Title

EARLY-PREG: Preconception, longitudinal, bidirectional, and counterfactual open cohort of women trying to conceive for the characterization of maternal–embryonic molecular crosstalk during the first weeks after conception.

Running Title:

Ultra-early pregnancy preconception cohort protocol.

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Abstract

STUDY QUESTIONS

Embryo–maternal crosstalk involves intense and complex molecular exchange between the early embryo and the mother. This interaction begins after fertilization but is poorly understood before implantation. EARLY-PREG is a preconception open cohort that aims to research the proteome signature of maternal–embryonic communication interrogating a growing biorepository of maternal fluids and tissues collected during the first two weeks of a natural conception.

WHAT IS KNOWN ALREADY

To understand the mother-embryo communication in humans, *in vivo*, *ex vivo*, and *in vitro* biological models have been developed to simulate certain phases of the implantation process and its related events. Mass spectrometry for proteomic profiling in longitudinal cohort studies has emerged as a modern method for understanding complex biological processes directly *in vivo*. Proteomic profiles have been investigated during pregnancy in women from the 8th week of gestation, but not before.

STUDY DESIGN, SIZE, DURATION

In three waves of recruitment from 2017 to 2024 so far, healthy women seeking to conceive have been recruited in Concepcion, Chile. Participants completed a survey with health, lifestyle, and sociodemographic data and their menstrual cycles with ovulation and fertile window ascertainment (ultrasound, fertility monitor, and/or LH strips) were followed prospectively until pregnancy was achieved or for a maximum of six consecutive cycles. The follow-up of every cycle includes systematic day-by-day sampling of cervicovaginal fluid (CVF), urine, saliva, and blood. In addition, a cervicovaginal brushing between days 12th and 14th post-ovulation is collected. The day of ovulation and other time windows of interest in each cycle is retrospectively corrected by hormonal curves (LH, oestradiol, progesterone and beta-hCG) on stored urine samples. Blood

samples and cervical brushings are collected on day 21 post-ovulation and in each trimester of pregnancy.

PARTICIPANTS, MATERIALS, SETTING, METHODS

At the present, 1,183 women has been contacted, of whom 223 met all eligibility requirements; 129 participants who were trying to conceive completed the protocol for at least one complete cycle, of whom 35 participants achieved full-term pregnancies and 20 had early pregnancy losses; 40 abstinent and 5 sterilized women entered and completed the protocol. A total of 292 menstrual cycles have been fully documented and sampled; 238 cycles have been classified as non-conception cycles, and 54 as conception cycles. The biorepository comprehends maternal fluids and tissues throughout the first 2 weeks after ovulation and during early pregnancy for all 292 cycles so far. Biospecimen collection compliance has been high. The study is currently analysing the proteome of CVF samples from women who conceived and their counterfactual non-conception cycles to characterize the proteome signature of early pregnancy in this maternal fluid. A fourth recruitment wave to characterize changes of the immunophenotype in maternal peripheral blood mononuclear cells (PBMC) during ultra-early pregnancy is planned to begin during 2025.

STUDY FUNDING/COMPETING INTEREST

The EARLY-PREG preconception open cohort has been supported by multiple research grants awarded by the FISAR Foundation (www.fisachile.org). The pilot study and the first wave of recruitment was supported by grants #MEL109112011 and #MEL109112011R4 awarded to E.S.K, C.V. and J.F.S. The second wave of recruitment was supported by supplemental grants #MEL109112011R5 and #MEL131032017R1 awarded to E.S.K. The third wave was supported by grant #MEL205062018 awarded to E.S.K and M.H. Current funding for the design of the fourth recruitment wave and mass spectrometry research on maternal CVF is supported by grant No. REH042024-01 awarded to M.H., G.N., and E.S.K. As a senior scientist, E.S.K. has served as an honorary research consultant and/or reviewer on research applications for the FISAR Foundation since 2015. No other conflicts of interest are reported.

TRIAL REGISTRATION NUMBER

Not applicable.

TRIAL REGISTRATION DATE

Not applicable.

DATE OF FIRST PATIENT'S ENROLMENT

Not applicable.

WHAT DOES THIS MEAN FOR PATIENTS?

Previous studies have shown that a successful pregnancy relies on an exchange of signals between the embryo and the mother. This complex communication begins just after the sperm meets the egg—before the embryo attaches to the uterus—during a stage called the preimplantation period. Although it plays a critical role in preparing the maternal body for pregnancy, the mechanisms behind this communication are still not fully understood.

The unique “dialogue” between the embryo and the mother is a two-way exchange known as embryo–maternal crosstalk. Research has suggested that this interaction, mediated by molecules in the uterine fluid, helps the embryo implant properly and modulates the maternal immune system to ensure acceptance of the embryo.

To date, most of what we know about this process comes from animal studies and laboratory experiments, which may not accurately reflect what happens in spontaneous human pregnancies. To better understand this embryo–maternal crosstalk, we designed the EARLY-PREG preconception open cohort. This study follows healthy women who are trying to become pregnant, following them from before conception through the first two weeks of a natural conception and continuing into pregnancy and childbirth. It includes the daily collection of maternal body fluid samples—such as saliva, urine, blood, and cervicovaginal fluid—during key phases of the menstrual cycle. The samples are processed, preserved and stored in a biorepository for research with *omic* techniques.

Using advanced techniques to study proteins through mass spectrometry in these fluids, EARLY-PREG aims to explain how pregnancy begins and why some pregnancies succeed while others do not. This knowledge may reveal key biological clues to improve fertility care, assisted reproduction and support early pregnancy health, and deepen our understanding of how life begins.

Introduction

Embryo–maternal crosstalk is critical for the establishment of a successful pregnancy. The period after fertilization and before implantation, known as the *preimplantation* period, was previously thought to be silent. However, evidence suggests that this communication lays the groundwork for pregnancy (Barnea *et al.*, 2012). During this period, intense and complex molecular exchange occurs between the early embryo and the maternal endometrium (Lane *et al.*, 2014). At present, researchers have not fully characterized this interaction (Lynch *et al.*, 2006; Benagiano *et al.*, 2023a, 2023b). This embryo–mother dialogue involves a complex interaction between the embryo and the mother starting at fertilization, termed the ultra-early stages of pregnancy (Hill, 2001; Barnea, 2004; Singh *et al.*, 2011). Through this communication, the embryo can modulate the maternal response during key phases, such as apposition, adhesion, invasion, and decidualization in the endometrium, through extensive exchange of signals (Somerset *et al.*, 2004; Barnea, 2007; Lédée *et al.*, 2007; Fazeli, 2011). The success of each of these events is essential for advancing to the next stage. However, the regulatory mechanisms that govern this embryo–mother interaction are still not completely understood (Cha *et al.*, 2012).

During the preimplantation period, uterine fluid potentially facilitates the transfer of vital information between mother and embryo (Zhang *et al.*, 2017). In this context, the presence of molecules involved in the interaction between the embryo and the maternal environment, such as EPF (early pregnancy factor) (Morton *et al.*, 1982), PIF (preimplantation factor) (Barnea *et al.*, 1994) and PAF (embryo-derived platelet-activating factor), has been reported (O'Neill, 1992, 2005). EPF and PIF are involved in the modulation of the maternal immune response, which is crucial for the viability of early embryos and implantation (Nahhas and Barnea, 1990; Morton, 1998; Barnea *et al.*, 2012, 2014; Santos *et al.*, 2021). It has been postulated that EPF originates from the mother during the preimplantation period and shifts to embryonic origin after implantation (Nahhas and Barnea, 1990; Morton, 1998; Barnea *et al.*, 2012, 2014; Santos *et al.*, 2021). PIF is secreted by the embryo, and it has recently been suggested that it enhances the decidualization process and the production of endometrial factors that limit trophoblastic invasion (Santos *et al.*, 2021). Moreover, a third factor known as PAF has been described; its activity as an embryotrophin would mediate the transport of the embryo to the uterus (O'Neill, 1992, 2005). In addition, recent studies have shed light on new forms of embryo–maternal communication via the delivery and/or exchange of extracellular vesicles (EVs) and mobile RNAs. Furthermore, extracellular vesicles play a role in generating an immunosuppressed environment during embryo–mother interactions (Simon *et al.*, 2018; Kaminski *et al.*, 2019; Das and Kale, 2020), allowing the embryo to counteract the maternal immune response (Burnett and Nowak, 2016). Nevertheless, further investigation is needed to fully understand the embryo–mother dialogue.

Embryo–mother communication in humans is a unique and challenging-to-model process. Commonly, *in vivo*, *ex vivo*, and *in vitro* models have been developed to simulate certain phases of the implantation process and its related events (Dimova *et al.*, 2024). Despite advances in experimental strategies, understanding the mechanisms of embryo–maternal crosstalk faces inherent limitations that affect how well their findings can be extrapolated to humans. Notably, variation between humans and animal models has been observed in decidualization, the timing

and type of implantation, attachment, trophoblast subpopulations, and the depth of extravillous trophoblast invasion (Muter *et al.*, 2023; Dimova *et al.*, 2024). Moreover, *in vitro* models designed to replicate the implantation process have been developed using immortalized cell lines or cancer cells, which may not accurately represent normal physiological conditions (Shibata *et al.*, 2024). Thus, the maternal–embryonic dialogue during early stages requires further research using novel strategies tailored to human physiology.

In recent years, proteomic profiling in longitudinal cohort studies has emerged as a modern strategy for understanding complex biological processes (Romero *et al.*, 2017; Aghaeepour *et al.*, 2018; Yohannes *et al.*, 2022). These advancements have been driven by innovations in mass spectrometry (MS), which have allowed the integration of proteomic data with pathway analysis. Proteomics has proven to be a reliable and highly sensitive method for biological research (Cox and Mann, 2011; Geyer *et al.*, 2017), particularly because of its ability to analyse biological fluids (Bader *et al.*, 2023). Proteomic profiling, combined with longitudinal sampling of biological fluids, is a non-invasive alternative to monitoring processes, such as the embryo–mother molecular exchange of signals.

Longitudinal proteomic profiles during pregnancy in healthy women have been investigated in a few cohort studies, starting from the 8th week of gestation. Although these studies have performed proteomic analyses during pregnancy, to date, no studies have characterized the first two weeks of gestation from the time of conception (Romero *et al.*, 2017; Hedman *et al.*, 2020). Therefore, the early stages of this interaction are not yet fully understood, especially without altering, manipulating, or intervening in its natural environment. To address this gap, we proposed the design of a preconception, longitudinal, bidirectional, and counterfactual cohort study. EARLY-PREG aims to research maternal–embryonic communication during the first two weeks after conception through a biorepository of biological maternal fluids and tissues that will be studied via high-throughput proteomic techniques. These samples are collected frequently throughout the preimplantation period until the end of pregnancy. This cohort profile outlines the study design and provides an overview of the data collected so far, including baseline information of participants, outcomes, characteristics of menstrual cycles, biological samples, and protocol performance.

Cohort description

Study design

The preconception study employs an open longitudinal, bidirectional, and counterfactual cohort design, which is prospective when successive samples are collected and retrospective for analysis once pregnancy is confirmed. In addition, this design allows each participant to serve as their own control (counterfactual), enabling the comparison of non-conception and conception cycles while minimizing interindividual variability, as detailed in Figure 1.

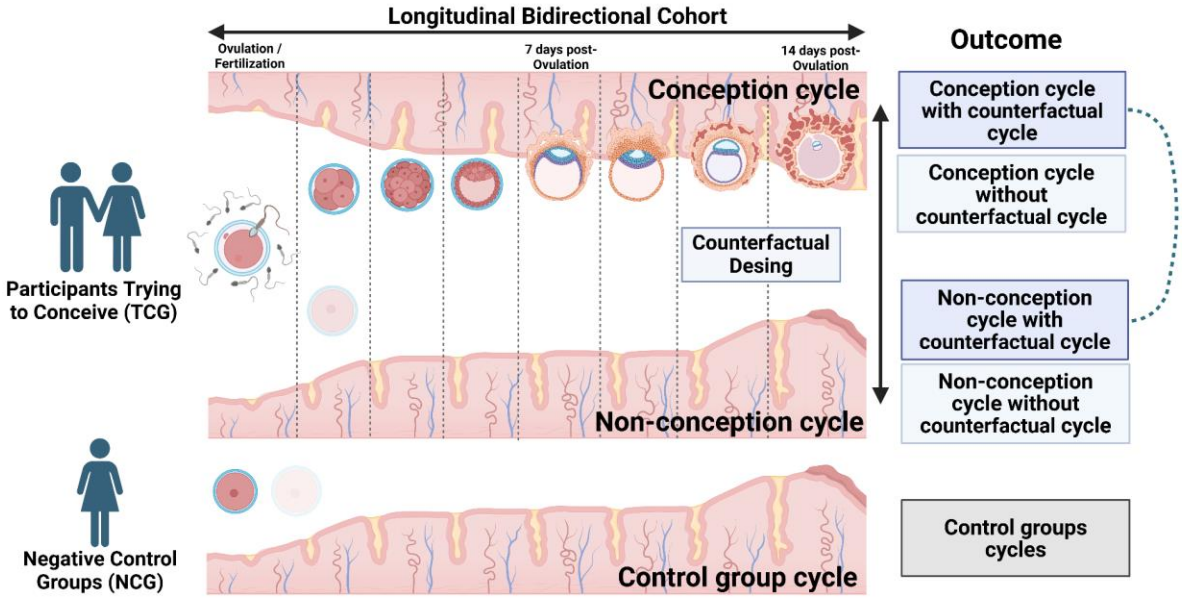


Figure 1. EARLY-PREG cohort design.
The figure represents our two main study groups: Participants trying to conceive (TCG) and a negative control group (NCG) and illustrates the endometrium in three distinct scenarios, along with the anticipated outcomes. Created in BioRender. Nova Lamperti, E. (2025) https://BioRender.com/y8d2fpw

Bidirectional longitudinal design

Given the objective of the study, a longitudinal prospective follow-up with repeated measures was established, beginning prior to the onset of pregnancy in the study participants. Measurements were planned to continue throughout the menstrual cycles while couples sought pregnancy, during pregnancy, and until delivery. A bidirectional or two-way component was incorporated into longitudinal follow-up for sampling. Consequently, the occurrence of pregnancy serves as a milestone for the retrospective hormonal analysis (LH, oestradiol, progesterone and beta-hCG) of the stored samples to correct and synchronise specific time periods of interest, such as the fertile window in general and the preimplantation and implantation windows in particular.

Counterfactual approach

Counterfactual models use hypothetical scenarios to estimate the outcomes that individuals would have experienced if they had been exposed to a different intervention than the one they received. The counterfactual design of EARLY-PREG enables a comparison of the systemic and localized effects of embryo appearance and implantation in an individual (the factual) against the physiological state of the same individual without experiencing pregnancy (the counterfactual).

Comparison of an early-in-life event with a late-in-life event was restricted to a range of up to six menstrual cycles from the start of active pregnancy seeking (Eichler *et al.*, 2016).

Setting and recruitment

The EARLY-PREG cohort consists of healthy couples trying to conceive and women not trying to conceive. Since 2017, a total of 1,183 women were invited to participate through word of mouth, invitations from gynaecologists, and midwives in private practices, public hospitals, and Family Health Centres (CESFAMs) in the Biobio region of Chile.

Women who are interested in participating in the study went through a pre-selection phase to receive general information about the study in a consultation room at the *Sanatorio Aleman* Clinic in Concepcion, Chile. Potential participants were subsequently interviewed by the midwife to address any questions from the recruitment team and to request participation in the study from the women and their partners (in the case of couples trying to conceive), which was formalized through corresponding informed consent.

Selection Criteria

Eligibility requirements common to all women include being between 18 and 40 years of age, not being pregnant, normal colposcopy, body mass index (BMI) between 18 and 29, having regular menstrual cycles (21–35 days), and absence of chronic diseases (hypertension, diabetes mellitus, cancer, depression, personality disorder, thyroid pathology, polycystic ovary syndrome, or hyperprolactinemia). Pregnant women and those with a history of alcoholism, infertility treatment, endometriosis, pelvic inflammatory disease or pelvic surgery, or allergy to latex or silicone are excluded. In addition, eligibility criteria have been established for the partners of women trying to conceive. Women were included if their partners were males between 18 and 40 years old, without pathologies (diabetes mellitus, depression, personality disorder, or cancer), were not taking chronic medication, without erectile disorders, BMI between 18 and 29.9, moderate alcohol use, and no recreational drug use. The exclusion criteria were working in contact with pesticides, a history of erectile dysfunction, mumps in adulthood, chronic diseases, and psychological problems.

Study groups

The figure 2 shows the recruitment flowchart for the entire EARLY-PREG cohort. The women enrolled so far in the study are divided into two main categories: women trying to conceive (TCG) and women who are not seeking pregnancy, which represents a negative control group (NCG). The negative control group is further categorized into two subgroups: women practising abstinence and women who were sterilized. Within the sterilized women, there are additional subdivisions: women with sexual abstinence and women without sexual abstinence. These negative control groups will be used to establish proteome libraries under initial conditions differ from those of conception or ultra-early pregnancy stage.

Throughout the study, participants are monitored during their cycles through the collection of various biological samples, including blood, urine, saliva, cervicovaginal fluid (CVF), and cervicovaginal brushing, for both groups. The NCG is followed for a single complete cycle, whereas the TCG is monitored over multiple cycles (up to six cycles), with serial collection of biological samples. If a pregnancy is identified in the TCG study group, monitoring continues until the pregnancy concludes.

A total of 1,183 women have been contacted so far and recruited during the screening phase. A total of 223 participants were selected and agreed to participate in the study through consent forms and enrolment in the protocol. During the study, 49 participants withdrew from the protocol (21.97%) (Figure 2). The main reasons for withdrawal were classified as personal or medical. A total of 292 menstrual cycles from 174 women have been monitored so far, 35 of whom achieved full-term pregnancies.

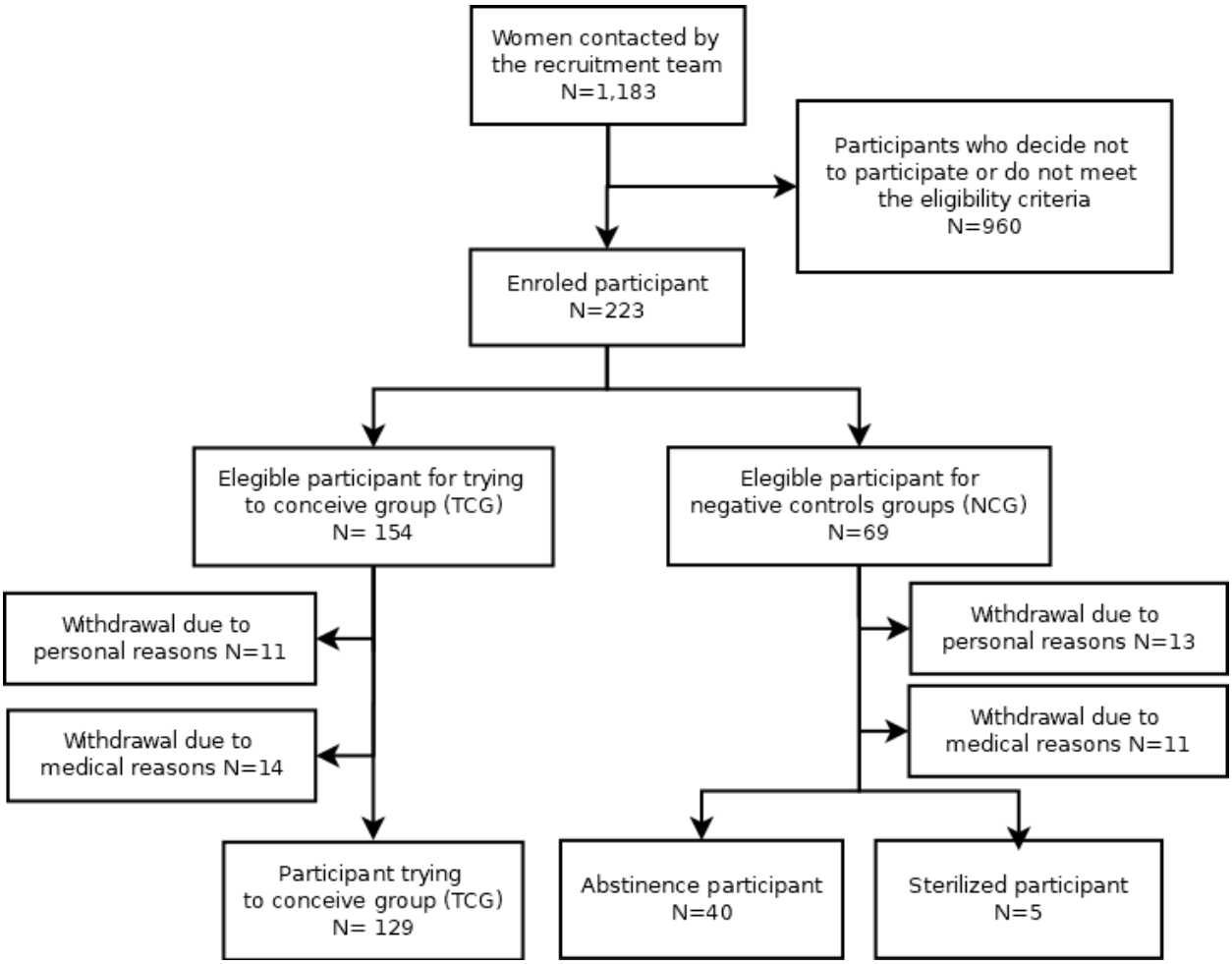


Figure 2. Flow chart of the EARLY-PREG open cohort design.

Patient and public involvement

The study participants were not involved in the design, conduct, reporting, or dissemination of our research. They were not directly involved in the study's design, the development of the research questions and outcome measures, or the recruitment and execution.

The protocol was reviewed and approved by the ethics committee of the Servicio de Salud Concepcion, Biobio region, Chile (CODE: 17-03-06). The committee's role was to advise on the ethical and operational aspects of the study.

Questionnaire survey

At enrolment, both female and male participants complete a baseline questionnaire survey, which is conducted and recorded by a midwife from the research team. The questionnaire includes sections for female, male, and sociodemographic information. Both women and their male partners (when applicable) are asked about their health (e.g., BMI, allergies, chronic diseases, and use of chronic medications) and lifestyle (e.g., smoking habits, alcohol consumption, drug use, coffee drinking habits, and physical activities). Additionally, women are asked about contraceptive use, reproductive and general obstetric history, and dietary habits. The sociodemographic section includes questions on marital status, age, educational level, occupation, and income.

Methods

Main Outcomes

The two main outcomes in the cohort are menstrual cycles in which conception is achieved and menstrual cycles in which conception is not achieved. Below are their respective clinical definitions:

- **Conception cycle** refers to the cycle in which the ovum is fertilized, leading to pregnancy. The conception cycle requires beta-hCG levels above the clinical threshold for a positive pregnancy test, which is determined in peripheral venous blood on the 14th day post-ovulation.

- **Non-conception cycle** refers to a menstrual cycle in which pregnancy does not occur. When referring to the same individual, it is, by definition, considered the counterfactual to the conception cycle described above. The non-conception cycle requires a clinically negative beta-hCG test, which is determined in peripheral venous blood on the 14th day post-ovulation. This cycle is characterized by the absence of a clinical pregnancy.

- **Negative control cycle** refers to cycles in which conception did not occur because of abstinence and/or sterilization.

Recruitment, biospecimen collection and follow-up

Recruitment phase

Approval by the ethics committee of the Servicio de Salud de Concepcion was obtained prior to the recruitment stage. Participants were recruited through medical consultations, health centres, and referrals from doctors and midwives. An initial interview was conducted to explain the details of the study and assess eligibility based on the inclusion and exclusion criteria. The study was discussed in a private room at the respective health centres, and informed consent was obtained.

Each participant undergoes a clinical evaluation, including a physical examination and speculoscopy. A Papanicolaou test was administered to women who had not recently undergone one to screen for cervical cancer. Instructions for cycle follow-up were provided both orally and in writing.

Data collection

Personal, clinical, and follow-up data have been collected in individual files. Each participant is anonymized and assigned a unique code to ensure data confidentiality.

Additionally, the midwives responsible for recruitment and follow-up transfer the information from the physical records to a digital document on password-protected computers belonging to MELISA Institute.

The collected samples are registered in a digital inventory stored on password-protected computers belonging to MELISA Institute. Each sample is registered using the unique code assigned to each participant to ensure confidentiality.

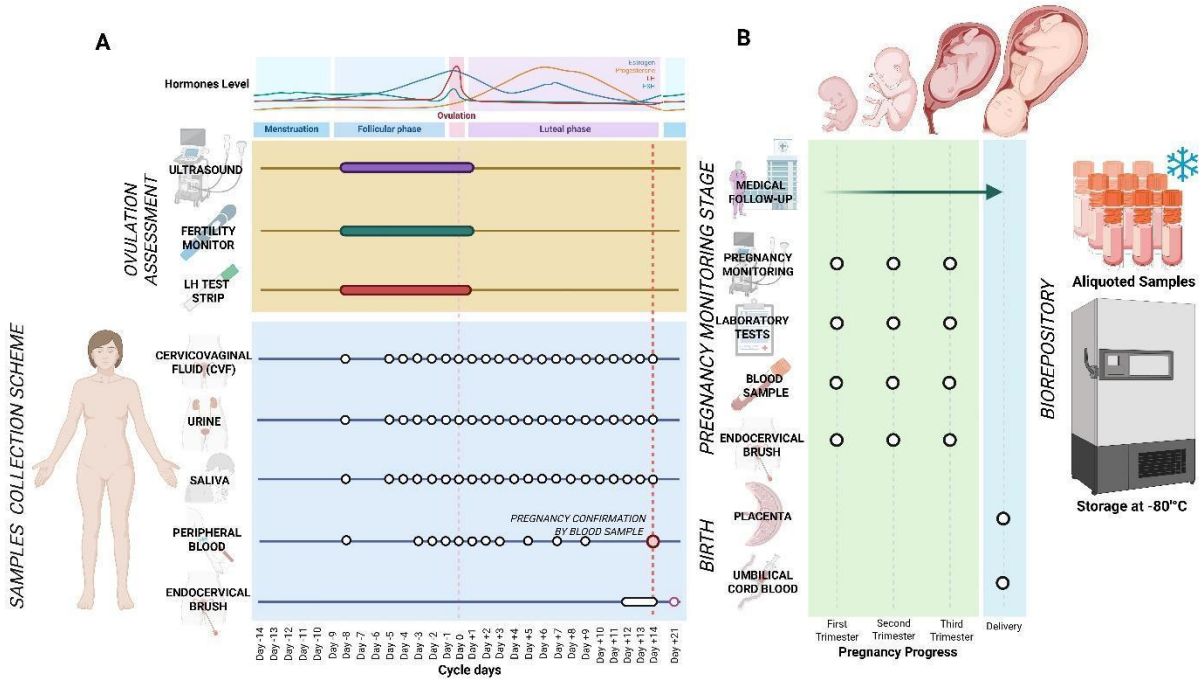


Figure 3. EARLY-PREG cohort workflow scheme.
A. Systematic collection of biological samples (CVF, saliva, blood, cervicovaginal brushing, and urine) from participants actively trying to conceive and participants not trying to conceive. In addition, cervical brushing is performed on day 21 post-ovulation for each participant with a clinically confirmed pregnancy. Cycle days are expressed in distance from ovulation day (day 0). B. For participants who achieve pregnancy, additional samples, such as umbilical cord blood and placenta, are obtained at delivery. Created in BioRender. Nova Lamperti, E. (2025) https://BioRender.com/t8ecf5u.

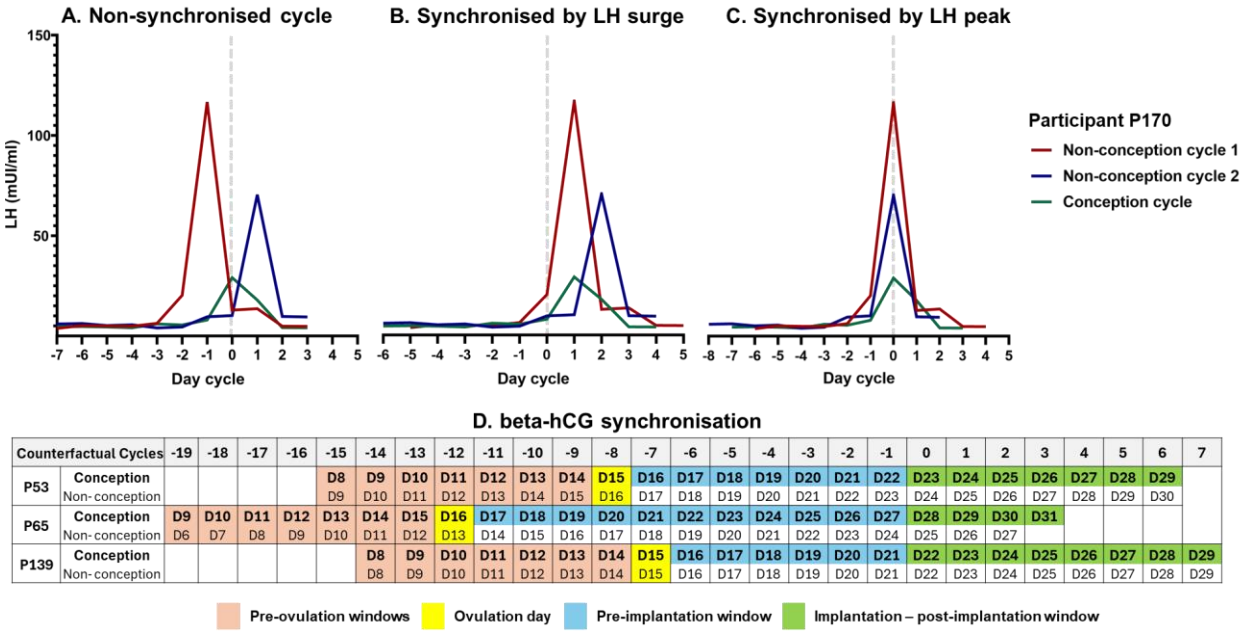
Ovulation and fertile window ascertainment

Although conception cannot be directly observed, estimating the day of ovulation allows researchers to narrow the time frame during which fertilization is most likely to occur (Wilcox *et al.*, 1995). Furthermore, the estimation of the ovulation day enables the targeted collection of biological samples to characterize and investigate the peri-implantation window in conception cycles, as well as the fertile window in non-conception cycles. The EARLY-PREG cohort has undergone three recruitment waves so far, each defined by variations in the clinical method used to estimate the day of ovulation and fertile window, following the proposed schema on Figure 3A. The pilot study and first wave relied on ultrasound to determine ovulation. The second wave employed a portable fertility monitor (Clearblue Digital Ovulation test). In the third wave, commercial LH strips were used to infer ovulation from urine samples. In addition, in all recruitment waves, a professional instructor trained women in the Billings method to estimate the fertile window for each menstrual cycle. Detailed, hand-filled fertility charts were used for each

individual cycle. A nurse or midwife in daily contact with each couple provided reproductive counselling.

Correction and synchronisation of counterfactual cycles

Given the high variability of menstrual cycles, identifying the day of ovulation is a key issue in the EARLY-PREG cohort. In fact, this day, estimated with a precision margin of +/- 12 hours, is considered as the most reliable proxy for the day of conception, or day 0, in the cohort of pregnant women. The two-way or bidirectional design used in the present preconception cohort offers a critical temporal framework for studying ultra-early pregnancy events with improved accuracy for multiple non-conception and conception menstrual cycles in a single individual following a counterfactual model. The fertile window typically includes the five days preceding ovulation, the day of ovulation itself, and the day following the estimated ovulation (Wilcox *et al.*, 1995). While all of the above methods offer a pragmatic approach to clinically estimating the fertile window and providing reproductive counselling at the specific period of the menstrual cycle during which conception is possible, these methods are, however, inaccurate and do not allow synchronising multiple cycles for research purposes. Therefore, to address this major issue, comprehensive laboratory analyses of urinary hormone curves —including LH, oestradiol, progesterone, and beta-hCG— are retrospectively performed on stored samples to more accurately synchronise the day of ovulation across multiple counterfactual cycles and/or specifically identify the pre-implantation window within a conception cycle. An example of a multi-cycle correction method used to synchronise ovulation (LH based approach) and implantation (beta-hCG based approach) used in the cohort is presented in Figure 4. The combination of these methods allows for a more precise isolation of specific time windows of interest in counterfactual cycles within the same individual.



Counterfactual Cycles		-19	-18	-17	-16	-15	-14	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4	5	6	7
P53	Conception					D8	D9	D10	D11	D12	D13	D14	D15	D16	D17	D18	D19	D20	D21	D22	D23	D24	D25	D26	D27	D28	D29	
	Non-conception					D9	D10	D11	D12	D13	D14	D15	D16	D17	D18	D19	D20	D21	D22	D23	D24	D25	D26	D27	D28	D29	D30	
P65	Conception	D9	D10	D11	D12	D13	D14	D15	D16	D17	D18	D19	D20	D21	D22	D23	D24	D25	D26	D27	D28	D29	D30	D31				
	Non-conception	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16	D17	D18	D19	D20	D21	D22	D23	D24	D25	D26	D27					
P139	Conception						D8	D9	D10	D11	D12	D13	D14	D15	D16	D17	D18	D19	D20	D21	D22	D23	D24	D25	D26	D27	D28	D29
	Non-conception						D8	D9	D10	D11	D12	D13	D14	D15	D16	D17	D18	D19	D20	D21	D22	D23	D24	D25	D26	D27	D28	D29

Figure 4. Synchronisation methods based on ovulation and implantation process using urine samples.

The figure illustrates two types of synchronisations: one based on luteinising hormone (LH) concentration for ovulation assessment, and the other on beta-hCG concentration for embryo implantation. The red, blue, and green lines represent three LH concentration profiles from cycles of the same participant. Panels A, B and C show synchronisation based on ovulation day and LH concentration for two consecutive non-conception cycles and a third conception cycle in the participant P170 of the cohort. In panel A, the cycles are non-synchronised. In panel B, the cycles are synchronised using the LH surge strategy proposed by Godbert et al. (2015). In panel C, the cycles were synchronised through LH peak concentration. Panel D presents synchronisation based on beta-hCG presence in counterfactual cycles of three participants P53, P65, and P139 of EARLY-PREG open cohort. The cells represent a specific day (D) of menstrual cycle for each participant, alignment according to beta-hCG synchronisation approach. The colours in the table indicate different phases of the cycle: pre-ovulation window (orange), ovulation day (yellow), pre-implantation window (blue) and implantation/post-implantation window (green). This alignment enables a standardised comparison of parallel time windows across counterfactual cycles in the same individual.

Biological material handling and storage

The strategy for collecting biological samples used in the EARLY-PREG cohort is intensive and challenging. The protocol is based on daily serial collection of CVF, urine, saliva and blood. A cervicovaginal brushing sample is collected between day 12 and 14 post-ovulation in all participants. In addition, only in pregnant women, blood samples and cervical brushings are collected on day 21 after ovulation and in each trimester of pregnancy. Finally, placenta and umbilical blood are obtained at childbirth (Figure 3B). Serial sampling has been performed during the phases of interest, including the follicular, periovulatory, and luteal phases. The specific details about the collection protocols for each sample are described below:

Blood

Four tubes of venous blood are drawn using BD Vacutainer tubes: two with EDTA (ethylenediaminetetraacetic acid) for proteomic analysis, peripheral blood mononuclear cell extraction, and platelet count, and two with a separator gel and coagulation activator for beta-hCG and hormone analysis. The samples are collected by nursing staff and transported to the laboratory in a cooler box with cold gel packs. The plasma is obtained by centrifugation at 2000 × g for 15 minutes and stored at -80 °C.

Urine

The participants are trained to collect morning urine at home in a sterile 60 mL container, discarding the initial stream. Instructions are given to keep the samples refrigerated (~4 °C) until daily collection. The urine samples were transported to the laboratory in a cooler box with cold gel packs. In the laboratory, the samples were centrifuged (600 × g, 10 minutes), and the supernatants were stored at -80 ºC for hormone analysis (oestradiol, FSH, LH, progesterone, and beta-hCG).

Saliva

The participants collect ~1 mL of saliva at home using Salimetrics SalivaBio passive collectors before eating and after brushing their teeth in the mornings. Instructions from the manufacturer are provided to participants for the collection of samples. Saliva samples are collected daily and transported to the laboratory in a cooler box with cold gel packs. The samples are stored directly at -80 °C in cryotubes.

Cervicovaginal Fluid (CVF)

The participants collected CVF using a silicone menstrual cup worn for two hours during the first hours of the morning. Cups are washed with a pipette using a buffer that contains PBS, physiological serum, and protease inhibitors and then transferred into a borosilicate tube. The samples are retrieved daily and transported to the laboratory in a cooler box with cold gel packs. These samples are subsequently processed (centrifuged and aliquoted) and then stored at -80 °C.

Cervicovaginal Brushing

Cervicovaginal brushing samples are collected from the cervical canal area by trained staff via a Rovers Cervex-Brush. The brush is then placed in a 50 mL tube containing medium enriched for trophoblastic cells (RPMI) with foetal bovine serum (FBS) and antibiotics. The samples are transported to the laboratory within 4 hours of collection via a cooler box with cold gel packs. Brushes are washed with PBS and then centrifuged. The collected pellet is resuspended in 400 µL of PBS and transferred to a microcentrifuge tube. Subsequently, 100 µL of 4% paraformaldehyde is added, and the sample is finally stored at 4 °C for further analysis.

Umbilical cord blood

At birth, the obstetrician collected blood from the cord, umbilical vein, and umbilical artery using BD Vacutainer tubes with EDTA. The sample collection times are recorded, and the samples are then immediately sent to the laboratory. The umbilical cord blood is transported at room

temperature (20 ºC–25 ºC) in a transport box. Sample processing involved centrifugation to separate peripheral blood mononuclear cells using Ficoll gradients. The cells are stored in RPMI medium supplemented with 20% FBS and 5% DMSO. Initially, the samples were stored for two weeks at -80 °C and then transferred to liquid nitrogen for long-term storage.

Placenta

Obstetricians collected placenta samples at the time of birth. The tissue was sectioned under sterile conditions, placed in a sterile container, and then transported to the laboratory in a cooler box with cold gel packs. The samples were preserved in RPMI medium supplemented with 20% FBS and stored at -80 °C for further analysis.

Follow-up stage

The length of follow-up is variable between individuals and study groups. The TCG is monitored for up to six menstrual cycles or until pregnancy, whichever comes first. In the case of pregnancy, follow-up is extended to the end of the pregnancy. The NCG is followed for a single menstrual cycle.

For all the participants, the follow-up phase begins on the first day of menstrual bleeding after enrolment, which is reported by the participant to the team via a phone call. This day marks the establishment of the cycle start date and serves as a milestone for scheduling visits and daily phone calls with participants. The first visit takes place between the third and fifth days of the cycle, when trained staff collect urine and peripheral blood samples to exclude pregnancy through a beta-hCG test. An ultrasound is performed during this visit for gynaecological evaluation of uterine and ovarian structures (this assessment is performed once). Additionally, the clinical staff provides counselling to couples regarding sexual intercourse during the fertile window of each menstrual cycle.

For monitoring, days 6 and 9 of the cycle include visits for collecting peripheral blood samples, CVF, urine, and saliva. After day 9 of the cycle, daily collection of urine, CVF, and saliva continues until the end of the menstrual cycle. In accordance with one of the ovulation assessment methods, post-ovulatory monitoring is scheduled. After this estimated point, alternate-day blood sampling is performed until 10 days post-ovulation. Fourteen days post-ovulation, a visit is scheduled that includes blood sampling for beta-hCG hormone measurement and cervical brushing. At this stage, the participants in the NCG conclude their study participation.

The TCG repeats the same follow-up protocol above for up to six cycles. Throughout these cycles, clinical pregnancies are assessed via beta-hCG levels, with clinical pregnancy monitoring commencing upon detection and continuing until childbirth. Once beta-hCG has been detected, the participant moves on to the pregnancy monitoring phase. One cervical brushing is performed on day 21 post-ovulation. Following this, the participants underwent ultrasound at 7, 11–14, 24–26, and 32–34 weeks. In addition to ultrasound monitoring, blood samples and brushings are collected during each trimester, specifically at weeks 11–14, 24, and 36. Standard prenatal care, in accordance with Chilean guidelines, is provided either by the obstetrician from the research

team or by the obstetrician chosen by the participant based on their preference (Minsal, 2015). At childbirth, placental tissue, umbilical cord blood, and newborn data are collected.

Recruitment numbers to date

The EARLY-PREG open cohort provides a dynamic characterization of menstrual cycle patterns and reproductive outcomes through a two-way longitudinal study. The demographic and general population characteristics of the EARLY-PREG open cohort at present are detailed in Table 1.

Table 1. Demographic and reproductive characteristics of the enrolled participants in the EARLY-PREG cohort.

	Total	Trying to Conceive (TCG)	Negative Controls (NCG)	
			Abstinence	Sterilized
Participants, N (%)	223 (100)	154 (69)	62 (28)	7 (3)
Age, mean [SD]	29.21 [4.85]	30.30 [4.36]	25.95 [4,35]	35.29 [2.25]
between 18 and 25, N (%)	52 (23.32)	21 (13.64)	31 (50.00)	0
between 26 and 30, N (%)	82 (36.77)	59 (38.31)	22 (35.48)	0
between 31 and 35, N (%)	71 (31.84)	60 (38.96)	9 (14.52)	3 (42.86)
between 36 and 40, N (%)	18 (8.07)	14 (9.09)	0 (0)	4 (57.14)
Age at menarche, mean [SD]	12.66 [1.56]	12.68 [1.53]	12.41 [1,64]	14.29 [0.88]
BMI, mean [SD]	24.80 [2,91]	24.88 [2.9]	24.47 [2.89]	24.8 [3.47]
Gynaecological history				
Gravidity, N (%)				
0	114 (51.12)	67 (43.51)	47 (75.81)	0 (0)
1	69 (30.94)	61 (39.61)	8 (12.9)	0 (0)
2	30 (13.45)	20 (12.99)	6 (9.68)	4 (57.14)
>3	10 (4.49)	6 (3.9)	1 (1.61)	3 (42.86)
Parity, N (%)				
Nullipara	134 (60.09)	86 (55.84)	48 (77.42)	0 (0)
1	69 (30.94)	59 (38.31)	10 (16.13)	0 (0)
2	17 (7.62)	8 (5.19)	4 (6.45)	5 (71.43)
>3	3 (1.35)	1 (0.65)	0 (0)	2 (28.53)
Miscarriage, N (%)				
0	319 (78.96)	246 (75.46)	58 (93.55)	6 (85.71)

1	65 (16.09)	63 (19.33)	2 (3.23)	1 (14.29)
2	19 (4.7)	17 (5.21)	1 (1.61)	0 (0)
>3	1 (0.25)	0 (0)	1 (1.61)	0 (0)
Educational level				
Secondary school, N (%)	24 (10.76)	21 (13.64)	2 (3.23)	1 (14.29)
Technical education, N (%)	64 (28.7)	46 (29.87)	15 (24.19)	3 (42.86)
Incomplete university level, N (%)	36 (16.14)	11 (7.14)	23 (37.10)	2 (28.57)
Complete university level, N (%)	93 (41.7)	73 (47.40)	19 (30.65)	1 (14.29)
Postgraduate, N (%)	6 (2.69)	3 (1.95)	3 (4.84)	0 (0)

% is the proportion of women/N within the sample, where N is the number of participants in the category.
BMI, body mass index.
Abstinence controls refer to women without sexual activity who contributed menstrual cycle data to the EARLY-PREG cohort.
The sterilized control refers to women who underwent tubal sterilization. Sterilized participant cycles can be with or without sexual activity.

In Table 2, we present the total number of cycles contributed by participants in the NCG and TCG.

Table 2. Number of cycles contributed by participants per group.

	Total	Trying to Conceive (TCG)	Negative Controls (NCG)	
			Abstinence	Sterilized
1 cycle, N (%)	124	64 (51.61)	53 (42.74)	7 (5.65)
2 cycles, N (%)	57	47 (82.46)	9 (17.54)	0 (0)
3 cycles, N (%)	23	23 (100)	0 (0)	0 (0)
4 cycles, N (%)	6	6 (100)	0 (0)	0 (0)
5 cycles, N (%)	9	9 (100)	0 (0)	0 (0)
6 cycles, N (%)	5	5 (100)	0 (0)	0 (0)

N represents the number of participants who contributed between 1 cycle and 6 cycles in their respective categories.
% is the proportion of contributors per group/individuals per cycle category.

Menstrual Cycle Description and Outcomes

We provide an overview of the participants' menstrual cycle characteristics (Table 3). A total of 407 menstrual cycles have been documented to date, classified as either non-conception (n = 352) or conception cycles (n = 55). Among the conception cycles, 35 resulted in full-term

pregnancies, whereas 20 ended in early pregnancy loss (EPL), which is defined as a miscarriage until 12 6/7 weeks (American College of Obstetricians and Gynecologists' Committee on Practice Bulletins-Gynecology, 2018).

Table 3. Menstrual cycle characteristics according to outcome in the EARLY-PREG cohort.

	Total	Non-conception cycles	Conception cycles	
			Pregnancy	EPL
Total cycles, n	407	352	35	20
Complete cycles, n (%)	292 (71.74)	238 (67.61)	34 (97.14)	20 (100)
Total cycle length, mean days [SD]	-	28.69 [3.75]	268.26 [11.27]*	40.61 [10.36]†
Follicular phase, mean days [SD]	15.72 [3.25]	14.66 [3.23]	15.83 [3.57]	15.10 [3.29]
Luteal phase, mean days [SD]	-	13.98 [2.67]	250.74 [11.97]**	24.61 [9.53]††
Menstruation, mean days [SD]	4.01 [1.37]	4.02 [1.41]	4.0 [1.16]	3.90 [1.29]

N refers to the number of elements per category.
Non-conception cycles include NCG cycles.
Complete cycles were defined as those involving longitudinal monitoring and sample collection from the first day of menstruation until the beginning of the next cycle (non-conception cycles) or until miscarriage or full-term pregnancy (conception cycles), with minimal data loss during the follow-up stage.
The cycle length and duration of each phase (follicular, luteal, and menstrual) were calculated using complete cycles and are presented as the mean days and corresponding standard deviations.
EPL: early pregnancy loss.
*The length of pregnancy is defined as the number of days from the beginning of the cycle to delivery.
** During pregnancy, length of the luteal phase is defined as the number of days from the day after ovulation to delivery.
† The length of EPL is defined as the period from the beginning of the cycle to the first day of bleeding after a positive beta-hCG test.
†† The length of luteal phase is defined as the period from the day after ovulation to the first day of bleeding after a positive beta-hCG test.

Biorepository and sample collection performance

A total of 407 menstrual cycles have been monitored in the EARLY-PREG cohort biorepository so far, which includes 6,406 CVF samples, 5,120 urine samples, 1,467 saliva samples, 928 blood samples, and 293 brushing samples collected throughout the menstrual cycles. Additionally, 17 placenta and umbilical cord blood samples have been obtained from term pregnancies, providing a valuable resource for studying perinatal outcomes.

Out of the monitored cycles, a total of 292 complete menstrual cycles have been biologically sampled: 43 belonging to the NCG and 249 to the TCG. The NCG samples collected from the EARLY-PREG cohort included 208 blood samples, 832 CVF samples, 876 urine samples, 279 saliva samples, and 26 cervical brushing samples. The cohort cycles have been further classified into counterfactual and non-counterfactual groups to facilitate reproductive studies on pregnancy, early pregnancy loss, and non-conception. Table 4 presents the total number of samples collected from the complete TCG cycles (excluding those from NCG). Within the TCG, 78 cycles were classified as counterfactual, and 171 were classified as non-counterfactual.

Table 4. Classification of complete cycles of the TCG in the EARLY-PREG cohort.

	Total	Counterfactual			Non-Counterfactual		
		Conception cycle		Non conception cycle	Conception cycle		Non conception cycle
		Pregnancy	EPL		Pregnancy	EPL	
Cycles							
Complete	249	18	15	45	17	8	146
Samples							
CVF	4,533	376	251	797	312	152	2,645
Blood	1,307	92	82	204	125	57	747
Urine	4,838	408	256	875	314	136	2,849
Saliva	1,043	129	58	200	95	0	561
Cervical Brushing	195	20	32	32	18	6	87

EPL: early pregnancy loss; CVF: cervicovaginal fluid.

Biospecimen Collection by Cycle Day and Type

The collection of biospecimens has been categorized by cycle day and type so far, as shown in Figure 5. The timeframes enabled the evaluation of samples of non-conception and conception cycles (pregnancy and early pregnancy loss). We highlight the presence of samples collected during the first two weeks after conception in this longitudinal cohort study.

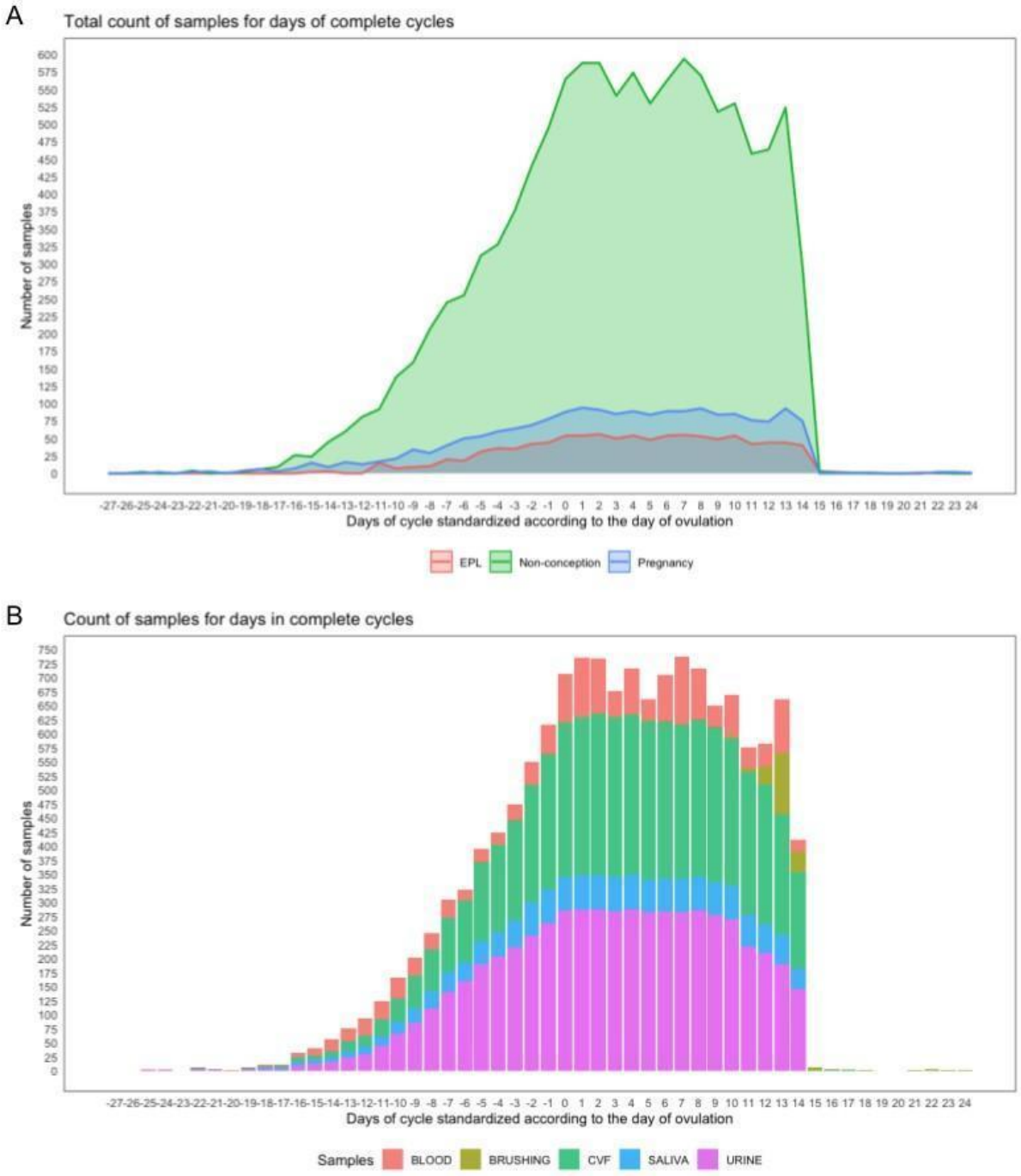


Figure 5. Total sample count per day across cycles in complete cycles.
The x-axis represents the days relative to the cycle standardized according to the day of ovulation, whereas the y-axis indicates the total number of collected samples. A. Total sample count for EPL, pregnancy, and non-conception cycles. B. Counts of samples per day in total cycles. Sample types are colour-coded: CVF (green), blood (red), urine (magenta), and saliva (light blue). EPL: early pregnancy loss; CVF: cervicovaginal fluid.

Biospecimen Collection Performance

We have evaluated so far the performance of the sample collection by comparing the number of expected samples to the actual samples obtained during the specified windows of interest. The efficiency of sample collection is determined by calculating the percentage of completeness within the windows via Formula (1), and we adjusted the CVF and urine according to Formula (2).

$$Performance(\%) = \left(\frac{samples\ collected\ in\ 14\ days}{expected\ samples\ in\ 14\ days}\right) \times 100 \quad (1)$$

$$Performance(\%) = \left(\frac{samples\ collected\ in\ cycle\ length}{expected\ samples\ in\ cycle\ length}\right) \times 100 \quad (2)$$

Overall compliance with sample collection during defined windows of interest has been high for most biospecimens. For CVF, 3,956 out of 4,380 expected samples were collected (90.3%) in the window from day 0 to 14, and 96.15% were collected in the window adjusted for each patient's luteal phase length; for urine samples, 3,880 out of 4,380 samples were collected (88.6%) during the period from days 0 to 14 across study cycles, and 94.29% were collected in the window adjusted for each patient's luteal phase length. In contrast, compliance was lower for saliva (collected in wave three and, in some cases, in wave two), with 89.22% in the window adjusted for each patient's luteal phase length (2). For blood, 1,499 of the 3,600 expected samples were collected (41.6%) across seven scheduled collections from day 0 to 10 of the window.

Throughout the study, a total of 287 out of 462 brushing samples have been collected from TCG and NCG. Brushing was expected at least once per participant, depending on the study aim and reproductive outcome. During the pregnancy monitoring stage of TCG, 72 out of 105 expected brushings were collected, resulting in a compliance rate of 68.6%. Among conceptions and EPL, brushing was expected once or twice (±14 and ±21 days post-ovulation), depending on gestational progression. Of the 347 expected samples, 215 were obtained, yielding a compliance of 62.0%.

Strengths and limitations

- To our knowledge, the EARLY-PREG cohort is the first study design aiming to research embryo–mother molecular exchange with a preconception, longitudinal, bidirectional, and counterfactual approach. This unique strategy allows each participant to serve as their own control to compare local and systemic effects of the presence or absence of a naturally conceived embryo, minimizing interindividual variability during ultra-early pregnancy.
- A key strength of this study is its prospective open design, which allows follow-up across participants for one or more menstrual cycles, providing detailed data across critical reproductive phases, especially the first two weeks after conception.

- We established a comprehensive multisample biobank including CVF, urine, blood, saliva and cervical brushings samples, as well as placenta and umbilical cord blood to support future research in human reproduction.
- In the EARLY-PREG cohort, biological samples were collected exclusively from women. Similar to other preconception and pregnancy cohorts, our data on male partners are limited to information from participating women or a short baseline survey.
- Another limitation is the lack of prior clinical records for the participants. During the recruitment and baseline surveys, the participants self-reported their medical conditions and family medical history; however, this information was not verified against existing medical records.
- Some fertilization events may have occurred without successful implantation, which would not be detected through standard beta-hCG testing. Advances in proteomic profiling in CVF could allow the identification of markers that recognize these events from our biorepository.
- Like many voluntary longitudinal studies, the cohort is susceptible to selective recruitment, which, combined with geographical constraints, may limit the representativeness of the findings for broader populations.

Future plans

The EARLY-PREG cohort biorepository has reached a sufficient size and number of biological samples to begin characterizing the longitudinal proteomes of the conception and non-conception cycles. The extensive collection of women's and maternal biospecimens opens the possibility for future advances in the study of longitudinal changes in the proteome throughout key stages of female human reproduction. Additionally, biological samples have been collected from full-term pregnancies, providing a valuable resource for further research on perinatal outcomes.

We project to identify local and systemic maternal responses associated with the embryo–mother molecular dialogue in the early stages of pregnancy. To this end, we will analyse biological samples from the EARLY-PREG cohort using a proteomics approach, following the proposed design and workflow of Figure 6, in which consecutive days during the early pregnancy stage are compared between participants who achieved pregnancy and their respective previous non-conception counterfactual cycles.

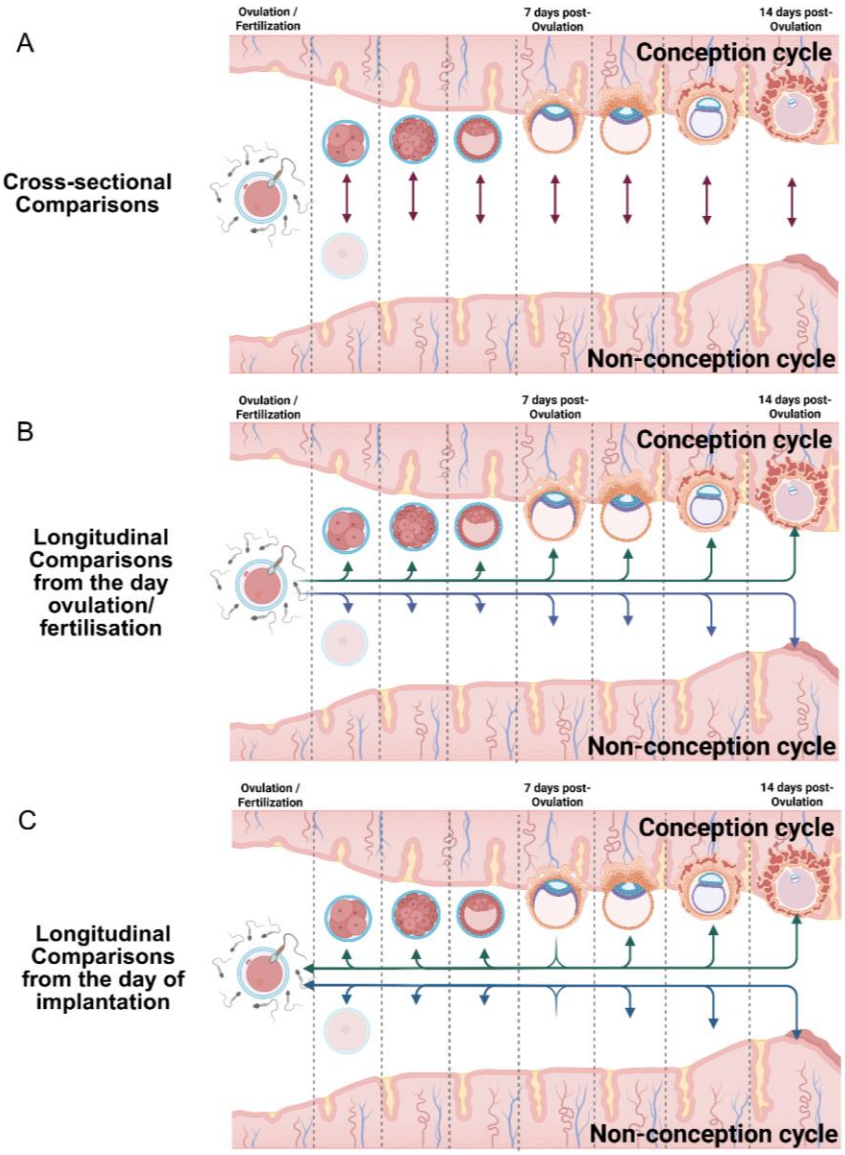


Figure 6. Proposed designs for analytic comparisons across the cycles and samples of the EARLY-PREG cohort.

Panel A illustrates a cross-sectional comparison approach, where each day is compared with its equivalent in both conception and non-conception cycles, following synchronisation (based on LH surge or beta-hCG). Panels B and C present longitudinal comparison approaches. In Panel B, days are aligned according to the day of ovulation/fertilisation, synchronised using the LH surge, in both conception and non-conception cycles. Panel C uses the same approach but aligns days based on implantation. This method employs beta-hCG as an implantation marker, which serves as the reference point for synchronisation under this standard. All comparisons presented are based on a counterfactual model of the EARLY-PREG open cohort. Created in BioRender. Nova Lamperti, E. (2025) https://BioRender.com/3s5qr6s.

Based on our work in standardizing proteomic workflows for CVF samples using optimized mass spectrometry data-independent acquisition approach (unpublished manuscript, submitted to preprint server), we will characterize the molecular communication between the embryo and the mother. This will be complemented by subsequently analysing various biospecimens. Our study is intended to cover the period from ovulation to post-embryo implantation (day 0 to 14). The primary objective of this stage is to identify the proteome signature of conception by comparing the CVF proteome of participants who successfully conceived with their respective counterfactual non-conception cycles while also quantifying associated biological processes and potential biomarkers. The biospecimens collected will also enable us to characterize additional key periods of human reproduction, including the fertile window (-5 to 0 days) (Wilcox *et al.*, 1995), the periovulatory window (-3 to 3 days), and the implantation window (7 to 10 days), synchronised according to the day of ovulation or embryo implantation.

Finally, a new recruitment wave of the EARLY-PREG study is set to begin to explore maternal immunoregulation and the microbiome during the ultra-early stages of pregnancy. This new wave will involve a multi-omic approach, incorporating advanced techniques such as flow cytometry, proteomic, metaproteomic, and metagenomic analyses. We will collect cervical fluid, endometrial biopsy, and stool samples, in addition to baseline samples, including CVF, urine, blood, saliva, and cervical brushing. This design facilitates comparisons following the proposed counterfactual framework. The results obtained may contribute to future research on maternal health and fertility and represent an important step towards future studies of biomarkers of ultra-early pregnancy.

Collaboration

The datasets generated and/or analysed during the current study are not publicly available because of the sensitivity of the participant material. Data requests can be sent to the corresponding author.

Contributors

E.S.K. conceived the original study using a two-way open longitudinal design. C.V. and J.S. contributed to implementation of all three recruitment waves. M.H., E.C. and B.A. contributed to the implementation and maintenance of the biobank. V.L. and G.N. and C.V. had the main responsibility for database construction and data analysis. D.A., V.L., G.N. and B.A., drafted the working manuscript, and E.S.K, C.V. and G.N. reviewed and commented on drafts. All the authors approved the final manuscript. E.S.K. is the guarantor of the study, accepts full responsibility for the research, had full access to the data, and controlled the decision to publish.

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Conflict of interest statement

As a senior scientist, E.S.K has served as an honorary research advisor and/or reviewer on research applications for the FISAR Foundation since 2015. No other conflicts of interest are reported.

Data availability statement

The data are available upon reasonable request. All data relevant to the study are included in the manuscript.

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