PROTOCOL of the study

Versie 1 – 21 december 2023

Integrated genetic and functional analysis of the influence of menstrual products on intimate female health (abbreviated as 'Luna' project).

Research study at Antwerp University Hospital and the University of Antwerp, in a collaboration between Faculty of Medicine and Health Sciences and Faculty of Science, Department of Bioengineering

Inclusion/exclusion criteria

Volunteers
Age: 18+
Gender: F
Not pregnant
Not menopausal
Natural menstrual cycle, copper IUD or combination pill
Regular menstrual cycle
No reproductive disease (endometriosis, PCOS,..)
No history ofn Toxic Shock Syndroom (TSS), sensitivity or allergy to menstrual hygiene products

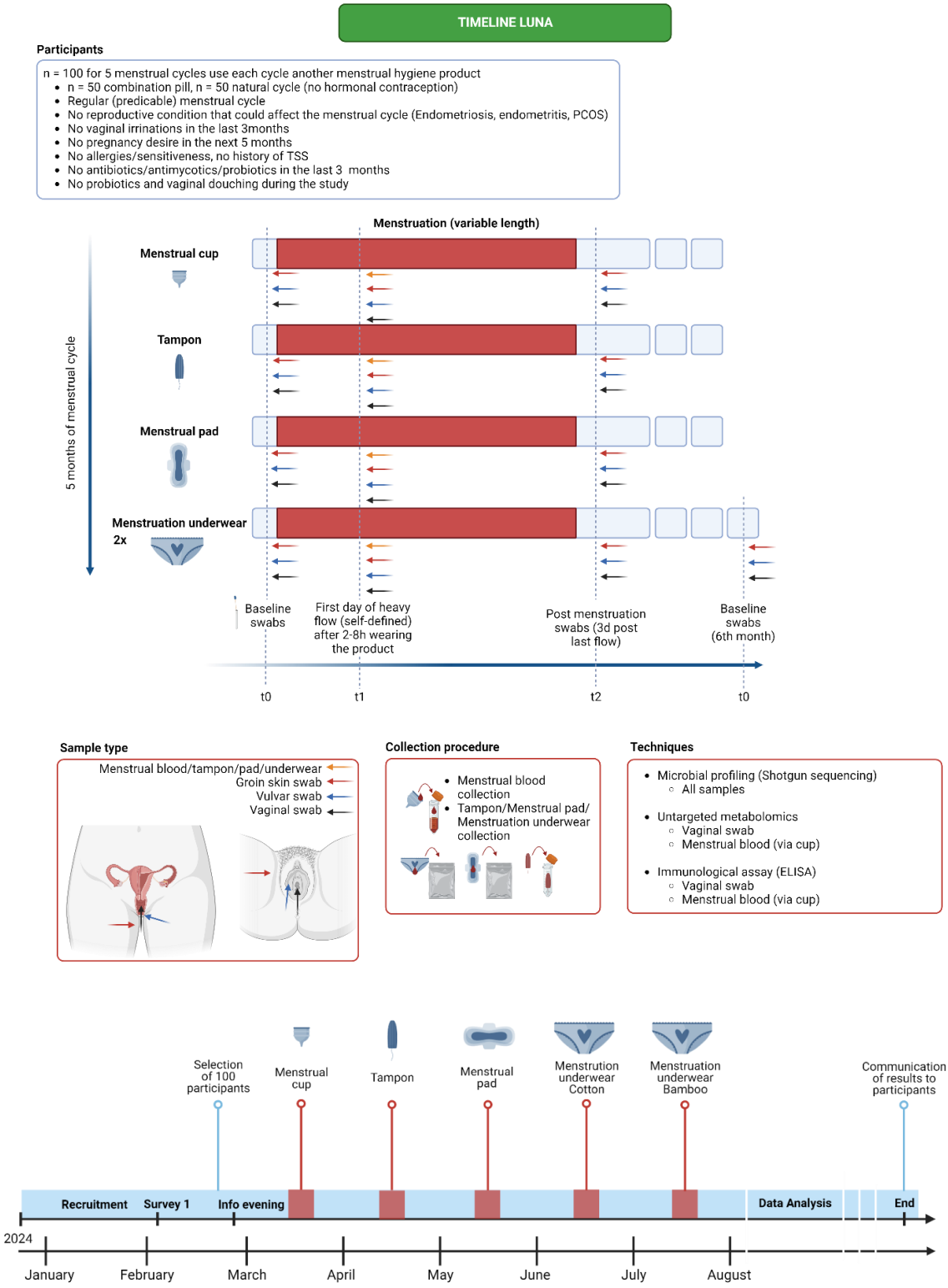
Furthermore, selections will be made based on the questionnaire in order to achieve a balanced study population.

Recruitment of participants

The volunteers (n=500) will be recruited through the channels built up in the Isala project (EDGE000471), such as the website (www.isala.be), instagram (Isala_UAntwerp), twitter (Isala_UAntwerp) and Linkedin (Isala). Thus, a large community has already been built, consisting of women who are very eager to participate in follow-up research. These channels will be used to obtain a representative group. Staff and students of the UAntwerpen and the UZA will also be recruited through posters, ad valvas and mails. No students will be recruited who have yet to take exams from the relevant members of the study. The volunteers will receive the information about the study and a consent form during information sessions, during which the self-sampling kit and menstrual products can also be distributed.

Sample collection

Samples will be self-sampled by the selected volunteers (n = 100). The self-sampling kit contains a detailed manual, information brochure and all materials needed by the volunteers for sample collection, storage and delivery to Prof. Lebeer's lab. Participants will have 3 sample collections for each menstrual product. This will consist of a vaginal, vulvar and groin swab, and during menstruation the menstrual blood will also be collected, this in the case of the cup directly and in the case of the other products as the product itself, this via non-transparent sterile sealed bags provided for this purpose. These products are a tampon, a sanitary pad, a menstrual cup and 2 types of menstrual underwear, 1 with a cotton lining and 1 with a bamboo lining.



Sample size calculation

Power analyses are challenging for microbiome studies because the magnitude and variance of effects on the microbiome are unknown and are highly individual-dependent and different for each bacterium. However, from literature review and our experience with the Isala study, we assume that the sample size selected is reasonable. Thus, initially recruited participants (n=1500) will complete a

comprehensive questionnaire regarding their health, lifestyle, menstrual hygiene and vaginal hygiene. Based on this questionnaire, a selection will be made (n = 100) of participants who will participate in the actual intervention study, in order to obtain a balanced study population, as well as based on location to make the study logistically feasible.

Thus, with 50 participants per condition (half natural cycle, half combined pill) in time series, we can perform paired analyses that allow us to account for intra-personal variation and increase power. Due to the nature of the study, no wash-out period will be necessary during the different interventions, as this is already natural between 2 menstruations, this also makes each participant a separate control and does not require varying the order of menstrual products. From our own experience with longitudinal studies, we take into account 5-10% dropout. If it is larger, we will recruit additional participants to avoid undermining the study.

We examine the influence of menstrual products at different levels. A PERMANOVA test will allow us to examine the overall effect of the intervention on the composition of the microbiome. Furthermore, we will investigate the effect on alpha diversity, for which health associations are known in the vaginal microbiome. This can be examined with a mixed effect model. Differential abundance of individual taxa will also be tested. It has been found that test tools for differential abundance often do not agree in reported results. To compensate for this, we have developed a consensus-based way in which several differential abundance testing tools are used and a taxon/condition is considered significant only if multiple tools agree. Three tools are capable of including random effects for paired analysis. Finally, for the vaginal microbiome, we test the effect on taxa modules known to be associated with lifestyle factors. This multi-level approach gives us a broad view to detect effects that may not be visible at a single individual level.

Analysis of samples

Laboratory of Applied Microbiology and Biotechnology Prof. Sarah Lebeer, Faculty of Science, Department of Bioengineering, UAntwerpen

<u>Part 1: Microbiome Analysis</u>

Microbial DNA and RNA will be isolated from swabs and menstrual blood (collected via menstrual cups and extracted from the other menstrual products) via a commercial extraction kit such as the Qiagen PowerFecal DNA kit and the Qiagen RNeasy kits (cfr. Human Microbiome Project). The isolated DNA and RNA (converted to cDNA) will then be analyzed via shotgun sequencing and (meta)transcriptomics. Shotgun sequencing

and (meta)transcriptomics allow to study the functional profiles and composition of these microbial communities. Combined, these techniques will provide valuable insights into better understanding the role of microbial communities in the context of health and the influence by menstrual products. The Illumina sequencing platform will be used.

Milestone: Identification of the (functional) microbiome composition of swabs and menstrual blood, with the aim of mapping the influence of menstrual products

Part 2: Quantitative analysis of concentration of specific microorganisms

Using quantitative real-time polymerase chain reaction (qPCR), species- or genus-specific primers will be used to estimate the concentration of specific microorganisms. In qPCR, the amount of a specific piece of DNA is measured in "real time. This allows the initial concentration of this specific DNA to be determined. The results then provide a picture of the concentration of different microorganisms in a sample. For processing these data, the "Minimum Information for Publication of Quantitative Real-Time PCR Experiments" (MIQE) guidelines will be used.

Milestone: Absolute abundance of microorganisms in swabs and menstrual blood, with the aim of mapping the influence of menstrual products

Part 3: Identification and quantification of metabolites from swabs or menstrual blood via a cup

In addition to the identification and quantification of specific microorganisms from swabs and menstrual blood, it is also interesting to look at what metabolites are produced by these microorganisms, and whether any toxins or allergens might be found in the fluid/blood. These could be from menstrual products. This can be found out using metabolomics. Specifically, liquid chromatography and mass spectrometry will be applied to supernatant solutions that will be further prepared by extraction with a cold organic solvent after addition of an internal standard mixture. The MassHunter Quantitative/Qualitative Analysis Software will then be used for identification and quantification of metabolites. Finally, the Mass Profiler Professional will be used for peak alignment, allowing metabolite annotation and statistical analysis.

Milestone: Identification and quantification of metabolites from swabs and menstrual blood, with the goal of mapping the influence of menstrual products

Part 4: Cultivating microorganisms from swabs and menstrual blood

Besides studying the composition of the microbiome through DNA sequencing technology, studying a microbiome through culture techniques remains a relevant way. Namely, low abundant members that might be missed by DNA sequencing techniques can be found, as well as individual strains examined in detail. This may be relevant to find differences in niche-specific strains adapted to conditions on the skin, in the vagina and specifically occurring in menstrual blood. Furthermore, this is expected to isolate a large proportion of lactic acid bacteria, which are of interest because they have a GRAS status ("Generally Recognized As Safe") and numerous health effects have been extensively described. Consequently, we will plate swabs and menstrual blood from the volunteers on both selective media for lactic acid bacteria, e.g. MRS (de Man Rogosa and Sharpe) medium and blood agar, and rich general media for other bacteria and fungi such as nutrient agar and yeast extract peptone medium (YPD). For this purpose, swabs or menstrual blood will be dissolved in PBS (Phosphate Buffered Saline) and after thorough mixing, a dilution series will be plated out. Several colonies will be highlighted and further identification at species level will be done via colony PCR with universal primers for the 16S rRNA gene (e.g. 8F-1525R) for bacteria and ITS primers for fungi, followed by Sanger sequencing and alignment against a reference genome (e.g. BLAST and EZBioCloud).

Milestone: Cultivation of niche-specific microorganisms.

Part 5: Identification of pro-inflammatory cytokines in swabs and menstrual blood

Besides changes in the microbiome composition due to menstrual products, it is also important to look at possible effects on the immune system of the participants. To investigate this, various immunoassays will be performed on the samples, including ELISAs for antibodies and pro-inflammatory cytokines, chemokines and growth factors (e.g. osteopontin, TGF-b superfamily, VEGF, CXCL10, CCL3, GM-CSF, IL-1β, IL-2, IL-6, IL-8, IL-12, IL-15, IL-18, XCL1, TNF, IFN-γ, perforin and granzyme etc.) In addition, relevant immune cell populations (e.g. neutrophils, T-cells, …) and cell-surface receptors will be quantified using flow cytometry.

Milestone: Identification of differentially expressed immune factors, such as cytokines, antibodies and surface molecules.

Part 6: isolation of MAIT cells

A unique goal in this study is the isolation and quantification of white blood cells in menstrual blood, also called "menstrual blood mononuclear cells" or MBMCs. To this end, the blood samples (obtained using the vaginal cup) are processed in analogy to standard blood samples (separation based on a density gradient (e.g. Sepmate tube)

and freezing). The MBMCs are used purely for the quantification of immune responses after co-incubation with microbial (vaginal) isolates: the quantification of cell-surface receptors (via e.g. flow cytometry, ELISA, fluorescence microscopy), cytokines/chemokines and gene expression. In no case will the material be used for elaboration of cell lines.

Milestone: Isolation of MBMCs with enrichment of MAIT cell subpopulation for studying immune responses (cell surface receptors, cytokine production, gene expression of immune signaling).