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Protocol Title:

Menstrual Cycle Phases and Exposure to Environmental Contaminants—Menstrual cycle and Ovulation Study 2 (MOS2)

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Menstrual Cycle Phases and Exposure to Environmental Contaminants-Menstrual Cycle and Ovulation Study 2 (MOS2)

Funding: (primary objective only)
Health Canada Contract 2019-2022

I. Summary/Abstract:

In 2006-2008, 610 premenopausal, spontaneously menstruating women in the Metro Vancouver region participated in a Canadian Institutes of Health Research (CIHR)-funded single-cycle in which they collected first morning urine specimens for estrogen and progesterone metabolites[1]. Following that study, after analyses, we retrieved the remaining urine specimens from the analyzing laboratory (University of Washington). We sorted data so that samples from all those women who were anovulatory by the two combined urinary steroid evaluation methods[2,3], plus from those who were ovulatory with the highest and the lowest urinary hormone values were shipped to Health Canada (HC) via Dr. Warren Foster's laboratory at McMaster University.

Those data on flame retardant contaminants in women's urine have been published[4], but the cycle-phase specific data are still in analysis (personal communication, S Kalyan, 2019). In 2017, HC basic scientists launched applications to HC to fund a similar study to assess flame retardant excretory changes in the same population/locale 10 years later. This application was funded in 2018 at Health Canada with Dr. JC Prior as a collaborator.

Extensive negotiations by CeMCOR and HC scientists ensued about funding the process of obtaining these follow-up specimens. CeMCOR managed to obtain a HC agreement to fund the minimal cost of recruiting, training and obtaining two menstrual phase-specific urine specimens from 250 Metro Vancouver women. But Health Canada provided no additional COVID-related support. We revised our goal to 125 based on available funding.

Because of the lack of a progesterone threshold for ovulation, we will collect one follicular and one luteal/premenstrual urine sample per woman. However, this time we will better characterize the ovulatory cycle using a validated quantitative basal temperature (QBT)method[5,6] that can assess luteal phase length as well as the presence/absence of evidence for ovulation. In addition, we will collect serial salivary progesterone and estradiol values measured by the state-of-the-art sensitive and specific tandem mass spectrometry (LC-MS/MS) methods[7] to use as the gold standard for an ovulatory cycle.

There is increasing evidence that many variables differ across women's two main menstrual cycle phases: follicular and luteal[7-10]. These real and potential differences in metabolism may alter the susceptibility of women to environment exposures, and also could change their urinary elimination. Those are the root reasons for doing this study.

II. Background and Significance

Literature Review

Numerous of women's physical and emotional experiences are likely to be variable across the cycle phases that differ dramatically in their two primary ovarian steroid hormone levels, estradiol (E2) and progesterone(P4)[8]; strong evidence says that these two steroids interact in every tissue as part of a system in which E2 promotes proliferation and P4 stimulates maturation[9]. Evidence now supports different, complementary and essential balancing actions of these two fundamental women's steroids in bone physiology[10] and osteoporosis prevention/treatment[11].

This HC contract provides the opportunity to do a number of discrete investigations given the opportunity provided by the recruitment, training of 250 community dwelling women who are

spontaneously menstruating. They will be collecting data of various kinds across a documented menstrual cycle.

Collected basic data include:

- A comprehensive general, medical, reproductive and lifestyle history (using the Canadian Multicentre Osteoporosis Study [CaMos] questionnaire with which local population-based data from about 200 age-similar local women from the BC CaMos Centre have been collected and archived)[12];
- Menstrual Cycle Diary©[13] data with which we have already collected and published phase-specific data related to "fluid retention"[14] and negative moods[15]; will be collected from the start of one cycle through to the end of flow in the next cycle.
- First morning urines (once/week over six weeks) for environmental contaminants and salivary specimens (three consecutive days per week over about six weeks) to be analyzed for steroid hormones including cortisol, progesterone, estradiol, testosterone and potentially others;
- Plus, the physical measures of height, weight, waist circumference.
- Quantitative basal temperature© (QBT) to additionally assess ovulatory status and determine the luteal phase length.
- Emailed questionnaire about COVID-19 vaccination and COVID-19 illness during the study (approved by Ethics).

This protocol will provide specific literature backgrounds as part of each of the sub-protocols for each of the ancillary studies related to this primary one. The protocol will be complete for the primary outcome, including objectives, research questions, hypotheses and methods and references. However, is only as complete as currently possible for the similar research questions, hypotheses, methods and references for each of the secondary objectives. We will only provide preliminary listings for these sub-study characteristics and methods here. The primary purpose of this protocol is to provide the major outline for the entire scope of all of these projects.

III. Study Aims

Justification:

Objectives

1. Primary outcome:

Research questions

- Will the excretion of breakdown products of flame-retardants over all cycle phases differ between 2019-20 samples and earlier collections from the Metro-Vancouver region?
- Will the excretion of breakdown products of flame-retardants in 2019-20 differ within or between women in the follicular and luteal phases of ovulatory cycles and from those in the premenstrual phase of anovulatory cycles?

Methods

Our aim is to create a study that is convenient, interesting and empowering (because each participant is learning tools that allow her increased understanding of herself). We will provide as much flexibility as is possible (as limited by the time of research staff and our funding) and do entirely non-invasive testing. We will provide each woman with her own study results.
We will recruit women using recognizable branding (graphic artist assistance) through the Centre for Menstrual Cycle and Ovulation Research website (www.cemcor.ca), local news media and public services announcements, with posters (and tear-off tabs) in community centres, coffee

shops, religious establishments, gyms/stores and neighborhood centres, transit ads and paid print/magazine ads as needed.

Inclusion criteria will be minimal to ensure generalizability. They will be individuals ages 19-35 years who have menstruated in the last three months but ideally with reasonably regular cycles of 21-35 days in length (3-5 weeks). Participants will be ineligible if they have taken exogenous hormones (CHC, estrogen or progestin or progesterone) within the last three months.

Education in data collection involves women's learning to keep the Menstrual Cycle Diary[13] (Diary) each evening for about one and a quarter cycles. This instrument, that has a research number and both the actual date and the cycle day, asks women to record actual information about the number of soaked normal sized sanitary products per day, and their assessment on a 0-4 scale of other experiences such as fluid retention, constipation, sleep problems. At the bottom of the Diary are experiences for which there is no true 0; for these participants will record U (for usual) with letters higher and lower for increased or decreased experiences respectively from each woman's self-assessed usual.

The menstrual cycle teaching will occur through access to three already created, online 10-minute videos (https://www.youtube.com/watch?v=6K9LB6afKxE) as well as printed plus personalized instructions.

The bottom of the Diary has reminders for when to collect the (once weekly) first morning urines and the (3-times weekly) first morning salivary levels. There are also spots for a check mark for each collected specimen.

In addition, women will be provided with a digital thermometer and taught to obtain their first morning basal temperature (when they first wake up having done no more activity that getting up to go to the washroom) and to record the temperature in the evening when they completing the rest of the Diary.

Duration of data collection will total about 1.2 menstrual cycles (approximately a continuous record and data collection over 30-38 days). First contact will be any time in the woman's cycle. If she has read and agreed to sign the consent, passes the brief telephone screening questionnaire, she will be sent (via email, fax or postal mail) the Diary and the coordinator will provide the online links to the Menstrual Cycle Diary adapted for MOS2, the written instructions and the videos. The coordinator will also mail a couple of study-specific Diaries and the digital thermometer as well as the consent form.

First in-person visit—Actual data collection will begin early in the next flow (Cycle 1) with an in-person visit in which the signed consent and then the interviewer-administered General and Reproductive CaMos questionnaire completed, and physical measurements obtained (height, weight, waist circumference).

Data collection will continue through to the end of flow in the next cycle (Cycle 2).

Second in-person visit—In Cycle 2 at the end of flow, the participant will bring (on ice packs) her completed Diary forms and the urine and saliva samples that she has kept frozen in her home freezer. We will then ask her a brief final questionnaire about her preferences and knowledge. Also indicating to her that we will share the QBT results of her cycle and results from the whole study in a password protected section of the CeMCOR website.

Handling of research specimens

Note—we will use the urine specimens only for the measurement of environmental contaminants per HC scientists. The initial documentation of ovulation will be by QBT and the final (ultimate) assessment will be from analysis of free P4 in a robust-to-pulsatility progesterone sample from the mid-luteal phase [16].

Urine—Each participant will be provided with 7 30-ml urine containers (chemistry) and an opaque large zip-lock bag and asked to collect first morning urine as early in the Cycle 1 as possible and weekly thereafter including cycle day 1 (first of flow) for Cycle 2 and on the last day of flow before

they return the specimens. At home, they will immediately place each labelled sample container (date and ID#) into a zip-lock bag in their domestic freezer.

We will send the specimen nearest to Cycle 1 days 3-7 (follicular phase specimen) to HC as well as one specimen from the Cycle 1 days 5-10 days after ovulation by QBT (luteal phase specimen). If the cycle is non-ovulatory by QBT, we will send the specimen during the 3rd week of the cycle to HC as a "premenstrual phase" specimen.

Once returned to the lab, all urines will be initially stored for 1-2 weeks frozen at -20ºC in a refrigerator freezer in a locked room in the CeMCOR area. We will then transport them on ice for storage at -70ºC in a locked scientific freezer in a locked room, in a locked floor of a limited access Research Pavilion at the Vancouver General Hospital for 3-6 months. We shipped all urines on dry ice in a single batch to Health Canada in December 2021.

Saliva—Each participant will be provided with 24 5-ml saliva containers (chemistry) to begin collecting a salivary specimen three times weekly from the day after their visit early in Cycle 1 through to the end of flow in Cycle 2. At home, the labelled saliva sample will be stored in a domestic freezer in a provided zip-lock bag. For the final visit, the participant will return to the lab with the frozen specimens on an ice pack. If that final visit is inconvenient for the participant or not possible, a research assistant will pick up the specimens with a cooler and ice packs, and administer the final questionnaire.

Like the urine, the saliva specimens will temporarily (1-2 weeks) be stored in a refrigerator freezer (-20ºC) and then stored at -70ºC for 3-6 months before sample selection to represent the mid-follicular and mid-luteal phases.

Because of the pulsatility of steroid hormone production, 3 specimens from the same early cycle week will be thawed, and from each an identical 0.5 ml aliquot will be obtained and the combination of these three aliquots will create a single reliable mid-follicular phase specimen. We will follow the same process for three specimens after the QBT onset of the luteal phase to provide the mid-luteal phase specimen. We shipped cycle representative specimens for a given woman (~4) in a single batch to Germany (Dresden Lab Service GmbH) for analysis. Given inappropriate quality control and validation, we have instead started collaboration with the MX Chen laboratory in Victoria BC (see NOTE page 6) who now are creating reproducible liquid chromatography tandem mass spectroscopy (LC-MS/MS) assays that we will work with them to validate. The pooled mid-luteal salivary P4 will eventually provide the gold standard for ovulation for this cycle; in the meantime, twice-validated QBT is the gold standard.

We will analyze all saliva from a given woman in the same steroid analysis run by published methods[17].

Local regulatory approvals

As soon as the first installment of Health Canada funding has been received and an account has been set up through University Industry Liaison Organization, we will recruit and hire of a research assistant (RA) with excellent interpersonal skills, knowledge of the menstrual cycle, documented ability to organize a study, recruit participants and complete a project. (This person will ideally have CCRP qualifications and some biology-based degree).

This RA first job will be to apply for UBC Clinical Research Ethics Board approval of the project. In the Informed Consent document we will highlight the primary outcome with the secondary outcomes B, C and D listed as optional and specific consent or protocol not provided for each. Outcomes C and D involve an additional visit and outcome B requires a relatively sensitive physical measurement. We can accomplish all the other secondary outcomes with the data collected and necessary for the primary outcome.

2. **Secondary outcomes:** (NOTE—we will fund these with other-than HC contract monies as they are available from co-investigator sources or specific donations).

A. What are the sensitivity and specificity of Quantitative Basal Temperature (QBT, analyzed by the Mean Temperature Method[5,18]) for the presence of ovulation (yes/no) versus the gold standard mean of 3/week (cycle days 20 onward and at least 3 days before flow) salivary progesterone and estradiol levels as measured by liquid chromatography, tandem mass spectrometry (LC-MS/MS) methods that will eventually be appropriately validated (*currently in progress, 2022/10/20*).*

*We are working with a new pathology/biochemistry collaborator, Dr. Michael X. Chen, UBC Translational Omics Laboratory at Victoria General Hospital, Victoria, BC, to develop a new LC-MS/MS salivary steroid hormone assay for progesterone, estradiol, cortisol, and testosterone. We sent the remaining samples to this new lab; the cortisol method has so far been developed and refined.

Co-Investigators: Drs. Azita Goshtasebi and Sonia Shirin, Dhani Kalidasan MSc

We have previously validated a Quantitative Basal Temperature (QBT) analysis (by two different statistical methods—Maximina© or least mean squares [although that programme needs updating into a currently usable software] and Vollman Mean Temperature method) [5, 16].

This will be more reliable validation than against either the midcycle serum LH peak day [5] (since not every LH peak is detected and not every detected LH peak is followed by ovulation [19]). It will also be more robust than comparison with the urinary 3-fold increase in PdG2 that appears to be relatively over-sensitive for diagnosis of ovulation and also differs in Asia women[20] as well as being without any absolute threshold.

(NOTE—This German lab (Kirschbaum and Gao), although led by a former academic and originally expressing a willingness to collaborate, has subsequently refused to repeat out-of-range values. There were no differences between cycle days 21-24 progesterone levels in their laboratory when the cycle was anovulatory versus normally ovulatory cycles as indicated by twice-validated QBT.

B. Breast maturation sub-study: What is the normal horizontal (medial-lateral) diameter of a breast's areola (R and L) in healthy, ovulatory premenopausal women?

Co-Investigators: Dr. Azita Goshtasebi, Constance Bos

There are fairly standardized breast maturation stages of pubertal developmental (Tanner Breast Stages) [21]. However, there is appears to be confusion in the literature between Tanner 3 and Tanner 5 stages, both of which have a nipple that arises above the plane of the areola ("up-standing"), but, in our hypothesis, differ dramatically in the diameters (superior/inferior and medial/lateral) of the areolae.

Our hypothesis is that progesterone is required to transform the Tanner 3 with its small areola (independent of breast size as we have shown in a transgender woman)[22] into the larger areola (estimate ≥3 cm) of a woman who has established ovulation and is consistently ovulating. Will the areola diameter differ in women with any gravidity or use of CHC in the past versus those without, who may have never ovulated?

We will need to take into account gynecological age, history of irregular cycles/ amenorrhea, age at menarche, and ever pregnancy (not just parity) and/or use of progestin-containing combined hormonal contraceptives—CHC or Progestin-only pills. It is apparent that medroxyprogesterone has similar actions on the areolar size as P4[22] but this action is not known for the various chemistries of androgen-derived and third and fourth generation progestins in versions of CHC.

A woman physician will obtain the bra size (chest circumference and cup size) and assess the areolar diameters bilaterally with the woman lying quietly (covered with her own clothing) in a warm room. We will measure all participating women who agree. We can perform these measurements at any visit, as it is not a phase-specific finding.

If this physical finding were shown to be sensitive and specific for having established ovulatory cycles, it would be an excellent further tool to indicate women's past ovulatory versus ovulatory-

disturbed history and thus their risks for infertility, osteoporosis[11], early cardiovascular disease[23] or breast24/endometrial[25] cancers.

C. Connective tissue sub-study: Do tendon/ligament and muscle strength characteristics differ within-woman in the follicular versus in the luteal phase of a normal-length, normally ovulatory menstrual cycle?

Special Additional Co-Investigators: Dr. Alexander Scott, Dr. Charlotte Waugh, Dr. Jackie Whittaker and Kipling Squier (all trained in physical therapy) in the Centre for Hip Health and Mobility at UBC. With collaboration from Dr. Alexander Scott, UBC Professor of Physical Therapy, Charlotte Waugh, Postdoctoral Fellow at the Centre for Hip Health and Mobility (CHHM), Dr. Jackie Whittaker, UBC Associate Professor of Physical Therapy and Kipling Squier PhD Candidate, we plan to measure patellar tendon biomechanical properties and hand grip by menstrual cycle phase. The CHHM has a MyotonPro®, a hand-held tendon biomechanical testing device[26], and also a knee arthrometer (that assesses laxity of knee ligaments)[27] plus we can use the CaMos hand-grip strength instrument (hydraulic hand dynamometer-JAMAR #30911078) to assess grip strength[28-30]. We will perform this test bilaterally unless there is specific lateral injury (such as known knee osteoarthritis, surgery, joint replacement or severe pain) disqualifying one side.

We would do the first test when the woman comes to learn the menstrual cycle tracking methods (Menstrual Cycle Diary, QBT), complete the interviewer- administered questionnaire and pick up her specimen-collecting materials. This will ideally occur within 4-8 days after the first day of flow=follicular phase). The second testing would be at an additional visit after the participant's temperature has increased and stayed higher for three days.

(The disadvantage of this testing for women is that it requires an extra visit. However, some women will be willing if we are flexible about timing and could potentially accommodate them on a weekend plus provide an additional visit honourarium).

Any data we collect will be new information and can be considered at least pilot data for a postulated positive relationship of endogenous progesterone with tendon, joint and muscle function. These data will complement progesterone's now clear role in bone formation[11].

D. Heart electrical pattern sub-study: Does the electrocardiographic rate-corrected Q to T interval (QTc) differ within-woman between the follicular and the luteal phases of the ovulatory menstrual cycle? Does it correlate with mean 3-day salivary E2 or P4 or some ratio of these? Does the QTc in the luteal phase relate to luteal phase length (via QBT).

Special Additional Co-Investigators are Dr. Andrew Krahn (head of UBC cardiology and an international expert in genetic Long QTc Syndrome[31] and co-investigators in his laboratory.

Hypotheses:

- The luteal phase QTc will shorter than the mid-follicular phase QTc.
- P4 will correlate negatively with QTc length.
- E2 will correlate positively with QTc length.
- The mean luteal phase length will be the best predictor of QTc.

Using a CeMCOR donation, we subsequently purchased the remote electrocardiogram 6-lead testing device, **KardiaMobile 6L©**, that was administered twice/cycle by the MOS2 study coordinator in women volunteering for this substudy. We have coordinated with research personnel working with Dr. Krahn to evaluate the ECG data sent anonymously to them. They have assessed the heart-rate adjusted QTc for comparison with the phases of the MOS2 cycles. We are now collaborating with them in comparison of differences between follicular and luteal phases in women with normally ovulatory cycles versus in women in whom data were collected when they were not in a luteal phase (with short luteal phase and anovulatory cycles). Assessing the hypothesized relationships of QTc with salivary hormone values will require further work by the Chen lab and each hormone's validation. Cardiology researchers will be provided with the primary

data, evaluate it for rate-adjust QTc by cycle phase before being provided with the cycle phase documentation. They will also write up and be first authors on the publication on which appropriate MOS2 collaborators will become co-authors.
This sub-study will require that participants come for an extra visit when their temperature has increased in addition to the two required for the base study. They would receive an additional honourarium for that visit. All testing will be non-invasive.

E. Do women's menstrual cycle, life experiences and estradiol levels differ in ovulatory and anovulatory cycles (between and not within-woman)?
Research questions:
- Are there reliable between-women differences in the Menstrual Cycle Diary© phase-specific records for women who have an ovulatory and women who have an anovulatory cycle?
- Are there differences in the temporal patterns of stretchy of cervical mucus, or the pattern of breast front/anterior and lateral/axillary tenderness between ovulatory and anovulatory cycles?

Hypotheses:
- Anovulatory cycles will show midcycle stretchy mucus that does not totally disappear or that increases prior to flow.
- Ovulatory cycles will show mild axillary breast tenderness (without front breast tenderness) in the week prior to starting flow; it will have no stretchy mucus before flow.

F. Do the menstrual cycle experiences by Menstrual Cycle Diary© of women with diagnosed PCOS or diagnosed endometriosis differ from those with similar length cycles without those diagnoses?
We do not know how many of the 125 women with PCOS or endometriosis we will enroll in MOS2. If we have at least 10 of each, it will provide us with the opportunity to describe the salivary hormone patterns in comparison with normally ovulatory cycles. This would require us to find funding for analysis of more salivary samples in those women with PCOS or endometriosis and two similar ovulatory controls for each case than we have funds to analyze in the basic study.

G. Women's Preferences for Menstrual Cycle and Ovulation Monitoring Methods
Ascertain women's attitudes toward, acceptance of and suggestions for improvement of the various methods for teaching and data collection we have used in this cycle-plus one week-long study:
- Collection of daily Menstrual Cycle Diary© data;
- Digital thermometer measurement (first morning) and recording (in the evening) of basal temperatures;
- Collection of weekly first morning urine specimens; and
- Collection of morning salivary samples three times a week.

H. This sub-study became not possible during the pandemic.

I. This sub-study became not possible during the pandemic.

J. To collect early follicular phase urine and saliva samples immediately following a hormonally documented cycle for use in developing a future non-invasive test of ovulation.
Hypotheses:
We will be able to discover and reliably measure some directly progesterone-related compound or substance that persists after the progesterone levels have already decreased during the flow of the next cycle. We will figure out a way that is non-invasive and inexpensive to document the ovulatory status (ovulatory/anovulatory) of the preceding cycle.

We could use this this test, if convenient and sufficiently inexpensive, over many cycles to help women decide about whether or not to seek current pregnancy or to wait until a more personally appropriate times in their work/social lives. Alternatively, it could help to indicate whether or not there is a need to evaluate and correct risks for osteoporosis, early heart attacks and endometrial and breast cancers.

Materials to be used: The Cycle 2 urines (ideally 2 specimens) and saliva collected 3 days during the first week of the new cycle will be stored at -70 for use in a validation study of whatever ovulation-detecting method is being assessed.

K. To determine whether living within the SARS-CoV-2 Pandemic altered the expected pattern of menstrual cycle lengths and ovulatory characteristics

a. Were there differences in cycle lengths and ovulatory characteristics in ***comparison with age-appropriate women in Menstruation Ovulation Study (MOS)*** conducted in 2006-7 and who completed the same CaMos questionnaire, had the same physical measurements (height, weight, waist circumference), and recorded in the same Menstrual Cycle Diary as MOS2? We recognize that ovulation was assessed differently in the two cohorts; in MOS it was assessed using a three-fold increase in urinary excretion of pregnanediol glucuronide (PdG) from the follicular to the luteal phases rather than by Quantitative Basal Temperature© (QBT) as in MOS2 followed by salivary progesterone back-up. We ***hypothesized*** that MOS2 women during the pandemic versus MOS would have more short luteal phase and anovulatory cycles associated with increased Diary-documented negative moods, sleep problems and outside stresses. (A*bstract podium-presented Endocrine Society Conference 2022, and published in J. Endoc Soc* 2022;6).

b. Comparing the women **within MOS2** having normal cycle lengths with those having cycles longer than 21-35-day length cycles to see whether changes that we ***hypothesized*** are related to the duress of the pandemic (such as increased feelings of frustration, depression and anxiety [negative moods], increased sleep problems and "outside stresses") are important. In addition, we will compare **within MOS2** those who have normally ovulatory menstrual cycles with those having ovulatory disturbances (short luteal phase, and anovulatory cycles) again making the same hypotheses as above about differences between those with normally ovulatory versus those with ovulatory disturbed menstrual cycles.

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