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Influence of gender and menopausal status on gut microbiota

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ABSTRACT

Objectives: We explore the differences in the gut microbiota associated with gender and hormonal status.
Study design: We included 76 individuals in this study: 17 pre-menopausal women, 19 men matched by age, as a control group for the pre-menopausal women, 20 post-menopausal women and 20 men matched by age as a control group for the post-menopausal women; all 4 groups were also matched by body mass index (BMI) and nutritional background.
Main measurements: We analyzed the differences in the gut microbiota, endotoxemia, intestinal incretins, pro-inflammatory cytokines, and plasma levels of energy homeostasis regulatory hormones between pre- and post-menopausal women and compared them with their respective male control groups.
Results: We found a higher *Firmicutes/Bacteroidetes* ratio, a higher relative abundance of *Lachnospira* and *Roseburia*, and higher GLP-1 plasma levels in pre-menopausal women than in post-menopausal women, who had similar levels to men. In contrast, we observed a lower relative abundance of the *Prevotella*, *Parabacteroides* and *Bilophila* genera, and IL-6 and MCP-1 plasma levels in pre-menopausal women than in post-menopausal women, who had similar levels to the men. We also found higher GiP and leptin plasma levels in women than in men, irrespective of the menopausal status of the women. In addition, adiponectin levels were higher in pre-menopausal women than in their corresponding age-matched male control group.
Conclusions: Our results suggest that the differences in the composition of gut microbiota between genders and between women of different hormonal status may be related to the sexual dimorphism observed in the incidence of metabolic diseases and their co-morbidities.

1. Introduction

The incidence of metabolic diseases and their co-morbidities is sexually dimorphic [1]. Sexual asymmetry in glucose homeostasis has also been described. The prevalence of pre-diabetic syndromes, such as impaired fasting glucose (IFG) and impaired glucose tolerance (IGT), differs by sex, with IFG more prevalent in men, while IGT is more prevalent in women [2]. Moreover, the prevalence of type 2 diabetes mellitus (T2DM) is also marked by sexual dimorphism: there are more diabetic men before puberty and more diabetic women after menopause [2]. Furthermore, it has been suggested that females have stronger immune responses, on the basis of the higher frequency and severity of infectious diseases in males than in females, which in turn makes females more likely to develop autoimmune diseases [3].

The gut microbiota is a symbiotic community that acts as an organ which is fully integrated in the host's metabolism [4]. Interestingly, studies performed over the last few years have shown that the gut microbiota seems to be related with development of metabolic diseases [5], in which sexual dimorphism has been described [2]. Moreover, it has been suggested that gut microbiota composition can be regulated by

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estrogen levels [6–8]. We therefore hypothesized that differences in the gut microbiota, between gender and hormonal status, may be a determinant of the sexual dimorphism in metabolic diseases.

Several studies have suggested that differences in the gut microbiota may trigger the pathogenic mechanisms that involve the development of obesity and insulin resistance [5]. In fact, gender differences in fat distribution have been reported, and these are related with differences in sex hormone levels [9]. In line with this, the sexual dimorphism in total body fat content seen in rodents has been to shown to be absent in germ-free animals, which suggests that the gut microbiota plays a role in host adiposity [10]. In addition, microbiota has also been shown to act by regulating innate and adaptive immunity [4], which may be involved in the higher prevalence of autoimmune diseases in women than in men.

Microbial exposure and sex hormones seem to exert potent effects on autoimmune diseases, such as type 1 diabetes mellitus [11] and autoimmune encephalomyelitis [12], whereas other studies have also related gut microbiota with phospholipid metabolism and cardiovascular risk [13], in which sex hormones are thought to play an important role [14]. On the basis of the potential role of gut microbiota in the sexual dimorphism of metabolic diseases, we aimed to explore the differences in the gut microbiota associated to hormonal status between pre- and post-menopausal women (estrogen depletion), and to compare these with their respective male control groups with a similar age, body mass index and nutritional background.

2. Material and methods

2.1. Study participants

The current work was conducted on a subgroup of patients from the healthy control group (without oncological diseases) included in the ONCOVER study (http://www.proyecto-oncover.es/), which is focused in the development of a volatile compounds detection system for the early diagnosis of lung, colon, breast and prostate cancer. Recruitment was carried out among the free-living population without oncological diseases or disabling diseases or whose severity implied a life expectancy of less than three years. These inclusion and exclusion criteria were assessed according to their medical history, biochemical measurements and physical examination by clinicians. The use of antibiotics in the 3 months prior to sampling was included as an exclusion criteria for the current study. We analyzed 76 patients (39 men and 37 women): 17 pre-menopausal women (estradiol = 109.40 ± 41.46 pg/mL), 19 men matched by age as a control group for the pre-menopausal women, 20 post-menopausal women (years after last menstrual cycle: 6.38 ± 1.14; Spearman correlation between estradiol levels and years after menopause: R = –0.575 P = 0.025; estradiol = 11.49 ± 4.47 pg/mL) and 20 men matched by age as a control group for the post-menopausal women; all 4 groups were also matched by BMI. All the patients gave their written informed consent to participate in the study. The protocol was approved by the Human Investigation Review Committee of Reina Sofia University Hospital (2012/000069), following the Helsinki declaration and good clinical practice. Diet assessment and Clinical plasma parameters were determined as previously [15]. The metabolic characteristics of the subjects in the study are shown in Table 1.

2.2. Measurement of pro-inflammatory cytokines, energy homeostasis regulatory hormones and intestinal incretins in plasma

The patients had fasted (food/drugs) for 12 h and were asked to refrain from smoking during the fasting period and from alcohol intake during the preceding 7 days. They were also asked to avoid strenuous physical activity the day before the test and pre-menopausal women were asked not to attend during the days of menstruation. A fasting blood sample was taken in tubes containing EDTA to give a final concentration of 0.1% EDTA. The plasma was separated from the red cells by centrifugation at 1500 × *g* for 15 min at 4 °C, and then frozen until the biomolecular measurements were taken. The plasma levels of MCP-1, IL-6 and TNF-alpha levels were measured by an IL-1 beta/IL-1F2 Quantikine HS ELISA Kit, IL-6 Quantikine HS ELISA Kit and TNF-alpha Quantikine ELISA Kit, from R&D Systems, Inc.™ (MN, USA), following the manufacturer's instructions. The level of estradiol was determined using the commercial kit: Estradiol EIA kit (Cayman Chemical; Cat. No. 582251), according to the manufacturer's instructions. The plasma levels of adiponectin, leptin and resistin levels were measured by Total Adiponectin/Acrp30 Quantikine ELISA Kit, Leptin Quantikine ELISA Kit, and Resistin Quantikine ELISA Kit, from R&D Systems, Inc.™ (MN, USA), following the manufacturer's instructions. The plasma levels of GLP-1 and GiP were measured using the GLP-1 (7–36)-Amide EIA kit and GiP EIA kit (Phoenix Europe GmbH, Germany), according to the manufacturer's instructions. The endotoxin lipopolysaccharide (LPS) was measured using the limulus amebocyte lysate test (QCL-1000 Chromogenic LAL (Lonza Iberica S.A., Spain), as previously described [16]. LPS Binding Protein (LBP) measurements were made using the human LBP ELISA kit (Hycult biotech, Netherlands), according to the manufacturer's instructions.

2.3. Sequencing the V1-V2 microbial 16S rRNA gene on the Illumina MiSeq

DNA extraction from fecal samples was performed by the QIAamp DNA kit Stool Mini Kit Handbook (Qiagen, Hilden, Germany), following the manufacturer's instructions. The 76 samples were amplified in triplicate by polymerase chain reaction (PCR) to generate an amplification library. The bacterial 16S rRNA gene V1-V2 hypervariable regions were amplified in polymerase chain reactions (PCR) using universal bacterial primers 8F (AGAGTTTGATCMTGGCTCAG) and 357R (CTGCTGCCTYCCGTA) complemented with 8 nt index and Illumina adapter sequences. PCR was performed with Smart-Taq Hot Red 2X PCR Mix (Naxo, Estonia), 1 μl of extracted DNA (10 ng/μl), 0.2 μM of each primer, using the following cycling parameters: 15 min denaturation at 95 °C followed by 3 cycles (30 s at 95 °C, 30 s at 50 °C, 60 s at 72 °C), 28 cycles (30 s at 95 °C, 30 s at 65 °C, 60 s at 72 °C) and a final extension at 72 °C for 7 min. Oligonucleotides were removed from pooled PCR product library using the QIAquick® PCR Purification Kit (Qiagen, Inc.). Single end sequencing of V2 hypervariable region was performed on Illumina MiSeq next generation sequencing platform using the v3 kit (a service provided by The Estonian Genome Center Core Facility).

2.4. Upstream analysis of the 16S rRNA gene sequences

The 16S rRNA gene sequences obtained were analyzed using QIIME 1.9.1 with default parameters unless indicated otherwise [17]. Raw sequencing data was de-multiplexed and low quality and short (< 150 nt) readings were discarded. The readings were clustered using a closed-reference OTU picking protocol that assigned readings to reference sequences. Briefly, the processing involved the following steps: (1) demultiplexing and filtering of short (< 150 nt) and low quality readings; (2) de novo clustering of the sequences into operational taxonomic units (OTUs) with the USEARCH61 program using a 97% similarity threshold [18] and the Greengenes v13-8 database [19]; (3) taxonomical assignment of each OTU by running the RDP Classifier [20] at 85% bootstrap confidence on a selected representative sequence from each OTU; (4) alignment of representative sequences using PyNAST [21] with the Greengenes core-set alignment template.

The differences between the bacterial communities were calculated in QIIME using rarefaction curves of alpha-diversity indexes including estimates of community richness (such as the Chao1 estimator and the observed number of OTUs present in each sample and Phylogenetic diversity (PD) or the length of the phylogenetic branch observed in each sample). The upper limit of rarefaction depths was 16,998 sequences per sample. Beta diversity was estimated using weighted and