GuVa PCOS study

Changes in the gut- and vaginal microbiome composition in association with PCOS clinical phenotypes. (Version 3.0, d.d.23-9-2022)

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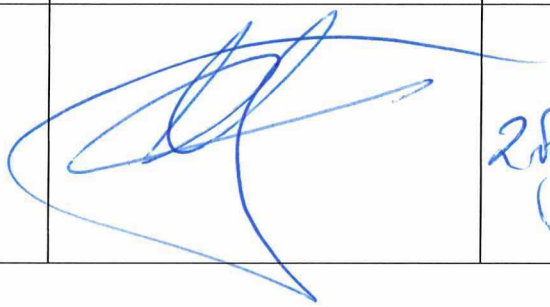
Principal Investigator Department of Obstetrics and Gynecology: Dr. S. Schoenmakers, MD PhD		28/9/22

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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABR	**General Assessment and Registration form (ABR form), the application form that is required for submission to the accredited Ethics Committee; in Dutch: Algemeen Beoordelings- en Registratieformulier (ABR-formulier)**
AE	**Adverse Event**
CA	**Competent Authority**
CCMO	**Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek**
CV	**Curriculum Vitae**
DSMB	**Data Safety Monitoring Board**
EU	**European Union**
GCP	**Good Clinical Practice**
GDPR	**General Data Protection Regulation; in Dutch: Algemene Verordening Gegevensbescherming (AVG)**
HA	**Hyperandrogenism**
IC	**Informed Consent**
METC	**Medical research ethics committee (MREC); in Dutch: medisch-ethische toetsingscommissie (METC)**
OD	**Ovarian Dysfunction**
PCOM	**Polycystic ovarian morphology ovarian dysfunction (OD)**
(S)AE	**(Serious) Adverse Event**
Sponsor	**The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation, or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidising party.**
UAVG	**Dutch Act on Implementation of the General Data Protection Regulation; in Dutch: Uitvoeringswet AVG**
WMO	**Medical Research Involving Human Subjects Act; in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen**

SUMMARY

Rationale:

Polycystic ovary syndrome (PCOS) is an endocrine disorder that affects up to 10% of reproductive-aged women worldwide. According to the Rotterdam criteria the syndrome is diagnosed based on two of the following three criteria: ovarian dysfunction (OD), hyperandrogenism (HA) (clinical or biochemical), and/or polycystic ovarian morphology (PCOM) (1) and there are four different PCOS phenotypes based on the features (2).
In PCOS women, not only is infertility a concern but the syndrome is also associated with obesity, insulin resistance, type 2 diabetes mellitus, metabolic syndrome, and cardiovascular risk factors (3).
Various factors have been reported to contribute to the pathophysiology of PCOS, but due to the heterogeneity of the syndrome, the pathophysiology remains unclear. However, studies emerged on the crucial role of the gut microbiome in human health. Recent studies confirmed that the gut microbiome is altered in women with PCOS compared to healthy women (4-7), and some of these alterations have been linked to the clinical markers of PCOS (4, 5). Different PCOS phenotypes may show different gut microbiome features due to the heterogeneity of this syndrome, which makes it hard to detect the significant changes in the gut microbiome. Age, obesity, androgen status, and insulin resistance or glucose intolerance should all be taken into account while studying the gut microbiome of PCOS patients.
Even though studies have proven that PCOS patients have an altered gut microbiome, the characteristics of microbiome diversity and the specific types of gut bacteria have remained inconsistent. Studies reported on specific alterations of alpha and beta diversity in PCOS patients. Although some studies have shown that decreased alpha diversity and altered beta diversity were associated with PCOS (4, 5, 8), many studies have reported that microbial diversity remained unchanged (9-11).

Studies have investigated correlations between the clinical features of PCOS and the gut microbiome. Features of PCOS such as hyperandrogenism, hirsutism, insulin resistance, and obesity can all have an impact on the gut microbiome to some extent. The decrease in α-diversity has been linked to total testosterone levels and hirsutism (9, 12) ,and changes in β-diversity were also correlated with hyperandrogenism (5). Also, some even suggested that the hyperandrogenism seen in the PCOS rodent model may affect the gut microbiome independently of diet (13). The role of metabolic factors, such as obesity and insulin resistance, in the PCOS patient's gut microbiome,e remains unclear. Although another study showed that obesity itself is linked to a decrease in gut microbial diversity (12), most studies on the gut microbiome in PCOS have included BMI-matched controls to exclude the

influence of body weight. In another study, BMI was found to positively correlate with three bacterial species, while waist-to-hip ratio and fat mass were also found to significantly correlate with the levels of multiple species (9, 12).
While the etiology of the microbiome-mediated disease is yet unknown, one possible pathway is microbial metabolism. Using metabolomics technology to identify metabolites that constitute chemical fingerprints of several metabolic pathways relevant for the pathogenesis of PCOS. While PCOS affects metabolites from various metabolic pathways, this primarily includes abnormalities in the metabolism of lipids, steroid hormones, carbohydrates, and amino acids. Identifying disrupted pathways enables PCOS-specific molecules that could be used as biomarkers and future metabolomic research targets.
Fewer studies are available investigating the relationship between PCOS and the vaginal microbiome, due to challenges in translating the vaginal microbiome of rodent models to humans. However, more consistent results were observed for the vaginal microbiome of PCOS patients (14, 15). The reason could be that the vaginal microbiome has lesser biomass and biodiversity than the gut, which makes it easier to get affected by PCOS.

As far as we are aware, they are no studies investigating the vaginal microbiota and its relation with sex hormone concentrations and PCOS phenotypical, diagnostic, and metabolic characteristics. This study aims to get a better understanding of the relationship between PCOS phenotypical, diagnostic, and metabolic characteristics (hirsutism, hyperandrogenism, ovarian dysfunction, polycystic ovary) and the gut- or vaginal microbiome in overweight/obese and lean patients. Hopefully, this may lead to a better understanding of the role of this microbiota in the pathogenesis of PCOS and future perspectives on treatment options.
The hypothesis is that PCOS patients have a less diverse microbiome in comparison with non-PCOS patients. Obese patients with PCOS will have a more severe dysbiosis of the gut microbiome than lean patients with PCOS.

Objective:
To analyse, characterise and determine the gut- and vaginal microbiome and its association with PCOS.
Main objectives

- To analyse whether there is an association between the gut microbiome and PCOS as such and its diagnostic and metabolic characteristics.

Secondary objectives

- To analyse whether there is an association between the *vaginal* microbiome and PCOS as such and its diagnostic and metabolic characteristics.
- To analyse whether there is a correlation between the composition of the gut- and vaginal microbiome in PCOS patients
- To analyse the difference in gut- and vaginal microbiome composition between overweight/obese and lean (with/without PCOS).
- To analyse potential metabolic profiles characterizing different phenotypes of PCOS

Study design: A prospective observational case-control study, conducted at the Department of Obstetrics and Gynecology (Division of Reproductive Medicine) at the Erasmus University Medical Center Rotterdam

Study population: Patients with PCOS diagnosed based on the Rotterdam criteria must be Caucasian women, aged 18 - 45 years old (premenopausal), either overweight or obese (BMI>25) or lean (BMI18-25).

Main study parameters/endpoints:
The main parameter of this study is the differences in community structure (presence or absence of bacteria, alpha, and beta diversity) of the gut microbiome between PCOS patients and controls.
Secondary study parameters: 1. Differences in community structure (presence or absence of bacteria, alpha, and beta diversity) in the *vaginal* microbiome between PCOS patients and controls. 2. The differences and similarities between the vaginal- and gut microbiome composition in PCOS patients 3. The differences and similarities between the vaginal- and gut microbiome composition between overweight/obese and lean patients (with/without PCOS). 4. Metabolic profiles from different phenotypes of PCOS
Nature and extent of the burden and risks associated with participation, benefit, and group relatedness:
For all participants, the risks involve primarily the burden of participating in a study, which in the current study proposal means 1 additional hospital visit and assessments. Women from the study group have already undergone the regular physical examination (transvaginal ultrasound) and laboratory tests for the standard outpatient clinic for COLA (Cycle disorders, Oligomenorrhea and Amenorrhea) screening. Patients will be asked to collect a stool sample at home and bring or send this to the hospital. A vaginal sample will be obtained by a physician without using a speculum. The risks of participation are considered to be minor and there are no obvious risks associated with participation in the study. The assessment and determination of relevant gut and vaginal microbiome may lead to a better understanding of

the pathophysiology of PCOS and might provide future intervention strategies eventually leading to new therapeutic options reducing the risk of metabolic risk factors. Therefore, this project will have an impact on research, individual clinical care as well as on future medical health care costs.

1. INTRODUCTION AND RATIONALE

Polycystic ovary syndrome (PCOS) is an endocrine disorder that affects up to 10% of reproductive-aged women worldwide. According to the Rotterdam criteria the syndrome is diagnosed based on two of the following three criteria (2): ovarian dysfunction (OD), hyperandrogenism (HA) (clinical or biochemical), and/or polycystic ovarian morphology (PCOM) (1). PCOS phenotypes are currently classified as phenotype-A (HA+ OD+ PCOM), phenotype-B (HA+OD), and phenotype-C (HA+PCOM). Phenotype-A or full-blown phenotype is present in half of the patients who present in the clinic. See Supplementary Table 1.
In PCOS women, not only is infertility a concern, but the syndrome is also associated with obesity, insulin resistance, type 2 diabetes mellitus, metabolic syndrome, and cardiovascular risk factors (3). Various factors have been reported to contribute to the pathophysiology of PCOS, such as lifestyle, metabolic status, and genetic and epigenetic variants. However, studies emerged on the crucial role of the gut microbiome in human health. Recent studies confirmed that the gut microbiome is altered in women with PCOS (4-6). The gut microbiome composition of PCOS patients is altered compared to healthy women and some of these alterations have been linked to the clinical markers of PCOS (4, 5). PCOS patients seem to have a leaky gut: the gut bacteria change the intestinal lining, resulting in the release of bacterial endotoxins into the bloodstream. This could activate pro-inflammatory cytokines, promoting insulin resistance, hyperandrogenism, and fat storage, all of which are linked to PCOS (16).
Even though studies have proven that PCOS patients have an altered gut microbiome, the characteristics of microbiome diversity and the specific types of gut bacteria have remained inconsistent. Studies reported on specific alterations of alpha and beta diversity in PCOS patients. Alpha diversity refers to the number of different taxa within a specific community; it indicates the richness of the gut microbiome. Beta diversity is a measure of how comparable the gut microbiome is between healthy people and PCOS patients. Although some studies have shown that decreased alpha diversity and altered beta diversity were associated with PCOS (4, 5, 8), many studies have reported that microbial diversity remained unchanged (9-11). Of note, a recent study with more than 300 subjects did not find microbial diversity in PCOS patients compared to healthy patients. Although, this was a population-based cohort study, and subjects were asked retrospectively whether they had oligomenorrhea, hirsutism or were diagnosed with PCOM. Several other reasons could have led to an unchanged microbial diversity and inconsistent outcomes, such as small sample size, and differences in sequencing techniques and analytical methods.
Unlike gut microbial diversity, most human studies have found differences in taxa between healthy women and PCOS patients. The phyla *Bacteroidetes, Firmicutes,* and *Tenericutes*,

as well as the families *Bacteroidaceae, Ruminococcaceae, Lachnospiraceae, Prevotellaceae,* and *Streptococcaceae,* are mostly represented. In many studies, significant changes in *Bacteroides* abundance were discovered (6, 7, 10, 17, 18). In addition, a metagenomic analysis revealed the existence of specific species of *Bacteroides spp*. in PCOS patients. The most typical aspect of the gut microbiome in patients with PCOS, according to Qi et al., is an increase in the abundance of *Bacteroides vulgatus* (6). More notably, administration of *B. vulgatus* mimics PCOS characteristics in mice (6). Additionally, *Bacteroides fragilis* was shown to be more abundant in another metagenomic study, and its abundance was positively linked with luteinizing hormone (LH) and antimullerian hormone levels (AMH) (17). According to these studies, *Bacteroides spp* plays an important role in PCOS patients. More research is needed to compare the pathogenic role of several species of *Bacteroides spp.*

Studies have investigated on correlations between the clinical features of PCOS and the gut microbiome. Features of PCOS such as hyperandrogenism, hirsutism, insulin resistance and obesity can all have an impact on the gut microbiome to some extent. The decrease in α-diversity has been linked to total testosterone levels and hirsutism (9, 12) ,and changes in β-diversity was also correlated with hyperandrogenism (5). Also, some even suggested that the hyperandrogenism seen in the PCOS rodent model may affect the gut microbiome independently of diet (13). The role of metabolic factors, such as obesity and insulin resistance, in the PCOS patient's gut microbiome remains unclear. Although another study showed that obesity itself is linked to a decrease in gut microbial diversity (12), most studies on the gut microbiome in PCOS have included BMI-matched controls to exclude the influence of body weight. In another study, BMI showed positive correlations with three bacterial species and waist-to-hip, ratio and fat mass was also correlated with several species levels (9, 12).

While the etiology of microbiome-mediated disease is yet unknown, one possible pathway is microbial metabolism. Using metabolomics technology in order to identify metabolites that constitute chemical fingerprints of several metabolic pathways relevant for the pathogenesis of PCOS. Hyperandrogenism appears to be the major pathological component in the etiology of PCOS, according to a rodent study. In this work, eliminating the gut microbiota of DHEA-treated rats did not prevent PCOS phenotypes, however transplanting microbiota from androgen-treated rats into pseudo germ-free animals caused hepatic glucolipid metabolic disruptions and reproductive hormone imbalance (19).

Human studies have shown that levels of metabolites from different biochemical pathways are altered in women with PCOS, but abnormalities in hormone and lipid metabolism were mainly involved in the onset of PCOS (20). Also, metabolites connected to amino acids as well as energy metabolism such as citric acid cycle appear to be the most common. Glycerophospholipid metabolism was shown to be down-regulated in PCOS, while glucose metabolism was found to be up-regulated. Zhao et al. found that in PCOS, all of the measured fatty acids are up-regulated when compared to control patients (21). According to Zou et al., certain carbohydrates and fatty acid metabolites are up-regulated in PCOS compared to controls (22). Identifying disrupted pathways enables of PCOS-specific molecules that could be used as biomarkers and future metabolomic research targets. Finding relevant biochemical indicators may help in early diagnosis and future targeted pharmacological therapies.

Fewer studies are available investigating the relationship between PCOS and the vaginal microbiome, due to challenges in translating the vaginal microbiome of rodent models to humans. However, a recent study found lower abundances of *Lactobacillus spp.* and elevated levels of *Gardnerella, Prevotella,* and *Mycoplasma* (15). In addition, the authors found highly comparative microbiome composition in vaginal and cervical canal samples in the same patient. In correspondence to another case-control study, they found that women with PCOS have higher alpha diversity, lower abundances of *Lactobacillus crispatus*, and higher abundances of *Mycoplasma* and *Prevotella* (14), after adjusting for BMI. These studies suggested a more consistent conclusion in the vaginal microbiome of PCOS patients. The reason could be that the vaginal microbiome has lesser biomass and biodiversity than the gut, which makes it easier to get affected by PCOS.

As far as we are aware, they are no studies investigating the vaginal microbiota and its relation with sex hormone concentrations and PCOS phenotypical, diagnostic and metabolic characteristics. This study aims to get a better understanding of the relationship between PCOS phenotypical, diagnostic and metabolic characteristics (hirsutism, hyperandrogenism, ovarian dysfunction, polycystic ovary) and the gut- or vaginal microbiome in overweight/obese and lean patients. Hopefully, this may lead to a better understanding of the role of these microbiota in the pathogenesis of PCOS and future perspectives on treatment options.
The hypothesis is that PCOS patients have a less diverse microbiome in comparison with non-PCOS patients. Obese patients with PCOS will have a more severe dysbiosis of the gut microbiome than lean patients with or without PCOS.

2. OBJECTIVES

To analyse, characterise and determine the gut- and vaginal microbiome and its association with PCOS clinical phenotypes.

Main objectives

- To analyse whether there is an association between the gut microbiome and PCOS as such and its diagnostic and metabolic characteristics.

Secondary objectives

- To analyse whether there is an association between the vaginal microbiome and PCOS as such and its diagnostic and metabolic characteristics.
- To analyse whether there is a correlation between gut- and vaginal microbiome in PCOS patients and controls
- To analyse the difference in gut- and vaginal microbiome composition between obese and lean (with/without PCOS).
- To analyse potential metabolic profiles characterizing different phenotypes of PCOS

3. STUDY DESIGN

This is a prospective observational case-control study, conducted at the department of Obstetrics and Gynecology (Division of Reproductive Medicine) at the Erasmus University Medical Center Rotterdam. In this study changes of the gut- and vaginal microbiome will be analysed in correlation with PCOS phenotypes.

Patients: women diagnosed by the division of Reproductive Medicine with PCOS in West- or Central Europe*
Sample size: n=50 PCOS overweight/obese and n=50 PCOS lean patients
Moments of inclusion: after PCOS diagnosis/ COLA screening (Cycle disorders, Oligomenorrhea and Amenorrhea screening)
Controls: n=50 overweight/obese and n=50 lean non-PCOS healthy women
Test sample: vaginal swab, stool sample and blood sample
Study period: 1-11-2022 – 1-12-2024

* The diet is strongly associated with the gut microbiome. The reason to only include women from Western and Central Europe, is because these women have a different diet compared to women from Asian, Mediterranean and African countries (recently reviewed in:(23)) . We want to include women who have a Western diet. In addition, the vaginal microbiome is structurally different across ethnicities (24). To avoid getting too small groups in sub-analyses based on ethnicity or diet, we chose to include patients from Western and Central Europe.

4. STUDY POPULATION

4.1 Population (base)

Patients who have had COLA screening at the outpatient clinic of Reproductive Medicine.
Study group: overweight/obese women (BMI>25) and lean women BMI (18-25) with PCOS
Control group: overweight/obese women and lean women without PCOS.

4.2 Inclusion criteria

In order to be eligible to participate in this study, a subject must meet all of the following criteria:

- Age 18-45years (premenopausal)
- Caucasian
- Willing to provide vaginal swab and stool sample
- Willing to provide informed consent
- Sufficient command of the Dutch language
- Diagnosed with PCOS at Erasmus MC using the Rotterdam criteria 2003 (2) (see also H.15 Supplementary 1)

4.3 Exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:

- BMI <18
- Smoking
- Diabetes Mellitus or use of insulin sensitizer
- Chronic and acute infection diseases
- Endometriosis (American Fertility Score (AFS) III/IV)
- Elevated prolactin levels, thyroid disease, Cushing disease or gastro-intestinal disease
- The use of hormonal contraceptives, other steroid hormones in last 3 months
- Use of antibiotics, probiotics or laxatives in the last 3 months

Control group:

- After a COLA screening, subjects who have not been diagnosed with PCOS and have a regular menstrual cycle without hyperandrogenism or PCOM will have their dossiers individually evaluated to see whether they can participate in the control group.
    - COLA screening volunteers who have already agreed to be approached for any future studies, will receive the PIF and be asked to participate in this study. These candidates are healthy and have a regular menstrual cycle.
    - Online advertisement will be published to recruit volunteers.
- Inclusion criteria control group:
    - Age 18-45years (premenopausal)
    - Caucasian
    - Willing to provide vaginal and stool sample
    - Willing to provide informed consent
    - Regular menstrual cycle (25-35days)
    - Sufficient command of the Dutch language
    - No history of diagnosed PCOS and do not meet any of the Rotterdam criteria.
- Exclusion criteria: see above.

4.4 Sample size calculation

Sample size is estimated on the basis of sample availability and literature. An important study is by Torres et al. (5), where they included 48 controls and 73 women with PCOS. In figures 1a and 1b they show that with these numbers they find a significant difference between the operational taxonomic units (OTUs) and the

Faith PD. The Faith PD is a measure of diversity based on the phylum of the bacteria. There are several studies that show no difference with a small sample size (n=10-30), but the study by Lindheim et al.(4) shows that with 24 PCOS women and 19 controls a decreased diversity was found with also significantly decreased abundances of *Tenericutes* and *Bacteroidetes*. Due to the novelty of this field of study, the pilot data set is not publicly accessible. In order to analyze the differences in microbiome composition between gut and vaginal microbiome, between study and control group, 100 patients will be included (n=50 with a BMI > 30 kg/m2 and n=50 with BMI 19-25 kg/m^2) in study group. Also, 100 healthy women will be included in the control group.

TREATMENT OF SUBJECTS

5.1 Investigational product/treatment

Not applicable

5.2 Use of co-intervention (if applicable)

Not applicable

5.3 Escape medication (if applicable)

Not applicable

6 INVESTIGATIONAL PRODUCT

6.1 Name and description of investigational product(s)

Not applicable

6.2 Summary of findings from non-clinical studies

Not applicable

6.3 Summary of findings from clinical studies

Not applicable

6.4 Summary of known and potential risks and benefits

Not applicable

6.5 Description and justification of route of administration and dosage

Not applicable

6.6 Dosages, dosage modifications and method of administration

Not applicable

6.7 Preparation and labelling of Investigational Medicinal Product

Not applicable

6.8 Drug accountability

Not applicable

7 NON-INVESTIGATIONAL PRODUCT>

7.1 Name and description of non-investigational product(s)

Not applicable

7.2 Summary of findings from non-clinical studies

Not applicable

7.3 Summary of findings from clinical studies

Not applicable

7.4 Summary of known and potential risks and benefits

Not applicable

7.5 Description and justification of route of administration and dosage

Not applicable

7.6 Dosages, dosage modifications and method of administration

Not applicable

7.7 Preparation and labelling of Non Investigational Medicinal Product

Not applicable

7.8 Drug accountability

Not applicable

8 METHODS

8.1 Study parameters/endpoints

8.1.1 Main study parameter/endpoint

- Differences in community structure of the gut microbiome between PCOS patients and controls as such and its diagnostic and metabolic characteristics.

8.1.2 Secondary study parameters/endpoints

- Differences in community structure in the vaginal microbiome between PCOS patients and controls as such and its diagnostic and metabolic characteristics.
- Differences and similarities in community structure of the vaginal- and gut microbiome in PCOS patients
- Differences and similarities between the vaginal- and gut microbiome composition between overweight/obese and lean patients (with/without PCOS)
- Metabolic profiles from different phenotypes of PCOS

8.1.3 Other study parameters (if applicable)

Not applicable

The analyses for differences in community structure in the gut and vaginal microbiome are explained here:
A common strategy is to group reads at some level of similarity into representative sequences of hypothetical species called Operational Taxonomic Units (OTUs), where all reads within, 97% similarity are clustered together and represented by a single OTU sequence. This is done because bacterial taxonomy is incomplete and there are sequencing errors in the Next Generation Sequencing (NGS) reads.

Alpha diversity refers to the number of different taxa within a specific community; it indicates the richness of the gut microbiome. Beta diversity is a measure of how comparable the gut microbiome is between healthy people and PCOS patients. Shannon diversity index is a way to measure the diversity of species in a community. The higher the value of H, the higher the diversity in a particular community. Another measure of alpha diversity could be the species richness, which is the count of the number of species or OTUs present in that sample.

UniFrac will be used to compare the (dis-)similarities among the microbial communities samples (beta-diversity). Unweighted UniFrac analyses is based on presence/absence of difference taxa and abundances and weighted UniFrac will take the abundances of different taxa into consideration.

8.2 Randomisation, blinding and treatment allocation

Not applicable

8.3 Study procedures

Patients are eligible for the study after they are diagnosed with PCOS at the regular COLA screening. The abbreviation COLA stands for: Cycle disorders, Oligomenorrhea and Amenorrhea. During the regular COLA screening visit, patients will undergo a physical examination, including a transvaginal ultrasound. Three to four weeks after the COLA screening patients will receive their COLA screening results. Patients who are diagnosed with PCOS and are eligible for the study will be informed about the details of the study. Patients will receive a patient information leaflet with the informed consent form, together with the contact information of the clinical coordinator in case they might have any remaining questions.

The main investigator will contact them by phone 1 week after their appointment for their COLA screening result and ask if they are interested in participating in the study. If the patient agrees to participate, the material for stool sample collection will be sent to her. Patients will be instructed to collect the stool sample after signing the informed consent (preferably on the day of the second appointment, but no more than 2 days before) On the day of the second appointment, the researcher will sign the informed consent and provide a copy to the patient. A vaginal swab will be obtained by the physician and the stool sample will be handed in by the patient.

Vaginal samples and blood samples will be collected in the non-menstrual period during the appointment. Patients will be asked to fill in an online Food Frequency Questionnaire (max 45min).

Also, PCOS patients in the COLA database who are eligible will be approached to participate in this study. They will be contacted by phone, and if they are interested in participation, a PIF will be sent to their home address.

Participants in the control group will be informed by the main investigator. When they meet the inclusion criteria and they decide to participate in the study, the same COLA screening will be conducted, in combination with this study protocol.

COLA screening volunteers who have already agreed to be approached for any future studies, will receive the PIF and be asked to participate in this study. These candidates are healthy and have a regular menstrual cycle.

Online advertisement will be published to recruit volunteers.

After collection the vaginal swab and stool sample will be split in different portions to be analysed using the 16S rRNA gene sequencing. In both the stool and vaginal swab samples the following parameters will be measured: both the microbiome profile patterns (three different groups of microorganisms: (1) Firmicutes/ Actinobacteria/ Verrucomicrobia (2) Bacteroidetes and (3) Proteobacteria) by the NGS and another high throughput technique for the Lactobacillus, Staphylococcus, E coli and Bacillus RG4 composition as percentage of the total microbiome content.

16S ribosomal RNA amplicon sequencing
Both gut and vaginal microbiome will be analysed by using 16S ribosomal RNA sequencing (rRNA). For each sample the V4 hypervariable region of the 16S rRNA gene will be amplified by polymerase chain reaction (PCR) with primers. The steps in the PCR process are as follow and each step occurs at a different temperature. First denaturation at 94°C occurs and the two DNA strands get separated out. Then amplification at different temperatures occurs. Finally, the enzyme Polymerase is added which make the primers extend along the length of the DNA strand.

Laboratory measurements includes: estradiol, total testosterone, androstenedione, dyhydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), dehydrotestosterone (DHT), serum cortisol, thyroid-stimulating hormone (TSH), prolactin, insulin, anti-Muellerian hormone (AMH), sex hormone-binding globulin (SHBG), serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), 17-hydroxyprogesterone (17OH-P), plasma total cholesterol, high-density lipoprotein (HDL)- cholesterol, triglycerides and glucose. These laboratory measurements are already included in the COLA screening. Addition measurements that will be obtained include but will not limited to: LH, FSH, estradiol, progresteron, hsC-reactive protein (CRP), interleukin-22 (IL-22), IL6, tumor necrosis factor-a (TNF-a), serum endotoxin (=lipopolyssacharide, LPS), adiponectin, serum zonulin, sCD14: soluble CD14 (endotoxin co-receptor), bile acids in serum and in stool, stool calprotectin and stool zonulin.
Vaginal samples will be taken using a cotton swab: eSwab™ (COPAN Italia S.p.A.) The swab will be inserted 5-6 cm beyond the vaginal orifice, and moved around along the vaginal wall for 10–15 s. The vaginal samples will be stored at -20°C and transported to a freezer at -80°C twice per week. Stool samples will be collected using a stool collection tube and stored in a cold transport container. Until further processing, samples will be stored in a freezer at -

20 °C immediately by the patient and at -80°C at the day the stool sample has arrived at Erasmus MC.

8.4 Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The investigator can decide to withdraw a subject from the study for urgent medical reasons.

8.4.1 Specific criteria for withdrawal (if applicable)

Not applicable

8.5 Replacement of individual subjects after withdrawal

Subjects who opt to withdraw will not be replaced.

8.6 Follow-up of subjects withdrawn from treatment

Not applicable

8.7 Premature termination of the study

A premature termination of the study is not expected. However, in case of a premature termination of the study no consequences for the study subjects are to be expected.

9 SAFETY REPORTING

9.1 Temporary halt for reasons of subject safety

In accordance to section 10, subsection 4, of the WMO, the sponsor will suspend the study if there is sufficient ground that continuation of the study will jeopardise subject health or safety. The sponsor will notify the accredited METC without undue delay of a temporary halt including the reason for such an action. The study will be suspended pending a further positive decision by the accredited METC. The investigator will take care that all subjects are kept informed.

9.2 AEs, SAEs and SUSARs

9.2.1 Adverse events (AEs)

The interventions performed in this pilot study are blood collection and a vaginal swab. Adverse events are defined as any undesirable experience occurring to a subject during the study. Events considered related to the experiment, reported spontaneously by the subject or observed by the investigator or his staff will be recorded. Regular laboratory screening is part of the standard care on the PICU, although unlikely, adverse reactions from blood collection can occur. Personnel collecting blood specimens know what can occur and how best to manage the reactions. This is an observational study, adverse drug events are not caused by study intervention. All adverse events due to blood collection, vaginal swab collection or faeces collection will be registered.

9.2.2 - Serious adverse events (SAEs)

A serious adverse event is any untoward medical occurrence or effect that

- results in death;
- is life threatening (at the time of the event);
- requires hospitalisation or prolongation of existing inpatients' hospitalisation;
- results in persistent or significant disability or incapacity;
- is a congenital anomaly or birth defect; or
- any other important medical event that did not result in any of the outcomes listed above due to medical or surgical intervention but could have been based upon appropriate judgement by the investigator.

An elective hospital admission will not be considered as a serious adverse event

In line with the CCMO guidelines, the SAEs will be reported to the MEC via yearly line listings together with the progress report.

9.2.3 Suspected unexpected serious adverse reactions (SUSARs)

Not applicable

9.3 Annual safety report

Not applicable

9.4 Follow-up of adverse events

All AEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist. SAEs need to be reported till end of study within the Netherlands, as defined in the protocol

9.5 Data Safety Monitoring Board (DSMB) / Safety Committee

Not applicable

10 STATISTICAL ANALYSIS

10.1 Primary study parameter(s)

Statistical analyses

The statistical analyses will be conducted using the statistical software R.

Continuous variables will be expressed as mean and standard deviation for normally distributed variables, median and interquartile range for not normally distributed variables.

To analyse the association between the gut microbiome and PCOS compared to controls, reads with some level of similarity will be grouped into operational taxonomic units (OTUs). Alpha and beta diversity analyses will be calculated to estimate the abundance and diversity of species within samples.

Alpha diversity comparisons will be analysed with Shannon diversity index and richness as continuous variables. Higher Shannon diversity index is associated with a more diverse and rich vaginal microbiome. It measures the number of species and the differences between species abundances. A logistic regression model will be used to test the changes of alpha diversity in PCOS and non-PCOS women. Same analyses will be conducted for changes of alpha diversity in overweight/obese and lean women with or without PCOS. Linear regression analyses will be conducted to study the correlation between the diversity of the microbiome (Alpha and Beta diversity) and parameters such as hyperandrogenism, rated as serum total testosterone level or Free Androgen Index (FAI). Other PCOS-associated variables included in the univariable linear regression analyses are: hirsutism, acne, BMI, LH/FSH, testosterone, androstenedione. Significant variables from the univariable regression analyses will be tested in a multivariable linear regression model. To correct for multiple testing, Bonferroni's correction will be conducted.

UniFrac will be used to compare the (dis-)similarities among the microbial communities samples (beta-diversity). Unweighted UniFrac analyses is based on presence/absence of difference taxa and abundances and weighted UniFrac will take the abundances of different taxa into consideration. The results from Unifrac analyses will be represented using Principal Coordinate Analysis (PCoA) plots. PCoA plots will represent the dissimilarity of microbiome samples. The PCoA will be based on the overall structure of the gut microbiome in all samples for PCOS and controls.

10.2 Secondary study parameter(s)

To analyse the association between the vaginal microbiome and PCOS compared to controls, the bacterial taxa will be first classified to the genus level. The relative abundance will be quantified based on each taxa contribution to an individual sample. Operational taxonomic unit (OTU) abundance will be noted for all samples.
Alpha and Beta diversity will be calculated. Associations between the vaginal microbiome (Alpha and Beta diversity) and PCOS-associated variables as BMI, hirsutism, acne, testosterone, androstenedione, LH/FSH, insulin, will be tested in a linear regression model.

To investigate the association between the bacterial diversity and markers for gut permeability and inflammation in PCOS patients, a logistic regression model will be used. Leaky gut markers as calprotectin, zonulin, lipopolysaccharide and soluble CD15 and inflammation markers as CRP, IL-22, IL6 and TNF-a will be tested in the logistic regression model. Also the results of the Food Frequency Questionnaire (FFQ) will be analysed in the logistic regression model.

Differences on clinical characteristics and metabolic biomarkers will be evaluated using t-test or a one-way analysis of variance (ANOVA) for normally distributed variables or non-parameter Wilcoxon test for non-normally distributed variables. Two-sided tests will be used, and p-values less than 0.05 will be considered statistically significant.

As for the metabolomic analysis, the raw data will be pre-processed first. Log-transformation and centering are techniques used to make the data normal distributed. To investigate differences between the PCOS and controls, univariate analysis will be performed to examine each metabolite feature separately.

10.3 Other study parameters

Not applicable

10.4 Interim analysis (if applicable)

Not applicable

11. ETHICAL CONSIDERATIONS

11.1 Regulation statement

The investigators will ensure that this study is conducted in agreement with the Declaration of Helsinki (version of October 2013, www.wma.net) and all relevant national guidelines and regulations, whichever provides the greatest protection for the patient. The study protocol will be submitted to the ethics committee.

11.2 Recruitment and consent

Women who are eligible for the study will be informed by the clinician about the the GuVa PCOS study and when the patient is interested in participation, the main investigator will contact the patient The main investigator will inform the patient. about the voluntary nature of their participation as well as by means of the information leaflet and informed consent form. Full comprehensive information will be given to all patients according to the regulations of the competent authorities that need to approve the clinical study.
Participants in the control group will be recruited via online advertisement and via the outpatient clinic of Reproductive medicine.

Informed consent:
The study will be performed according to written informed consent procedures and this study protocol that are approved by local and/or national ethics committees. The informed consent procedures ensure personal data protection and confidentiality. Data will be coded, i.e. information will not be directly traceable to an individual person.
Patients who signed the informed consent, agree on sharing unexpected findings with the specialist or general practitioner. Patients who do not want their physician and or general practitioner to be informed about the following, cannot participate in the study.

11.3 Objection by minors or incapacitated subjects (if applicable)

Not applicable

11.4 Benefits and risks assessment, group relatedness

Participants will not benefit directly from the study. The only study procedure for the subjects is the collection of stool samples by themselves and a vaginal sample by the physician. The participation will not influence any standard care provided.

11.5 Compensation for injury

The sponsor/investigator has a liabity insurance which is in accordance with article 7 of the WMO.

Dispensation from the statutory obligation to provide insurance has been obtained because participating in the study is without risks.

11.6 Incentives (if applicable)

Not applicable

12. ADMINISTRATIVE ASPECTS, MONITORING ,AND PUBLICATION

12.1 Handling and storage of data and documents

Each subject will be assigned a study number. Data on microbiome composition will be stored anonymously and coded on basis of that study number. Only the coordinating investigators, project leader, and the principal investigator(s) will have access to patient identifying data and their respective study numbers. This is according to the EU General Data Protection Regulation and the Dutch Act on Implementation of the General Data Protection Regulation.

Storage of samples

Vaginal and stool samples will be frozen at -80°C and stored up to a maximum of 25 years.

Storage of data

Every patient will have to sign an informed consent form. All data will be stored and handled in the same way and according to the same rules and regulations as other patient-related data at the Erasmus MC. It will be stored as files on a separate research storage platform. Access will be limited to authorized study personnel. Should data transfer to other parties be necessary for testing or validation purposes, we will code the datasets and remove all personal data before the transfer. Data and information about the patient will be extracted from the clinical file. The signed consent forms must accompany the rectal and vaginal swab samples.

Data extracted from the clinical file are:

- Age
- Body Mass Index (BMI)
- Ethnicity
- Hirsutism or not (and Modified Ferriman Gallwey score)
- PCOM (polycystic ovarian morphology) or not
- Amenorrhea or oligomenorrhea
- Smoking / non-smoking
- Alcohol consumption
- Previous treatments for fertility
- Gynaecological history
- General health status and diseases
- Operations before treatment

- Menstrual cycle
- Previous use of antibiotics (estimated dates)
- Use of hormonal contraceptives

Data will be stored in a databank file in the Erasmus MC using Castor. Data will be further analyzed using analyzing and statistical software (e.g. R). The originally informed consent will be archived. Data sets used for analysis will be stored along with data analysis output and syntax files to facilitate control measures. These sets of data and files will be stored together with the versions of scientific publications reporting the findings of the study for an unlimited time.

12.2 Monitoring and Quality Assurance

To guarantee high-quality research and reliability of data a minimal intensive form of monitoring will be performed in compliance with Good Clinical Practice (GCP) and other rules and regulations. Monitoring services will be provided by the qualified monitoring-unit of the OZBS (Onderzoeksbureau Sophia). This trial was classified as having 'negligible risk' according to the Dutch Federation of Universities (NFU) guidance 'Quality Assurance of Human Subject Research', requiring minimal monitoring. Using guidelines from the NFU, the monitor has established a specific monitoring plan. Based on this plan, the monitoring visit will take place a year after the first inclusion. To assure the quality of research data this monitor will have access to the data and source documents of the trial. For details, see the provided NFU risk classification form in Section K of the METC.

12.3 Dossier Amendments

Amendments are changes made to the research after a favourable opinion by the accredited METC has been given. All substantial amendments will be notified to the METC and the competent authority.
Non-substantial amendments will not be notified to the accredited METC and the competent authority, but will be recorded and filed by the sponsor.

12.4 Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

12.5 Temporary halt and (prematurely) end of study report

The investigator/sponsor will notify the accredited METC of the end of the study within a period of 8 weeks. The end of the study is defined as the last patient's last visit.
The sponsor will notify the METC immediately of a temporary halt of the study, including the reason of such an action.
In case the study is ended prematurely, the sponsor will notify the accredited METC within 15 days, including the reasons for the premature termination.
Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC.

12.6 Public disclosure and publication policy

This trial will be registered on public trial registry ClinicalTrials.gov.
The results will be published in a scientific journal and be made available through a press release. The data will remain accessible upon request. The project will, if necessary, be communicated at the Erasmus MC at the outpatient clinic, University communication boards and the intranet.
All data will be available for verification and re-use for at least 15 years. Full permission to access the data will be granted only to the project leader / coordinating investigator and the principal investigator. In case of an additional research question, this research can only be done with permission of all members of the research team and a signed statement ensuring confidentiality must be obtained.

13. STRUCTURED RISK ANALYSIS

Not applicable

13.1 Potential issues of concern

Not applicable

13.2 Synthesis

Not applicable

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15. Supplementary I

PCOS diagnose using the Rotterdam criteria by a presence of at least two of the following criteria:

- Oligomenorrorrhea or amenorrhea
- Clinical or biochemical hyperandrogenism (modified Ferriman-Gallway score ≥5; testosterone level ≥2nmol/L, Free Androgen Index ≥ 2.9)
- Polycystic ovaries

Table 1

Phenotypes of PCOS according to the Rotterdam 2003 criteria

Features	Phenotypes			
	A	B	C	D
Ovulatory dysfunction	X	X	X	
Hirsutism and/or hyperandrogenemia	X	X		X
Polycystic ovaries	X		X	X

X, Features included in phenotypes