cessation.

Study Participant Compensation. All participants will receive $10 at the screening visit, $20 at the baseline visit, $10 at the interim visit, $20 for Follow-up Visit on Day 6, $20 for Smoking Lab Session on Day 7 and $50 for the Nicotine Exposure Lab Session on Day 8. They will also receive $5 for each EMA completed with a bonus of $20 for not missing any EMAs, and $1 for each cortisol salivary sample collected with a bonus of $20 for not missing any of the five samples. A bonus of $100 will be given for completing all study procedures and $120 for returning the study supplied EDCD. Thus, all participants in Sample 1 will be compensated a total of $510 for completing the study. The participants in Sample 2 will have the potential to earn $ 900 by completing the testing week twice.

Study Measures. The independent measures will be the serum levels of progesterone (Prog), allopregnanolone (Allo), and the estradiol/progesterone (E2/P) ratio. Measurement of hormones will be done by collecting a blood sample on the baseline,follow-up, and nicotine nasal spray lab visits (Day 0, 6, and 8). We have opted to collect blood samples three times to allow for average hormone levels during the testing period to be obtained. Blood (20 cc) will be drawn then centrifuged. The serum stored at -20°C in sealed storage tubes to prevent evaporation. Approximately two mL of serum will be analyzed by the Fairview University Medical Laboratories for progesterone and estradiol samples using chemiluminescence. The expected values for these tests are displayed in Figure 6. Approximately two mL of serum will be sent to Dr. Richard Hauger's lab at the University of Califorina, San Diego (see Section 2.14 for letter of support) for Allo level measurement using radioimmunoassay techniques previously described. [47] The remaining serum (approximately two to four mL) will be stored as back-up.

For Aim 1, the outcome is the level of smoking-related symptomatology during ad libitum smoking. Items 1-7 below will be measured an average of three times a day for seven days via the EMAs. We selected the seven day testing period given that the hormones fluctuate fairly rapidly; thus, measuring symptomatology for longer than seven days would make the association between sex hormones and symptomatology more difficult to assess. For each item, participants will be instructed to rate their symptoms since the last assessment. The following nine dependent measures will be used (Appendix B):

(1) Minnesota Nicotine Withdrawal Scale (MNWS): Participants rate their nicotine withdrawal symptoms on eight-items on a scale of '0' (not present) to '4' (severe) resulting in summary scores overall withdrawal and craving. [25]
(2) Questionnaire on Smoking Urges-Brief (QSU-Brief): This ten-item version of the original form (QSU) was developed by Tiffany and Drobes [26] and has an excellent level of reliability (α = .97). Two factors are obtained: 'Factor 1' is a measure of primary intention and desire to smoke and 'Factor 2' is a desire to smoke in anticipation of relief from negative affect.
(3) Modified Cigarette Evaluation Questionnaire (mCEQ): This 12-item questionnaire uses a seven-point Likert-type scale to assess the reinforcing effects of smoking resulting in five domains including smoking satisfaction, psychological reward, aversion, enjoyment of respiratory tract sensations and craving reduction. [27]
(4) Positive and Negative Affect Scale (PANAS): Participants rate 20 words associated with positive or negative affect using a five-point Likert-type scale. This scale has been shown to have high internal consistency. [48]
(5) Cohen Perceived Stress Scale (PSS): This ten-item questionnaire has been validated in a variety of populations including pregnant and postpartum women. It uses five-items to assess ten questions resulting in two factors negative feelings and inability to handle stress. It has high internal reliability (alpha = 0.75). [49]
(6) Center for Epidemiologic Studies – Depression (CES-D): This form contains ten-items to assess symptoms typically associated with depression. The items are ranked using a four-point Likert-type scale. It has high levels of sensitivity and specificity. [50-51] We have opted to use this item rather pregnancy-specific item to ensure that all participants had comparable data.
(7) Leisure Time Exercise Questionnaire (LTEQ): This questionnaire has four-items to assess strenuous, moderate and mild exercise completed during leisure time and has high reliability (r=0.83, 0.85). [52]
(8) Monitoring Subjective State Scale (mSSS, Day 5 only): This form is identical to the Subjective State Scale (SSS) [53-54] except it also includes variables to assess possible confounders such as coffee consumption. Participants will be asked to mark each rating scale at the point that best describes how they felt over the past 30 minutes.
(9) Salivary Cortisol (Day 5 only): Five saliva samples over the course of the day given that cortisol, a physiological measure of stress released by the hypothalamic-pituitary adrenal axis (HPAA), has a clear diurnal variation. Its peak activity occurs about 8:00 am in the normal sleeper, followed by steady decline until noon. The lowest activity occurs around 8:00 pm. The frequent sampling in the morning will be used to assess free cortisol responses to awakening. Awakening cortisol increase is considered a promising marker of adrenocortical functional status. [55-57]

For Aim 2, the outcomes will be assessed following overnight abstinence: (1) the change in smoking-related symptomatology (items 1-6 above), and (2) response to nicotine (described below). We have a nearly identical timed series to assess nicotine response with success in our ongoing study. All items will be self-administered per direction of study staff via a computer program (REDCap; http://project-redcap.org/) to improve the quality of data (by avoid missing data, illegible data, etc) and limit amount of time spent on data entry and cleaning. The following seven *dependent measures* were selected to assess seven domains of nicotine response: [41]

(1) Physiological Measures: Blood pressure (BP) and heart rate (HR) will be measured using a Dinamap S X/P. This device is an automated device that will read BP and HR simultaneously by placing a blood pressures cuff above the antecubital fossa region of the arm.
(2) Subjective Response: The Subjective State Scale (SSS) questionnaire is comprised of 24-items to assess positive affect, distress and nicotine withdrawal symptoms. [53-54] Participants will rank each item based on how they felt over the past 5-30 minutes (depending on time of last completion) using a seven-point scale ranging from 'not at all' to 'very strong.' This form takes approximately two minutes.
(3) Motor Speed: During the Finger Tapping (FT) task, [58] participants are instructed to use the index finger on the dominant hand to tap a key on a computer as quickly as possible for 30 seconds. This task takes about 1.5 minutes.
(4) Episodic Memory: The Word Recall (WR) task presents 25 concrete nouns individually on a computer screen for three seconds each. Upon completion of the display participants will be given a short distractor of counting out loud from "20" to "0." They then complete a written recall of the words within two minutes. The total number of words correct is each participant's score. [59] This task is designed to assess short-term episodic memory and is estimated to take four minutes.

(5) Working Memory: The 2-Back (2B) task presents stimuli (letters on a computer screen) for two seconds with a half-second between stimuli. Participants are instructed to press a key when a letter was repeated with one intervening letter (i.e. matched a letter two back in the series). The percent correct responding and mean response times are recorded.[60] This task takes approximately six minutes.

(6) Attention/Impulsivity: The Immediate Memory Task (IMT) is a continuous performance task that displays a series of randomly generated 5-digit numbers (e.g., 54983) on a computer monitor for ½ a second at a rate of one per second. The participant is instructed to click a button when the number displayed is identical to the preceding number. Responses to the target stimuli (i.e. correct identification of duplicate numbers) and filler stimuli (i.e. all other random 5-digit) are indicators of response inhibition and continuous attention. The higher the 'catch stimuli' (i.e. a number that differs from the preceding number by only one digit) indicates a more impulsive pattern. [24] It is estimated that this task will take approximately six minutes.

(7) Delayed Memory: The Delayed Memory Task (DMT) is a continuous performance task functions nearly identical to the IMT except a series of distracter sequences (i.e. 12345) are presented between target stimuli. Participants are instructed to ignore these distracters and click the right mouse button in response to target stimuli. Therefore, this task measures delayed memory. [24] It is estimated that this task will take approximately six minutes

(8) Plasma Nicotine (Day 8 only): We will also collect two blood samples (30 cc) through an intravenous catheter one minute prior to nicotine nasal spray and five minutes after. The intravenous catheter will be placed by trained study staff approximately 45 minutes prior to the use of nicotine nasal spray to avoid influencing data collected during the baseline (Time -40 or -1 minutes) assessment. These samples will be frozen and stored to be analyzed for plasma nicotine and sex hormones. These time points were selected to allow for maximum concentrations of nicotine to be captured based on data from our prior work (Allen et al, 2012).

Information on *potential covariates* is collected at the baseline visit. These items include number of cigarettes smoked per day during ad libitum smoking, Fagerstrom Test for Nicotine Dependence (FTND), [61] self-reported number of past quit attempts, past longest quit attempt, motivation to quit smoking, social influences such as partner smoking and sociodemographic variables (Appendix B). Participants in both samples will also complete an adverse events form along with the *Columbia-Suicide Severity Rating Scale* at Screening, Day 0, and Day 8 to assess possible adverse events associated with the study medications. The C-SSRS (Posner et al, 2009) assesses suicidal ideation, given this is listed as an SAE on the Prometrium label. If any item of suicidality is endorsed referrals to the appropriate mental health care will be made.

Sample Size & Power Analysis. In Sample 1, we expect an approximate drop-out rate of 25% based on our current, more burdensome protocol with a sample of 18-40 year-old non-pregnant females. Therefore, we will enroll a total of 112 participants (56 at 12-22-20 weeks and 56 at 32-37 weeks gestation) to ensure we have 84 participants to complete the nicotine exposure lab session. Based on a linear regression model, sample size of 84 will have 81% power to detect a correlation coefficient of 0.30 for a test of the association between Prog and self-reported craving with a two-sided 0.05 significance level. In Sample 2, we expect approximately 25% will drop out and an additional 20% will want to maintain smoking abstinence after overnight abstinence, therefore we will enroll a total of 78 participants to ensure a final sample size of 52. Based on a paired t-test (mean difference in craving outcome), this sample size will have 80% power to detect an effect size of 0.40 with a 0.05 two-sided significance level. These detectable effect sizes are realistic given observations in our current study (e.g. Study #1 in Section D.1, d=0.42) and other published literature (d=0.44 to 0.77). [63-65]

Statistical Analysis. The analyses are intended to test each hypothesis from the specific aims (**Table 3**). Descriptive statistics will be used to summarize the data. For all analyses, assumptions regarding distributions, outliers and covariates (see details in Section E.5 under 'potential covariates') will be evaluated. We will use an average of self-reported levels of craving (primary), urges to smoke, stress and mood collected at Day 0, Day and during the seven-day EMA as the outcomes. For nicotine exposure outcomes, the changes in physiological measures (BP, HR [primary]), subjective measures (Subjective State Scale) and behavioral measures (Finger Tapping, Word Recall, 2 -Back, Immediate and Delayed Memory Task) will be calculated taking levels at specific time points after nicotine nasal spray administration minus the baseline measures at Time -1 (BP, HR) or Time -30 (all measures except BP, HR). The averages of the Day 0 and Day 7 Prog, Allo, and E2/P levels will be used as the independent variables.

For Sample 1, Hypothesis 1: We will investigate the association between Prog and self-reported craving with a linear regression model. A multiple linear regression model will be used to see if the relationship changes with consideration of other baseline measures (including cigarettes/day). Similar analyses will be performed comparing the change in Prog with the change in self-reported craving from Day 0 to Day 7. Hypothesis 2a: For this analysis, we will investigate the association between Prog and self-reported craving after overnight abstinence with a linear regression model. A multiple linear regression model will be used to see if the relationship changes with consideration of other baseline measures, such as cigarettes/day and FTND score. Hypothesis 2b: We will investigate the association between Prog and heart rate outcome at five minutes post nicotine nasal spray using a linear regression model. A multiple linear regression model will be used to see if the relationship changes with consideration of other baseline measures (including cigarettes/day). We will also explore how Prog impacts the nicotine exposure outcomes over time using repeated measures models to account for within-subject correlation. For Hypothesis 1, 2a, and 2b, similar analyses will be performed for the secondary outcomes and the independent variables (Allo, E2/P).

Table 3. Data & Statistical Analysis Comparison of the Two Study Samples

	Sample 1 *Pregnant Women (n=84)*	Sample 2 *Oral Contraceptive (OC) Users (n=52)*
Independent Variable Collection	Both samples complete *identical procedures*: • Serum progesterone, allopregnanolone, and estradiol (Day 0, Day 7)	
Dependent Variable Collection	Both samples complete *identical procedures*: • Aim 1 - Smoking-related symptomatology collected via EMA (Day 1-7) • Aim 2 - Smoking-related symptomatology collected during *ad libitum* smoking (Day 0, Day 7) and after overnight abstinence (Day 8). Nicotine response measures collected during three-hour lab session (Day 8).	
Statistical Analysis	• Aim 1 – Linear regression • Aim 2 – Linear regression	• Aim 1 – Repeated measures model • Aim 2 – Repeated measures model
	Comparison of Clinical Models – Linear regression with propensity scores (baseline characteristics) and clinical model indicator variable	

For Sample 2, Hypothesis 1: We will analyze self-reported craving (primary) using a repeated measures model. A random intercept will be included to model within subject correlation. OC level ("low" or "high" dose), testing period (first or second) and sequence will be included in the model as fixed effects. Hypothesis 2a: For this analysis, we will use self-reported craving, urges to smoke and stress collected after overnight abstinence. As in Hypothesis 1, repeated measures models will be used to compare the self-reported craving outcome between hormonal OC level groups. Hypothesis 2b: Again, repeated measures models will be used to compare the heart rate outcome between hormonal OC level groups at the first time point after baseline. We will also explore the heart rate outcome over time by using a covariance structure to model repeated measures on subjects at each visit. For Hypothesis 1, 2a, and 2b, similar analyses will be performed for the secondary outcomes and the independent variables (Allo, E2/P).

To compare the two study samples we will use all data from Sample 1 and data from the first testing period only for Sample 2. To compare outcomes between Sample 1 and Sample 2, we will use a propensity score [65] to balance the baseline covariates in the two groups. A linear regression model will be used to investigate the association between Prog and the self-reported craving outcome. An indicator variable for group, the propensity score quintile, Prog and a group by Prog level interaction will be included in the model as independent variables. Similar analyses will be conducted for the secondary outcomes and the independent variables (Allo, E2/P)

Strengths & Limitations. This study has several strengths. First, and foremost, is the use of two unique clinical models to investigate the effects of sex hormones on smoking-related symptomatology. This study will be among the first to utilize this type of study design. Further, the cross-over study design used with Sample 2 will limit confounding given that each participant is compared to herself. An additional strength is the detailed measurement of smoking status, sex hormones, smoking-related symptomatology, withdrawal symptoms and nicotine response. Finally, the study team has extensive experience and expertise, which provides unique qualifications to pursue this research project. Three limitations are worth noting. First, we do not have a sample of males for comparison. The relevance for needing this comparison group may be of lesser importance considering that female sex hormones are the main interest. Second, due to the experimental design and the use of a convenience sample, we will have limited generalizability. Finally, given that we will be completing the nicotine exposure session among pregnant smokers who report current daily smoking, it is possible that we will have a biased sample. However, the ethical concerns of administering nicotine to a pregnant woman who has quit smoking, makes this limitation unavoidable. Therefore, to investigate the possibility of a biased sample difference in associations between sex hormones (Prog, Allo, E2/P) with smoking-related symptomatology and nicotine response we will also look at a subgroup of women who reduced their smoking during pregnancy (>50% reduction) versus those who maintained their self-reported pre-pregnancy smoking rate. Specifically, we will include an indicator variable for smoking reduction and the interaction between this indicator and sex

hormone in the models. Despite these limitations, this study provides a unique opportunity and excellent conditions for testing our overall study objective.

Study Team. Our research team has unique breadth and depth of expertise in the following areas: effects of ovarian hormones (menstrual phase, hormone replacement therapy) on smoking cessation in women (Allen, PI); nicotine dependence, smoking cessation, and tobacco toxin exposure (Hatsukami, Co-I); cortisol measurement of stress (al'Absi, Co-I) and mental health and pregnancy (Lewis, Co-I). We will also be utilizing several experts (see Section 2.14 for letters of support): Dr. Neal Benowitz (safety of smoking cessation medications in pregnancy), Dr. Mehmet Sofoglu (sex hormones and addiction), Dr. Virginia Lupo (obstetrician), and Dr. Steve Heishman (neuropsychological nicotine response).

Table 4. Study Timeline

	Year 1							Year 2				Year 3				Year 4				Year 5			
Training Staff & Study Preparation	X	X	X																				
Participant Recruitment			X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X					
Participant Follow-up				X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X				
Data Entry/Cleaning				X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X			
Analysis/Report Writing																		X	X	X	X	X	X

Study Timeline. The timeline is displayed in **Table 4**.

Future Directions. The expected outcomes of this project will be a better understanding of the relationship between sex hormones and smoking-related symptomatology. Upon completing this project, we intend to continue our exploration into the role of sex hormones on smoking behavior. Specifically, if our expectations are confirmed, we will conduct additional studies to investigate the possibility of exogenous progesterone as a treatment for smoking cessation and relapse prevention in several different populations including pregnant women, during the postpartum period and non-pregnant women.

INCLUSION OF WOMEN AND MINORITIES

This study will only be recruiting women because the research question addressed is only relevant to one sex. We are looking specifically at the effects of female sex hormones on smoking behavior and nicotine response in pregnant and oral contraceptive users. The study will use participants recruited from the greater metropolitan area of Minneapolis and St. Paul, via two clinical sites and mass media advertisements. The Twin Cities metropolitan area has a sufficiently large female smoking population to ensure an adequate sample. We have been successful in recruiting similar numbers of women in similar periods of time in previous studies. Estimates indicate that as of 2009 the Minneapolis and St. Paul, Minnesota Twin Cities metro area included approximately 50.5% female and 20.2% of the population in the racial minority groups (Black= 11.0%, Asian = 5.8%, American Indian = 1.2%, other = 4.0%) and the Hispanic population is 6.4%. If minority women do not respond to the advertisements, we will make special efforts to solicit their participation by advertising in local neighborhood newspapers with high minority readership (such as the Southside Pride, Phillips/Powderhorn, and Riverside Editions); by posting flyers in free clinics in the metro area who service minorities (e.g., Community University Healthcare Center, Pilot City); and by identifying contacts in churches, health centers, and community centers with high minority participation and disseminating information regarding the study opportunity; and once we have garnered initial contact with participants we receive multiples word of mouth referrals. Our current study has been successful in recruiting a diverse sample in that of the 212 participants enrolled to date 48% are White, 29% are Black, 9% are more than one race and 14% are other.

INCLUSION OF CHILDREN

Children (under 18 years old) are not included since both menstrual cycle irregularities (e.g., anovulation) and pregnancy complications are more common in adolescents. Further, since this study requires the continuous and regular use of cigarettes, it is unlikely adolescents will meet this criterion. Young women (aged 18-20 years) will be recruited for participation in our study. The trial site is at the University of Minnesota, which has a large undergraduate population. Campus media will be also used to recruit young women. We will also target venues and institutions frequented by young non-college women (bars, festivals, coffee shops, and community events) to ensure the sociodemographic diversity of the youngest component of our participant sample.

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