Abstract: The dynamics of the human gut microbiome in PCOS and its therapeutic potential

Duha AlAwad & Nada Al-Emadi

Background

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders affecting 6-10% of women of reproductive age. Due to the complex nature of the disease, its pathophysiology is poorly understood, limiting the development of treatments. PCOS hallmarks are hyperandrogenism and anovulation. It is also often accompanied with metabolic dysfunction which is thought to play a role in its etiology. Dysbiosis, an imbalance in gut microbiota, has been linked to the development of different metabolic diseases. Recent studies have shown that women with PCOS suffer from gut dysbiosis. However, the gut microbiota (GM) in PCOS is not well characterized due to the limited number of studies and the substantial variability in results. Evidence linking GM with PCOS development indicate that restoring the GM using fecal microbiota transplant (FMT) could provide a treatment for PCOS. We hypothesize that gut dysbiosis can initiate and aggravate hormonal imbalances seen in PCOS and re-establishing a healthy GM through FMT could provide a novel therapeutic strategy.

Aims

1. Characterize the composition and function of the GM in PCOS in an ethnically diverse cohort using a multi-omics approach and screen for PCOS biomarkers.
2. Utilize mouse models to study the effect of modifying the GM using FMTs on the development and resolution of PCOS.
3. Explore the use of FMT colonoscopy as a therapeutic strategy for the treatment of PCOS.

Methodology

Study participants will include healthy women and PCOS patients who will be selected according to the Rotterdam criteria. Stool and blood samples will be collected and analyzed. Metagenomics, metaproteomics, and metabolomics will be used to characterize the GM. Blood samples will be analyzed for a comprehensive hormone panel, HbA1c, and metabolites. Mice trials will be conducted using pre-pubertal germ-free female mice in different treatment groups receiving: FMT from healthy women, FMT from PCOS patients, or letrozole. The mice will be monitored for PCOS development and metabolic syndrome features for four weeks. After which, stool samples will be collected, and mice with a PCOS phenotype will receive FMTs from healthy women by means of oral gavage. Mice will be monitored similarly until the end of treatment. Lastly, PCOS patients will partake in clinical trials to evaluate the efficacy of FMT colonoscopy as a potential therapy for PCOS. The patients will be monitored for metabolic changes and changes in the GM for 6 months.

Expected results

We expect PCOS patients to have GM dysbiosis with decreased microbial diversity and unique metabolic/proteomic profile. Mice studies will reveal the possibility of inducing and treating PCOS in mice by modifying the GM using FMTs. Clinical trials on PCOS patients will establish evidence on the use of FMT as a novel therapeutic strategy for PCOS. Our study will provide valuable insight on the gut microbiome-PCOS interplay and demonstrate FMT as a novel treatment for PCOS.

Character count = 2993

Research Proposal

Targeting the gut microbiome to treat Polycystic Ovary Syndrome

Background

Polycystic ovary syndrome (PCOS) is the most common female endocrine disorder affecting 6-10% of women of reproductive age [1]. It is characterized by metabolic and reproductive dysfunctions. There are several diagnostic criteria for PCOS, however, Rotterdam criteria is most frequently used. For a patient to be diagnosed following Rotterdam criteria, they must present with at least two of the following three manifestations: oligo/anovulation, clinical and/or biochemical hyperandrogenism, and polycystic ovary morphology on ultrasound. In addition, underlying conditions that could cause any of the previous symptoms must be excluded [2]. In addition, women with PCOS often suffer from reproductive dysfunctions leading to infertility [3]. The impact of PCOS extends beyond the reproductive system as PCOS patients often suffer from metabolic syndrome features such as insulin resistance, obesity, and/or low-grade inflammation [1]. As a result, women with PCOS have an increased risk of developing cardiovascular disease, type 2 diabetes mellitus, non-alcoholic fatty liver disease and cancer [1]. Moreover, the diagnosis of PCOS is challenging due to a lack of consensus on the diagnostic criteria and the variability of the clinical features in women of different ages [4]. Furthermore, the etiology of PCOS is poorly understood due to the complexity of the syndrome. The heterogeneity of the disorder points towards the involvement of developmental, genetic, epigenetic, and environmental factors [5]. Lastly, we are yet to find a cure for PCOS. Current treatment methods rely on the management of symptoms and monitoring risk factors associated with the disease. Overall, these factors emphasize the need to better understand the pathophysiology of PCOS, identify reliable biomarkers, and develop novel therapeutic strategies to combat this challenging syndrome.

The gut microbiome (GM) is a collection of a diverse community of archaea, bacteria, protozoa, viruses, and fungi that are continuously interacting with the gut of host. Each person has a unique microbial composition dictated by their health, diet, and age [6]. The bacteria are the most abundant microorganisms found in the GM. The most abundant bacterial phyla seen in healthy individuals are Firmicutes and Bacteroides [7]. Disturbances in healthy microbiota that lead to adverse effects on the host are termed dysbiosis. Changes in the microbiome are often assessed by α diversity, which indicates the number of species and their abundance in a sample, and β diversity, which is a measure of similarity between two different sample populations [8]. Research highlighting the potential role of gut dysbiosis in the development of different metabolic disorders led to the birth of a hypothesis linking it to PCOS development. In 2012, Tremellen & Pearce formed a hypothesis that, GM imbalances could result in insulin resistance which ultimately alters the function of the ovaries leading to hyperandrogenemia [9]. Following this theory, in 2016 the first evidence for GM changes in PCOS emerged. Mice induced with PCOS using letrozole (PCOS inducer) showed a significant decrease in both α and β diversity when compare to the control group [10]. This study indicated the potential role of host steroid hormone levels (including androgens) on modulating GM composition. In addition, PCOS patients displayed a similar decrease in α and β diversity of their GMs when compared to healthy women[11]. A recent systemic review assessing all studies done on human PCOS patients identified a total of 10 publications comparing PCOS patients with healthy controls [12]. It revealed substantial variability in the results that are likely due to small sample sizes and the use of different analytical methods. Some studies show significant decrease in α diversity and β diversity of PCOS when compared to healthy women, while others show no significant difference in either one or two of the parameters between PCOS patients and healthy women [12]. Although these studies cumulatively indicate a change in microbial composition in PCOS patients, there is no consensus on the observed

alterations, or an identification of specific microbial taxa that is significantly altered in PCOS. The previously mentioned studies rely on metagenomics to identify the microbial composition of the GM in PCOS. This method only provides information on the taxonomical structure of the microbiota. Utilizing metaproteomic and metabolomic approaches complementary to metagenomics would provide a powerful insight on the mechanisms underlying the observed changes in microbial communities in PCOS.

Considering the established relationship between gut dysbiosis and disease development, a variety of therapeutic strategies have been developed to restore a healthy GM to treat disease. Among these methods is fecal microbiota transplant (FMT) which employs the transfer of fecal microbes from a healthy donor to a recipient to restore a healthy GM composition. FMT is currently considered one of the treatment strategies used for *Clostridium difficile* infections [13]. Guo *et al.* investigated the effect of FMT from healthy women on letrozole treated rats. PCOS rats showed an improved estrous cycle and restored normal ovarian morphology after FMT treatment [14]. Additionally, to understand the role of GM in PCOS development, Qi *et al.* used FMTs from PCOS patients and healthy women on antibiotic treated mice. Mice receiving FMTs from PCOS patients developed PCOS-like phenotype with dysregulated ovarian function and insulin resistance. Although this study is the first to provide a potential causal role of GM dysbiosis in PCOS development, the gut microbial compositions in mice after antibiotic treatment and before FMT was not reported [15]. Despite the scarcity of research and variability between studies, one can infer that the GM could be involved in the development of PCOS. Consequently, the main objectives of our study are to characterize and functionally define the GM in PCOS patients using a multi-omics approach and explore FMT as a novel therapeutic strategy to treat the disease. We hypothesize that gut dysbiosis can initiate and aggravate hormonal imbalances seen in PCOS and re-establishing a healthy GM through FMT is a potential novel therapeutic strategy.

Aims

1- Characterize the composition and function of the GM in PCOS in an ethnically diverse cohort using a multi-omics approach and screen for PCOS biomarkers.
2- Study the effect of FMTs from PCOS patients on PCOS development in germ-free mice and the reversal of these effects using subsequent FMTs from healthy women.
3- Explore the use of FMT colonoscopy as a therapeutic strategy for the treatment of PCOS.

Methodology

Aim 1: Characterize the composition and function of the GM in PCOS in an ethnically diverse cohort using a multi-omics approach and screen for PCOS biomarkers.

We will recruit 500 healthy women as well as 500 PCOS patients aged 18 to 35 from ethnically diverse backgrounds from Canada, Qatar, and Kenya. PCOS patients diagnosed based on the Rotterdam criteria describes earlier will be recruited for this study. In addition, volunteers will be required to fill background and dietary questionnaires. Participants taking antibiotics, birth control pills, or antidiabetic drugs will be excluded from the study. Each participant will be required to submit stool and fasting blood samples at weeks 1, 4, 8, 12 of the study. Fecal samples will be used to collect metagenomics, metaproteomics, and metabolomics data from each sample. Genomic material will be extracted from fecal samples for shotgun sequencing. In addition, high-performance liquid-chromatography mass-spectrometry (HPLC-MS) will used to detect the metabolites and proteins present in emulsified fecal samples. Blood samples will be used to measure the levels of androgens, HbA1c, insulin, luteinizing hormone (LH), follicle stimulating hormone (FSH) and metabolites. Measurements of HbA1c and insulin will allow us to detect

probable features of metabolic syndrome, while androgens, LH and FSH will help us monitor hormonal imbalances relative to PCOS. Hormonal levels will be compared with clinical reference ranges to allow accurate comparison between normal and abnormal levels. Stool and blood samples are collected at 4 different timepoints in the span of 3 months to ensure that the microbiomes characterized are stable microbiomes.

Aim 2: Utilize mouse models to study the effect of modifying the GM using FMTs on the development and resolution of PCOS.

A total of 60 germ-free (GF) C57BL/6 pre-pubertal (21 days old) female mice will be divided into four groups. Group A, the control group, will consist of 10 mice which will receive PBS. Group B consists of 10 mice which will receive FMT from healthy volunteers. Group C will have 20 mice that will receive FMT from PCOS patients. Lastly, group D with 20 mice will receive letrozole, an aromatase inhibitor that is used to induce PCOS phenotypes in female mice by inhibiting the conversion of testosterone to estrogen [16]. Fecal samples will be collected and cultured to ensure the mice are germ-free prior to the experiment. On day 1, after the mice pass the culture test, they will be sorted into their respective groups and will receive PBS oral gavage, FMT oral gavage, or PBS oral gavage + a letrozole subcutaneous pellet. Fecal samples will be collected from the mice at weeks 0, 1, 2, 3, 4, 8, 12, and 16 to detect the establishment of the microbiome and follow its changes. Blood samples will be collected and ultrasound biomicroscopy will be performed on weeks 0, 1, 3, 8, 12, and 16. Daily vaginal smearing on all test groups will be done to monitor the estrous cycle by microscopic analysis. At week 8, a subgroup of 10 mice from group C and 10 mice from group D will be selected to receive oral FMT gavage from heathy women volunteers. These subgroups will be referred to as subC and subD respectively. Fecal samples from mice in subC and subD will be collected at weeks 8, 9, 10, 11, 12, and 16. Blood samples collection and ultrasound biomicroscopy will be performed on weeks 8, 9, 11, 12, and 16 for these subgroups. Shotgun sequencing and HPLC -MS will be performed on fecal samples for metagenomic, metabolomic, and metaproteomic analysis. Blood samples will be analyzed for androgen levels, HbA1c, insulin, LH, FSH, and metabolites to monitor hormonal and metabolic changes over time. The time intervals between consecutive blood collections from mice is spaced out in order to allow the mice to recover from the blood loss and prevent the development of anemia. Multi-omics approach coupled with blood hormones and metabolites will allow for the detection of potentials associations between these parameters. Ultrasound biomicroscopy is used to safely monitor cyst development on the ovaries of the mice.

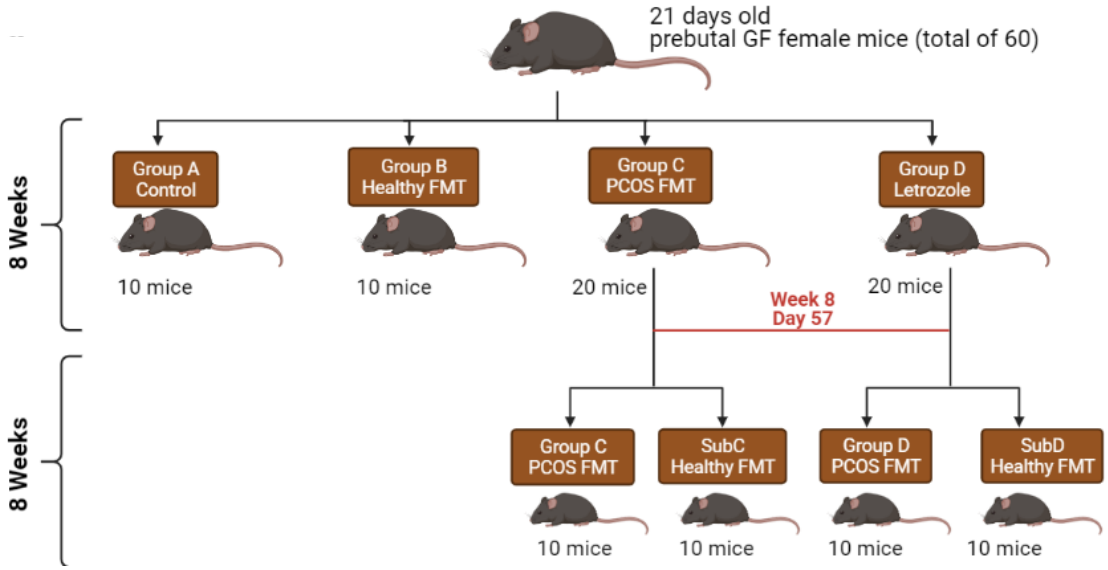

Figure 1: Experimental setup for mouse trials. 60 GF C57BL/6 pre-pubertal female mice are divided into 4 experimental groups and monitored for 8 weeks. At the end of week 8, mice in groups C and D are further divided into subgroup subC and subD and monitored for another 8 weeks. *GF; Germ-free.* The illustrations were adapted from Biorender.

Aim 3: Explore the use of FMT colonoscopy as a therapeutic strategy for the treatment of PCOS.

200 PCOS Qatari patients between the ages of 18 and 25 from will be recruited for this clinical study. Qatar has a high prevalence of PCOS estimated at 12.1%, making it an ideal location to conduct this trial. Patients diagnosed based on Rotterdam criteria will be recruited for this study. Patients will receive FMT colonoscopy and their gut microbiomes and PCOS symptoms will be monitored over the course of 6 months. The selection and screening of healthy female FMT donors as well as FMT sample preparation will be done following the methodology described by OpenBiome [17]. Briefly, multiple samples will be collected from screened healthy donors. These samples will be extensively cultured and bacterial strains will be isolated to make concentrated FMT preparations. These preparations will undergo rigorous quality control testing and identification. In addition to OpenBiome selection criteria, we will select for women with who have a BMI between 18.5 and 24.9 with no current or risk of metabolic disorders. Fecal samples will be collected from patients before the colonoscopy, and at weeks 1, 2, 4, 8, 12, 16, 20, and 24. Blood samples will be collected and ultrasounds performed at weeks 1, 4, 12, 16, 20 and 24. Shotgun sequencing and LC-MS will be performed on fecal samples for metagenomic, metaproteomic, and metabolomic analyses. Blood samples will be analyzed for androgen levels, LH, FSH and metabolites to monitor hyperandrogenemia, and menstrual hormones (FSH and LH). Ultrasounds are used to monitor cyst development on the ovaries.

Bioinformatic pipelines

Metagenomic analysis will be performed using MicrobiomeAnalyst, a web-based user-friendly framework created specifically for microbiome data analysis [18]. Metaproteomic analysis will be performed following the MetaProteomeAnalyzer and Prophane workflow described by Schiebenhoefer et al. [19]. Untargeted metabolomics data will be analyzed using MetaboAnalyst [20]. It is worth to mention that data analysis will be limited by the information present in the databases at our disposal, however, we believe that despite this limitation we will be able to decipher an intricate interplay between the gut microbiome and PCOS. Additionally, we will use in-house scripts to assist in specific tasks during data analysis as needed. Lastly, we will explore the possibility of building a model that is capable of assisting physicians in diagnosing PCOS patients promptly and accurately using supervised machine learning based on the parameters obtain from our results in aim 1.

Expected Results

Dissecting the gut microbiome in healthy women compared to PCOS patients will reveal complex interactions between the microbiota and PCOS. We expect to find characteristic difference in α diversity, β diversity and relative abundance of bacterial species between PCOS patients and healthy women. Due to the discrepancies in previous research, however, we cannot deduce anticipated increase/decrease in α or β diversities, however, we expect to see decreased abundance of lactobacilli. Furthermore, by performing metaproteomics and metabolomics on stool and blood samples from PCOS patients we will be able to determine the identity and quantity of different proteins/metabolites in these samples. It is very likely that we will identify a potential biomarker that is modulated by the gut microbiome and is specific to PCOS. Additionally, recruiting a large cohort from three different

countries will account for population diversity and aid in differentiating microbiome alterations induced by PCOS from those attributed to lifestyle and dietary habits.

In accordance with previous studies, we expect the mice treated with FMT from PCOS patients to develop phenotypes resembling those seen in PCOS [14]. We also expect that treatment with FMT from healthy women will ameliorate the PCOS phenotype in both mice treated with PCOS-FMT and those exposed to letrozole [14,15]. Closely monitoring the microbiome and the development of PCOS in mice will aid in investigating the potential causative relationship between the GM symbiosis and PCOS development. If we observe a decrease in severity of PCOS symptoms after treating PCOS patients with healthy FMT we will have further confirmation of a causative relationship. Furthermore, changes in the functional capacity of bacterial populations as a healthy GM is established in PCOS patients could be helpful in determining associations between bacterial populations and the development/reversal of disease specific phenotypes. For example, a certain bacterial strain may be highly abundant during high androgen blood levels, however, as this strain decrease in abundance after FMT treatment, a decrease in androgen levels maybe observed. Unfortunately, this is merely a speculation as there are not enough studies done on the subject to reach such a conclusion. We aim to use the data collected from this study to provide the cornerstone for such speculations to become conclusions.

We believe FMT colonoscopy will lead to marked decrease in androgen levels, regular ovulation, and a reduction in ovarian cysts in PCOS patients. This study will also provide detailed analysis of the metabolic and microbiome changes that occur as the severity of PCOS symptoms is reduced. Further proving the effectiveness of restoring a healthy GM in treating PCOS patients.

Research Impact

PCOS is the most common female endocrine disorder. However, the diagnostic criteria for the disease are variable leading to both overdiagnosis in females of reproductive age and underdiagnosis in females at age extremes (childhood and perimenopause) [21]. The identification of a biochemical marker for PCOS would provide a rapid diagnostic tool which will aid in early treatment. The etiology of the disease is not yet identified due to the complex pathophysiology and different disease phenotypes [1]. Thus, to date, there is no cure for PCOS, and the available treatments usually target the symptoms of the disease [21]. Therefore, it is imperative to identify the etiology of the disease to aid in the discovery of targeted long-term treatments. The current literature linking PCOS and GM dysbiosis is limited and has considerable variation [22]. Our study will provide a detailed characterization of the GM of a large cohort from multiple countries, and it will shed light on the possible metabolic pathways/markers involved which could provide the groundwork for further studies. We will also put forward a potential therapeutic strategy for PCOS using FMT. These results will help improve the diagnosis of the disease as well as its treatment resulting in lower morbidity and enhanced quality of life for women suffering from PCOS.

Expected Timeline

Overall, the estimated duration of the study is between 3-5 years. We estimate that it will take 1-2 years to complete phase I depending on the speed of volunteer recruitment. Phase II will require 6 months to 1 year to complete the preclinical trials on mice. Depending on the recruitment efficiency, we estimate that phase III will require a duration of 1-2 years to be completed.

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