Protocol Title:

Association between menstrual cycle phases and exposure to environmental contaminants—Menstrual Cycle and Ovulation Study 2 (MOS2)

Investigators:

Principal Investigator	Jerilynn C. Prior BA MD FRCPC Scientific Director, Centre for Menstrual Cycle and Ovulation Research (CeMCOR); Professor of Endocrinology, University of British Columbia (UBC)
Co-Investigator	Azita Goshtasebi MD, MPH, PhD, CCRP
Co-Investigator	Dharani Kalidasan MSc
Co-Investigator	Sonia Shirin, MD, PhD
Co-Investigators	Dr. Andrew Krahn, Dr. Tara Sedlak, Dr. Karin Humphries
Co-Investigators	Dr. Alexander Scott, Dr. Charlotte Waugh, Dr. Jackie Whittaker

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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. 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 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 data include:

- A comprehensive general, medical, reproductive and lifestyle history (using the Canadian Multicentre Osteoporosis Study [cam*os*] questionnaire with which local population-based data from about 200 age-similar women 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];
- First morning urine and salivary specimens;
- Plus, the physical measures of height, weight, waist circumference and automated mean of 5 blood pressure/pulse assessments (BPTru®).

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 with be complete for the primary outcome, including objectives, research questions, hypotheses and methods and references. However, it will be 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, and paid print/magazine ads as needed.

Inclusion criteria will be minimal to ensure generalizability. They will be biological women 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, levonorgestrel-releasing IUD, estrogen or progestin or progesterone) within the last six 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 that she will be sent (via email, fax or postal mail), and passes the brief telephone screening questionnaire, 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 questions will be obtained, the interviewer-administered General and Reproductive CaM*os* questionnaire completed and physical measurements obtained (height, weight, waist circumference, blood pressure and heart rate). 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 her 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 6 30-ml urine containers (*chemistry*) 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 day they return the specimens. At home, they will immediately place each labelled container 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 the Cycle 1 days 7-12 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 will ship urines on dry ice in a single batch to Health Canada in August of 2020.

Saliva—Each participant will be provided with 20 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. For the final visit, the participant will return to the lab with the specimens on ice packs. 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 will ship cycle representative specimens for a given

woman (~4) in a single batch to Germany (the Kirschbaum lab) for analysis. The pooled mid-luteal salivary P4 will provide the ultimate gold standard for ovulation for this cycle.
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 not provided for each. Outcomes B and C involve an additional visit and outcome D 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 the Kirschbaum new steroid mass spectrometry methods[7]?**

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 PdG[2] 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—Current there is, as yet, no P4 Ovulatory Threshold determined with the new salivary method for P4 (per conversation with C. Kirschbaum, 3/2019). Therefore, with different women and in a different study (with the analysis of saliva samples provided free by Kirschbaum, agreement via email March 2019) we will validate a threshold level of salivary P4 the new LC-MS salivary P4 method[7]. We will assess optimal sensitivity and specificity against evidence of ovulation by follicle rupture as documented with serial midcycle vaginal ultrasounds in spontaneously menstruating women in collaboration with Dr. Anthony Cheung of Fertility with Grace clinic and Dr. Sheila Pride.)

B. 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, and likely also Dr. Jackie Whittaker (all trained in physical therapy) in the Centre for Hip Health and Mobility at UBC.

With collaboration from Dr. Alexander Scott, UBC Associate Professor of Physical Therapy and Charlotte Waugh, Post-Doctoral Fellow at the Centre for Hip Health and Mobility (CHHM), and new UBC faculty member Dr. Jackie Whittaker, we plan to measure patellar tendon biomechanical properties and hand grip by menstrual cycle phase. The CHHM has a MyotonPro® hand-held tendon biomechanical testing device[21], and also a knee arthrometer (that assesses laxity of knee ligaments)[22] plus we can use the CaMOS hand-grip strength instrument (hydraulic hand dynamometer-JAMAR #30911078) to assess grip strength[23-25]. 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].

C. 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?

Special Additional Co-Investigators—Dr. Andrew Krahn (head of UBC cardiology and an international expert in Long QTc Syndrome [26]. Dr. Tara Sedlak who has reviewed ovarian steroids and QTc [27] and Christopher Chang who ideally will be the coordinator of this sub-study.

Hypotheses:

- The luteal phase QTc will shorter than the follicular phase QTc.
- P4 will correlate negatively with QTc length.
- E2 will correlate positively with QTc length.

This would require us to borrow the appropriate equipment (to be determined) and to collaborate with experts in cardiology. Ideally a cardiology fellow or resident this sub-study will run this study and coordinate with other MOS 2 data collection, collate with the hormonal and menstrual cycle data and write up and be first author on the publication.

This sub-study will require that participants come for an extra visit when their temperature has increased in addition to the one-two required for the base study. They would receive an additional honourarium for that visit. All testing will be non-invasive.

D. What is the normal horizontal diameter of a breast's areola (R and L) **in healthy, ovulatory premenopausal women**?

Co-Investigators: Dr. Azita Goshtasebi,

There are fairly standardized breast maturation stages of pubertal developmental (Tanner Breast Stages)[28]. 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)[29] 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[29] but this action is not known for the various chemistries of progestins in versions of CHC.

A woman physician will assess the areolar diameters bilaterally in a warm room for 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[30] or breast[31]/endometrial[32] cancers.

E. Do women's menstrual cycle and life experiences and estradiol levels differ in ovulatory and anovulatory cycles?

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 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 and 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?

I do not know how many of the 250 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 and recording (in the evening) of first morning basal temperatures;
- collection of weekly first morning urine specimens; and
- collection of morning salivary samples three times a week.

H. Do women with Type 1 Diabetes Mellitus (T1DM) and reasonable metabolic control (HbA1c 5-8) **have a similar point prevalence of ovulation, luteal phase lengths, salivary progesterone levels and mean increase in quantitative basal temperature as women without T1DM in this study?**

Women with DM (of T1 or T2) have higher rates of early myocardial infarctions than do women without DM. Those with DM also are at higher risk of normal cardiac output heart failure those who are similar but without DM.
As a "wild" ***hypothesis***, given the apparently positive roles that progesterone apparently plays in the cardiovascular system, I would guess that progesterone levels are lower and/or luteal phase lengths by QBT data are shorter in women with T1DM.

I. In women with T1DM who have an ovulatory cycle, do fasting capillary glucose levels, insulin requirements and hypoglycemia experiences vary across the hormonal phases of the cycle?

Special Additional Co-Investigators: Dr. Tricia Tang, endocrine psychologist

For secondary objectives **H** and **I** we plan to intentionally recruit women for MOS2 who have TIDM in endocrine offices and online [Canadian Diabetes Association, CeMCOR website etc.].
We will create and administer an additional questionnaire that assesses their HbA1c, insulin therapy patterns, vision, renal function, neuropathy and past experiences of pregnancy, hospitalizations, and ketoacidosis. This questionnaire will also ask several open ended questions about their previous experiences with diabetes control related to their menstrual cycles. We will also administer the ***Diabetes Distress*** questionnaire (in collaboration with Dr. Tricia Tang, Associate Professor Endocrinology. In addition, we will ask them to provide their daily capillary glucose and their insulin therapy records during the study).

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 make a decision about whether or not to seek current pregnancy or to wait until 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.

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