THE ROLE OF OBESITY AND METABOLIC SYNDROME IN COUPLE INFERTILITY

EDITED BY: Elisabetta Baldi, Sara Marchiani, Nicole McPherson and Lara Tamburrino

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THE ROLE OF OBESITY AND METABOLIC SYNDROME IN COUPLE INFERTILITY

Topic Editors:
Elisabetta Baldi, University of Florence, Italy
Sara Marchiani, University of Florence, Italy
Nicole McPherson, University of Adelaide, Australia
Lara Tamburrino, University of Florence, Italy

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EDITORIAL
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Editorial: The Role of Obesity and Metabolic Syndrome in Couple Infertility

Sara Marchiani[1], *Lara Tamburrino*[2], *Nicole McPherson*[3,4,5] *and Elisabetta Baldi*[2]*

[1] Department of Clinical and Experimental Biomedical Sciences, University of Florence, Florence, Italy, [2] Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy, [3] Faculty of Health and Medical Sciences, School of Biomedicine, University of Adelaide, Adelaide, SA, Australia, [4] Freemasons Centre for Male Health and Wellbeing, University of Adelaide, Adelaide, SA, Australia, [5] Robinson Research Institute, University of Adelaide, Adelaide, SA, Australia

Keywords: obesity, metabolic syndrome, infertility, reproduction, spermatozoa, oocyte

Editorial on the Research Topic

The Role of Obesity and Metabolic Syndrome in Couple Infertility

Metabolic syndrome (MetS) is a pathological condition characterized by abdominal obesity, insulin resistance, hypertension, and hyperlipidemia. It is estimated that in the USA MetS has reached an overall prevalence of more than 30% of adults older than 18 years, with incidences higher in some races and sociodemographic backgrounds (1). Together with obesity, the prevalence of MetS is increasing worldwide every year, with some of the biggest growths occurring in developing countries, gaining the terminology of a global pandemic (2).

MetS and obesity have a strong negative impact on reproductive function in both females and males causing hormonal imbalances and gonadal dysfunction. In women, both MetS and obesity are among the causes of polycystic ovarian syndrome (PCOS) (3) which, in turn, may result in alterations to endometrial receptivity. In men, obesity is related to hypogonadism and other sexual and reproductive dysfunctions, including alterations to gamete production and sperm function (4).

Several papers included in our research topic focused on PCOS, clarifying some aspects of the syndrome and giving some hints on possible treatments. Yang et al. demonstrated that serum prolactin levels are decreased in PCOS women and are positively associated with high-density lipoprotein cholesterol and, negatively, with body mass index (BMI), waist circumference, and reproductive hormone levels, likely contributing to the infertility problem of these women. Di Stasi et al. identified sex-hormone-binding globulin (SHBG) as a possible diagnostic parameter for the occurrence of non-alcoholic fatty liver disease associated with PCOS, as well as a possible marker of metabolic impairment in PCOS women. Barros de Melo et al. used an animal model to study the influence of high sucrose diet (HSD) administered during puberty on the ovarian hormonal milieu. They show that HSD determined MetS-like characteristics in these animals, which showed an increase in the prevalence of PCOS leading to ovarian dysfunction. Cao et al. showed that by evaluating three-dimensional (3D) genome interactions in ovary tissue from a PCOS mouse model, STON1 and FSHR genes were identified as potential targets for the PCOS-susceptible locus rs13405728, providing insights into the pathogenesis of PCOS. Two papers are related to therapeutic approaches for PCOS. Liu et al. reviewed published randomized control studies regarding insulin sensitivity and pregnancy rate in infertile PCOS women. Overall, these studies demonstrate that various non-surgical therapeutic strategies aimed to improve insulin sensitivity in PCOS women increase the possibility of successful pregnancy and that the improvement of insulin

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Jeff M. P. Holly,
University of Bristol, United Kingdom

***Correspondence:**
Elisabetta Baldi
elisabetta.baldi@unifi.it

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sensitivity might be even more important than reduction of BMI for ameliorating pregnancy rate. Zhu et al. employed a PCOS animal model to study the possible mechanisms of action of Chinese herbal medicine used in China to treat PCOS women. They found that the medicine decreases inflammation and improves insulin resistance in these animals by regulating intestinal flora.

Regarding obese/MetS men and reproductive function, Tančić-Gajić et al. evaluated testosterone levels on severely obese men affected by obstructive apnea. They found that both free and total testosterone levels were decreased in men with elevated apnea/hypopnea index, severe obesity, and MetS. Further, they found that obstructive sleep apnea is an independent determinant of serum testosterone concentrations in severely obese, highlighting the link between sleep quality and hormone production. Pini et al. reviewed the evidence of rodent dietary models of male obesity and reproductive dysfunction and the importance of assessed outcomes using a practical application approach such as Nutritional Geometry. Nutritional Geometry assesses outcomes of interest over an extended range of dietary macronutrient compositions rather than an individual macronutrient effect and, therefore, provides a promising tool for the development of evidence-based pre-conception nutritional guidelines for men. Finally, Zhao et al. assess the combined and independent impacts of both male and female body mass index (BMI) on cumulative pregnancy outcomes in a large Asian assisted reproduction cohort (15,972 couples). They found that both increasing female BMI and overweight males independently reduced cumulative pregnancy rates, with pregnancies rates further decreased if both partners were overweight, indicating a synergistic effect from couple overweight. They concluded that a couples approach to lifestyle change was warranted.

In summary, this Research Topic provides a large range of evidence that supports the collaborative efforts of endocrinologists, gynecologists, and reproductive biologists to continue to work together for the management of male and female reproductive health and the interplay of obesity and MetS.

AUTHOR CONTRIBUTIONS

All authors contributed to writing and approved the submitted version of this editorial by resuming the results of all scientific articles included in the Research Topic "*The Role of Obesity and Metabolic Syndrome in Couple Infertility*".

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Guizhi Fuling Wan, Chinese Herbal Medicine, Ameliorates Insulin Sensitivity in PCOS Model Rats With Insulin Resistance via Remodeling Intestinal Homeostasis

Ying Zhu[1], *Yin Li*[1], *Min Liu*[1], *XiaoDan Hu*[1] *and Hongqiu Zhu*[2]*

[1] School of Clinical Medical, Chengdu University of Traditional Chinese Medicine, Chengdu, China, [2] College of Medicine and Life Sciences, Chengdu University of Traditional Chinese Medicine, Chengdu, China

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Edited by:
Lara Tamburrino, University of Florence, Italy

Reviewed by:
Gonzalo Cruz, Universidad de Valparaiso, Chile
Jürgen Michael Weiss, University of Lucerne, Switzerland
Francesco Elia Marino, University of Pennsylvania, United States

***Correspondence:**
Hongqiu Zhu
zhuhongqiu@cdutcm.edu.cn

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Polycystic ovary syndrome (PCOS) is a common endocrine disease with reproductive dysfunction and metabolic disorder in women of childbearing age. Gastrointestinal microbiome contributes to PCOS through mediating insulin resistance. Guizhi Fuling Wan, Chinese herbal medicine, can treat PCOS with insulin resistance (PCOS-IR), but the underlying mechanism is not clear. The aim of this study was to characterize the exact mechanism of Guizhi Fuling Wan action and whether it is related to the regulation of intestinal flora structure. We induced PCOS-IR rat model by means of letrozole sodium carboxymethyl cellulose (CMC-na) solution combined with high-fat emulsion administration and randomly divided it into blank control group (K), model control group (M), low dose of Guizhi Fuling Wan group (D), middle dose of Guizhi Fuling Wan group (Z), high dose of Guizhi Fuling Wan group (G) and positive drug (Metformin) control group (Y). After 36 days of modeling and treatment, serum and stool samples from all rats were collected for a follow-up analysis. The data display that, compared with K group, elevated testosterone and HOMA-IR, turbulent estrous cycles and polycystic ovaries in M group, indicating the PCOS-IR rat model is successfully established. Increased fasting insulin is associated with higher inflammation(plasma TNF-α, IL-6, and HS-CPR concentration were determined) in M group, and the altered intestinal flora (compared with the K group, in M group the relative abundance of *Alloprevotella* was decreased significantly, while the relative abundance of *Lachnospiraceae UCG-008*, *Lachnospiraceae NK4A136*, *Lactobacillus, Ruminiclostridium 9,* and *Ruminococcaceae UCG-003* was increased significantly) induced the secretion of inflammatory markers. On the other hand, Guizhi Fuling Wan can alleviate inflammation, improve insulin resistence: Lower inflammation decreased fasting insulin can be seen in G group compared with M group, this effect is related to the regulating effect of Guizhi Fuling Wan on intestinal flora (in G group, the relative abundance of *Alloprevotella*, *Ruminococcaceae UCG-003,* and *Lachnospiraceae UCG-008* was increased significantly, compared with M group).

This research demonstrates Guizhi Fuling Wan improve insulin resistance in polycystic ovary syndrome with the underlying mechanism of regulating intestinal flora to control inflammation. It would be useful to promote the therapeutic effect of Guizhi Fuling Wan on PCOS-IR.

Keywords: polycystic ovary syndrome, Guizhi Fuling Wan, gastrointestinal microbiome, insulin resistance, homeostasis

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common reproductive endocrine disorders in women of childbearing age, and it is also the leading cause of anovulatory infertility (1). Its etiology is complex and multiple factors contribute to the development of polycystic ovary syndrome. The prevalence of PCOS in women of reproductive age ranges from 6 to 20% around the world (2), and the incidence rate is 5.6% in China (3). Clinical and/or biochemical hyperandrogenemia (HA), oligo-anovulation (OA), and polycystic ovarian morphology (PCOM) are three main characteristics of PCOS, the diagnosis of PCOS, according to the Rotterdam Consensus criteria (2003), requires two of the above (4). In addition to reproductive dysfunction, PCOS patients often have metabolic disturbances characterized by insulin resistance (IR) and display an inflammatory state, with an increased risk of developing type 2 diabetes (T2D), dyslipidemia, hypertension, and cardiovascular disease (5–9). Besides, PCOS-related insulin resistance and chronic inflammation is independent of obesity (10, 11). Insulin resistance affecting about 44–70% of PCOS patients (12), is believed to be a key factor in the development of PCOS, closely related to chronic inflammation (1, 13, 14).

The gut is the body's largest organ for digestion and detoxification where hundreds of millions of bacteria inhabit. Abnormal gut microflora is closely related to the occurrence of diseases. Pedro and Bryan performed a cohousing study using a letrozole-induced PCOS mouse model, the results suggest that dysbiosis of the gut microbiome may contribute to PCOS (1). In human studies, scholars found the structure of intestinal flora in PCOS patients is significantly different from that in normal women and a subtle link can be seen between the metabolic abnormality of polycystic ovary syndrome and the gut microbiome changes (1, 15, 16). In 2004 Bäckhed et al. had confirmed that gut microbiota is associated with the onset of insulin resistance (17). The change of gut microbiota can directly and indirectly affect immune cells in the gut to mediate insulin resistance, the indirect effects are achieved via gut microbial products (LPS, metabolites, and SCFAs) which are closely related to inflammation (18). So the above studies suggest that gut microbiota contributes insulin resistance with the underlying mechanism of the inflammation induced by gut microbiota.

In China, Traditional Chinese medicine is still one of the main methods to treat PCOS. Originated from the *Synopsis of the golden chamber · treatment of pregnancy diseases* written by Zhang Zhongjing, Guizhi Fuling Wan, a traditional Chinese Medicine formula composed of *Gui Zhi*, *Fu Ling*, *Tao Ren*, *Bai Shao*, *Dan Pi*, has been widely used to treat multiple gynecological diseases (19). According to the basic theory of traditional Chinese medicine, it can activate blood, resolve blood stasis, and dissipate phlegm. Based on modern medical researches, Guizhi Fuling Wan has an excellent anti-inflammatory effect and the ability to improve insulin resistance, usually used to treat chronic pelvic inflammatory disease and polycystic ovary syndrome (20–23). *Gui Zhi*, *Fu Ling*, *Tao Ren*, *Bai Shao*, the compositions of Guizhi Fuling Wan, are frequently used to treat PCOS, Besides, *Bai Shao* contains paeoniflorin which is an effective compound in the treatment of PCOS (24), and the efficacy of paeoniflorin in improving insulin resistance has been proved in both mammals and vitro experiments (25, 26). Researchers also found paeoniflorin plays an active role in the regulation of beneficial bacteria in intestinal flora. We speculate that Guizhi Fuling Wan containing *Bai Shao* could also regulate intestinal flora and its efficacy in improving PCOS-IR and PCOS related inflammation is achieved by affecting gut microbiota. In this study, we choose letrozole combined with high fat diet to induce a PCOS-IR rat model (27, 28) to investigate the assumptions we mentioned above.

MATERIALS AND METHODS

Animals and Animal Husbandry

Six-week-old specific-pathogen free (SPF) level female Sprague-Dawley (SD) rats [Laboratory animal license number: SCXK (chuan) 2015-030] with-body mass of 180–200 g were provided by Chengdu Dossy Experimental Animals Co., Ltd. All animals raised in SPF laboratory of experimental animal center of Chengdu University of TCM and the whole animal experiment operating process in accordance with the Animal Care Committee of Chengdu University of TCM, China (SYXK2018-0126). In this experiment, 72 rats were randomly divided into six groups of 12 rats each, including blank control group (K), model control group (M), low dose of Guizhi Fuling Wan group (D), middle dose of Guizhi Fuling Wan group (Z), high dose of Guizhi Fuling Wan group (G), and positive drug (Metformin) control group (Y). The blank control group rats received a gavage of normal saline once a day (10 ml/kg). Rats in the other groups were given a gavage of letrozole (Jiangsu Hengrui pharmaceutical Co., Ltd., China) at a concentration of 1 mg/kg dissolved in aqueous solution of carboxymethylcellulose (CMC [10 g/L]) and high-fat emulsion (76.9% lard and 23.1% cholesterol) at concentrations of 15 ml/kg once a day. At the same time, rats in D, Z, G groups were administered with Guizhi Fuling Wan (Chengdu Jiuzhitang Jinding Pharmaceutical Co., Ltd., China) at concentrations of 0.31, 0.62, and 1.24 g/kg by gavage, respectively, once a day, Y group rats was performed

metformin (Sino-American Shanghai Squibb pharmaceutical Co., Ltd., China) by gavage once a day at a concentration of 270 mg/kg. The entire modeling and administration process lasted successive 35. From the 6 day on, vaginal smears were collected daily. On 35 day all rats fasted at 8 p.m. On 36 day, all rat fecal samples were collected and stored in a stool box (−800°C) for 16S rDNA amplicon sequencing. Then all rats were sacrificed by cervical dislocation. All blood samples and ovarian tissue samples were collected for following analysis.

Vaginal Smear and Ovarian Morphological Observation

From the 6 day on, vaginal smears were collected daily and the determination of estrous cycle was evaluated microscopically with hematoxylin-eosin (HE) staining. On the 36 day, ovaries were obtained, weighed, fixed in 10% paraformaldehyde for 48 h, and then embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin and analyzed using an Olympus DP73 microscope.

Biochemical Assays

All rats' fasting plasma glucose (FPG) was measured by bayer blood glucose meter 1455 (Bayer HealthCare LLC). Serum INS, and testosterone (T) was determined by radioimmunoassay (RIA) using RIA kits (Mibio, Shanghai, China). According to calculation formula: $HOMA - IR = \frac{FINS \times FPG}{22.5}$, we used FPG and FINS to calculate Homeostasis model assessment of insulin resistance index (HOMA-IR) which is used to evaluate insulin sensitivity and to observe the efficacy of Guizhi Fuling Wan in improving insulin resistance. Plasma TNF-α were determined using enzyme-linked immunosorbent assay (ELISA) kit (Elabscience Biotechnology, Wuhan, China), plasma TNF-A, IL-6, and HS-CRP were determined using enzyme-linked immunosorbent assay (ELISA) kits [MultiSciences (LiankeBio), Hangzhou, China].

16S rRNA Gene Sequencing and 16S rRNA Gene Sequence Analysis

Genomic DNA from rat feces samples was extracted using a specific DNA extraction kit, and then the DNA was detected by 0.8% agarose gel electrophoresis. With the diluted genomic DNA as the template, The 16S rRNA V4 region of the sample was amplified by PCR using 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (29, 30), three PCR technique repeats were performed for each sample. The resulting amplicons were recovered, purified with the QIA quick Gel Extraction Kit (QIAGEN) and quantitated with the qubit@2.0 Fluorometer (Thermo Scientific). Illumina TruSeq DNA PCR-Free Sample Prep Kit (FC-121-3001/3003) was used for libraries construction and PE250 sequencing was performed using Illumina Hiseq Rapid SBS Kit V2 (FC-402-4023 500 Cycle). According to the overlap relationship between PE reads, the double-ended sequence is spliced into a sequence using FLASH8, that is, Raw Tag. In order to obtain high-quality sequences, it is necessary to carry out quality control on the sequences, and the sequences obtained after quality control are called Clean Tag. UCHIME9 algorithm was used to remove chimerism and Effective Tag was obtained. The following bioinformatics operation is completed using Usearch (version 7.1) and QIIME. In order to facilitate the study of the species composition and diversity information of samples, the taxon in the clustering generation operation of sequences, namely OUT (Operational Taxonomic Units), is needed. OTU cluster analysis is usually performed at a similar level of 97%. Each OTU represents a set of similar sequences, and the OTU abundance table can be obtained through the OTU cluster analysis, which is the basic file for subsequent analysis. To obtain the corresponding classification information of each OTU, UCLUST taxonomy, and SILVA database (Rlease_123) were used to annotate the classification of the representative sequences of OUT. The richness, evenness, and diversity of the microbial community can be reflected by calculating the alpha-diversity index (Simpson index) of the samples.

Statistical Method

Statistical analysis was performed using IBM SPSS25.0 software. Data were expressed as Mean ± sem. Normality test was performed by Shapiro-Wilk test and homogeneity of variance test was performed by Levene test. One-way ANOVA with LSD's *post-hoc* test was used to determine the significance of the data which is satisfied the normal distribution and the equal variance among groups. If the data is satisfied non-normal distribution and/or unequal variance, Kruskal-Wallis non-parametric tests were used to determine the significance of the data. Because the data is not binormal, Spearman correlation analysis is used to assess the correlation between the data. $P < 0.05$ means the difference is statistically significant.

RESULTS

Letrozole and High-Fat Emulsion Administration Resulted in Typical Endocrine and Ovarian Morphology and Function Changes of PCOS

Letrozole and High-Fat Emulsion Administration Resulted in Elevated Serum Testosterone Levels

The serum testosterone concentrations was significantly higher in M group rats than those in K group rats. After administration of Guizhi Fuling Wan, the concentration of plasma testosterone is decreased, but without significant difference. Metformin can significantly inhibit testosterone secretion (**Figure 1A**).

Letrozole and High-Fat Emulsion Administration Resulted in Disturbed Polycystic Ovarian Morphology and Estrou Cycle

The ovaries of the K group showed normal morphology (**Figure 1B**). Rats in M group appeared increased vesicular follicles, atretic follicles, and thin granular cell layer (**Figure 1C**).

As the results showed, the estrous cycle of rats in the blank control group showed regular periodic changes, while the estrus

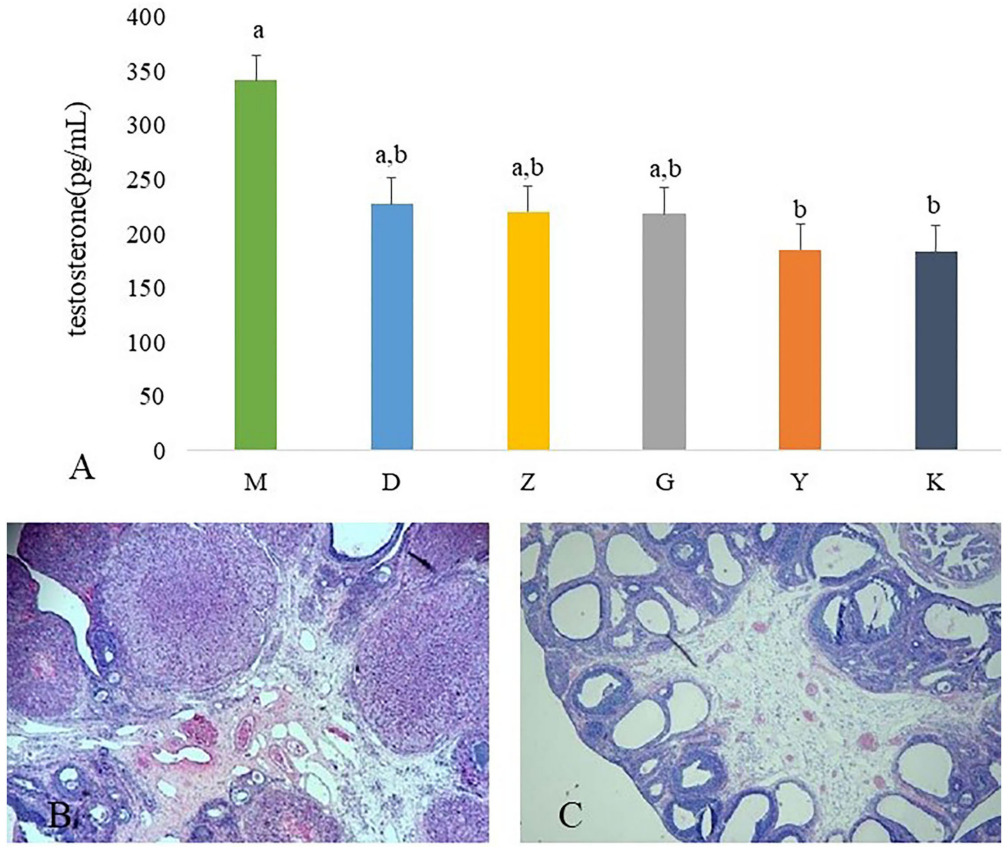

FIGURE 1 | Results of ovarian tissues morphology changes and the plasma concentration of testosterone. The plasma concentration of testosterone in model control group (M), low dose of Guizhi Fuling Wan (L), middle dose of Guizhi Fuling Wan (Z), high dose of Guizhi Fuling Wan (G). Different superscript letters indicate significant differences ($P < 0.05$) in data according to Kruskal-Wallis statistical analysis **(A)**. The ovarian tissue morphology of blank control group (K) **(B)**, the ovarian tissue of model control group (M) **(C)**.

cycle of rats in the model group was disordered and basically maintained in the diestrus. The vaginal smear in the model group showed a large number of spot-shaped white blood cells and a few patchy keratinocytes (**Figure 2C**). Guizhi Fuling Wan could improve the estrous cycle of rats, and its effect was similar to that of metformin: the estrus cycle of rats in the low, middle, high dose of Guizhi Fuling Wan groups and the positive drug group basically recovered and showed periodic changes (**Figures 2A,B,D**)

Guizhi Fuling Wan Treatment Could Ameliorate IR

Compared with the K group, plasma fasting blood glucose, fasting insulin level, and the insulin resistance index were significantly increased in the M group ($P < 0.01$). Compared with the M group, the plasma fasting blood glucose, fasting insulin level and insulin resistance index of D, Z, G, Y groups rats were significantly decreased ($P < 0.01$; **Table 1**).

Guizhi Fuling Wan Treatment Reduce the Plasma Concentration of Inflammatory Markers to Control Inflammation

In order to better link the changes in intestinal flora of PCOS model rats with insulin resistance, we measured the serum concentration of HS-CPR, IL-6, and TNF-α. And statistics indicate that a significant increase in plasma concentration of HS-CPR, IL-6, TNF-α was found in M group, compared with K group (**Figures 3A–C**). Although it did not completely inhibit inflammation, Guizhi Fuling Wan can reduced the release of HS-CPR, IL-6 and TNF-α to alleviate the systemic inflammatory state (**Figures 3A–C**).

In addition, we further analyzed the correlation between inflammatory cytokines and fasting insulin. The results showed that the serum fasting insulin concentration increased with the increase of il-6 and HS-CRP (**Figures 3D,E**).

Alpha Diversity Analysis of Intestinal Flora

Simpson: one of the indices used to estimate microbial alpha diversity, taking into account the abundance and evenness of

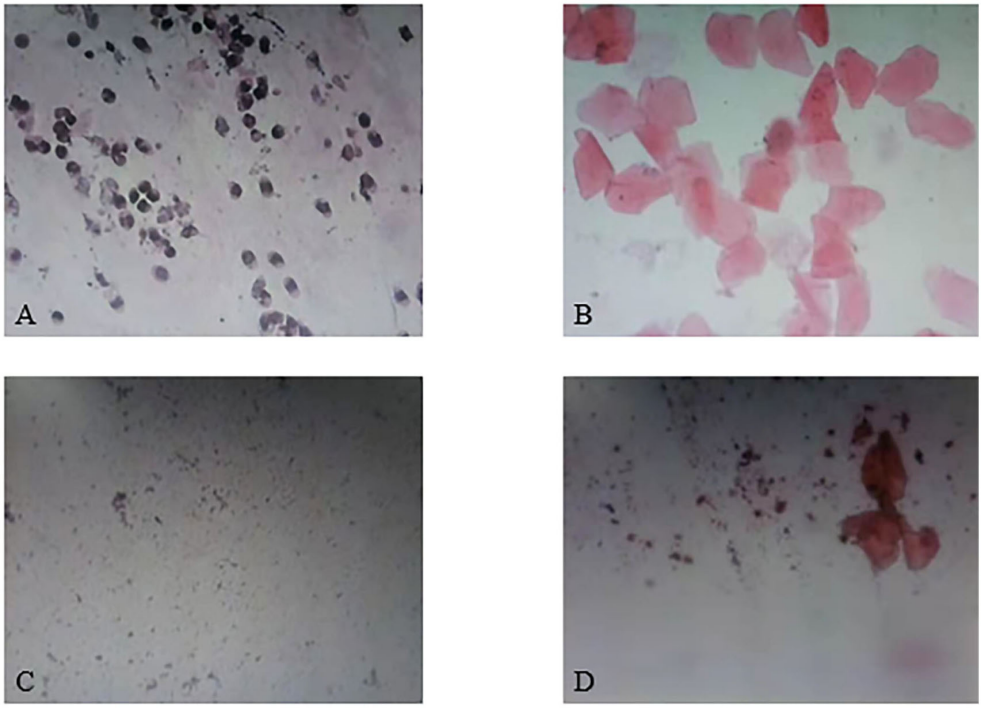

FIGURE 2 | Results of vaginal smear. **(A)** Proestrus. **(B)** Estrus. **(C)** Diestrus. **(D)** Metestrus.

TABLE 1 | Fasting blood glucose and insulin levels of rats in each group.

Group	FPG (mmol/L)	FINS (mU/L)	HOMA-IR
K	4.34 ± 0.21	25.70 ± 5.60	5.08 ± 1.14
M	6.94 ± 0.41$^{\Delta}$	46.95 ± 7.93$^{\Delta}$	14.81 ± 2.56$^{\Delta}$
D	6.25 ± 0.39*	32.71 ± 6.18*	9.21 ± 1.80*
Z	5.61 ± 0.64*	32.24 ± 6.33*	8.37 ± 1.61*
G	5.31 ± 0.22*	31.81 ± 4.09*	7.50 ± 0.94*
Y	5.51 ± 0.34*	31.93 ± 6.80*	8.16 ± 1.88*

$^{\Delta}P < 0.01$ *data are significantly different vs. blank control group (K).*
*$P < 0.01$ *data are significantly different vs. model control group (M).*
Blank control group (K), model control group (M), low dose of Guizhi Fuling Wan group (D), middle dose of Guizhi Fuling Wan group (Z), high dose of Guizhi Fuling Wan group (G), and positive drug (Metformin) control group (Y).

species. Here the result is 1-D (D is Simpson's Index), and the higher 1-D, the higher α diversity of gut microflora. There was no significant difference in 1-D between the model group and the blank control group ($P > 0.05$). Compared with the model group, the 1-D of D and Z group was significantly lower than that in the model group ($P < 0.01$, $P < 0.05$; **Table 2**), however, there was no significant difference in 1-D of G and Y group (**Table 2**).

As *Firmicutes*, *Bacteroidetes,* and *Proteobacteria* are the three main types of bacteria with the highest relative abundance in intestinal flora, the changes in the number of these three bacteria will have a certain impact on the structure and function of intestinal microecology. Compared with M group, there was a decrease can be seen in the number of *Firmicutes*, *Bacteroidetes,* and *Proteobacteria* in D group (without a significant difference) (**Table 3**). The number of *Firmicutes* and *Bacteroidetes* in Z group had a slight elevation **(Table 3)**, but the relative abundance of *Proteobacteria* was significantly decrease, compared with M group. So in general, the relative abundance of the three bacteria in D and Z group had decreased, which leading to a decrease in intestinal diversity. In G group, compared with M group, the number of *Firmicutes* was significantly increased, while the *Bacteroidetes* was significantly decreased. A rise and fall can be seen in G group, so the diversity of intestinal flora had no difference between G and M group. We can speculate from the results that the higher the dose of Guizhi Fuling Wan, the higher the α-diversity of intestinal flora.

Intestinal Flora Abundance Analysis

Changes of Intestinal Flora in PCOS-IR Model Rats

Both M group and K group were mainly composed of *Bacteroidetes*, *Firmicutes,* and *Proteobacteria*. In the M group, *Bacteroidetes* accounted for 49.18%, *Firmicutes* for 45.78% and *Proteobacteria* for 3%. At the phylum classification level, compared with K group, the relative abundance of *Bacteroidetes* was decreased with no significant difference **(Figure 4A**, **Table 4)**, and the relative abundance of *Firmicutes* and *Proteobacteria* were increased with no significant difference **(Figure 4A**, **Table 4)**, it is worth mentioning that the *P*-value between the number of *Firmicutes* in M group and K group is just equal to 0.05, which is statistically

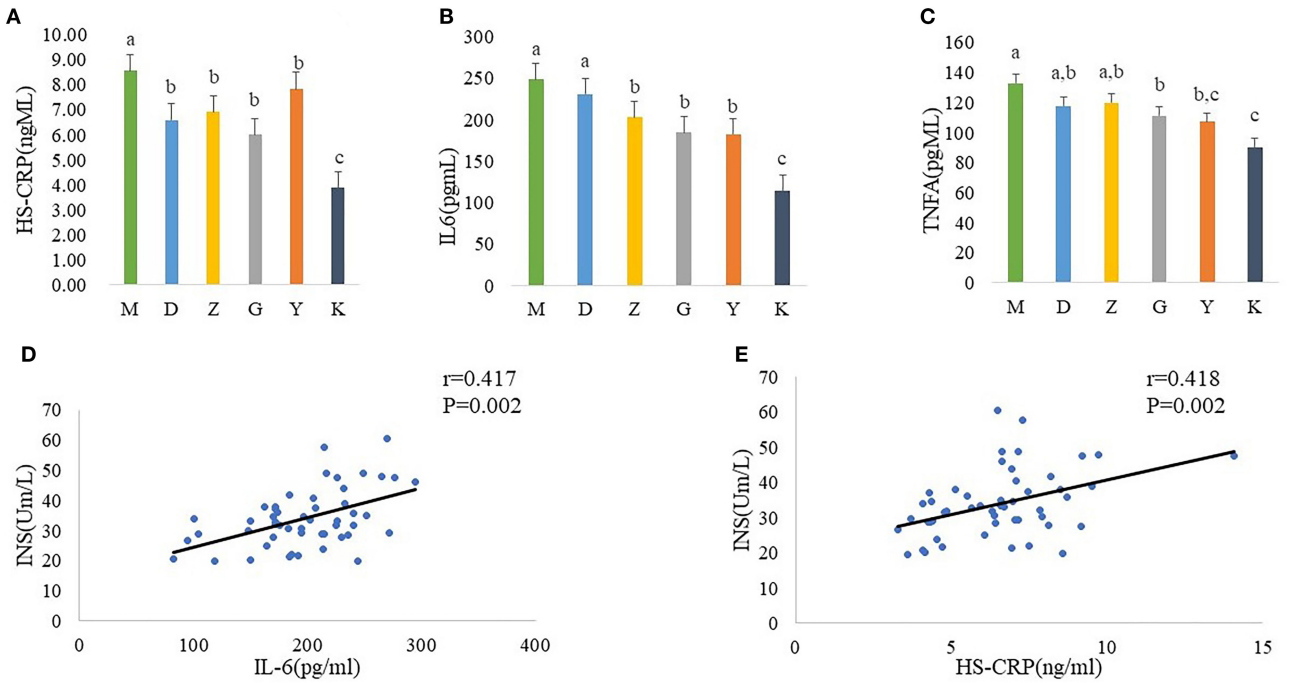

FIGURE 3 | Plasma concentration of HS-CPR **(A)**, IL-6 **(B)**, and TNF-α **(C)** in model control group (M), low dose of Guizhi Fuling Wans (L), middle dose of Guizhi Fuling Wans (Z), high dose of Guizhi Fuling Wans (G). Different superscript letters indicate significant differences ($P < 0.05$) in data according to Kruskal-Wallis and the *post-hoc* ANOVA statistical analysis. Correlation analysis between inflammatory cytokines and fasting insulin: A positive correlation can be seen between inflammatory cytokines (IL-6, HS-CRP) and INS **(D,E)**. The "*r*" refers to the Spearman correlation coefficient, and the $P < 0.05$ indicate differences are significant.

TABLE 2 | Simpson index of intestinal flora of rats in each group.

Group	Case number	1-D
M	11	0.9867 ± 0.0007
D	10	0.9533 ± 0.0058**
Z	9	0.9630 ± 0.0023*
G	10	0.9862 ± 0.001
Y	9	0.9877 ± 0.0005$^{\Delta\Delta}$
K	11	0.9647 ± 0.0029

$^{\Delta\Delta}P < 0.01$ *data are significantly different vs. K group.*

*$P < 0.05$, **$P < 0.01$ *data are significantly different vs. M group.*

Blank control group (K), model control group (M), low dose of Guizhi Fuling Wan group (D), middle dose of Guizhi Fuling Wan group (Z), high dose of Guizhi Fuling Wan group (G), and positive drug (Metformin) control group (Y).

significant in theory but not in practice. Therefore, we believe that there is no significant difference in the number of *Firmicutes* between group M and group K. At the genus classification level, compared with the K group, the relative abundance of *Alloprevotella* was decreased significantly ($P < 0.01$; **Figure 4B**, **Table 4**). While the relative abundance of *Lachnospiraceae UCG-008*, *Lachnospiraceae NK4A136*, *Lactobacillus*, *Ruminiclostridium 9,* and *Ruminococcaceae UCG-003* was increased significantly ($P < 0.01$; **Figure 4B**, **Table 4**). The remaining bacteria had no significant difference between the K and M group (**Table 4**).

Gut Flora Is Related With Inflammation

Alloprevotella, *Lachnospiraceae UCG-008*, *Lachnospiraceae NK4A136*, *Lactobacillus*, *Ruminiclostridium 9,* and *Ruminococcaceae UCG-003* were choose for further analysis on the basic of results that the abundance of these five intestinal bacteria showed a significant difference between group M and group K. Results indicate the plasma concentration of IL-6,HS-CRP, TNF-α was associated with the abundance of *Alloprevotella*, *Lachnospiraceae UCG-008*, *Ruminococcaceae UCG-003*: there was a negative correlation between *Alloprevotella* and inflammatory markers (IL-6,HS-CRP) **(Figures 5A,B)**; a positive correlation can be found in *Ruminococcaceae UCG-003* and inflammatory markers (IL-6,HS-CRP) **(Figures 5C,D)**, as well as in *Lachnospiraceae UCG-008* and inflammatory markers (IL-6, HS-CRP, TNF-α) **(Figures 5E–G)**.

Guizhi Fuling Wan Treatment Alter the Relative Abundance of Multiple Intestinal Flora

Combining the results given above, we chose *Alloprevotella*, *Ruminococcaceae UCG-003,* and *Lachnospiraceae UCG-008* to deeply discuss Guizhi Fuling Wan alleviate inflammation and improve insulin resistance by changing the structure of intestinal flora: in G group, the relative abundance of *Alloprevotella* was increased significantly, and *Ruminococcaceae UCG-003*, *Lachnospiraceae UCG-008* was decreased significantly ($P < 0.05$), compared with M group (**Table 5**). On the other hand, the effect of metformin (insulin sensitizer)

TABLE 3 | The relative abundance of the three main types of bacteria in low, medium, and high dose of Guizhi Fuling Wan group, model group and positive drug group.

	Classfication	M	D	Z	G	Y
Firmicutes	Phylum	0.4578 ± 0.0168	0.4467 ± 0.0117	0.4692 ± 0.0216	0.6082 ± 0.0168*	0.5411 ± 0.0097*
Bacteroidetes	Phylum	0.4918 ± 0.0195	0.4362 ± 0.0079	0.5071 ± 0.0227	0.3522 ± 0.0204*	0.4224 ± 0.0071*
Proteobacteria	Phylum	0.0300 ± 0.0033	0.0254 ± 0.0098	0.0119 ± 0.0017*	0.0177 ± 0.0019*	0.0202 ± 0.0024*

Compared with model group $^{*}P < 0.01$.

blank control group (K), model control group (M), low dose of Guizhi Fuling Wan group (D), middle dose of Guizhi Fuling Wan group (Z), high dose of Guizhi Fuling Wan group (G) and positive drug (Metformin) control group (Y).

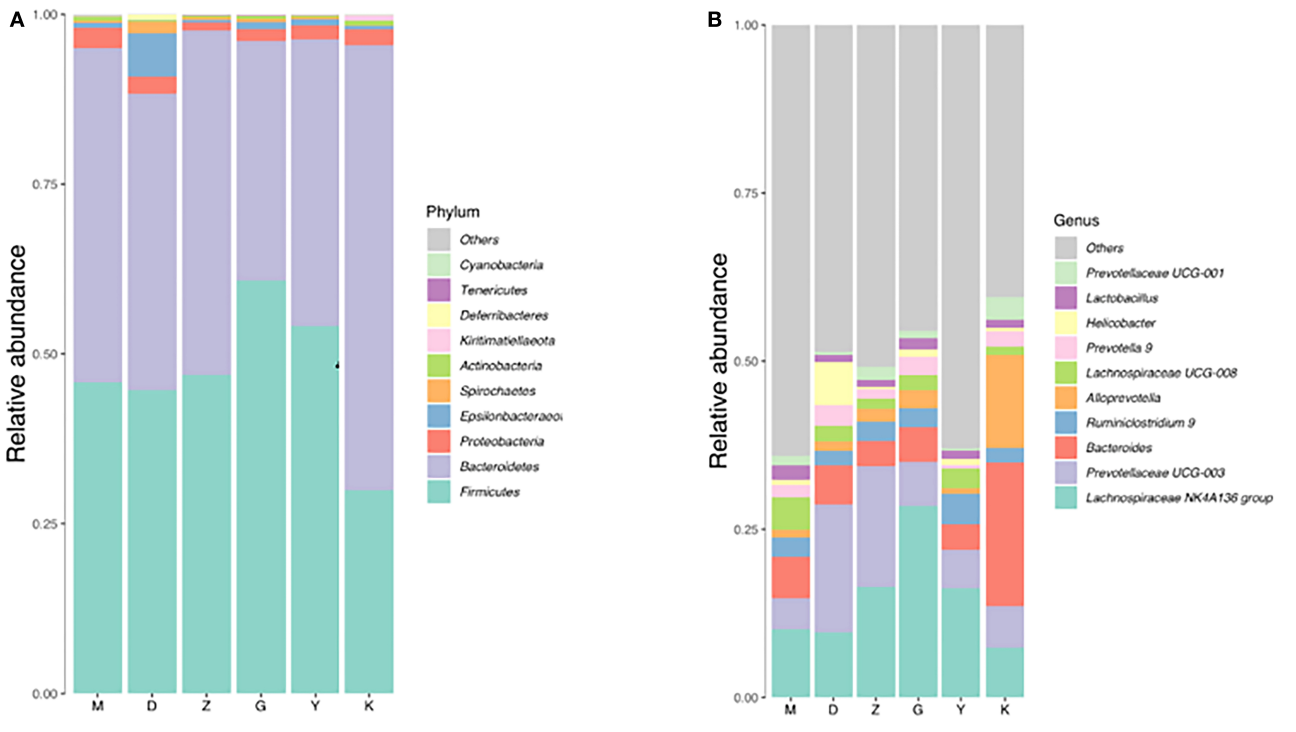

FIGURE 4 | Barplot of relative abundance at phylum level **(A)** and Genus level **(B)**.

on the relative abundance of the three bacteria was not significant **(Table 5)**.

DISCUSSION

Polycystic ovary syndrome (PCOS) is a complex multi-organ disorder associated with metabolic disorder as well as inflammation, seriously affecting the physical, and mental health of affected women. The gut is the largest microecological environment in the human body and the gut microbiota is regarded as an endocrine organ including the maintenance of energy homeostasis and host immunity, the imbalance of gut microbiota has a decisive influence on human health including metabolism and immunity of the body (31).

The increasing knowledge of the role of microbiota in PCOS has provided new perspectives and methods to understand and treat PCOS (32, 33). Yanjie et al. found excess androgen production in PCOS rat model is related with dysbiosis of gut microbiota (16). Besides being related to the changes of serum hormone of PCOS, gut microbiota is also associated with occurrence of insulin resistance. As mentioned earlier, Bäckhed et al. had proved the correlation between gut microbiota and insulin resistance via fecal microbiota transplantation (FMT) (17). This study was later extended to humans, Kootte found at 6 weeks after lean donor (allogenic) fecal microbiota transplantation, insulin sensitivity of recipients with the metabolic syndrome was significantly improved, accompanied by altered microbiota composition. While no change can be observed in metabolism of recipients at 18 weeks after own (autologous) FMT (34). Collectively, these studies demonstrated the regulation of gut microbiota on insulin sensitivity. Reduced insulin sensitivity can lead to insulin resistance and compensatory hyperinsulinemia. Emerging as an important contributor to PCOS, on the one hand, IR

TABLE 4 | Relative abundance of intestinal flora of rats in model group and blank group.

	Classification	M	K	Tendency
Firmicutes	Phylum	0.4578 ± 0.0168*	0.2995 ± 0.0283	↑
Bacteroidetes	Phylum	0.4918 ± 0.0195	0.6551 ± 0.0293	↓
Proteobacteria	Phylum	0.0300 ± 0.0033	0.0229 ± 0.0015	↑
Lachnospiraceae NK4A136 group	Genus	0.1013 ± 0.0085*	0.0742 ± 0.0111	↑
Prevotellaceae UCG-003	Genus	0.0465 ± 0.0056	0.0614 ± 0.0052	↓
Bacteroides	Genus	0.0610 ± 0.0058	0.2136 ± 0.0461	↓
Ruminiclostridium 9	Genus	0.0287 ± 0.0013*	0.0216 ± 0.0036	↑
Alloprevotella	Genus	0.0112 ± 0.0011*	0.1383 ± 0.0068	↓
Lachnospiraceae UCG-008	Genus	0.0489 ± 0.0042*	0.0124 ± 0.0021	↑
Prevotella 9	Genus	0.0175 ± 0.0025	0.0226 ± 0.0047	↓
Helicobacter	Genus	0.0077 ± 0.0014	0.0053 ± 0.0011	↑
Lactobacillus	Genus	0.0216 ± 0.0020*	0.0121 ± 0.0010	↑
Prevotellaceae UCG-001	Genus	0.0143 ± 0.0026	0.0335 ± 0.0030	↓
Ruminococcaceae UCG-003	Genus	0.0187 ± 0.0021*	0.0042 ± 0.0010	↑
Bifidobacterium	Genus	0.0012 ± 0.0004	0.0006 ± 0.0002	↑
Enterobacteriaceae	Family	0.0014 ± 0.0005	0.0031 ± 0.0010	↓

*Compared with the blank control group *P < 0.01.*
Blank control group (K), model control group (M), low dose of Guizhi Fuling Wan group (D), middle dose of Guizhi Fuling Wan group (Z), high dose of Guizhi Fuling Wan group (G), and positive drug (Metformin) control group (Y).

can promote the luteinizing hormone (LH) and androgen secretion, reduce the content of serum sex hormone binding protein and aggravate hyperandrogenemia and endocrine disorder, on the other hand, it also can directly induce abnormal follicular development and atresia, causing polycystic ovarian morphology (35, 36). Inflammation is an established factor in the etiopathogenesis of insulin resistance. Groot had mentioned that studies in the early twentieth century with the results that large doses of salicylate can reduce glycosuria in patients, provided early clues that insulin resistance secondary to inflammation (37). Escobar summarize from the literatures that the chronic low-grade inflammation of PCOS woman is a key contributor to IR in PCOS (8). What's more, the mechanism of gut microbiota mediating insulin resistance lies in gut microbial-derived inflammatory responses (38).

Regarding PCOS, there is no record in ancient books of traditional Chinese medicine (TCM), but since the 1980s, the research on traditional Chinese medicine and PCOS has emerged (39). Guizhi Fuling Wan, a traditional Chinese formula with good effects of anti-inflammatory as well as improving IR, is widely used to treat PCOS in China, but whether these effects of Guizhi Fuling Wan are related to regulating gut flora is unclear. In this study, we chose letrozole, a non-steroidal aromatase inhibitor, combined with high-fat emulsion to induce PCOS rat model for investigating the association of Guizhi Fuling Wan alleviating inflammation and improving IR with gut microbiome. Firstly, the results of elevated plasma testosterone and INS, disturbed estrous cycle and polycystic change of ovarian tissue in M group were consistent with the typical characteristics of PCOS-IR, indicating that modeling successfully.

In the letrozole-induced PCOS model rats, Kelley et al. (40) found that the relative abundance of *Firmicutes*, *Lachnospiraceae,* and *Ruminococcaceae* is increased, while the relative abundance of *Bacteroidetes* is decreased. Our results are basically consistent with those results mentioned above: compared with K group, the relative abundance of *Alloprevotella* was decreased significantly, while the relative abundance of *Lachnospiraceae UCG-008*, *Lachnospiraceae NK4A136*, *Lactobacillus*, *Ruminiclostridium 9,* and *Ruminococcaceae UCG-003* was increased significantly. Besides altered gut microbiome, the concentrations of plasma INS, IL-6, HS-CRP, and TNF-α are increased in M group. In order to further analyze the gut microbiome mediated insulin resistance by inducing inflammation, we also analyzed the correlation between inflammatory factors and gut microbiome, as well as the correlation between inflammatory factors and fasting insulin. *Alloprevotella* is short-chain fatty acids (SCFAs)-producing bacteria (41). The SCFAs are the end products of dietary fiber fermented by the gut microbiota, having strong anti-inflammatory effects (42). Therefore, the *Alloprevotella* has the same effect on anti-inflammation with SCFAs, and our results confirm this conclusion. Our results presented that there was a negative correlation between *Alloprevotella* and inflammatory markers (IL-6, HS-CRP), which indicates that the concentrations of plasma IL-6, HS-CRP will decrease with the increase of the number of *Alloprevotella*. On the contrary, a positive correlation can be found in *Ruminococcaceae UCG-003* and inflammatory markers (IL-6, HS-CRP), as well as in *Lachnospiraceae UCG-008* and inflammatory markers (IL-6, HS-CRP, TNF-α), suggesting that reducing the number of *Ruminococcaceae UCG-003* and *Lachnospiraceae UCG-008* has certain benefits for the control of inflammation. However, previous literatures reported that

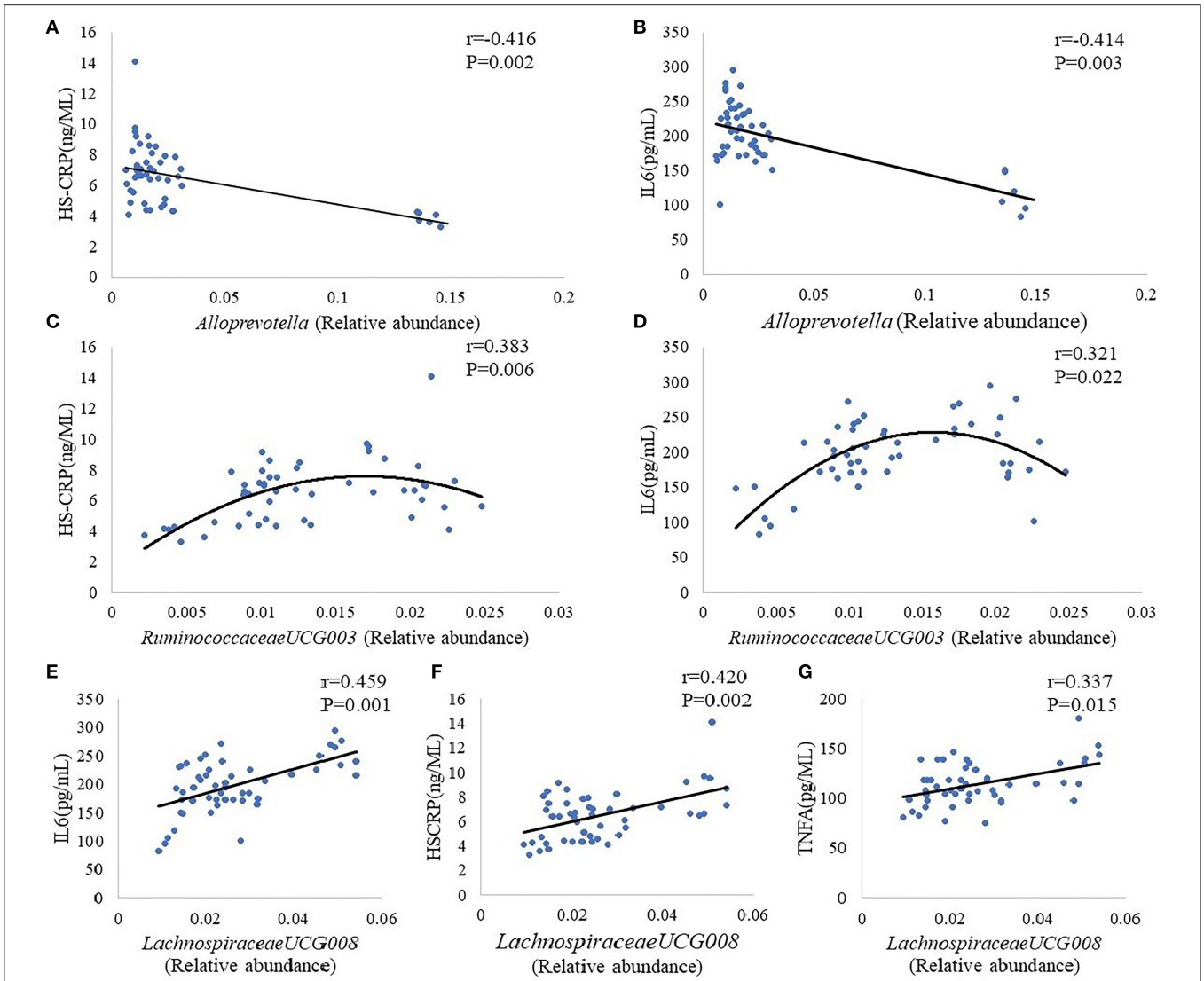

FIGURE 5 | Correlation analysis between *Alloprevotella* and inflammatory markers (HS-CRP, IL-6) **(A,B)**. Correlation analysis between *Ruminococcaceae UCG-003* and inflammatory markers (HS-CRP, IL-6) **(C,D)**. Correlation analysis between *Lachnospiraceae UCG-008* and inflammatory markers (IL-6, HS-CRP, TNF-α) **(E–G)**. The "*r*" refers to the Spearman correlation coefficient, and the $P < 0.05$ indicate differences are significant.

TABLE 5 | The relative abundance of selected bacterial in low, medium and high dose of Guizhi Fuling Wan group, model group, and positive drug group.

	Classification	M	D	Z	G	Y
Lachnospiraceae UCG-008	Genus	0.0489 ± 0.0042	0.0232 ± 0.0048$^{\Delta}$	0.0154 ± 0.0016*	0.0229 ± 0.0018$^{\Delta}$	0.0294 ± 0.0018
Ruminococcaceae UCG-003	Genus	0.0187 ± 0.0021	0.0103 ± 0.0011	0.0116 ± 0.0015	0.0090 ± 0.0012*	0.0214 ± 0.0015
Alloprevotella	Genus	0.0112 ± 0.0011	0.0146 ± 0.0015	0.0189 ± 0.0023	0.0266 ± 0.0031*	0.0079 ± 0.0016

*Compared with model group *P < 0.01, $^{\Delta}$P < 0.05.*
Blank control group (K), model control group (M), low dose of Guizhi Fuling Wan group (D), middle dose of Guizhi Fuling Wan group (Z), high dose of Guizhi Fuling Wan group (G), and positive drug (Metformin) control group (Y).

Lachnospiraceae and *Ruminococcaceae* attenuates inflammation, the anti-inflammatory effect of *Ruminococcaceae* is dependent on secondary bile acids which can be produced by *Ruminococcaceae* (43, 44), the reason probably lies in the effect of *Lachnospiraceae* and *Ruminococcaceae* on anti-inflammatory may be species and strain specific. In addition, the serum fasting insulin concentration increased with the increase of il-6 and HS-CRP, indicating that *Ruminococcaceae UCG-003* and *Lachnospiraceae*

UCG-008 may have a positive relationship with serum fasting insulin concentration, and the *Alloprevotella* may have a negative relationship with serum fasting insulin concentration. Based on our results, Guizhi Fuliing Wan can increase the relative abundance of *Alloprevotella*, decrease the relative abundance of *Ruminococcaceae UCG-003* and *Lachnospiraceae UCG-008*. Simultaneously, Guizhi Fuliing Wan can also inhibit the release of inflammatory factors and improve insulin sensitivity. Considering of the correlation between the three kinds of bacteria and inflammatory factors, and the correlation between inflammatory factors and fasting INS, thus we have a reason to believe Guizhi Fuliing Wan is capable to regulate gut microbiome positively to reduce the release of inflammatory factors and achieves the goal of improving insulin resistance ultimately.

In conclusion, we found Guizhi Fuling Wan can regulate the structure of gut microbiome, and this regulatory effect of Guizhi Fuling Wan is the basis of alleviating inflammation and improving insulin resistance. Our study provides a basis for promoting the treatment of PCOS with Guizhi Fuling Wan and lays a solid foundation for the treatment of PCOS with TCM. Recently, Qi et al. (45) found that bile acid, a metabolic product of intestinal bacteria, is involved in improving PCOS-IR. Our present results showed that Guizhi Fuling Wan could reshape the intestinal flora. Whether Guizhi Fuling Wan can interfere with the generation of bile acids through regulating intestinal flora remain unclear. If possible, whether interfering with bile acid metabolism is another way of Guizhi Fuling Wan to improve insulin resistance, which needs further experiments to verify.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

This animal study was reviewed and approved by the Animal Care Committee of Chengdu University of TCM, China (SYXK2018-0126).

AUTHOR CONTRIBUTIONS

HZ, YL, ML, YZ, and XH contributed conception and design of the study. YL and ML organized the database. YZ performed the statistical analysis and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00575/full#supplementary-material

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Prolactin Is Associated With Insulin Resistance and Beta-Cell Dysfunction in Infertile Women With Polycystic Ovary Syndrome

Haiyan Yang[1†], Jie Lin[1†], He Li[2], Zhangwei Liu[1], Xia Chen[1] and Qianqian Chen[1*]*

[1] Reproductive Medicine Center, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China, [2] Shanghai Ji Ai Genetics and IVF Institute, Obstetrics and Gynecology Hospital, Fudan University, Shanghai, China

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****Correspondence:***
Qianqian Chen
785446163@qq.com
Xia Chen
gracewzivf@163.com

[†]These authors have contributed equally to this work

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Background: Our study aimed to investigate if serum prolactin (PRL) levels associated with insulin resistance and beta-cell dysfunction in infertile patients with polycystic ovary syndrome (PCOS).

Methods: This was a retrospective cross-sectional study performed in the reproductive medicine center of the first affiliated hospital of Wenzhou Medical University. From January 2007 to August 2018, a total of 792 PCOS and 700 non-PCOS infertile women were included. All patients' prolactin levels were in the normal range. PCOS was diagnosed according to the Rotterdam Criteria. Anthropometric parameters, blood pressure, serum prolactin levels, sex hormones, fasting lipids, fasting plasma glucose (FPG), fasting insulin (FINS) and hepatic biological parameters were measured in all subjects.

Results: Serum prolactin levels in PCOS women were significantly decreased compared with levels in non-PCOS women after adjusting for age and BMI ($P < 0.05$). Moreover, we found that prolactin levels were positively associated with high-density lipoprotein cholesterol (HDL-C) and negatively associated with age, BMI, waist circumference (WC), hip circumference (HC), luteinizing hormone/follicle stimulating hormone (LH/FSH), estradiol (E_2), FINS, homeostasis model assessment of insulin resistance (HOMA-IR), homeostasis model assessment of β (HOMA-β), triglyceride (TG) and alanine aminotransferase (ALT) ($P < 0.05$). After adjusting for age and BMI, multiple linear regression analysis revealed that LH, LH/FSH, E_2, FINS, HOMA-IR, and HOMA-β were negatively associated with serum PRL ($P < 0.05$).

Conclusions: Low serum PRL levels within the normal range associates with a higher incidence of insulin resistance and beta-cell dysfunction in infertile women with PCOS.

Keywords: prolactin, infertility, polycystic ovary syndrome (PCOS), insulin resistance, beta-cell function

BACKGROUND

Prolactin (PRL) is a multifunctional polypeptide that stimulates insulin secretion, beta-cell proliferation and survival (1–5). It is reported that circulating PRL can support islet growth *via* enhancing hepatic insulin sensitivity and the secretion of 5-hydroxytryptamine and serotonin (6). Moreover, numerous studies have documented that PRL affects metabolism homeostasis through the regulation of key enzymes and transporters related to insulin resistance, hypertension or coronary syndrome (7–12).

It is reported that the role of PRL on glucose metabolism and insulin resistance depends on its circulating concentration. In the clinic, PRL improves glucose homeostasis by increasing beta-cell mass under certain conditions such as pregnancy, whereas excessive high PRL levels in serum indicate a high-risk of obesity and dysmetabolism, such as decreased insulin sensitivity, abnormal glucose tolerance or progressive insulin resistance (13–17). It has previously been observed that high levels of PRL exacerbate insulin resistance and impair the insulin-secretory capacity in diabetic mice, in contrast to the normal adaptive increases in glucose stimulated insulin secretion through expanded beta-cell mass and insulin sensitivity realized with moderately increased PRL levels (18). Additionally, increasing evidence links low PRL levels within the normal range with markers and outcomes of metabolic dysfunction (19–21). Previous studies have shown that serum PRL levels were negatively correlated with insulin sensitivity and glucose in young individuals (22). Low PRL levels may have an adverse effect on the JAK2/STAT5 signaling pathway and depress the function of beta-cells (8, 23–27). The maintenance of high serum PRL levels within the physiological range can improve insulin sensitivity and promote the proper distribution of fat, which ultimately modifies the metabolic dysfunction (20).

Polycystic ovary syndrome (PCOS) is a prevalent endocrine and metabolic condition characterized by the disturbance of reproductive hormones, insulin resistance, abnormal glucose tolerance, hypertension and cardiovascular disease (28, 29). It has been reported that serum PRL levels were significantly decreased in patients with PCOS, possibly leading to insulin resistance and damage of beta-cells (23). Nonetheless, the function of beta-cells and status of metabolism remain unknown in infertile patients with PCOS exhibiting normal serum PRL levels.

Thus, we analyzed the association between serum PRL levels and clinical parameters, such as waist circumference (WC), hip circumference (HC), luteinizing hormone (LH), triglyceride (TG), or the homeostasis model assessment of insulin resistance (HOMA-IR), in infertile PCOS patients by a retrospective cross-sectional study, to explore the status of PRL secretion and association with insulin resistance and beta-cell function.

Abbreviations: PRL, Prolactin; PCOS, Polycystic ovary syndrome; FPG, Fasting plasma glucose; FINS, Fasting insulin; HDL-C, High-density lipoprotein cholesterol; LDL-C, Low density lipoprotein-cholesterol; WC, Waist circumference; HC, Hip circumference; LH, Luteinizing hormone; FSH, Follicle stimulating hormone; E_2, Estradiol; HOMA-IR, Homeostasis model assessment of insulin resistance; HOMA-β, Homeostasis model assessment of β; TC, Total cholesterol TG, Triglyceride; ALT, Alanine aminotransferase; HPL, Hyperprolactinemia; MS, Metabolic syndrome; T2D, Type 2 diabetes; GnRH, Gonadotrophin-releasing hormone; NAFLED, Non-alcoholic fatty liver disease.

METHODS

Inclusion Criteria

The study was designed as a retrospective observational study of infertile women (792 with PCOS and 700 with tubal infertility) who were initially treated by IVF-ET and referred to the reproductive center, at the First Affiliated Hospital of Wenzhou Medical University during January 2007 to August 2018. All patients' prolactin levels were in the normal range. The detection lower limit for PRL was 20 mIU/L and if serum PRL is over 530 mIU/L, it is considered to be hyperprolactinemia (30, 31). Due to variation in detection assays and the different kit used, the normal range of PRL varies slightly among hospitals. Based on the assay and kit used at our hospital and published consensus on diagnosis and treatment of hyperprolactinemia, all patients had normal range prolactin levels below the upper reference limit (566.46 mIU/L) and above the lower reference limit (70.81 mIU/L). PCOS was diagnosed according to the Rotterdam criteria (Rotterdam, 2004). Written informed consent was obtained from all participating individuals and the study was approved by an Institutional Review Board that complies with all principles of the Declaration of Helsinki Principles Accord.

Exclusion Criteria

Patients with hormone therapy in three months, smoking, history of ovarian function damage by radiotherapy or chemotherapy, endometriosis, adenomyosis, thyroid disorders, liver disease, kidney disease, high blood pressure, pituitary microadenoma were excluded. Patients were also excluded if she had unexplained infertility, recurrent miscarriage or previous history of adverse pregnancy, congenital abnormalities such as chromosome aberration, congenital adrenal cortex hyperplasia, Cushing's syndrome or testosterone-secreting tumors.

Clinical Samples

Fasting blood samples were collected between 9 to 11 am in the morning at least 2 h after wake-up and 8 h after fasting on day 2–5 of a menstrual cycle. The body height (m) and weight (kg), waist-circumference and hip-circumference were measured by experienced nurses according to standard protocols. Body mass index(BMI) was calculated as body weight in kilograms divided by body height in meters squared. Blood pressure was taken twice in an interval of 2 min after at least 10 min rest using a mercury sphygmomanometer.

Assay

The PRL, FSH, LH, T, and E_2 levels in blood samples were measured using chemiluminescence assay on UniCel® DxI 800 Immunoassay System (Beckman Coulter, USA) with commercial

kits according to manufacturer's and supplier's instructions. The FPG, TG, TC, LDL-C, HDL-C and hepatic function were measured by a Cobas 8000 modular analyzer kits, and FINS by a Cobas E602 automatic electrochemical luminescence analyzer according to manufacturer's instructions.

Calculation

1. BMI = Weight (kg)/Height (m^2)
2. Waist-hip ratio = Waist circumference (cm)/Hip circumference (cm)
3. Waist-height ratio = Waist circumference (cm)/Height (cm)
4. HOMA-IR = Fasting blood-glucose (FPG, mmol/L) × Fasting insulin (FINS, mIU/L)/22.5
5. HOMA-β = 20 × Fasting insulin (FINS, mIU/L)/[Fasting blood-glucose (FPG, mmol/L) − 3.5] (%)
6. The normal range of prolactin levels: 70.81–566.46 mIU/L.

Statistical Analysis

Parameters were not normally distributed and were therefore described using medians and quartiles. The rank sum test was used to compare differences between patients and controls. The correlation among variables was analyzed using the Spearman correlation analysis. Univariate logistic regression and multiple linear regression analysis were applied to reveal the association between prolactin and the index. All statistical analyses were performed using the SPSS version 22. P values < 0.05 were considered statistically significant.

TABLE 1 | Clinical and biochemical data from PCOS and control patients.

Variables	PCOS (n = 792)	non-PCOS (n = 700)
Age	29(27–32.5)	31(28–35)**
Systolic pressur (mmHg)	116(106–126.5)	109(103–117)**[a]
Diastolic pressure (mmHg)	77(68–83)	72(67–77)**[a]
BMI (kg/m^2)	23.73(21.48–26.85)	21.64(19.53–23.88)**
WC (cm)	84(77–90.5)	75(70–81)**
HC (cm)	94(90–101)	90(86–96)*
Waist–hip ratio	0.88(0.85–0.91)	0.83(0.79–0.87)**
Basal serum PRL (mIU/L)	235.74(186.85–318.03)	275.13(213.60–355.84)*[b]
Basal serum LH (IU/L)	9.09(6.26–13.44)	4.65(3.48–5.98)**[b]
Basal serum FSH (IU/L)	6.62(5.86–7.81)	7.82(6.67–9.02)**[b]
LH/FSH	1.37(0.89–1.96)	0.59(0.42–0.77)**[b]
Basal serum E_2 (pmol/L)	180(113.5–238.5)	182.5(134.5–238)
Basal serum T (nmol/L)	2.15(1.59–2.75)	1.56(1.25–1.96)**[b]
FINS (pmol/L)	82.9(50.35–112.30)	52.75(37.6–75.6)**[a]
FPG (mmol/L)	5.2(4.9–5.5)	5.1(4.9–5.4)
HOMA-IR	2.74(1.75–3.76)	1.74(1.22–2.55)**[a]
HOMA- β	137.49(88.35–216.24)	93.45(67.08–128.42)**[a]
TG (mmol/L)	1.37(0.94–1.98)	0.93(0.69–1.24)**[b]
TC (mmol/L)	4.79(4.20–5.30)	4.48(3.99–4.98)**[a]
LDL-C (mmol/L)	2.74(2.31–3.28)	2.49(2.10–2.92)**[a]
HDL-C (mmol/L)	1.29(1.12–1.46)	1.40(1.23–1.61)**[a]
AST (U/L)	18(16–22.5)	16(14–19)**[b]
ALT (U/L)	21(15–28)	14(11–20.5)**[b]

Parameters were not normally distributed and data are presented as quartiles. The rank sum test was used to compare differences between patients and controls.
**P < 0.05 versus controls, **P < 0.001 versus controls.*
[a]P < 0.05 versus controls after correcting for age and BMI.
[b]P < 0.001 versus controls after correcting for age and BMI.

RESULTS

Baseline Characteristics of Study Population

Characteristics of 792 PCOS and 700 non-PCOS women are provided in **Table 1**. Prolactin, FSH, and HDL-C levels were significantly lower in PCOS compared with non-PCOS women (235.74 versus 275.13 mIU/L, 6.62 versus 7.82 IU/L, and 1.29 versus 1.40 mmol/L, respectively), whereas blood pressure, LH, LH/FSH, testosterone (T), FINS, HOMA-IR, HOMA-β, TG, TC, LDL-C, AST and ALT remained higher in PCOS versus controls after correcting for age and BMI.

Clinical, Hormonal, and Metabolic Characteristics in Patients With Polycystic Ovary Syndrome

Table 2 shows the clinical and biochemical characteristics of PCOS patients grouped according to the shown quartiles of prolactin levels. Patients' prolactin levels were inversely association with age, WC, HC, basal serum LH, LH/FSH, FINS, HOMA-IR, HOMA-β, and TG, but showed a positive association with HDL-C.

Associations of Serum Prolactin Levels With Sexual Hormonal and Metabolic Variables in Polycystic Ovary Syndrome Patients

Table 3 shows the analysis of bivariate associations between prolactin and hormonal and metabolic variables in patients with PCOS, which displayed a negative association between prolactin levels and age, WC, HC, Waist-hip ratio, basal serum LH, LH/FSH, E_2, FINS, HOMA-IR, HOMA-β, TG, and ALT ($P < 0.05$ or $P < 0.001$). Additionally, we found that prolactin levels were positively associated with HDL-C ($P < 0.05$).

Multiple Linear Regression Analysis on the Effect of Prolactin Upon Hormonal and Metabolic Outcomes in Patients With Polycystic Ovary Syndrome

Table 4 shows serum PRL was inversely associated with waist-hip ratio, LH, LH/FSH, E_2, FINS, HOMA-IR and HOMA-beta after excluding the influence of age and BMI in multiple linear regression analysis.

Associations of Serum Prolactin Levels With Sexual Hormonal and Metabolic Variables in Non-Polycystic Ovary Syndrome Patients

Table 5 shows serum PRL was negative association with age, BMI and waist-hip ratio ($P < 0.05$ or $P < 0.001$) and positively correlated with basal serum LH, LH/FSH and basal serum T ($P < 0.05$) in non-PCOS patients. In multiple linear regression analysis, serum PRL was not directly correlated with waist-hip ratio, basal serum LH, LH/FSH, T, FINS, HOMA-IR and HOMA-β after adjusting for age and BMI (**Table 6**).

TABLE 2 | Clinical and biochemical data from PCOS according to quartiles of prolactin levels.

Variables	PRL ≤ 186.85 (n = 175)	186.99–235.74 (n = 170)	235.77–318.03 (n=232)	PRL>318.03 (n = 215)	P-value
Age	31(28–34)	28.5(27–31)	29(27–31)	27(26–31)**	0.000
Systolic pressure (mmHg)	121(110–128)	113(98–124)	117(106–127)	115(106–12)	0.369
Diastolic pressure (mmHg)	78(68–83)	73(62–84)	77(70–81)	78(70.5–83)	0.841
BMI (kg/m^2)	26.13(22.83–27.89)	23.38(21.63–24.848)	22.86(20.45–25.81)	22.58(20.50–24.68)	0.095
WC (cm)	90(82–95)	82(74–88)	80(73–88)	81(78–86)**	0.000
HC (cm)	100(92–106)	92.5(88–100)	93(88–97)	93(90–98)*	0.001
Waist–hip ratio	0.89(0.85–0.92)	0.86(0.85–0.90)	0.86(0.81–0.89)	0.88(0.85–0.92)	0.110
Basal serum PRL (mIU/L)	137.11(107.06–155.76)	209.20(195.42–227.58)	275.91(265.71–287.79)	396.28(353.25–472.53)**	0.000
Basal serum LH (IU/L)	9.61(7.08–14.28)	9.83(6.21–13.94)	8.57(5.02–11.34)	9.0(6.07–12.03)*	0.001
Basal serum FSH (IU/L)	7.0(6.02–8.47)	6.45(5.30–7.16)	6.60(5.50–8.06)	6.58(6.03–7.59)	0.810
LH/FSH	1.40(1.02–1.94)	1.55(0.98–2.44)	1.21(0.76–2.03)	1.32(0.87–1.77)**	0.000
Basal serum E_2 (pmol/L)	162(108–217)	202(125–269)	186(143–252)	182(111–218.5)	0.345
Basal serum T (nmol/L)	2.17(1.64–2.67)	2.17(1.66–2.86)	2.15(1.72–3.02)	1.91(1.35–2.69)	0.074
FINS (pmol/L)	91.6(65.3–138.1)	87.1(50.9–145.4)	72(41.3–98)	81.6(52.3–91.25)*	0.003
FPG (mmol/L)	5.2(4.9–5.5)	5.2(4.9–5.6)	5.1(4.9–5.4)	5.1(4.9–5.6)	0.372
HOMA-IR	2.81(1.3–4.19)	2.39(1.59–3.78)	2.33(1.25–3.87)	2.20(1.02–3.34)*	0.006
HOMA-β	129.77(81.75–219.72)	112.29(78.08–164.75)	111.03(67.35–193.47)	104.48(58.59–163.14)*	0.001
TG (mmol/L)	1.43(1.09–2.26)	1.32(0.93–1.88)	1.03(0.88–2.08)	1.33(0.88–1.82)*	0.017
TC (mmol/L)	4.78(4.21–5.29)	4.79(4.120–5.29)	4.74(4.06–5.35)	4.98(4.49–5.33)	0.827
LDL-C (mmol/L)	2.74(2.37–3.20)	2.45(2.10–3.26)	2.73(2.30–3.10)	2.88(2.45–3.39)	0.810
HDL-C (mmol/L)	1.23(1.05–1.33)	1.30(1.14–1.58)	1.30(1.19–1.46)	1.31(1.16–1.54)*	0.005
AST (U/L)	18(15–24)	18(17–20)	19(15–20)	19(15.5–25.5)	0.256
ALT (U/L)	21(15–30)	22(15–28)	17(13–29)	21 (15–26)	0.123

Parameters were not normally distributed and data are presented as quartiles. The rank sum test was used to compare differences between patients and controls.
**P < 0.05 versus four groups, **P < 0.001 versus four groups.*

TABLE 3 | Bivariate associations between prolactin and hormonal and metabolic variables in patients with PCOS.

Variables	R	P
Age	–0.123*	0.001
Systolic pressure (mmHg)	–0.062	0.079
Diastolic pressure (mmHg)	–0.030	0.396
BMI (kg/m^2)	–0.086*	0.016
WC (cm)	–0.302**	0.000
HC (cm)	–0.313**	0.000
Waist–hip ratio	–0.074	0.356
Basal serum LH (IU/L)	–0.144**	0.000
Basal serum FSH (IU/L)	0.000	0.999
LH/FSH	–0.154**	0.000
Basal serum E_2 (pmol/L)	–0.071*	0.047
Basal serum T (nmol/L)	–0.035	0.320
FINS (pmol/L)	–0.152**	0.000
FPG (mmol/L)	0.042	0.234
HOMA-IR	–0.144**	0.000
HOMA-β	–0.165**	0.000
TG (mmol/L)	–0.107*	0.004
TC (mmol/L)	–0.014	0.702
HDL-C (mmol/L)	0.084*	0.025
LDL-C (mmol/L)	–0.027	0.463
AST (U/L)	–0.048	0.173
ALT (U/L)	–0.077*	0.030

*Data shown are Spearman's rank correlation coefficients, *P < 0.05, **P < 0.001.*

DISCUSSION

To the best of our knowledge, this is the first study to report the association between serum PRL levels within the normal range and insulin resistance and beta-cell dysfunction in infertile patients with PCOS. In the present study, we observed that serum PRL levels were correlated with insulin sensitivity and beta-cell function in infertile PCOS patients with normal PRL levels, through analysis of the association of PRL levels with WC/HC, glucose metabolism indexes, lipid metabolism indexes and sexual hormonal regulation indexes. Whereas the correlation between PRL levels and insulin sensitivity or beta-celll function was not observed in infertile non-PCOS patients with normal PRL levels. The consequences of PRL levels in PCOS patients showed a significant decline after excluding the influence of age and BMI ($P < 0.001$), compared with non-PCOS patients (exhibiting oviductal infertility).

As a clinical diagnostic standard of central obesity, WC reflects the addition of visceral and abdominal fat, which can predict obesity-related health risk and provide a key risk factor for metabolic syndrome (MS) involving the onset of insulin resistance (32–34). In addition, as an insulin resistance-related risk factor, serum PRL levels were found to have an inverse association with WC, similar to results for PCOS or hypertrichosis patients (23, 24). In addition to WC, many studies have investigated the association between HC and type 2 diabetes (T2D), cardiovascular disease, and hypertensive or dysmetabolism disorders (35–39). An epidemiologic survey of urban Tehranian women found that HC was independently and inversely associated with metabolic risk factors (40). However, recent studies have revealed that HC is an independent risk factor for MS and cardiovascular disease (41). A relatively small sample size or imprecision of the measurements cannot be excluded as possible explanations. Our findings showed that PRL levels in women with PCOS were inversely associated with WC, HC, HOMA-IR, or HOMA-β, but not with the

TABLE 4 | Regression analysis on the effect of prolactin upon hormonal and metabolic outcomes in patients with PCOS.

Variables	PRL	Age	BMI	R	R^2	Adjusted R^2
WC (cm)	–0.001	2.349	2.537	0.217	0.047	0.029
HC (cm)	–0.008	–0.325*	1.867**	0.777	0.603	0.596
Basal serum LH (nmol/L)	–0.009**	–0.168*	–0.311**	0.261	0.068	0.065
LH/FSH	–0.001**	–0.025*	–0.029**	0.216	0.047	0.043
E_2 (pmol/L)	–0.055*	1.245	–1.280	0.108	0.012	0.008
FINS (pmol/L)	–0.066*	–1.399	7.991**	0.334	0.112	0.108
HOMA-IR (log10)	–0.102*	–0.033	0.293**	0.210	0.044	0.041
HOMA-β	–0.121*	–2.877	11.709**	0.302	0.091	0.088
TG (mmol/L)	0.000	0.019	0.081**	0.319	0.102	0.098
HDL-C (mmol/L)	0.733	–0.007*	–0.031**	0.413	0.170	0.167
ALT (U/L)	–0.004	–0.046	1.819**	0.211	0.044	0.041

*Multiple regression analyses were performed with metabolic and hormonal outcomes as dependent variables and prolactin, age, and BMI as explanatory variables. Data are presented as B-values (P-levels): *P < 0.05, **P< 0.001.*

TABLE 5 | Bivariate associations between prolactin and hormonal and metabolic variables in non-PCOS patients.

Variables	R	P
Age	–0.171**	0.000
Systolic pressure (mmHg)	–0.025	0.505
Diastolic pressure (mmHg)	0.004	0.916
BMI (kg/m^2)	–0.130*	0.001
WC (cm)	–0.099	0.059
HC (cm)	–0.007	0.894
Waist–hip ratio	–0.142*	0.007
Basal serum LH (IU/L)	0.094*	0.013
Basal serum FSH (IU/L)	–0.040	0.292
LH/FSH	0.120*	0.001
Basal serum E_2 (pmol/L)	0.040	0.294
Basal serum T (nmol/L)	0.076*	0.046
FINS (pmol/L)	0.018	0.018
FPG (mmol/L)	–0.012	0.752
HOMA-IR	0.012	0.755
HOMA-β	0.034	0.365
TG (mmol/L)	–0.053	0.177
TC (mmol/L)	–0.017	0.676
HDL-C (mmol/L)	0.059	0.132
LDL-C (mmol/L)	–0.021	0.587
AST (U/L)	–0.063	0.100
ALT (U/L)	–0.065	0.177

*Data shown are Spearman's rank correlation coefficients, *P < 0.05, **P < 0.001.*

waist-hip ratio. Hence, we deduce that low prolactin levels within the normal range may be associated with increased WC and HC and a higher risk for insulin resistance.

Circulating PRL levels exert wide effects upon glucose metabolism. Previous studies showed that high PRL disrupted glucose homeostasis and led to metabolic abnormalities (19, 42). Patients with hyperprolactinemia exhibit more insulin resistance and glucose intolerance compared with normal individuals. However, there are also increased MS- and T2D-related risks when low prolactin levels fall within the physiological range (20, 22, 27, 43–45). Our findings support an inverse association between serum PRL levels and clinical parameters including FINS, HOMA-IR and HOMA-β in women with PCOS, after adjustment of age and BMI. Furthermore, the FINS, HOMA-IR and HOMA-β in infertile women with PCOS were significant increased compared with non-PCOS women with oviductal infertility. Thus, we propose that serum PRL levels in infertile women with PCOS may be a predictor for insulin resistance and a functional deficiency of beta-cells.

In the analysis of reproductive hormones, we found that serum PRL exhibited inverse associations with LH, LH/FSH and E_2 levels, but was not directly correlated with either T or FSH levels. Excessive PRL reduces the secretion of FSH and LH *via* suppression of gonadotrophin-releasing hormone (GnRH) synthesis and release (46–51). Therefore, we predict that higher PRL levels within the normal range may also decrease the production of gonadotrophins. Moreover, emotional changes and a reduced quality-of-life in PCOS patients may promote dopamine secretion, which may reduce PRL levels, and lead to the inverse association between prolactin and LH or LH/FSH (52, 53). Hence, we conclude that low PRL levels may increase the levels of LH and LH/FSH.

TABLE 6 | Regression analysis on the effect of prolactin upon hormonal and metabolic outcomes in patients with non-PCOS.

Variables	PRL	Age	BMI	R	R^2	Adjusted R^2
Waist–hip ratio	–0.427	–0.790	1.315	0.081	0.007	–0.002
Basal serum LH (nmol/L)	–0.569	–4.056**	–4.874**	0.38	0.057	0.053
LH/FSH	–0.348	–5.951**	–5.951**	0.230	0.053	0.049
T (nmol/L)	1.157	–3.468*	1.428	0.152	0.023	0.019
FPG (mmol/L)	–0.315	1.427	4.780**	0.192	0.037	0.037
FINS (pmol/L)	1.497	–1.986	12.772**	0.439	0.439	0.189
HOMA-IR(log10)	1.319	–1.483	12.514**	0.430	0.185	0.182
HOMA-β	0.945	–2.221*	9.423**	0.343	0.118	0.114

*Multiple regression analyses were performed with metabolic and hormonal outcomes as dependent variables and prolactin, age, and BMI as explanatory variables. Data are presented as B-values (P-levels): *P < 0.05, **P< 0.001.*

Furthermore, we found the PRL levels were also inversely associated with TG and positively associated with HDL-C. TG, TC and LDL-C were significantly higher in PCOS compared with non-PCOS women after correcting the influence of BMI. Thus, there is significant correlation between metabolic abnormalities and serum PRL. We suspect that lower prolactin levels within the normal range may lead to dyslipidemia. Additionally, the index of ALT and AST was higher in PCOS compared with non-PCOS patients (none exhibited hepatitis or liver dysfunction), after controlling for BMI, and PRL was inversely associated with ALT. Hence, low PRL levels within the normal range may have association with higher prevalence of liver damage in PCOS. A recent clinical study into the role of PRL in the development of non-alcoholic fatty liver disease (NAFLED) suggested that there was a negative association between PRL and the presence of NAFLED. Lower PRL levels were found in patients with severe hepatic steatosis compared with those displaying mild and moderate hepatic steatosis (54). Moreover, the results revealed a novel association between the central nervous system and liver, whereby PRL/PRLR improved hepatic steatosis *via* the CD36 pathway.

With regard to the research methods, some limitations need to be acknowledged. For instance, we cannot draw causality from simple correlations in a retrospective study. Prospective studies are needed to testify their correlation, and future research will allow a more detailed investigation of all parameters such as glucose tolerance testing, insulin releasing test or abdominal ultrasonography. In addition, considering that the secretion of PRL is pulsatile and follows a circadian rhythm with the highest plasma concentration reached during sleep, and the lowest observed in the morning about 2–3 h after waking up (55), several dynamic tests of PRL secretion may be needed.

CONCLUSIONS

Our clinical study lend support to the assumption that serum PRL levels within the normal range associates with glucose metabolism changes in infertile women with PCOS, suggesting that PRL may be a sensitive marker to predict insulin resistance and dysfunction of beta-cells. Further studies are warranted to confirm this association.

DATA AVAILABILITY STATEMENT

All relevant data are contained within the article: the original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of The First Affiliated Hospital of Wenzhou Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HY contributed to the conception, data analysis, and draft writing. JL was involved in the acquisition of data. ZL was involved in the execution. HL provided suggestions on the study design. XC contributed to the conception and design of study. QC contributed to conception and study design and revised the article. All authors contributed to the article and approved the submitted version.

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Address all correspondence and requests for reprints: QC, MD, Reproductive Medicine Center, The First Affiliated Hospital of Wenzhou Medical University, No.96 Fuxue Road, Wenzhou 325000, People's Republic of China; Telephone number: +86-13695885092; Fax number: +86-0577-88069786; Email: chancy1031@163.com.

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REVIEW
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Obesity and Male Reproduction; Placing the Western Diet in Context

Taylor Pini, David Raubenheimer, Stephen J. Simpson and Angela J. Crean *

Charles Perkins Centre, School of Life and Environmental Sciences, The University of Sydney, Sydney, NSW, Australia

There is mounting evidence that obesity has negative repercussions for reproductive physiology in males. Much of this evidence has accumulated from rodent studies employing diets high in fat and sugar ("high fat" or "western" diets). While excessive fats and carbohydrates have long been considered major determinants of diet induced obesity, a growing body of research suggests that the relationships between diet composition and obesity are more complex than originally thought, involving interactions between dietary macronutrients. However, rodent dietary models have yet to evolve to capture this, instead relying heavily on elevated levels of a single macronutrient. While this approach has highlighted important effects of obesity on male reproduction, it does not allow for interpretation of the complex, interacting effects of dietary protein, carbohydrate and fat. Further, the single nutrient approach limits the ability to draw conclusions about which diets best support reproductive function. Nutritional Geometry offers an alternative approach, assessing outcomes of interest over an extended range of dietary macronutrient compositions. This review explores the practical application of Nutritional Geometry to study the effects of dietary macronutrient balance on male reproduction, including experimental considerations specific to studies of diet and reproductive physiology. Finally, this review discusses the promising use of Nutritional Geometry in the development of evidence-based pre-conception nutritional guidance for men.

Keywords: obesity, male fertility, reproduction, diet, high fat, nutritional geometry

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Nicole McPherson, University of Adelaide, Australia

Reviewed by:
Iwona Krela-Kaźmierczak, Poznan University of Medical Sciences, Poland
Shaun Roman, University of Newcastle, Australia

***Correspondence:**
Angela J. Crean
angela.crean@sydney.edu.au

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INTRODUCTION

Obesity affects millions of people globally. Men of reproductive age (18–64 years) are no exception, with averages of 37.8% (1), 31.4% (2), and 29.9% (3) classified as obese (body mass index ≥ 30 kg/m^2) in the United States, Australia, and the United Kingdom, respectively. Because of its epidemiological prevalence, the impacts of obesity have been studied in the context of many biological processes, including reproduction. While much of the literature has focused on female reproduction, a growing body of evidence suggests that obesity and associated metabolic dysfunction can alter spermatozoa on a molecular level (4, 5), negatively affect sperm function (6–8), alter circulating levels of reproductive hormones (9), cause male sub-fertility (10, 11) and impart epigenetic changes to spermatozoa which ultimately decrease offspring metabolic health (12) and reproductive potential (13). In addition, a wide range of intrinsic [e.g., DAZ deletion (14), age (15)] and extrinsic [e.g., radiation exposure (16), tobacco use (17)] factors can contribute to male

infertility, and may interact with or compound the effects of obesity on male reproduction (**Figure 1**). While male obesity is generally recognized as an important concern in the context of reproductive medicine (18–20), some studies question the effects of obesity on semen parameters and male fertility (21–25). These inconsistencies and the seriousness of the potential consequences of obesity on male fertility necessitate continued research efforts in this field.

The root cause of obesity is a topic which has been debated for decades. While there is a growing number of risk factors associated with obesity, including sleep, genetic background, and physical activity, diet is the most significant contributor (26). Many have identified lipid as the major dietary determinant (27, 28), but this has been refuted by others who consider carbohydrate to be the major culprit (29–31). In contrast to these single-nutrient explanations, there is mounting evidence that obesity may instead be driven by an altered macronutrient balance in the diet, rather than by high dietary fat or carbohydrate alone (32–34). Despite this, animal studies employing purified diets with elevated concentrations of fat (typically referred to as "high fat" or "western" diets) remain a staple of research investigating diet induced obesity (35). As the understanding of what constitutes an obesogenic diet changes, there is a need to rethink the dietary models which are used to study obesity and its downstream consequences for such factors as reproduction.

In this review, we summarise the limitations of the traditional western diet approach and introduce Nutritional Geometry as a powerful framework for studying the relationships between diet, obesity, and male reproduction. We also highlight important experimental design considerations unique to studying male reproductive physiology. Finally, we pose potential applications for Nutritional Geometry in the context of male reproduction, including a path toward the development of pre-conception nutritional guidelines.

THE CURRENT APPROACH TO STUDYING OBESITY AND MALE REPRODUCTION

Evidence from animal studies indicates that obesity and associated metabolic disease are deleterious for male reproduction (**Table 1**). A variety of processes are reportedly impacted by obesity, including testosterone production (7), testicular gene expression (36–38), production of reactive oxygen species (6, 8), and maintenance of the blood-testis barrier (7). These studies compare the effects of diets which contain normal (10%–18% of total kcal) or high (40%–60% of total kcal) amounts of fat, with some also incorporating elevated levels of sucrose. Rodent diets high in the proportion of fat are excellent tools to create an obese phenotype, resulting in significantly larger adipose tissue depots (6) and a higher overall percentage body fat compared to lean mass (37, 42). These high-fat diets also often capture metabolic sequelae, including elevated serum cholesterol, triglycerides, glucose, insulin, and leptin (6, 37, 42), though this is not always the

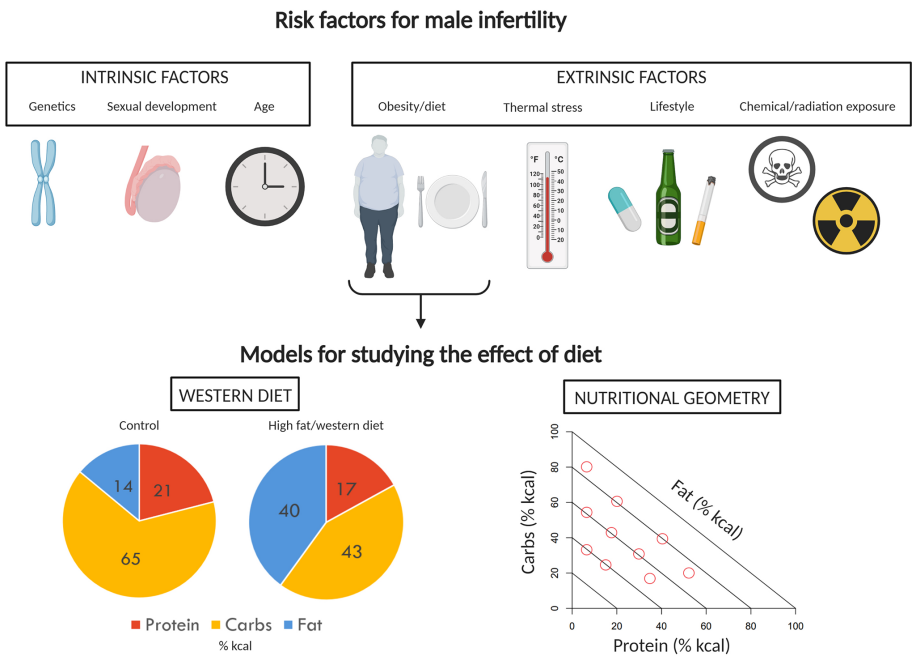


FIGURE 1 | Factors impacting male fertility and models to study effects of diet. A variety of intrinsic and extrinsic factors can contribute to poor reproductive performance and infertility in men. Of the extrinsic factors, the impact of diet and obesity on male reproduction has received a significant amount of attention. The effects of diet and obesity on male reproduction are typically investigated in rodents using the classic "western diet" approach, comparing control and treatment diet outcomes. Here, we instead propose the use of Nutritional Geometry, employing a range of diets which systematically differ in their proportions of protein, carbohydrates, and fat. Image created with BioRender.com.

TABLE 1 | Rodent studies employing a traditional high fat/western diet approach to study the impact of obesity on male reproduction.

Reference	Diet	Diet type	Species	Age at start	Treatment length	kcal/g	% protein	% carb	% fat	P:C	P:F	Unique ingredients	Outcomes			
													Testis	Epididymis/ Accessory sex glands	Sperm	Mating/ offspring
(7)	MD12031 10% fat, Mediscience Ltd	Grain-based	Mouse (C57BL/6)	5 wk	10 wk	–	20	70	10	3.5	0.5		Disrupted blood-testis barrier, decreased testosterone		Decreased sperm motility, normal morphology	
	MD12032 45% fat, Mediscience Ltd	Grain-based + purified					20	35	45	1.8	2.3	Sucrose, lard, cholesterol				
(36)	824050, Special Diet Services UK	Purified	Mouse (C57BL/6)	3 wk	8 wk	4.5	20	70	10	3.5	0.5		Increased *Cyp2e1*, *Cyp19a1*, *Pparg* and *Tnf* mRNA in testis		Increased sperm DNA fragmentation	
	824053, Special Diet Services UK	Purified				3.7	20	35	45	1.8	2.3	Lard				
(37)	D12450Bi, Research Diets Inc	Purified	Mouse (C57BL/6J)	5 wk	25 wk	3.8	20	70	10	3.5	0.5	Corn starch	Increased *Pparg* mRNA, decresed *Crem*, *Dhh*, *Igf1*, *Lepr*, *Sh2b1* mRNA in testis		Decreased sperm motility	Decreased pregnancy rates
	D12492i, Research Diets Inc	Purified				5.2	20	20	60	1.0	3.0					
(38)	SF04-057, Specialty Feeds	Purified	Mouse (C57BL/6)	5 wk	10 wk	3.8	21	65	14	3.1	0.7		Altered testis transcriptome, decreased global methylation of testis and spermatid DNA		Altered sperm miRNAs	
	SF00-219, Specialty Feeds	Purified				4.6	17	43	40	2.5	2.4	Ghee				
(6)	SF04-057, Specialty Feeds	Purified	Mouse (C57BL/6)	6 wk	9 wk	3.8	21	65	14	3.1	0.7				Decreased sperm motility, zona binding, increased ROS, DNA fragmentation	
	SF00-219, Specialty Feeds	Purified				4.6	17	43	40	2.5	2.4	Ghee				
(39)	SF04-057, Specialty Feeds	Purified	Mouse (C57BL/6)	5 wk	18 wk	3.8	21	65	14	3.1	0.7					Decreased total embryo cell number (TE and ICM), implantations, fetal weight, crown-rump length, placental weight
	SF00-219, Specialty Feeds	Purified				4.6	17	43	40	2.5	2.4	Ghee				
(13)	SF04-057, Specialty Feeds	Purified	Mouse (C57BL/6 NHsd)	5 wk	10 wk	3.8	21	65	14	3.1	0.7				Altered sperm miRNAs	
	SF00-219, Specialty Feeds	Purified				4.6	17	43	40	2.5	2.4	Ghee				
(4)	2016 Global, Envigo TD.88137, Envigo	Grain-based Purified	Mouse (C57BL/6)	10 wk	15 wk	3.0 4.5	22 15.2	66 42.7	12 42	3.0 2.8	0.5 2.8	Milk fat, sucrose	Decreased *Crisp4* and *Lepr* mRNA in testis	Decreased *Crisp4* mRNA in epididymis		Decreased fertilization, pregnancy rates
(40)	Meat free rat and mouse chow, Specialty Feeds	Grain-based	Mouse (C57BL/6)	6 wk	10 wk	3.3	23	65	12	2.8	0.5			Increased leptin, insulin, decreased estradiol in seminal vesicle fluid, altered seminal vesicle fluid metabolite composition	Increased sperm *Cox4il* mRNA	
	SF00-219, Specialty Feeds	Purified				4.6	17	43	40	2.5	2.4	Ghee, sucrose				
(8)	2018S Global, Teklad	Grain-based	Rat (Sprague-Dawley)	"Sexually mature"	4 wk	3.1	24	58	18	2.4	0.8				Decreased sperm activities of lactate and pyruvate dehydrogenases, citrate synthase, respiratory chain complexes, decreased ATP, increased ROS	
	TD.03584, Teklad	Purified				5.4	15	27	58	1.8	3.9	Lard				

(Continued)

TABLE 1 | Continued

Reference	Diet	Diet type	Species	Age at start	Treatment length	kcal/g	% protein	% carb	% fat	P:C	P:F	Unique ingredients	Outcomes			
													Testis	Epididymis/ Accessory sex glands	Sperm	Mating/ offspring
(41)	RM3, Special Diet Services D12451, Research Diets, New Brunswick	Grain-based Purified	Mouse (C57BL/6)	11 wk	21 wk	3.3 4.7	27.3 20	61.2 35	11.5 45	2.2 1.8	0.4 2.3	 Lard, sucrose	Altered testis proteome, decreased Sertoli cell numbers, meiotic index, numbers of post-meiotic round spermatids			

% protein/carb/fat = % of total kcal. P:C = ratio of protein to carbohydrate (as energy) in diet. P:F = ratio of protein to fat (as energy) in diet.

case (43–45). While it would be easy to conclude from these studies that avoiding a high-fat diet will safeguard reproductive potential, the reality is more complicated.

Nutritional studies have shown that it is not just the amount of energy consumed that matters, but from where this energy is sourced. Most dietary energy comes from the three principal classes of macronutrients—protein, carbohydrate, and fat. Carbohydrates are the main source of metabolic fuel, protein provides amino acids for growth, repair and a minimal contribution of metabolic energy, and fats provide a concentrated source of energy. This is reflected in the energy density of each macronutrient—whereas protein and carbohydrates have around 4 kcal/g, fats contain around 9 kcal/g. This at least partially explains why a high fat diet often leads to increased adiposity, because high fat diets generally contain more calories per gram of food (**Table 1**). Consequently, it is not clear whether the effects of western diets on male reproduction result from differences in fat or differences in calories. It is thus unclear whether men trying to conceive should be advised to simply eat less or to specifically avoid fats. This distinction is important, as dietary fats are also used in androgen production (46), and therefore the message to avoid fats may actually have negative consequences for male fertility.

Although this review mainly focuses on the impact of over-nutrition on male fertility, studies of undernutrition provide a different lens to examine the overall impact of nutrition on reproduction. Two approaches are commonly used; caloric restriction and low protein diets, respectively reflecting decreased food availability and a common dietary deficiency observed in undernourished children (47). With the focus shifted from fat to protein in these models, a host of interesting findings have come to light, leading to a growing recognition of the importance of dietary protein in health and disease. In the context of male reproduction, dietary protein has been demonstrated to impact weights of reproductive organs, reproductive hormone concentrations (48), testicular architecture and occurrence of apoptosis during spermatogenesis (49), testicular expression of DNA methyltransferases, and sperm DNA methylation (50). Further, the level of protein in a father's diet has also been shown to alter subsequent pre-implantation embryo gene expression, placental gene expression and imprinting, fetal bone growth (51), fetal and placental weights, placental structure (52), and adult offspring vascular function (53) and metabolism (50). These results highlight why it is important to consider the effects of dietary protein in addition to fats and carbohydrates when investigating the impact of dietary-induced obesity on reproductive outcomes. This is particularly important because in order to increase the percentage contribution of dietary energy of fat in the standard control versus western diet experimental design, the percentage of protein and/or carbohydrates must be decreased.

This problem of failing to consider macronutrient effects in concert is apparent from a comparison of the typical diets used in animal studies of obesity and reproduction. In addition to differences in the proportion of fat that is used to represent a "high fat" diet, the relative proportional reduction in proteins

and carbohydrates also varies widely across studies (**Table 1**). Hence, while results are interpreted in the context of the change in fat content, studies are actually comparing diets which differ across their percentages of protein, carbohydrate, and fat (e.g., see **Table 1**, diet SF04-057 compared to diet SF00-219, **Figure 1**). In some cases, studies fix protein and vary only in carbohydrate and fat (7, 36, 37). In this case, control diets (fat 10% of total kcal) are high in carbohydrate (70% of total kcal) and high-fat diets (fat 45%–60% of total kcal) are low in carbohydrate (20%–35% of total kcal). This type of experimental design gives very little opportunity to disentangle the effects of different macronutrients, as it is not possible to conclude whether results are due to high fat alone, or the combination of higher fat and lower carbohydrate and/or protein. In order to better understand the impacts of different diet compositions, a new approach is required, which allows macronutrient impacts to be considered in the context of the whole diet.

Another consideration is that in an important respect, the composition of an experimental dietary treatment is not necessarily the same thing as the consumed diet, even in a no-choice paradigm. This is because an animal restricted to a nutritionally imbalanced food theoretically has the option to eat any one nutrient at the required level, albeit at the cost of over- and/or undereating other nutrients. Thus, a "low protein" experimental treatment might in reality not represent protein deficiency at all, but rather carbohydrate and/or fat surplus. Many experiments either do not measure intake, or else do not analyze the data to distinguish these possibilities.

MOVING BEYOND THE WESTERN DIET; INTRODUCING NUTRITIONAL GEOMETRY

Background to Nutritional Geometry and Macronutrient Balance

Nutritional Geometry (NG) is a multi-dimensional nutritional framework which assesses how macronutrient balance, rather than an individual macronutrient effect (e.g., high fat alone), impacts a given variable. Animal NG studies related to reproduction have employed a large number of diets which systematically vary across protein, carbohydrate and fat [e.g., (54, 55), **Figure 1**]. While this can make practical application logistically challenging, it offers a robust experimental design for studying the effects of macronutrient balance, which is more relevant to human obesity. Originally developed in studies of insects (56, 57), NG has since been used to study impacts of diet across a range of invertebrate taxa, particularly locusts, flies, crickets, and cockroaches. Given the adaptability of the framework, NG studies have extended to include many vertebrate species [e.g., fish (58), mice (55), companion animals (59), and non-human primates (60–62)]. Using principles which have been well established in these animal studies, Nutritional Geometry has also been directed increasingly toward human health (63, 64).

One insight to emerge from NG is the "protein leverage hypothesis", a theory to explain why modern diets are driving the obesity epidemic (34). The PLH posits that food intake in humans is driven most strongly to fulfil a target intake for protein, which passively influences ("leverages") the intake of non-protein energy (34, 65). A nutrient-specific appetite for protein is widespread among animal species, and evidence that this powerful protein appetite has interacted with a decline in the density of protein in the industrialized food supply to drive human obesity has accumulated rapidly in recent years [e.g., see (33)]. This is seen particularly in the modern diet of Western countries, where commonly consumed ultra-processed foods are low in protein relative to fats and carbohydrates, driving increased overall energy intake (66, 67). The impact of dietary manipulations on food intake is therefore an important consideration and should be measured in studies of nutritional effects on reproduction.

Data Visualization Using Nutritional Geometry

One of the major advantages of the NG approach is that it provides a graphical visualization of the effects of macronutrients. Data for each response variable are mapped on to a multidimensional nutrient space, allowing for a generalized overview of how an outcome is impacted by different diet compositions (**Box 1**). This method allows the individual and interactive effects of nutrients to be explored and disentangled. Results can be interpreted on the basis of the dietary macronutrient proportions (% of total kcal from each nutrient) (68), or absolute macronutrient intakes (g or kcal eaten of each nutrient). Absolute macronutrient intakes are a function of diet composition and the amounts of food an individual consumes. The NG approach provides a platform both for examining the effects of dietary nutrient mixtures on outcomes of interest, and for developing a guide for how experimental diets can be adjusted to achieve a desired outcome. For example, using this framework, it can be seen why diets high in protein are effective for weight loss—less calories are consumed (69). However, this excess protein consumption comes at a cost—animals consuming these high protein/low carbohydrate diets show signs of metabolic disease and have shorter lifespans (70–72).

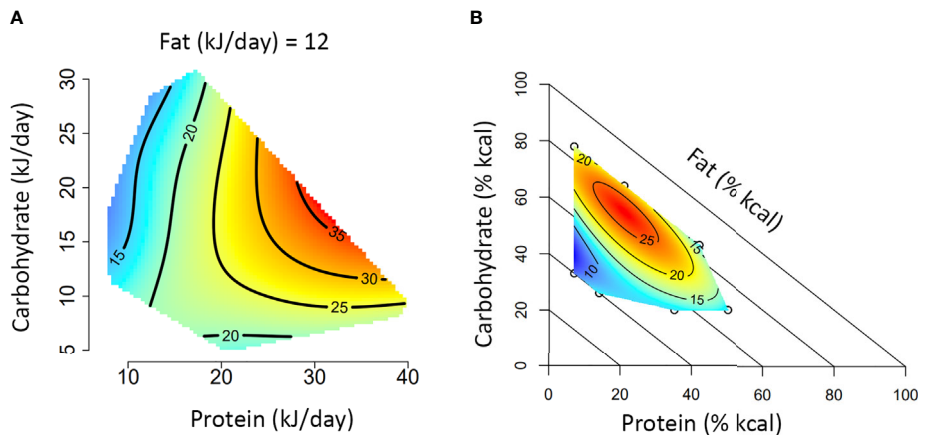
Nutritional Geometry in Action

NG has been used to understand how diet affects different aspects of health, including trade-offs between lifespan and reproduction (55, 70, 73). In general, these studies in mice and insects show that reproductive function is optimized by diets higher in protein content than diets that maximize lifespan. However, the strength of this response differs with sex (55, 70). This may be a true effect of differing nutrient requirements for reproduction in females versus males, or it may be an artefact of difficulties in assessing reproductive function in males (or likely a combination of both). Male reproduction in insects and fish is often assessed by an indirect measure of pre-mating investment in traits such as calling effort (70, 74), pheromone expression (75, 76), size of sexually-selected traits (77), and courting behavior (58). Other studies in both insects and mice have used measures of post-

BOX 1 | A guide to interpreting Nutritional Geometry surface figures.

The figures presented in **A** and **B** represent two different experimental approaches to using Nutritional Geometry. In **(A)**, the study design incorporates a range of different diet structures (protein/carbohydrate/fat %), which vary in their energy density. Such a design will significantly impact overall energy intake and is therefore analyzed according to intake of each nutrient (typically in kJ/day). In **(B)**, the study design incorporates different diet structures, but all diets have an equal energy density (isocaloric). This design typically only has minor effects on overall energy intake and is therefore analyzed according to the amount of each nutrient in the diet (typically as percentage of total kcal in the diet).

Both figures depict the impact of macronutrient balance on a given response variable (e.g., basal glucose). A color scale is used to indicate the level of the response variable (blue = minimum, red = maximum), with isolines giving numerical values. Isoline values vary by a fixed increment and the distance between isolines indicates the magnitude of nutrient effect over a given range (distant = minimal impact, close = substantial impact). In an intake-based model like **(A)**, responses are often compared based on protein versus non-protein (carbohydrate and fat) energy. Alternatively, a 3×3 panel figure can be used to show primary interactions at the lower, median and upper quartile of the third macronutrient (e.g., protein versus carbohydrate, at the 25th, 50th and 75th percentile of fat). If appropriate to the medium, a rotating animation could be created to display all three dimensions simultaneously. In a diet-based model like **(B)**, responses are compared based on diet structure, and the impacts of all three nutrients are analyzed simultaneously. Note that for the diagonal axis (in this case fat), the value increases from 0% at the diagonal axis to 100% at the origin.



mating investment including testes and accessory glands size (55), sperm number and quality (78), and mating success (75). Nutrient effects on pre- and post-mating sexual traits can differ (79), and may be age- and context-dependent (80). Therefore, to accurately measure male reproductive function, multiple measures may need to be assessed (81).

A more complete assessment of male reproductive function may also be obtained by examining offspring produced from mating trials. Insect studies examining the proportion of eggs that hatched after standardized females were mated to experimental males have found that protein has negative (82, 83) or non-linear (78) effects on male fertility. Similarly, studies in *Drosophila* assessing mating in a competitive context have found that male reproductive success is maximized on diets with intermediate levels of protein (84, 85), and a low protein to carbohydrate ratio (86). These studies suggest that while female reproduction may be enhanced by increased dietary protein, male reproduction may be enhanced on lower protein to carbohydrate ratios—a diet similar to that which maximizes lifespan and metabolic health. However, as dietary nutrients have different effects on different aspects of male reproductive function (79), dietary recommendations may need to be specific to the desired outcome. As many of these studies have shown, rarely is one macronutrient wholly responsible for an effect; instead, the balance of macronutrients has often proven to be the most significant factor determining an outcome. Therein lies the advantage of the NG approach in shifting from studying a single nutrient to the interactions of multiple nutrients.

IMPORTANT NUTRITIONAL CONSIDERATIONS TO IMPROVE AND EXPAND DIET STUDY DESIGNS

Caloric Density

As discussed above, the varying caloric densities of control and western diets present an issue for the interpretation of results. When diets differ in their energy density, whether effects are derived from calories or macronutrients cannot be ascertained—a common point of contention in nutrition research (72). However, energy density can be standardized (made isocaloric) using indigestible fibre (e.g., cellulose), so that while protein, carbohydrate, and fat are at different levels, diets provide the same amount of energy per gram. Thus, isocaloric diets are a useful tool to improve the clarity of results and are commonly used in NG studies [e.g., (87)]. To study the effects of calories using the NG approach, researchers have the option of analyzing data on the basis of calories consumed (which differs only with the amount of food eaten, not the macronutrient balance).

Alternatively, the study design can include a range of different macronutrient compositions at multiple calorie densities (e.g., low 3 kcal/g versus high 5 kcal/g for each combination of protein, carbohydrate, and fat).

Macronutrient Quality

Different foods differ in their biochemical profile of amino acids, fatty acids, and carbohydrate types (88, 89). As a result, the primary dietary sources of each macronutrient are likely to be just as important as overall macronutrient balance (90, 91), and a small number of studies support that this extends to reproduction. For example, when protein is supplied at a consistent level, vervet monkeys given animal protein (milk solids) had significantly poorer semen parameters than those fed plant protein (maize and legumes) (92). Similarly, dietary fat differentially impacts testicular enzyme activity depending on whether it is derived from virgin olive oil or butter (93). While virgin olive oil (monounsaturated fat) increased dipeptidyl peptidase IV activity, helping to maintain normal spermatogenesis, butter (saturated fat) increased the activity of gamma glutamyl transpeptidase, contributing to maintenance of the intracellular glutathione pool. While research into the effects of macronutrient source on reproduction is currently limited, it is likely to play an important role in response to diet and should also be a consideration in future studies.

Micronutrients

Beyond the macronutrients which provide dietary energy, Nutritional Geometry has also proven useful for studying the effects of dietary vitamins and minerals (79, 94). Many micronutrients, including calcium, sodium, zinc, potassium, and magnesium, have important roles in male reproduction, impacting testicular development, semen quality, and sperm biochemical processes (95). Micronutrient imbalances have also been suggested as a causal factor in unexplained female infertility (96). Reduced intake of dietary antioxidants including lycopene, vitamin C, folate, and carotenoids, has been associated with poorer semen parameters (97, 98). In addition, iodine intake outside the recommended range in men has been associated with increased time to conception (99), supporting a critical role for micronutrient balance in fertility. Supplementation of micronutrients, particularly those with antioxidant activity (e.g., vitamins C and E, selenium), has been widely studied as a tool for improving reproductive outcomes of infertile men (100, 101). However, results have varied widely depending on the andrological diagnosis and the type, quantity and duration of micronutrient supplementation. While micronutrients clearly play important roles in male reproductive physiology, there have been no studies which systematically evaluate the impacts of dietary micronutrient intake on reproductive success. Micronutrients are particularly important to consider in the context of obesity, as micronutrient deficiency appears to be common in obese individuals (102, 103). Further, micronutrient supplementation may be able to limit negative effects of obesity on sperm function (104). Overall, there is an ongoing need for systematic research into how dietary micronutrients impact reproduction in both lean and obese males.

Dietary Restriction

In studies using both western diets and a NG approach, food is generally provided *ad libitum*. However, another approach used in dietary studies is to restrict either the amount or timing of access to food. There is good evidence that the temporal pattern of intake, including caloric restriction, periodic and intermittent fasting can have important effects in addition to those of diet composition (105). In relation to male reproduction, caloric restriction has been demonstrated to impact testicular gene expression (106, 107), including expression of leptin and ghrelin receptors (108). Intermittent fasting has also been shown to affect testicular gene expression (106), as well as testosterone production (106, 109, 110). As caloric restriction and intermittent fasting remain popular (111) and recommended (112) weight loss strategies, future studies should also seek to investigate the effects of different intake patterns on reproductive health.

Genetics and the Human Context

One important consideration which spans both nutrition and reproductive biology, is the contribution of genetics. There are genetic factors, including copy number variants, gene mutations, single nucleotide polymorphisms and chromosomal abnormalities, implicated in obesity (113), and male infertility (114, 115). In the context of obesity, there is also the important consideration of nutrigenomics; the influence of nutrients themselves on gene expression (116). Given the important contribution of a unique genetic background to both response to diet and male fertility, this is a factor which poses a significant limitation in current studies. While C57BL/6 mice are used extensively as a model species in obesity research, this is an inbred sub-strain with limited genetic variability. Further, the consistent diet offered in animal studies does not reflect the depth of dietary variation in humans. While both constraints are inherent limitations of animal studies, they highlight the importance of moving from a single model to a variety of models (e.g., different mouse strains, non-human primates), and eventually to human studies. Making such a transition can allow for analyses based on populations with higher genetic variability. Further, Nutritional Geometry can be used to extend findings of animal studies into more complex human dietary patterns, analyzing either free-choice feeding from a selected range of foods (65) or dietary survey data (117).

FEATURES OF STUDY DESIGN AND MEASUREMENT SPECIFIC TO MALE REPRODUCTION

Investigations into how diet and obesity impact male reproductive function require the collaboration of two distinct research fields; nutrition and reproductive biology. In addition to considering the dietary aspect of animal studies, issues specific to studying male reproduction need to be considered. The first of these is the timing and length of diet treatments, which vary considerably in previous western diet-based studies (**Table 1**). In

agricultural species, a significant body of research shows that many effects of diet (including over and under feeding) observed in pre-pubertal males are different in sexually mature males (118, 119). In terms of treatment length, many studies apply dietary interventions for a minimum of one complete spermatogenic cycle [34.5 days in mice (120), 56 days in rats (121)], to ensure that mature spermatozoa in the ejaculate are "exposed" to treatment throughout the entirety of spermatogenesis. Conversely, some recent studies have indicated impacts of diet on sperm function in the short term (<2 weeks) (104, 122). There is no wrong answer here in terms of when to start and stop treatment, but the interpretation of results should consider whether treatments were applied pre or post-puberty, and how treatment duration relates to sperm development.

Another important consideration is the measurement of reproductive function. Fertility in humans is simply defined as natural conception within 12 months of unprotected intercourse (123), with time to conception commonly used to describe an individual's likelihood of fertility (11). Previous diet studies have employed a range of assessments, including basic observational measurements [e.g., testis size (55), sperm motility, histology (7)], molecular biology assays [e.g., miRNAseq (12), proteomics (41), enzyme activity (8), oxidative stress markers (6)], and direct measures of conception success [i.e., fertilization rate, blastulation rate, pregnancy rate (37, 39)]. While no one assay provides an infallible measurement of fertility, the combination of several variables will help to build a clearer picture of how diet and obesity impact male reproduction overall.

Finally, there should be a concerted effort to both capture and understand the unique impacts of diet on male compared to female reproductive physiology. It may be tempting to conclude that overall effects of diet on reproduction (i.e., increases or decreases in fertility) observed in one sex are equally applicable to the other sex. However, as discussed above, female and male reproductive performance appear to be optimized on different diets. Further, studies have indicated that female and male reproductive traits are differentially impacted by the same macronutrient ratios (55, 82, 83, 124). For example, male mice consuming a diet with an equal ratio of protein to carbohydrate had the largest testes and seminal vesicles, whereas female mice consuming the same diet had the largest uteri, but frequency of estrus, total follicle count and number of corpora lutea were reduced (55). Ultimately, future studies should endeavour to compare and contrast female and male reproductive responses to diet in order to determine whether the ideal macronutrient ratio to support reproduction is sex-specific.

DISCUSSION

As the prevalence of obesity continues to rise, and more negative implications for male reproductive physiology are discovered, its continued study remains a high priority. So far, research has provided strong evidence that a high fat diet negatively impacts male reproduction. However, as posited by the protein leverage hypothesis (34), dietary macronutrient balance rather than fat alone is likely to account for rising levels of obesity in the human population. In this context, the approach that is used to study obesity and the extent to which it captures the reality of the human experience must be considered. Using a tool such as Nutritional Geometry to study many different macronutrient combinations will not only provide information on which diets are detrimental but could also help guide research toward diets which may support reproductive function. This concept is particularly relevant when considering our approach to providing nutritional advice to men who are interested in conceiving.

Despite the observed impacts of obesity on male reproduction and the fact that men report >80% of pregnancies are planned (125), widespread, professional pre-conception nutritional guidance for men remains almost non-existent (126, 127). The advice most commonly given and acted upon by men is to lose weight and eat a healthy diet (126). This is undoubtedly good advice, given the clear negative impacts of obesity on male reproduction (18, 20) and the strong relationship between diet and obesity risk (34, 128). However, there is no clear definition of what a "healthy diet" for reproduction is.

Switching to a "healthy diet" for most men means reducing intakes of foods containing saturated fat and added salt and sugars, and eating a wider variety of unprocessed foods (as recommended by nutritional dietary guidelines). While nutritional guidelines from different countries also give recommendations for macronutrient proportions (e.g., USA; 10%–35% protein, 45%–65% carbohydrate, 20%–35% fat as % of total kcal) (129), it remains unclear whether this diet structure is optimal for male fertility. While observational studies in humans have identified associations between dietary patterns and semen quality (130), the ideal macronutrient balance to support male reproduction is far from being well defined. Importantly, this is not necessarily the same as a diet which supports overall health and longevity, nor the same diet which supports female reproduction (55). In addition, it is not clear whether different advice is required in different contexts—taking other extrinsic, intrinsic, and genetic factors into account. There is a clear need to further explore how diet impacts male reproductive function in order to develop evidence-based pre-conception nutritional guidance for men.

There are many exciting potential applications of Nutritional Geometry in the landscape of male reproduction, covering both fundamental and applied aspects of reproductive research. Beginning with fundamental research conducted in rodent models, results would inform more targeted pre-clinical animal research, as well as nutritional intervention based clinical trials in humans. The information gathered by this approach would provide strong evidence on which to build pre-conception guidelines. On a fundamental level, NG can be used to explore which macronutrient ratio best supports male reproduction, and whether this differs from a) what supports female reproduction and b) what supports overall health. As the impacts of a paternal high fat diet on offspring health and reproduction are rapidly being uncovered (131), NG will likely be useful in exploring new avenues of paternal effects. NG may

also be useful in exploring the effects of macronutrient source and weight loss strategies (e.g., caloric restriction, intermittent fasting) on reproductive function, and whether these factors alter the ideal macronutrient ratio. In the human context, it will be important to determine the impact of differing treatment durations to establish whether diet changes within the relatively short term pre-conception planning window (<12 months) are a feasible strategy. With the rising use of assisted reproductive technologies (e.g., IVF) for conception (132), more clinically focused research could use NG to examine whether the ideal dietary macronutrient ratio to support reproduction is applicable outside of natural conception.

Animal studies have used high fat and western diets for decades in the pursuit of understanding the many consequences of obesity. While this approach has produced a wealth of information on the physiological impacts of obesity, it doesn't tell the whole story and limits what interpretations can be made about the role of diet. Nutritional Geometry shifts the focus from the effect of fat alone to complex and interacting effects of dietary macronutrient balance. Adopting the NG approach in future studies will provide more information on how the overall diet composition impacts male reproduction. In turn, this will allow for the development of evidence-based pre-conception nutritional guidelines for men, to support natural conception and potentially limit negative effects on offspring.

AUTHOR CONTRIBUTIONS

TP and AC conceived the review. TP, DR, SS, and AC wrote and reviewed the paper. All authors contributed to the article and approved the submitted version.

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SHBG as a Marker of NAFLD and Metabolic Impairments in Women Referred for Oligomenorrhea and/or Hirsutism and in Women With Sexual Dysfunction

*Vincenza Di Stasi[1], Elisa Maseroli[1], Giulia Rastrelli[1], Irene Scavello[1], Sarah Cipriani[1], Tommaso Todisco[1], Sara Marchiani[1], Flavia Sorbi[2], Massimiliano Fambrini[2], Felice Petraglia[2], Mario Maggi[3,4] and Linda Vignozzi[1,4]**

[1] Andrology, Women's Endocrinology and Gender Incongruence Unit, Department of Experimental Clinical and Biomedical Sciences "Mario Serio," University of Florence, Florence, Italy, [2] Gynecology Unit, Department of Biomedical, Experimental and Clinical Sciences "Mario Serio," University of Florence, Florence, Italy, [3] Endocrinology Unit, Department of Experimental Clinical and Biomedical Sciences "Mario Serio," University of Florence, Florence, Italy, [4] I.N.B.B. (Istituto Nazionale Biostrutture e Biosistemi), Rome, Italy

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***Correspondence:**
Linda Vignozzi
linda.vignozzi@unifi.it

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PCOS is one of the most common endocrine disorders and NAFLD is one of its most dangerous metabolic consequences. The diagnosis of NAFLD is not a practical task and the condition is at risk of being overlooked. The use of simpler but still reliable surrogate markers is necessary to identify women with a high likelihood of NAFLD. The aim of this study was to evaluate the clinical correlates of NAFLD Liver Fat Score (NAFLD-LFS) in women with oligomenorrhea and/or hirsutism. Furthermore, the study aimed to evaluate whether, among the hormonal parameters evaluated in such women, possible hallmarks of NAFLD may be identified. To this purpose, 66 women who attended our Outpatient Clinic for oligomenorrhea and/or hyperandrogenism were included in the study. In order to validate the results obtained in the first cohort, a second independent sample of 233 women evaluated for female sexual dysfunction (FSD) was analyzed. In cohort 1, NAFLD-LFS positively correlated with metabolic and inflammatory parameters. Among the hormone parameters, NAFLD-LFS showed no significant relationships with androgens but a significant negative correlation with SHBG ($p<0.0001$) that therefore appeared as a candidate hallmark for pathologic NAFLD-LFS. The ROC analysis showed a significant accuracy (81.1%, C.I.69.1-93.0, $p <0.0001$) for SHBG in identifying women with a pathological NAFLD-LFS. In particular, a SHBG 33.4 nmol/l was recognized as the best threshold, with a sensitivity of 73.3% and a specificity of 70.7%. In order to validate this SHBG as a marker of metabolic impairment possible related with the presence of NAFLD, we tested this threshold in cohort 2. FSD women with SHBG <33.4 nmol/l had worse metabolic parameters than women with SHBG ≥33.4 nmol/l and a significantly higher NAFLD-LFS even after adjusting for confounders (B=4.18 [2.05; 6.31], p=0.001). In conclusion, this study provides a new evidence in the diagnostic process of NAFLD,

showing that the measurement of SHBG, which is routinely assessed in the workup of women referred for possible PCOS, could identify women at higher metabolic risk, thus detecting those who may deserve further targeted diagnostic assessment.

Keywords: sex hormone binding globulin (SHBG), non-alcoholic fatty liver disease (NAFLD), polycystic ovary syndrome (PCOS), metabolic syndrome, female sexual dysfunction

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is characterized by a spectrum of disorders ranging from the simple fatty liver to non-alcoholic steatohepatitis (NASH), with increasing fibrosis leading to cirrhosis (1–4). Nowadays, NAFLD is considered the hepatic hallmark of insulin resistance in several metabolic disorders. Hence, recognizing NAFLD may be of pivotal importance because it increased the risk of developing not only hepatic but also extrahepatic diseases, such as cardiovascular disease (CVD), chronic kidney disease (CKD) and type 2 diabetes mellitus (T2DM) (5–7). Growing evidence indicates a high prevalence of NAFLD in women with polycystic ovary syndrome (PCOS). In fact, in PCOS, NAFLD ranges from 34% to 70% as compared with a 14–34% in the female general population (8–15). This emerging association between NAFLD and PCOS was substantiated by evidence of common pathogenic mechanisms between the two conditions (16, 17). Recent studies confirmed that both insulin resistance and hyperandrogenism are key players of liver damage in women with PCOS, with a major role of the second one in non-obese patients (18, 19). PCOS, one of the most common endocrine disorders, affects 5-10% of reproductive-aged women (20), presenting not only a reproductive, but also an oncological and a cardio-metabolic burden. Among these, NAFLD is often overlooked. Therefore, due to this high frequency and its key consequences, the evaluation of hepatic health should be mandatory in patients with PCOS. However, the diagnosis of NAFLD is not an easy task. Liver biopsy remains the gold standard; however, it may not be considered the first line procedure in a prevalent condition such as PCOS. Therefore, the use of simpler, but still reliable, surrogate markers is necessary to identify women with a high likelihood of NAFLD. To this purpose, several algorithms, based on clinical and biochemical easily available information, have been introduced (21, 22). The best-validated steatosis algorithms are the SteatoTest®, the Fatty Liver Index (FLI) and the NAFLD Liver Fat Score. All these algorithms have been validated in the general population or in severe obese populations and variably predict hepatic and cardio-metabolic outcomes/mortality (21, 23).

In the last years, the FLI and the NAFLD –LFS algorithms were applied in large cohorts of patients showing great accuracy as markers of liver damage, in particular in metabolically susceptible populations (13, 24–26). In addition, a very recent study, comparing four non-invasive NAFLD score, i.e. FLI, NAFLD-LFS, Hepatic Steatosis Index (HIS) and Lipid Accumulation Product (LAP), concluded that, whereas FLI is the most accurate in a population-based setting, NAFLD- LFS performs better in the high-risk subjects (27).

The aim of the present study was to evaluate the clinical and biochemical correlates of NAFLD-LFS in women who refer to an outpatient endocrinology clinic for oligomenorrhea and/or hirsutism and thus undergoing further investigations for PCOS diagnosis. Furthermore, since NAFLD-LFS is more convenient than liver biopsy but still impractical for routine clinical practice, the study aimed to evaluate whether, among the hormonal parameters, routinely used for the diagnostic workup of PCOS, there are any that better predicts a pathological NAFLD-LFS. Essentially, we found that sex hormone binding globulin (SHBG) below 33.4 nmol/l was able to predict a high risk for NAFLD in women consulting for PCOS, thus suggesting it as a possible biochemical hallmark of this hepatic disease. Therefore, in the second part of the study we tested this cut-off in a larger cohort of women referring to our Unit for PCOS-unrelated reasons, such as sexual difficulties.

MATERIALS AND METHODS

Study Design

This study was designed as a cross-sectional prospective study including two cohorts of women attending our outpatient clinics. Data were retrospectively collected by revising the medical records.

Patients' Recruitment (Cohort 1 and Cohort 2)

The first cohort of this study included a consecutive series of 66 women who attended the Andrology, Women's Endocrinology and Gender Incongruence Outpatient Clinic at the University of Florence (Florence, Italy) seeking medical care for oligomenorrhea and/or clinical hyperandrogenism and, therefore, evaluated for possible PCOS. The study protocol was in accordance with the Declaration of Helsinki and was approved by the local ethics committee (protocol PCOSFLOWMETS-12/811 OSS, Careggi Hospital, Florence, Italy). Informed consent was obtained before the initiation of any clinical procedures (a parent's consent was obtained for underage patients). PCOS was diagnosed according to the Rotterdam Criteria (28, 29). Exclusion criteria were the differential diagnosis of PCOS (thyroid diseases, hyperprolactinemia, non-classical congenital adrenal hyperplasia, acromegaly and Cushing disease), uncontrolled psychiatric disorders and inability to provide study consent. At the first visit, demographic and clinical data were collected as part of routine practice, including information on menstrual cycle, sexual life, habit to perform physical activity, medications used and associated medical conditions. "Physical activity" is a dichotomous variable (yes/no) in which "yes" indicates that the patients performed physical activity (of any kind) and "no" indicates that the patients did

not perform any type of physical activity. "Hours of physical activity/week" is a categorical variable including 4 levels: No physical activity - 1-3 hours/week - 4-6 hours/week - more than 7 hours for week. Patients also underwent a physical examination with measurement of body weight, height, body mass index (BMI), waist circumference, bioimpedance analysis, systolic and diastolic blood pressure. Hirsutism was evaluated using the modified Ferriman Gallwey (mFG) Score (30). Hirsutism was defined by a mFG score ≥ 8 for caucasian women (100% of the analytical sample). Polycystic Ovarian Morphology (PCOM) was defined according to the criteria of *Androgen Excess and Polycystic Ovary Syndrome Society* (31). Specifically, new ultrasound machines (transducers with frequencies ≥8 mHz) allow diagnosis of PCOM in patients having at least 20 small follicles (2 to 9 mm) in the whole ovary while ovarian size at 10 mL remains the threshold for the definition of increased ovary size (preferred criterion when using transducer frequencies <8 mHz) (31, 32).

In order to validate the results obtained in the first cohort, a second independent sample was analyzed (Cohort 2). Cohort 2 included 233 consecutive patients who attended the Andrology, Women's Endocrinology and Gender Incongruence Outpatient Clinic seeking medical care for female sexual dysfunction (FSD). These women did not take drugs that could alter SHBG levels, such as antiepileptics and estroprogestins. Informed consent was obtained before the initiation of any clinical procedures. Exclusion criteria were history of drug or alcohol abuse and a diagnosis of uncontrolled or unstable disease. At the first visit, demographic and clinical data were collected as part of routine practice, including information on menopause, medications used, and associated medical conditions. Previous diagnoses of mental disorders were assessed using the *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition* criteria (33). Patients also underwent a physical examination with measurement of body weight, height, BMI, waist circumference, bioimpedance analysis, systolic and diastolic blood pressure. The assessment of NAFLD-LFS was possible in 26 patients (11.2% of total cohort). All patients underwent metabolic assessment and a Color Doppler Ultrasound (CDU) examination of clitoral vascularization.

Biochemical Parameters

In patients from both the cohorts, blood samples were drawn in the morning after an overnight fast in early follicular phase (in pre-menopausal women) for the measurement of metabolic and hormonal parameters. Among the metabolic parameters we measured: blood glucose (using the glucose hexokinase method; Dimension Vista 1500, Siemens Medical Solutions USA, Malvern, PA, USA), total cholesterol, high-density lipoprotein cholesterol, and triglycerides (using the automated enzymatic colorimetric method; Dimension Vista 1500), insulin levels (using an electro-chemiluminescence immunoassay; Roche Diagnostics, Mannheim, Germany), glycated hemoglobin levels (using high-performance liquid chromatography; Variant II, Biorad Laboratories, Hercules, CA, USA), AST and ALT (COBAS 8000, Roche). Low-density lipoprotein (LDL) cholesterol was estimated indirectly by the Friedewald formula, unless triglycerides were >400 mg/dl (34).

In addition, the following hormones were measured: LH, Follicle- Stimulating Hormone (FSH), Estradiol (E), prolactin, thyroid stimulating hormone (TSH), FT3, FT4 (using the chemiluminescence method; DIMENSION VISTA ® System, Siemens), testosterone (using the chemiluminescence method; CENTAUR, Siemens), 17 alpha-OH-progesterone (using the RadioImmunoAssay method; DIASOURCE, Belgium), delta 4-androstenedione (using RadioImmunoAssay method; BECKMAN COULTER), dehydroepiandrosterone sulfate, DHEAS (using the electro-chemiluminescence immunoassay; COBAS, ROCHE, Germany), SHBG (using the electro-chemiluminescence immunoassay; COBAS, ROCHE, Germany) and anti-Mullerian hormone, AMH (using the chemiluminescence method; BECKMAN COULTER). Free androgen index (FAI) was calculated as the total testosterone to SHBG ratio and then multiplied by 100. HOMA IR was calculated as (fasting plasma glucose*insulin/405) where glucose was expressed in mg/dl and insulin in mU/L (35).

Non-Alcoholic Fatty Liver Disease Assessment

The risk of being affected by NAFLD was estimated by the NAFLD Liver Fat Score (NAFLD-LFS) (36), according to the following formula:

NAFLD-LFS: -2.89 + 1.18 * metabolic syndrome (yes = 1/no = 0) + 0.45* type 2 diabetes (yes =2/no=0) + 0.15 * insulin (mU/L) + 0.04 *AST (U/L) - 0.94 * AST/ALT

Metabolic syndrome (MetS) was defined according to criteria of the International Diabetes Federation (IDF) (37). In addition, the IDF adult criteria can be used for adolescents aged ≥ 16 years, while a modified version of these criteria can be applied to those aged 10 to < 16 years (use 90th percentile cutoff point for waist and < 40 mg/dl of HDL) (38).

NAFLD-LFS above -0.640 predicted NAFLD with sensitivity of 86% and specificity of 71% (36).

Other possible causes of liver steatosis were ruled out by history taking.

Color Doppler Ultrasound Assessment

As for clinical practice, CDU was performed only in cohort 2 by an experienced operator blinded to the clinical data using the MyLabClass-C sonography system (Esaote SpA, Genova, Italy); a linear transducer (LA523, 6e13 MHz) was used. All women were scanned in a quiet room with consistent conditions of heating and lighting to decrease the impact of external factors on blood flow. For premenopausal women, CDU was carried out during the early follicular phase of the menstrual cycle (days 3-5). CDU was performed according to a previously published operating procedure (39–50). Briefly, the exam was performed after 12 hours of sexual abstention (sexual intercourse or masturbation), and immediately after bladder voiding. Patients were scanned in the lithotomy position, with a good quantity of sonographic jelly to avoid interference from air and without applying any significant pressure on the genital tissues, to minimize possible artifacts (51, 52). A cross-section of the clitoris was obtained by placing the probe transversally at the top of the vulva; this plane

allows for easy localization of the cavernous arteries, which appear well defined at the center of each clitoral body (51, 52). When adequate Doppler signals were detected, pulse-wave Doppler mode was activated and blood flow velocity waveforms were recorded, with automatic computation of the PI. The PI represents the difference between the peak systolic and the end-diastolic flows divided by the mean maximum flow velocity (53). Because it characterizes the shape of the spectral waveform, it is independent of the probe angle to the vessel (54). At least three similar sequential waveforms were sampled for each cavernous artery to define a mean PI value.

Statistical Analysis

Data were expressed as mean ± SD when normally distributed and as median (quartile) for parameters with non-normal distribution, unless otherwise specified. Furthermore, categorical variables were reported as number and percentage. Correlations were assessed using the Spearman method. Significant correlations at univariate analysis were tested at multivariate analysis after adjusting for confounding factors. Linear regression was applied for multivariate analysis. ROC curve analysis was used for the evaluation of the accuracy of SHBG in detecting the risk to have NAFLD, and the coordinates of the ROC curve have been evaluated for the identification of a possible threshold value. All statistical analyses were performed using SPSS 26.0 for Windows (SPSS Inc, Chicago, IL, USA).

RESULTS

Table 1 lists the main characteristics of the two samples and the comparisons for the available variables. In the first cohort, 55 out of 66 patients were diagnosed with PCOS (according Rotterdam criteria). Nine patients were diagnosed such as "not PCOS" (i.e. only oligomenorrhea or hyperandrogenism) and two patients had not a definitive diagnosis, at the time of the study analysis. At univariate analysis, NAFLD-LFS was positively correlated with BMI, waist circumference, fasting plasma glucose (FPG), insulin, glycated hemoglobin, HOMA index, HDL cholesterol, triglycerides, blood pressure (SBP and DBP), fat mass (FM), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), white blood cells (WBC) and FAI. Conversely, NAFLD-LFS negatively correlated with the time spent weekly for physical activity. Accordingly, women reporting regular physical activity had significantly lower NAFLD-LFS than physically inactive women did (-0.74 vs.-1.80 p=0.015). Among the hormone parameters, NAFLD-LFS showed a significant negative correlation only with SHBG, while there were no correlations between NAFLD-LFS with testosterone and other androgens (**Table 2**). Considering that the NAFLD-LFS algorithm includes, among its factors, waist circumference, SBP and DBP and that BMI largely overlaps with waist circumference measurement, SHBG was then identified as the candidate hallmark for pathologic NAFLD-LFS.

A ROC curve analysis (**Figure 1**) was performed and showed that SHBG has an accuracy of 81.1% (69.1-93.0, p <0.0001) in identifying women with a pathological NAFLD-LFS. In particular, a value of 33.4 nmol/l was found to be the best cut-off, with a sensitivity of 73.3% and a specificity of 70.7%. Using this threshold, the probability of having a pathological NAFLD-LFS score in women with SHBG <33.4 or >= 33.4 nmol/L was 47.8% and 12.1%, respectively (p=0.003). Subsequently, patients were categorized according to this SHBG threshold and the differences between patients with SHBG above or below 33.4 nmol/L in metabolic, hormonal and ultrasound parameters were evaluated (**Figure 2** and **Supplementary Table 1**). At univariate analysis, values of SHBG<33.4 nmol/l were positively associated with several cardio-metabolic risk factors [SBP, DBP, BMI, waist circumference, (FM), Free Fat Mass (FFM), insulin, HOMA index and triglycerides], and with androstenedione. **Figure 2** also reports the clear-cut and obvious association between SHBG and FAI, a parameter resulting from the TT to SHBG ratio. On the contrary, a negative association was found between SHBG and HDL cholesterol levels.

In order to further validate the identified SHBG cut-off, we retested the above-mentioned relationships in an independent cohort of women seeking medical care for FSD at the same outpatient clinic (cohort 2).

Table 1 also lists the main characteristics of this second cohort and the comparison with the cohort # 1.

At univariate analysis (**Figure 3** and **Supplementary Table 2**), women with SHBG <33.4 nmol/l had significantly higher BMI, waist circumference, fasting blood glucose, insulin, glycated hemoglobin, HOMA index, triglycerides, prolactin, FAI and lower HDL cholesterol. After adjusting for age and waist circumference, the associations among insulin, HOMA index, triglycerides, HDL cholesterol and FAI still retained statistical significance. In addition, also the association between SHBG levels and waist circumference retained significance, in an age-adjusted model. Women with SHBG <33.4 nmol/l had also a significantly higher NAFLD-LFS, both in unadjusted and adjusted models (p<0.0001 and p=0.001, respectively – data shown in **Supplementary Tables**).

In this second cohort, the association between SHBG values and clitoral ultrasound parameters was also evaluated (**Figure 4**). We observed a higher clitoral pulsatility index in women with SHBG < 33.4 nmol/l, even though a full statistical significance was not achieved.

In order to assess which SHBG value may be considered as "normal", women from cohort #2 of childbearing age and not affected by MetS (n = 86) were selected. In this population, the median SHBG value was 71.55 nmol/l with 90% of the population ranging from 39.2 to 166.7 nmol/L. Therefore, the value of 33.4 nmol/l, identified in women with hyperandrogenism and/or oligomenorrhea, may be considered below normality for healthy women of childbearing age.

DISCUSSION

The present study confirms the correlation between NAFLD and metabolic parameters in a cohort of patients with

TABLE 1 | Characteristics of the two samples.

	Cohort 1 (66 women with oligomenorrhea and/or hyperandrogenism)	Cohort 2 (233 women with FSD)	p-Value
Age (years)	20.0 [17.8–25.0]	48.0 [36.0–56.0]	**<0.0001**
BMI (kg/m^2)	23.7 [21.0–29.0]	24.4 [21.4–29.0]	0.462
Waist circumference (cm)	85.0 [74.0–99.3]	92.0 [84.0–103.2]	**0.001**
FPG (mg/dl)	85.5 [81.0–91.3]	90.0 [83.8–98.0]	**0.001**
Insulin (mU/L)	10.0 [6.2-15.5]	7.5 [5.2–14.2]	0.121
Glycated Hemoglobin (mmol/mol)	33.0 [31.0–35.0]	37.0 [34.0–41.0]	**<0.001**
HOMA IR	2.2 [1.3–3.3]	1.6 [1.1–3.3]	0.149
Total cholesterol (mg/dl)	166.9 ± 30.1	203.2 ± 41.1	**<0.0001**
HDL cholesterol (mg/dl)	56.8 ± 13.2	61.6 ± 16.0	**0.036**
Triglycerides (mg/dl)	72.0 [52.8–93.0]	79.0 [56–119.5]	0.088
LDL cholesterol (mg/dl)	89.5 [69.0–116.3]	119.0 [99.0–144]	**<0.001**
SBP (mm Hg)	113.5 [100.0–120.0]	120.0 [110.0–130.0]	**<0.0001**
DBP (mm Hg)	70.0 [63.8–80.0]	75.0 [70.0–80.0]	**0.041**
FM (kg)	15.7 [10.6–24.2]	18.9 [14.9–27.5]	0.125
FFM (kg)	47.3 [45.2–51.1]	43.1 [41.7–45.8]	**<0.0001**
PCOS (%)	83.3 [n = 55]	—	—
Not PCOS (%)	13.6 [n = 9]	—	—
Patients without definitive diagnosis of PCOS (%)	3.0 [n = 2]	—	—
Physical activity (%)	48.5 [n = 32]	30.5 [n = 71]	0.060
Menopause (%)	—	46.8 [109]	
Sexually active (%)	54.5 [n = 36]	—	—
Smoking habit (%)	6.1 [n = 4]	21.0 [n = 49]	0.467
ESR (mm/h)	16.0 [6.0–25.5]	—	—
Uric acid (mg/dl)	4.5 ± 1.0	—	—
CRP (mg/dl)	0.4 [0.1–0.9]	—	—
Ferritin (ng/ml)	30.0 [16.5–47.5]	—	—
WBC (mm3)	6.8 ± 2.1	—	—
LH (U/L)	7.3 [4.0–12.1]	11.5 [5.3–30.4]	**<0.0001**
FSH (U/L)	5.4 [4.3–6.4]	18.4 [7.0–70.2]	**<0.0001**
Estradiol (pg/ml)	38.5 [26.8–59.3]	28.0 [13.6–52.0]	**0.035**
Prolactin (ng/ml)	14.6 [8.8–17.8]	9.2 [7.2–15.3]	**0.001**
Testosterone (nmol/L)	1.5 [1.0–2.2]	0.8 [0.5–1.4]	**<0.0001**
SHBG (nmol/L)	36.1 [27.4–62.7]	58.7 [41.2–82.8]	**<0.0001**
FAI	3.6 [1.9–7.0]	1.5 [0.8–2.5]	**<0.0001**
Androstenedione nmol/L)	7.6 [5.4–11.8]	4.4 [2.5–6.5]	**<0.0001**
DHEAS (µmol/L)	6.4 [4.9–8.2]	2.8 [1.6–4.4]	**<0.0001**
AMH (ng/ml)	7.0 [4.4–10.2]	0.2 [0.1–2.4]	**<0.0001**

Data are expressed as mean ± SD when normally distributed, median (quartile) when not normally distributed, and percentage when categorical.
Differences in not normally distributed continuous variables were assessed by Mann–Whitney U test for comparison between the two groups. Differences in normally distributed continuous variables were assessed by unpaired T test. Differences in categorical variables were assessed by Chi squared test.
BMI, Body Mass Index; FPG, Fasting Plasma Glucose; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; PCOS, Polycystic Ovary Syndrome; ESR, Erythrocyte Sedimentation Rate; CRP, C-Reactive Protein; WBC, White Blood Cells; LH, Luteinizing Hormone; FSH, Follicle Stimulating Hormone; SHBG, Sex Hormone Binding Globulin; FAI, Free Androgen Index; DHEAS, Dehydro-epiandrosterone Sulfate; AMH, Anti Mullerian Hormone.
The bold values are statistically significant values.

oligomenorrhea and/or hirsutism and identify a cut-off of SHBG able to predict the risk of NAFLD in this population. Young women with SHBG below 33.4 nmol/L showed a higher probability of having pathological NAFLD-LFS score as compared to those with SHBG above this value. In an independent population of patients seeking medical care for sexual dysfunction, this cut-off was also able to discriminate women at higher cardio-metabolic risk. By analyzing in the first cohort the correlations between the NAFLD-LFS and several parameters, positive correlations were found with blood pressure, glyco-lipidic parameters, inflammatory indices, waist circumference and BMI, while a negative correlation was found with SHBG. The latter result confirms previously reported data, which identify SHBG as an indicator of liver metabolic impairment (55).

SHBG is a glycoprotein produced by the liver and it is involved in the transport of sex hormones in the bloodstream, having a major role in the regulation of their circulating free levels. Interestingly, this protein is increasingly recognized as a hepatokine and is involved in the occurrence and development of metabolic disorders and of their cardiovascular consequences (56, 57).

So far, the relationship between SHBG and NAFLD in patients with PCOS has been evaluated in a single retrospective study. As compared with age- and BMI-matched control women, PCOS patients had higher serum testosterone and reduced SHBG levels, being both associated with an increased NAFLD hazard (58). Interestingly, this study also showed that SHBG <30 nmol/L was associated with five-fold higher NAFLD risk than SHBG >60 nmol/L (58). This previous finding corroborates our

TABLE 2 | Associations between NAFLD-LFS and clinical and laboratory parameters.

	NAFLD-LFS		
	n	Univariate analysis*	
		r	P value
Age (years)	66	0.133	0.288
BMI (kg/m^2)	64	0.657	**<0.0001**
Waist circumference (cm)	55	0.598	**<0.0001**
FPG (mg/dl)	66	0.427	**<0.0001**
Insulin (mu/L)	66	0.850	**<0.0001**
Glycated hemoglobin (mmol/mol)	57	0.275	**0.035**
HOMA IR	59	0.867	**<0.0001**
Total cholesterol (mg/dl)	66	0.037	0.768
HDL cholesterol (mg/dl)	66	–0.286	**0.022**
Triglycerides (mg/dl)	66	0.333	**0.006**
LDL cholesterol (mg/dl)	61	–0.029	0.822
SBP (mm Hg)	66	0.404	**0.001**
DBP (mm Hg)	66	0.473	**<0.0001**
FM (kg)	42	0.579	**<0.0001**
FFM (kg)	42	0.260	0.097
Physical activity	65	–0.271	**0.029**
Hours of physical activity/week	65	–0.287	**0.020**
ESR (mm/h)	49	0.527	**<0.0001**
Uric acid (mg/dl)	45	0.180	0.237
CRP (mg/dl)	47	0.375	**0.009**
Ferritin (ng/ml)	53	0.236	0.088
WBC (mm3)	59	0.317	**0.014**
LH (U/L)	65	0.195	0.120
FSH (U/L)	65	0.066	0.601
Estradiol (pg/ml)	54	0.033	0.815
Prolactin (ng/ml)	62	0.168	0.192
Testosterone (nmol/L)	63	0.127	0.320
SHBG (nmol/L)	56	–0.557	**<0.0001**
FAI	55	0.487	**<0.0001**
Androstenedione (nmol/L)	60	0.224	0.085
DHEAS (µmol/L)	62	–0.188	0.142
AMH (ng/ml)	59	–0.162	0.220

**Unadjusted correlation coefficients (r) and levels of significance (P) were derived from Spearman analysis.*

BMI, Body Mass Index; FPG, Fasting Plasma Glucose; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; FM, Fat Mass; FFM, Free Fat Mass; ESR, Erythrocyte Sedimentation Rate; CRP, C-Reactive Protein; WBC, White Blood Cells; LH, Luteinizing Hormone; FSH, Follicle Stimulating Hormone; SHBG, Sex Hormone Binding Globulin; FAI, Free Androgen Index; DHEAS, Dehydro-epiandrosterone Sulfate; AMH, Anti Mullerian Hormone.

The bold values are statistically significant values.

results that, obtained with a different approach, identified a SHBG threshold of 33.4 nmol/L as the most appropriate to discriminate women with pathologic NAFLD-LFS.

In the first study cohort, including women with oligomenorrhea and/or hirsutism and enriched in PCOS patients, the assessment of metabolic parameters confirmed that SHBG <33.4 nmol/L identifies patients with adverse metabolic profile, including worse blood pressure, BMI, waist circumference, body composition (increased fat mass and reduced fat free mass), insulin, triglycerides and HDL cholesterol.

In order to confirm the reliability of SHBG <33.4 nmol/L as a marker of metabolic impairment and increased NAFLD risk, a second larger independent cohort of women with sexual dysfunction was analyzed. Also in this population, SHBG values lower than 33.4 nmol/l allowed to identify women with higher BMI and waist circumference, worse glyco-lipid profile and, notably, pathological NAFLD-LFS values. Furthermore, in this population, SHBG values lower than 33.4 nmol/l, independently of age, menopausal status and years since menopause, also identified patients with a numerical higher clitoral PI. This is in line with recent findings, which showed that, in women consulting for sexual dysfunction, the clitoral PI, an index of vascular resistance, is higher in patients with metabolic impairments (59–61).

Hence, SHBG <33.4 nmol/L is an effective marker for identifying women with altered metabolic parameters and higher NAFLD risk in either high risk groups, such as those with PCOS, or those consulting for conditions not directly linked to metabolic disease, such as female sexual dysfunction.

The role of SHBG as a marker of metabolic alterations is not completely understood. Some preclinical studies have been aimed at investigating whether reduced SHBG is a cause or a consequence of the metabolic dysregulation. Overexpression of SHBG, by creating a double transgenic mouse (SHBG-C57BL/ksJ-db/db), in a NAFLD model or in a diet–induced model of hepatic steatosis, significantly reduced liver fat accumulation through PPARγ modulation (62). On the contrary, an increased hepatic lipogenesis and pro-inflammatory cytokines

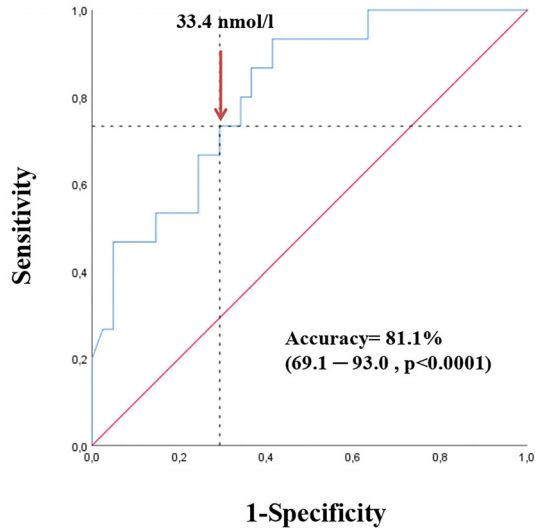

FIGURE 1 | Receiver operating characteristic (ROC) curve for SHBG in detecting NAFLD risk according to NAFLD-LFS (values > -0.640 predict NAFLD with sensitivity of 86% and specificity of 71 %) in the first cohort.

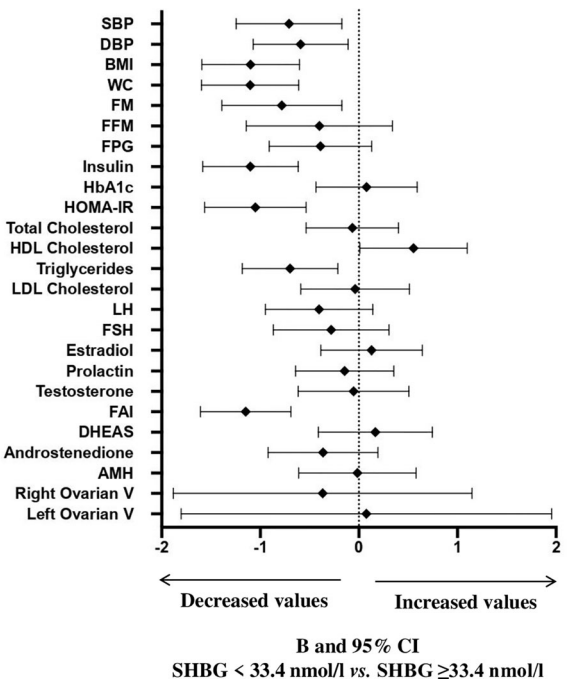

FIGURE 2 | B and 95% confidence interval (CI) for several metabolic and hormonal parameters as a function of SHBG <33.4 nmol/L as compared with SHBG ≥33.4 nmol/L in Cohort 1. Data are expressed as number of standard deviations from the mean value. The standardized values are based on log-transformed parameters. The statistics based on raw data are reported in **Supplementary Table 1**. SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; WC, waist circumference; FM, fat mass; FFM, free fat mass; FPG, fasting blood glucose; HbA1c, glycated hemoglobin; HOMA, Homeostatic Model Assessment for Insulin Resistance; HDL, High-Density Lipoprotein; LDL, low-density lipoprotein; LH, Luteinizing hormone; FSH, Follicle-stimulating hormone; FAI, Free androgen index; DHEAS, Dehydroepiandrosterone sulfate; AMH, Anti-müllerian hormone; V, volume.

secretion downregulates SHBG production (63). These data provide potential mechanisms by which SHBG may be either a cause or a consequence of NAFLD onset and progression (57). Clinical data also support a putative causal role of SHBG towards the development of NAFLD. In fact, in a recent study in a cohort of 3389 Chinese patients, lower baseline SHBG was associated with a higher occurrence of NAFLD, during a 3-year follow-up, and, conversely, higher SHBG at study entry predicted a more frequent recovery from NAFLD (64). However, due to the sex steroid-dependence of SHBG, its own role in describing the metabolic status is largely unclear. In men, it has been recently shown that SHBG, independently of T, is associated with worse lipid profile and blood pressure (65), thus supporting the value of SHBG as a pure marker of metabolic disorders. In women, increased testosterone levels are associated with lower SHBG, as well as with dyslipidemia and insulin resistance (66, 67). Indeed, this complicates the interpretation of lower SHBG levels. Interestingly, in both the experimental cohorts evaluated in the present study, SHBG below 33.4 nmol/L did not identify women with increased androgen levels, thus excluding that these hormones participate in worse metabolic pattern associated with low SHBG, at least when this threshold is used.

The relevance of our results relies on the recognition of SHBG as a single, and easy-to-obtain, parameter that can reliably identify patients with more adverse metabolic parameters and, even more importantly, establish which patients have higher probability of having NALFD. This may simplify the every-day clinical practice because, despite being a streamlining, the available scores take extra time during the visit for their calculation. For this reason, in a clinical setting, having a single, rapid, and low cost marker, such as SHBG, can be the first "alarm bell" to deepen liver conditions in these patients (55, 68). The confirmation of the reliability of this value as a cut-off indicative of a higher risk of NAFLD even in an independent and population with different clinical background, strengthen the generalizability of our results.

The systematic assessment of NAFLD risk is of pivotal importance because NAFLD is associated with an increased prevalence and incidence of T2DM and cardiovascular disease (69–71). The recognition of patients that, although young or apparently healthy, have hallmarks of NAFLD disease may help the physician in directing the diagnostic workup and further investigate liver disease thorough more accurate, albeit more expensive and invasive, tests and to implement more strict lifestyle or pharmacologic interventions.

This study has some limitations, including the small sample size and the heterogeneity of the cohort #1, which is not purely made of PCOS patients but it includes women with clinical features that deserve periodic assessment for a possible development of PCOS. In fact, it is known that subjects with even a single diagnostic factor are at higher risk of developing PCOS as well as MetS and NAFLD (72, 73). In addition, NAFLD was not diagnosed with imaging studies but only estimated with a clinical algorithm. Finally, in both the cohorts, androgens were not measured by the gold-standard

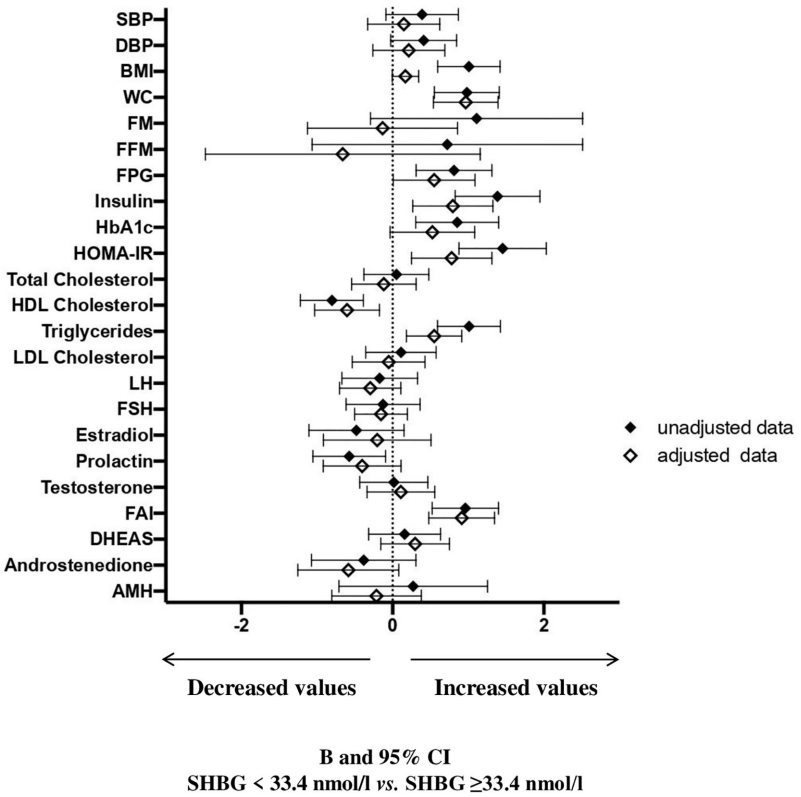

FIGURE 3 | B and 95% confidence interval (CI) for several metabolic and hormonal parameters as a function of SHBG <33.4 nmol/L as compared with SHBG ≥33.4 nmol/L in Cohort 2. Data are expressed as number of standard deviations from the mean value. The standardized values are based on log-transformed parameters. The statistics based on raw data are reported in **Supplementary Table 2**. Black diamonds: unadjusted data; white diamonds: data adjusted for age and waist circumference. SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; WC, waist circumference; FM, fat mass; FFM, free fat mass; FPG, fasting blood glucose; HbA1c, glycated hemoglobin; HOMA, Homeostatic Model Assessment for Insulin Resistance; HDL, High-Density Lipoprotein; LDL, low-density lipoprotein; LH, Luteinizing hormone; FSH, Follicle-stimulating hormone; FAI, Free androgen index; DHEAS, Dehydroepiandrosterone sulfate; AMH, Anti-müllerian hormone; V, volume.

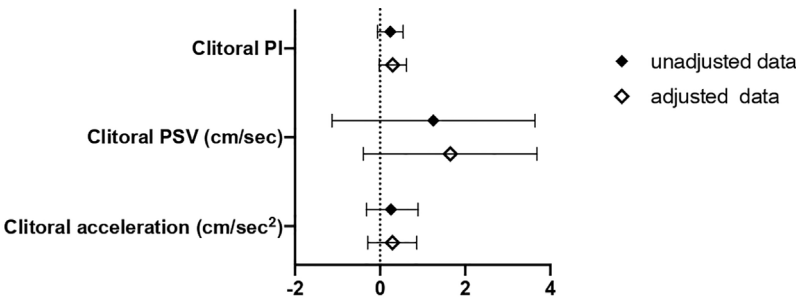

FIGURE 4 | B and 95% confidence interval (CI) for clitoral vascular parameters as a function of SHBG<33.4 nmol/L as compared with SHBG≥33.4 nmol/L in Cohort 2. Data are expressed as number of standard deviations from the mean value. Black diamonds: unadjusted data; white diamonds: data adjusted for age, menopausal status and years from menopause. PI, pulsatility index; PSV, peak systolic velocity; ACC, basal acceleration.

method, i.e. mass spectrometry, although a highly reliable immunoassay has been used.

The strength of this study is that the threshold value of SHBG that we analytically found in this small sample replicates values previously identified in a larger population. In our analytical sample, SHBG <33.4 nmol/L has good accuracy, sensitivity and specificity, and it is capable of identifying higher risk of NAFLD, either in a population of young women predisposed to metabolic

impairment or in young-adult/middle-aged women consulting for female sexual dysfunction.

A future perspective will be to evaluate whether treating NAFLD with lifestyle interventions (nutrition and physical activity) or with medications has consequences on SHBG values, thus confirming its role as a marker of liver health.

In conclusion, this study provides a new evidence in the complex diagnostic process of NAFLD in patients at a higher risk, which can be used as a first test to calibrate a subsequent targeted diagnostic assessment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee "Area Vasta Centro." Written informed consent to participate in this study was provided by the participants or by participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

VDS, GR, and LV conceptualized and designed the study. VDS acquired the data. VDS, EM, GR, and LV analyzed and interpreted the data. VS and EM drafted the article. VDS and LV revised the article for intellectual content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.641446/full#supplementary-material

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SYSTEMATIC REVIEW
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Improvement of Insulin Sensitivity Increases Pregnancy Rate in Infertile PCOS Women: A Systemic Review

Yuqi Liu[1,2†], Juan Li[1,2†], Zhe Yan[1,2†], Dan Liu[3], Jinfang Ma[1,2] and Nanwei Tong[1,2]*

[1] Department of Endocrinology and Metabolism, West China Hospital of Sichuan University, Chengdu, China, [2] Laboratory of Diabetes and Islet Transplantation Research, Center for Diabetes and Metabolism Research, West China Hospital of Sichuan University, Chengdu, China, [3] Department of Obstetrics and Gynecology, Nantong Rich Hospital of Nantong University, Nantong, China

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****Correspondence:***
Nanwei Tong
tongnw@scu.edu.cn

[†]These authors have contributed equally to this work

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Background: Polycystic ovary syndrome (PCOS) is the most common cause of infertility in reproductive-age women. Insulin increases steroidogenesis, deranges granulosa cell differentiation, and affects follicle growth. However, results from randomized control trials (RCTs) were heterogeneous, and little strong evidence associated actual achievement of insulin sensitivity (IS) improvement with reproductive outcomes.

Objectives: To identify evidence of the reproductive benefit of IS improvement in infertile PCOS women by analyzing eligible RCTs.

Search Strategy: Different search strategies with unlimited keywords, including treatment, therapy, intervention, polycystic ovary syndrome/PCOS, insulin resistance, pregnancy, conceive, live birth, and randomized controlled trials/RCT were used in databases including Pubmed, Embase, and Web of Science to November 20th, 2021.

Data Collection and Analysis: Two authors independently abstracted study details and assessed study quality.

Main Results: Ten RCTs that covered different races and met the inclusion criteria were included for analysis and discussion. Clinical pregnancy rate was increased in infertile PCOS women when they had significant improvement of IS after treatment regardless of the various interventions (non-surgical). The benefits of IS improvement appeared superior in PCOS women without severe obesity. The effect of IS improvement on pregnancy rate was independent of the change of BMI.

Conclusions: Nonsurgical therapeutic strategies that promote superior IS improvement may aid infertile PCOS women to increase their possibility of successful pregnancy regardless of the various interventions. The improvement of IS might be more important than the reduction of BMI in the improvement of pregnancy rate in infertile PCOS women.

Keywords: polycystic ovary syndrome (PCOS), insulin sensitivity, review – systematic, randomized controlled trials (RCT), insulin sensitizers

INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common endocrine disease in reproductive-age women, characterized by hyperandrogenism and olig-anovulation. It is considered a metabolic disorder because it is associated with high rates of insulin resistance (IR), dyslipidemia, obesity, and other metabolic abnormalities. The prevalence of PCOS can reach 18% in reproductive-age women (1) and those with PCOS have a higher risk of infertility and other health issues (2).

Depending on the pregnancy needs of individuals, therapies for women with PCOS differ. For women who do not desire conception, the aim of treatment is to improve symptoms including acne, hirsutism, and menstrual disorder. Regarding adult infertile women with PCOS, achieving successful pregnancy and live birth are the primary objectives. In PCOS patients with oligo-anovulation, the first-line ovulation induction agents are clomiphene citrate (CC), letrozole (LZ), and metformin (3). CC is an antiestrogen, nonsteroidal compound that can induce ovulation in anovulatory women (4). LZ is an aromatase inhibitor that inhibits the conversion of adrenal androgens to estrogens. It is generally employed for breast cancer therapy (5), and has been found to function as an ovulation induction agent. Metformin, a first-line drug recommended by guidelines for the treatment of diabetes (6), was shown to induce ovulation and improve pregnancy rate in infertile women with PCOS (7, 8), although not as effectively as CC, according to a meta-analysis (9).

The mechanism underlying metformin's effects in PCOS women remains unclear. Metformin improves insulin sensitivity (IS) in the liver and peripheral tissues, which may represent the mechanism explaining its effect in improving pregnancy in PCOS women. The prevalence of IR was reported as 44%–70% in women with PCOS when assessed by surrogate markers (10). PCOS has a multifactorial etiology including intra-uterine, genetic and environmental factors. Familial aggregation studies indicated that PCOS is an inherited disorder and gene variants associated with IR have been demonstrated in PCOS patients. And intra-uterine growth restriction (IUGR) and small for gestational age (SGA) might cause excess glucocorticoids which increased the risk of obesity and hyperinsulinemia during childhood and finally contributed to developing insulin resistance in PCOS women (11). Supraphysiological doses of insulin were found to increase steroidogenesis, derange granulosa cell differentiation, and affect follicle growth (12). However, evidence regarding reproductive outcomes of insulin-sensitizing drugs in PCOS was inconsistent (13) and few analyses focused on actual changes in IS after treatment, which reflects whether these drugs achieve improvement of IR in PCOS women. In another word, the inconsistencies may have been because of failure to achieve improvement of IS. Additionally, some randomized control trials (RCTs) observed beneficial effects of CC and other nondiabetic drugs on IS and also on clinical pregnancy rate (14, 15). Taken together, these observations suggest achieving improvement of IS may be the actual factor that promotes an increased rate of pregnancy.

In this review, we summarized data from RCTs that reported post-treatment changes in IS, with the objective of analyzing the correlation between improvement of IS and pregnancy rate in infertile women with PCOS who underwent various nonsurgical interventions, and exploring the reproductive outcomes of insulin-sensitizers, such as pioglitazone and exenatide, in PCOS women.

METHODS

Systematic Review Methodology

The systematic review was conducted according to the guidelines from PRISMA. We searched electronic databases, including MEDLINE from PubMed, Embase, and Web of Science with different search strategies and no limiting keywords, including treatment, therapy, intervention, polycystic ovary syndrome/PCOS, insulin resistance, pregnancy, conceive, live birth, and randomized controlled trials/RCT, to November 20, 2020.

Articles were screened by title and abstract. We established the following inclusion/exclusion criteria according to PICOS. 1) Population (P): reproductive-age women who were infertile because of PCOS rather than other reproductive diseases, and who were not complicated with diabetes or other endocrine disorders. 2) Interventions (I): administration of various reasonable nonsurgical interventions including lifestyle modification, monotherapies, and combined therapies was acceptable. Studies that used assisted reproductive technology or any surgical interventions were excluded. However, at least one parameter pertaining to IS (fasting blood glucose/insulin ratio, insulin sensitivity index, homeostasis model assessment of insulin resistance (HOMA-IR)) was reported before and after treatment. 3) Comparison/control (C): All head-to-head and placebo-controlled RCT were included. 4) Outcomes (O): clinical pregnancy rate and/or live birth rate. 5) Study (S): randomized controlled trials. 6) Additional inclusion/exclusion criteria: To assess IR, baseline fasting blood glucose in the different treatment groups of the included studies was not significantly different. And only English-language literature was included due to the language barrier.

To include as many relevant clinical trials as possible and without bias, the criteria did not limit treatment duration or sample size. The effects of treatment duration and sample size will be discussed.

Two authors independently abstracted study details and assessed the quality of RCTs in a blinded fashion.

Definition of Insulin Sensitivity and its Improvement After Treatment

Studies evaluated IS or IR according to different surrogate parameters, including fasting blood glucose/fasting serum insulin ratio, insulin sensitivity index, and HOMA-IR. Although some RCTs reported insulin levels (14–21) before and after treatment, and blood glucose levels, they did not report parameters that reflect IS or IR. Therefore, these trials were excluded from this review.

Each included trial reported whether there was a significant change in the IS parameter before and after treatment, and

whether it was significantly different between the groups in the trial. The group with significantly increased insulin sensitivity index or reduced HOMA-IR compared with other groups in the same trial was considered to have achieved an improvement of IS. Because of the lack of uniformity in the units of measurement of IS criteria, comparisons were only conducted within a single trial, and could not be conducted with meta-analysis.

Criteria for Overweight and Obesity Among Different Races

Owing to differences in race, diagnostic criteria for overweight and obesity differ. In America and most Western countries, BMI between 25.0 and 29.9 is considered overweight, BMI>30.0 is obese, and BMI≥35.0 is considered severely obese (22). However, in Asian populations, BMI between 23.0–27.5 is overweight, BMI>27.5 is obese, and BMI≥32.5 is severely obese (23). In this review, we followed the above criteria and classified PCOS women from the included trials as overweight, obese, or severely obese according to race.

Assessment for Quality of RCTs

The included RCTs were evaluated for quality by using the Cochrane Risk of Bias tool with RevMan 5.4. The assessment consists of seven aspects: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective outcome reporting, and other bias.

RESULTS

The preliminary search identified 959 unique citations. After screening on title and abstract, 101 eligible studies were identified by full text. In total, 10 studies were included in the final selection (**Figure 1**).

Ten RCTs from seven countries that covered different races and met the inclusion criteria were included for analysis (24–33). Sample sizes of included RCTs ranged from 25 to 626 (**Table 1**).

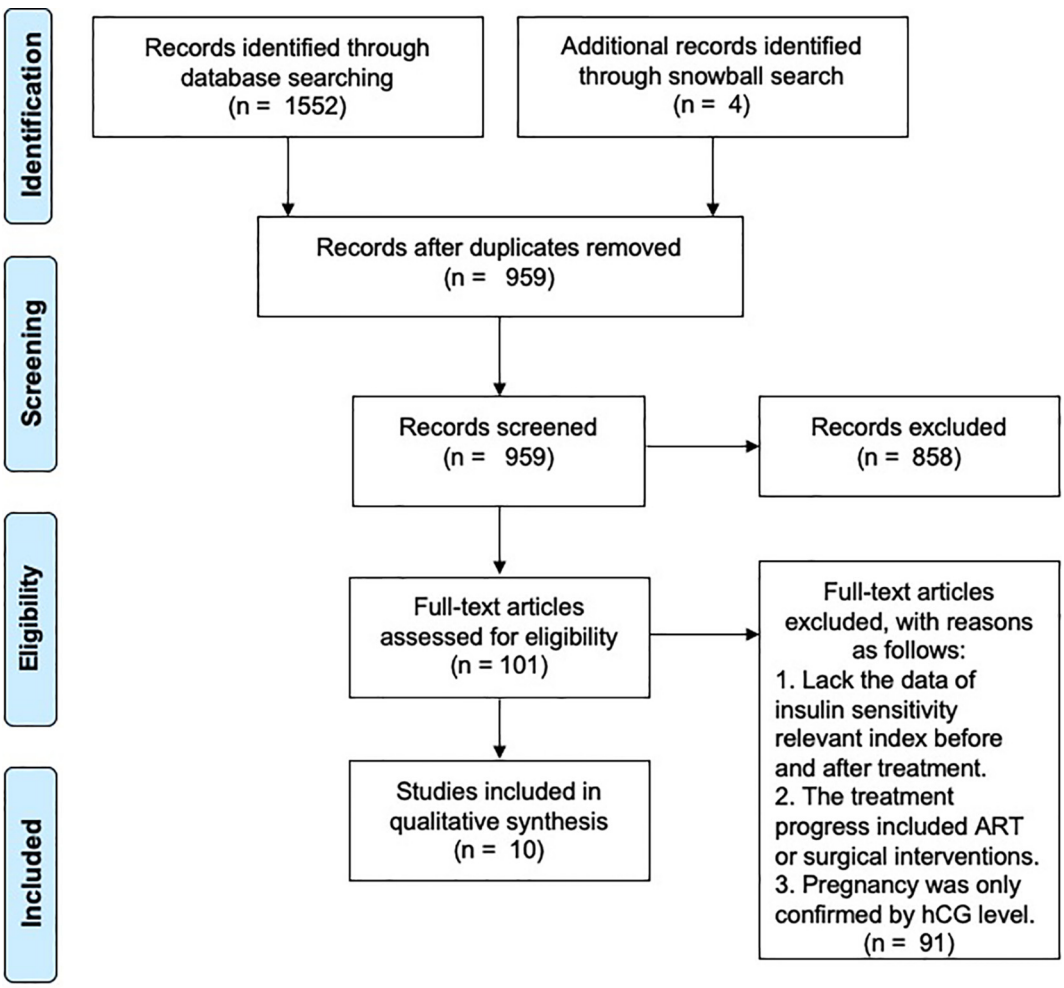

FIGURE 1 | Flow chart showing literature search and study selection.

TABLE 1 | The sample size, treatment duration, the formula and units of the IS parameters, and the basic condition of subjects in the included RCTs.

Trial No.	Study	Intervention Group (Subjects number in each group)	Sample size	Treatment Duration	CC resistant or not	BMI before treatment	Classification according to BMI[1]	Parameter of IS	The formula and Unit of the Parameter that reflects IS
1	Wang et al. (31)	Metf+CC (N=33)	78	up to 6 months	unknown	25.74±6.37	Over weight	HOMA-IR	FPG (mmol/L) × FINS (mIU/L) /22.5
		Exenatide+CC (N=45)				26.26±5.71			
2	Agrawal et al. (24)	Metformin+CC (N=60)	120	6 months	unknown	27.38±3.92	Over weight	HOMA-IR	mmol/L
		Metformin+MYO+CC (N=60)				27.71±3.60			
3	Ortega-González et al. (30)	Metformin (N=18)	47	6 months	unknown	34.1±1.6	Obese	HOMA-IR	FPG (mmol/L) × FINS (μIU/ml) /22.5
		Pioglitazone (N=17)				32.2±1.0			
4	Liu et al. (29)	24w Metf (N=80)	158	24 weeks	unknown	28.29±1.86	Obese	HOMA-IR	FPG (mmol/L) × FINS (mIU/L) /22.5
		12w Exenatide switch to 12w Metf (N=78)				29.16±3.11			
5	El Sharkwy and Sharaf El-Din (33)	Metf+CC (N=136)	274	3 months	CC-resistant	34.4±3.4	Severe Obese	HOMA-IR	FPG (mmol/L) × FINS (mIU/L) /22.5
		Metf+CC+L-carnitine (N=138)				35.5±3.2			
6	Legro et al. (27)	Metformin (N=208)	626	up to 6 months	unknown	35.6±8.5	Severe obese	Change in HOMA-IR	FPG (mg/dl) × FINS (μU/ml) /405
		CC (N=209)				36.0±8.9			
		Metformin+CC (N=209)				34.2±8.4			
7	Karimzadeh et al. (26)	Metformin (N=100)	200	3 months	unknown	28.80±3.18	Over weight	FPG/Insulin	The units were not reported
		Placebo (N=100)				29.49±4.75			
8	Yarali et al. (32)	Metformin (N=10)	25	6 weeks	CC-resistant	28.6±4.0	Over weight	FPG/Insulin	FPG (mmol/L) /FINS (pmol/L)
		Placebo (N=15)				29.6±4.8			
9	George et al. (25)	Metf+CC (N=30)	60	6 months	CC-resistant	25.7±3.9	Over weight	FPG/insulin	FPG (mg/dl) /FINS (μIU/ml)
		hMG (N=30)				25.9±2.9			
10	Legro et al. (28)	Lifestyle modification (N=50)	149	16 weeks	unknown	35.1±4.6	Severe obese	insulin sensitivity index	10,000/square root of (FPG x FINS) x (mean glucose x mean insulin during OGTT)
		OCPs (N=49)				35.1±4.2			
		Combination (N=50)				35.5±4.4			

1, The Asian criteria of overweight is BMI between 23-27.5, obesity is BMI>27.5, and severe obesity is BMI>=32.5.

BMI, Body mass index; CC, clomiphene citreate; Metf, Metformin; MYO, myoinositol; hMG, human menopausal gonadotropin; OCPs, Oral contraceptive pills; HOMA-IR, Homeostatic Model Assessment of insulin resistance; IS, Insulin sensitivity; FPG, Fasting Plasma Glucose; FINS, Fasting Insulin; OGTT, Oral Glucose Tolerance Test; RCT, Randomized Controlled Trial.

To avoid bias, we included RCTs with small sample sizes, the impact of which is discussed below.

Five RCTs used the HOMA-IR index as the parameter for evaluating IS (Trial No.1-6 in Tables) (24, 27, 29–31, 33), whereby larger value indicates more IR (less IS), whereas three studies reported the fasting plasma glucose (FPG)/insulin (INS) ratio (Trial No.7-9) (25, 26, 32), whereby larger value indicates improved IS. Only Legro et al. (Trial No.10 in Tables) used the insulin sensitivity index (28) (**Table 1**). Although the various parameters and units of measurement employed by each study made it difficult to compare IS status among different trials, changes in IS could be compared within a given trial (**Table 1**).

Only four trials among the included RCTs reported live birth rate (Trial No.2, 3, 6, 10) (24, 27, 28, 30), although all ten reported clinical pregnancy rate (**Table 2**). Therefore, the primary outcome of this review was pregnancy rate, and live birth rate was analyzed when it was reported.

Subjects could have been pregnant at any time during treatment, and therefore treatment was stopped when human chorionic gonadotropin level increased. Thus, treatment duration was defined as the longest treatment in a given trial. Treatment duration in the included trials was at least 3 months except for that in the report by Yarali et al. (32) (Trial No.8 in Tables) which was only up to 6 weeks. Among the 10 included RCTs, the longest treatment duration was up to around 6 months in five trials (Trial No.1, 3, 4, 6, 9 in Tables) (25, 27, 29–31). There were only two trials with relevantly large sample sizes (>100 subjects) and sufficiently long treatment duration (6 months or 24 weeks) (Trial No.4, 6) (27, 29) (**Table 1**).

Among the 10 included RCTs, one study did not describe the random sequence generation method (29), four trials did not describe the allocation concealment method (24, 29, 31, 32). Only three RCTs had a low risk of blinding participants and personnel (26, 32, 33), and Legro RS et al. could not use the blinding method due to lifestyle modification therapy (28). And because the outcome, clinical pregnancy rate, was not affected even without blinding method, all the ten studies had a low risk of outcome assessment. One study was rated high risk because of the high drop-out rate in the hCG group during follow-up (25) and three RCTs did not mention drop-out (26, 31, 33). All ten studies reported appropriate outcomes. Three studies were considered to have a high risk of other bias due to the small sample size (25, 30, 32), and one study was rated unclear risk because the sample size of the two groups was moderately different which were 33 in the metformin+CC group and 45 in the exenatide+CC group (31) (**Figure 2**).

DISCUSSION

Improvement of IS After Treatment Increased Pregnancy Rate in Infertile PCOS Women Regardless of Nonsurgical Intervention

Obesity is highly involved in the development of IR (34). To ensure consistency in our analyses, the included trials were analyzed by classifying body weight according to BMI at baseline.

Among RCTs that reported HOMA-IR, there were two in which mean BMI of groups was classified as overweight (Trial No.1, 2 in Tables) (24, 31), two where subjects were obese (Trial No.3, 4 in Tables) (29, 30), and two where subjects were severely obese (Trial No. 5, 6 in Tables) (27, 33). In all these trials, baseline HOMA-IR was not significantly different between groups in the same trial. In trials where subjects were overweight or obese, pregnancy rate increased in the group that had a significantly larger reduction of HOMA-IR after treatment (24, 29, 31). Furthermore, if there were no significant differences in HOMA-IR between groups, there were no significant differences in pregnancy rate between groups (30). Agrawal et al. (24) (Trial No.2 in Tables) and Ortega-González et al. (30) (Trial No.3 in Tables) also reported live birth rate and arrived at the same conclusion as that for pregnancy rate. Regarding two other trials where subjects were severely obese, the same observation was made by El Sharkwy and Sharaf El-Din (33) (Trial No.5 in Tables), but not by Legro et al. (27) (Trial No.6 in Tables). In the trial by *El* El Sharkwy and Sharaf El-Din (33) (Trial No.5 in Tables), the group with the addition of L-carnitine to the combination of metformin and CC achieved a further reduction of HOMA-IR and higher clinical pregnancy rate (33). In the trial by Legro et al. (27) (Trial No.6 in Tables), the three groups were administered metformin monotherapy, CC monotherapy, or combined metformin plus CC. Treatments were provided for up to 6 months, and although none achieved reduction of HOMA-IR and even significantly increased in CC group, HOMA-IR in the combined therapy group was significantly lower than in the CC group. However, the significant difference in HOMA-IR was because of the increase in CC monotherapy group. Regarding pregnancy rate, there was no significant difference between the CC and CC plus metformin groups. However, the pregnancy rate in the metformin group was significantly lower than in the other two groups (26). In the trial by Legro et al. (27) (Trial No.6 in Tables), no treatments (CC alone, metformin alone, or combined CC plus metformin) achieved reduction of IR, which suggested pregnancy rate was more closely associated with the ovulation-promoting effect of CC. The explanation for why metformin failed to achieve improvement of IS in the trial may have been the severe obesity of subjects. However, more evidence is necessary to confirm this theory.

Therefore, these trials suggested pregnancy rate was increased in the groups with a larger reduction of HOMA-IR.

For parameters that reflect IS (FPG/INS ratio or insulin sensitivity index), three trials recruited overweight PCOS women (Trial No.7, 8, 9 in Tables) (25, 26, 32), whereas one had severely obese subjects (Trial No.10 in Tables) (28). In overweight subjects, the pregnancy rate was significantly higher in the group where FPG/INS ratio was significantly higher compared with other groups in the same trial (26). Moreover, when IS did not differ significantly between groups, there was no difference in pregnancy rates (25, 32). In the trial by Legro et al. (Trial No.10 in Tables), subjects were severely obese and received lifestyle modification, oral contraceptive pills (OCPs), or combined therapy with lifestyle modification and OCPs for 16

TABLE 2 | The pre- and post- BMI, insulin sensitivity parameters in the RCTs and pregnancy rate and live birth rate in the included RCTs.

Trial No.	Study	Intervention Group	BMI before treatment	BMI after treatment	Sigificant change in BMI among groups	Parameter of IS	Parameter of IS before treatment	Parameter of IS after treatment[1]	Significant change of IS among groups[2]	Pregnancy rate	Significant changes of Pregnancy rate among groups[2]	Live birth rate	Significant change in Live birth rate among groups[2]
1	Wang et al. (31)	Metf+CC (N=33)	25.74±6.37	-		HOMA-IR	6.93±0.69	5.79±0.58	Y	9/33 (27.3%)	Y	-	-
		Exenatide+CC (N=45)	26.26±5.71	-			7.02±0.84	5.23±0.7*		24/45 (57.6%)		-	-
2	Agrawal et al. (24)	Metformin+CC (N=60)	27.38±3.92	25.45±3.22*	NS	HOMA-IR	2.83±1.29	1.62±0.59*	Y	20/60 (33.3%)	Y	16.60 (26.67%)	Y
		Metformin+MYO+CC (N=60)	27.71±3.60	25.77±3.48*			2.78±1.6	1.46±0.51*		38/60 (63.3%)		33/60 (55%)	
3	Ortega-González et al. (30)	Metformin (N=18)	34.1±1.6	32.9±1.7	Y	HOMA-IR	7.21±0.52	2.43±0.3	NS	3/18 (16.6%)	NS	2/18 (11.1%)	NS
		Pioglitazone (N=17)	32.2±1.0	34.0±1.2*			7.03±0.28	2.42±0.31		5/17 (29.4%)		2/17 (11.8%)	
4	Liu et al. (29)	24w Metf (N=80)	28.29±1.86	27.2±1.8*	Y	HOMA-IR	3.89±1.12	3.30±1.00	Y	15/80 (18.3%)	Y	-	-
		12w Exenatide switch to 12w Metf (N=78)	29.16±3.11	26.04±3.52*			4.21±1.89	2.92±1.31		34/78 (43.6%)		-	-
5	El Sharkwy and Sharaf El-Din (33)	Metf+CC (N=136)	34.4±3.4	32.5±3.2*	NS	HOMA-IR	2.83±0.7	2.22±0.34	Y	9/136 (6.6%)	Y	-	-
		Metf+CC+L-carnitine (N=138)	35.5±3.2	32.2±4.1*			2.72±0.38	1.8±0.43*		39/138 (28.2%)		-	-
6	Legro et al. (27)	Metformin (N=208)	35.6±8.5	-0.6±2.2	Y (Metf vs CC)	Change in HOMA-IR	5.6±8.9	+ (0.7±10.9)	Y (CC vs Metf +CC)	18/208 (8.7%)[a]	Y (Metf vs CC)	15/208 (7.2%)[a]	Y (Metf vs CC)
		CC (N=209)	36.0±8.9	+0.2±1.6	Y (Metf+CC vs CC)		5.2±5.3	+ (2.2±12.6)	NS (Other comparison	50/209 (23.9%)[b]	Y (Metf vs Metf+CC)	47/209 (22.5%)[b]	Y (Metf vs Metf+CC)
		Metformin+CC (N=209)	34.2±8.4	-0.5±1.4	NS (Metf+CC vs Metf)		5.6±10.2	- (0.1±11.8)		65/209 (31.1%)[b]	NS (CC vs Metf+CC)	56/209 (26.8)[b]	NS (CC vs Metf+CC)
7	Karimzadeh et al. (26)	Metformin (N=100)	28.80±3.18	28.45±2.8	NS	FPG/Insulin	4.67±0.9	6.07±1.4	Y	40/100 (40.0%)	Y	-	-
		Placebo (N=100)	29.49±4.75	29.29±4.8			5.03±1.3	5.05±1.3		11/100 (11.0%)		-	-
8	Yarali et al. (32)	Metformin (N=10)	28.6±4.0	28.0±3.4	NS	FPG/Insulin	0.10±0.03	0.12±0.07	NS	3/10 (30.0%)	NS	-	-
		Placebo (N=15)	29.6±4.8	29.8±4.9			0.09±0.04	0.10±0.06		1/15 (6.7%)		-	-
9	George et al. (25)	Metf+CC (N=30)	25.7±3.9	24.9±2.9*	Y	FPG/insulin	11.7±12.8	14.6±19.0	NS	5/30 (16.7%)	NS	-	-
		hMG (N=30)	25.9±2.9	25.9±2.9			4.8±2.7	11.6±6.5*		7/30 (23.3%)		-	-
10	Legro et al. (28)	Lifestyle modification (N=50)	35.1±4.6	-		Insulin sensitivity index	1.7 (1.0, 2.7)	+1.25 (1.09 –1.43)*	Y (Lifestyle modification vs OCPs	13/50 (26.0%)	NS	13/50 (26.0%)	NS
		OCPs (N=49)	35.1±4.2	-			2.0 (1.4, 2.6)	+0.89 (0.77–1.03)		7/49 (14.3%)		5/49 (10.2%)	(When Merge Life and combination group, Y)
		Combination (N=50)	35.5±4.4	-			1.9 (1.2, 3.2)	+1.21 (1.06 –1.39)*	Y (Combination vs OCPs)	13/50 (26.0%)		12/50 (24.0%)	

1, Mean±SD or Mean change (95% CI); 2, The significant change reported in the RCTs.

**, The change after treatment was significant compared with that in baseline.*

Y, The difference between groups was significant.

NS, Non-significant.

+, Value increased compared with baseline.

-, Value decreased compared with baseline.

a, b, the group with label a was significantly different from the group labeled b, and the same label means non-significant change between groups.

BMI, Body mass index; CC, clomiphene citreate; hMG, human menopausal gonadotropin; Metf, Metformin; MYO, myoinositol; OCPs, Oral contraceptive pills; HOMA-IR, homeostasis model assessment of insulin resistance; FPG, fasting plasma glucose; IS, Insulin sensitivity.

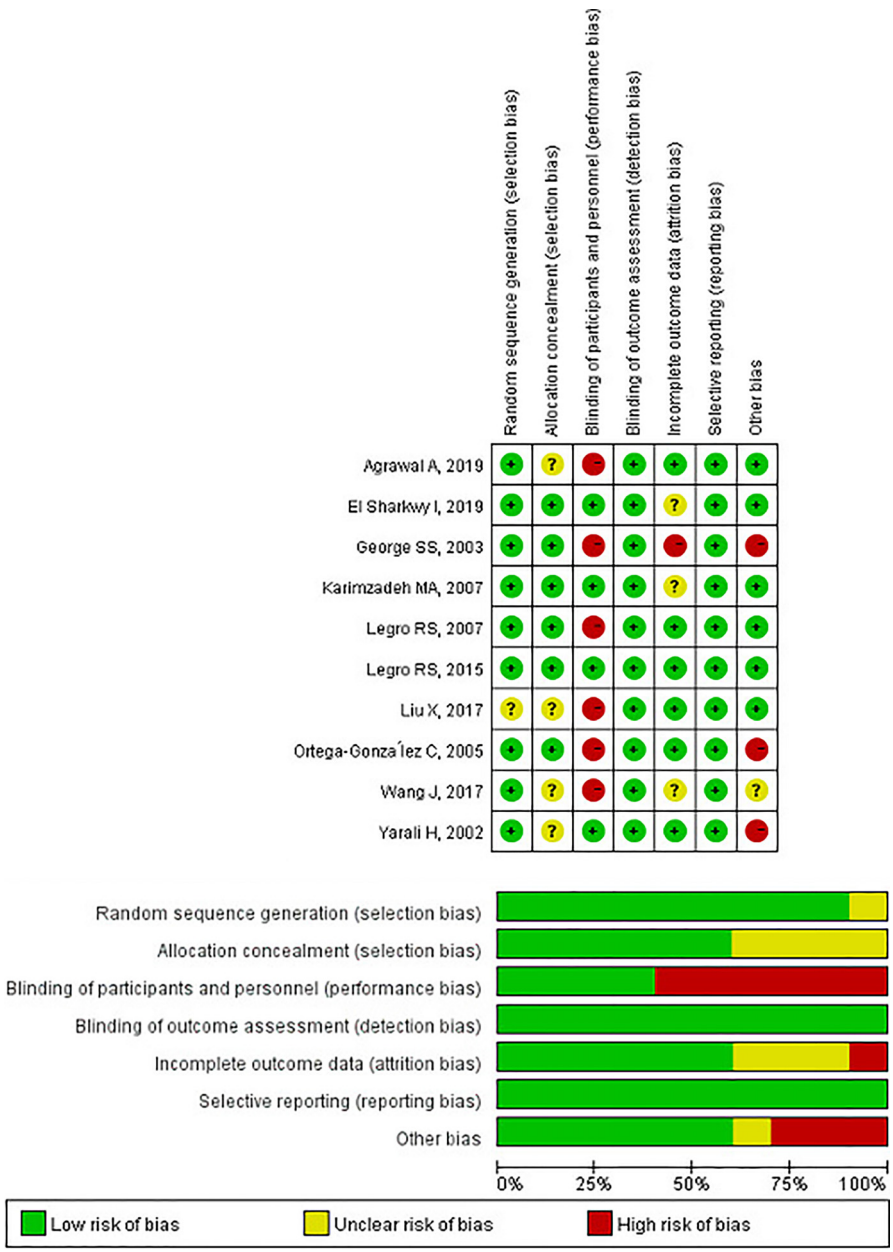

FIGURE 2 | The cochrane collaboration risk of bias evaluation for the included studies.

weeks (28). After treatment, insulin sensitivity index in the lifestyle modification and combined therapy groups was significantly increased and significantly higher than in the OCPs group. However, although pregnancy rate was not significantly different between the three groups, there was a trend toward a higher pregnancy rate in the lifestyle modification and combined therapy groups compared with the OCPs group. The authors believed this may have been because of the sample size. When they merged data from the lifestyle modification and combined therapy groups, they found the difference was significant between the merged and OCPs groups. This conclusion was also consistent with the previous discussion that pregnancy rate was increased in the groups with a larger improvement of IS. Live birth rate was reported by Legro et al. (28) (Trial No.10 in Tables), and the trend was the same as with pregnancy rate.

These results suggested pregnancy rate increased in the group with significant improvement of IS, even when the various parameters that reflect IS are considered.

All of these RCTs suggested achieving improvement of IS (including reduction of IR and increase of IS) after treatment is associated with increased pregnancy rate in infertile PCOS

women, regardless of nonsurgical intervention. Furthermore, the benefit of post-treatment improvement of IS was more pronounced in women without severe obesity. For severely obese individuals, effective improvement of IS was difficult to achieve, which may have been because of inflammation and other issues caused by severe obesity.

The Benefits of Improvement of IS was Inconsistent With the Reduction of BMI

Obesity is one of the most important risk factors for IR. However, it appears that improvement of IS after treatment was not consistent with the change of BMI in infertile PCOS women. BMI did not change after treatment compared with that before treatment and was not significantly different between groups in the trials by Agrawal et al. (24) (Trial No.2 in Tables), El Sharkwy and Sharaf El-Din (33) (Trial No.5 in Tables), and Karimzadeh et al. (26) (Trial No.7 in Tables). However, HOMA-IR or FPG/INS ratio was significantly changed, and pregnancy rate was different between groups in the three RCTs (24, 26, 33).

These data indicated that the reproductive benefit from the improvement of IS might be more important than that in the reduction of BMI.

PCOS Women Without Significant Insulin Resistance at Baseline Also Benefit From the Improvement of IS

To compare IS status in the different trials, significant IR was defined in this review as group mean HOMA-IR score >3.0, based on a large RCT where insulin resistance interventions after stroke were explored, and IR was identified as HOMA-IR score >3.0 (35). Although there are no globally standardized stratification criteria for the degree of IR, comparisons and analyses can aid in identifying evidence and trends regarding the effects of improvement of IS. HOMA-IR was reported in six studies, two of which included patients without IR (i.e., group mean HOMA-IR score <3.0) (Trial No.2, 5 in Tables). In the two studies, intervention groups achieved an improvement of IS, and the group with a larger reduction of HOMA-IR demonstrated a higher pregnancy rate.

This suggested improvement of IS can also increase pregnancy rate in PCOS women without IR. However, large, well-designed RCTs should be conducted to further validate this observation.

Benefits of Improvement of IS on Reproductive Outcomes Occurred With Various Nonsurgical Interventions but Were Not Dependent on Insulin Sensitizers

Two of the included RCTs evaluated the effect of metformin compared with placebo (26, 32). Treatment with metformin for 3 months in the trial by Karimzadeh et al. (26) (Trial No.7 in Tables) improved IS and increased pregnancy rate (26). This was consistent with the conclusion from a meta-analysis (13). However, 6-week metformin therapy in the trial by Yarali et al. (Trial No.8 in Tables) did not improve IS, and no difference was found between the metformin and placebo groups (32). This may have been because 6 weeks was too short to achieve significant improvement of IS.

Regarding combined therapy with metformin and CC, the meta-analysis did not find it was superior to CC monotherapy in PCOS infertile women, either in those with BMI<30.0 or BMI>30.0 (13). Similar observations were noted as part of this review. According to Legro et al. (27) (Trial No.6 in Tables), the combined therapy group demonstrated trends toward lower HOMA-IR and higher pregnancy rate compared with the CC monotherapy group, although they were not significant (28). This suggests the benefits of improvement of IS may be the secondary result of the potent effects of CC and other ovulation-inducing agents.

Other insulin sensitizers were also shown to effectively improve IS. Exenatide, a glucagon-like peptide-1 receptor agonist, was reported to reduce IR in type 2 diabetes patients (36). Compared with metformin, exenatide improved IS and pregnancy rate to a higher degree (29, 31). Wang et al. (31) (Trial No.1 in Tables) and Liu X et al. (29) (Trial No.4 in Tables) showed that exenatide improved IS and pregnancy rate, and the effect was superior to that of metformin, despite exenatide and metformin both being insulin-sensitizing agents. Pioglitazone, a typical insulin sensitizer, was proven superior to metformin at reducing IR (37, 38). However, other trials reported no difference between pioglitazone and metformin in improving IS in individuals with nonalcoholic fatty liver disease (39) or PCOS (40). Among the ten included RCTs, only one compared pioglitazone with metformin in PCOS infertile women, although no significant difference was found between the two groups (Trial No.3 in Tables) (30). However, this may have been because the sample size (N=47) was too small to demonstrate a statistical difference. More evidence is necessary to confirm the efficiency and safety of pioglitazone in PCOS women.

Adjuvant drugs, such as myoinositol (MYO) and L-carnitine, were not considered as traditional insulin-sensitizing agents but helped improve IS in PCOS women (Trial No.2, 5 in Tables) (24, 33). MYO is the precursor of inositol triphosphate, a second messenger that regulates thyroid-stimulating hormone, follicle-stimulating hormone, and insulin (41). A previous study indicated that MYO has an effect on improving IS (42) and estradiol levels in PCOS women (43). Previous studies suggested L-carnitine can reduce IR and BMI in PCOS women (44, 45). However, additional high-quality RCTs are required to confirm the effect and safety of these drugs in infertile PCOS women.

Only one RCT (with a small sample size (N=149) and short-term intervention) assessed the effect of lifestyle modification in infertile women with PCOS (Trial No.10 in Tables) (28). However, it was found that short-term lifestyle modification improved IS and increased pregnancy and live birth rates (28). Lifestyle modification may be among the most cost-efficient methods for significantly improving IS and reducing BMI, although clinical compliance is poor. All overweight or obese women should undergo lifestyle modification as a basic intervention.

To summarize, metformin and other related drugs, or lifestyle modification can increase pregnancy rate, but only if these interventions significantly improve IS. It appears exenatide had a better effect than metformin at increasing pregnancy rate and

improving IS. Metformin has been proven safe and is nonteratogenic, but additional evidence is necessary to confirm the safety of other insulin sensitizers (46).

Strengths and Limitation

By screening and analyzing existing RCTs, improvement of IR following nonsurgical interventions was strongly correlated with increased pregnancy rate in infertile women with PCOS. Although only two RCTs, Liu et al. (29) and Legro et al. (27) (Trial No.4,6), had relevantly large sample sizes and sufficient treatment durations, the conclusion and analysis were consistent in the studies. They were able to determine that pregnancy rate increased in the groups that achieved an improvement of IS. Therefore, nonsurgical therapeutic strategies that result in superior improvement of IS may aid infertile PCOS women to increase their possibility of a successful pregnancy.

This review had limitations. Evidence demonstrated that IUGR, SGA, and history of family diabetes contributed to PCOS patients with IR, and these factors might also affect the effectiveness of treatment on improving insulin sensitivity. However, few trials concerned about the factors so that there was insufficient information that can be obtained and discussed in the review. More evidence is required to fill the gap. Besides, most RCTs that explored the reproductive effects of nonsurgical interventions in PCOS women did not report changes in IR. Therefore, only some of these studies were included in this review, and some had small sample sizes. High-quality and large sample size RCTs are needed to confirm the results discussed in the systemic review.

CONCLUSION

Among the various nonsurgical interventions, the benefit of improvement of IS appeared to be superior in PCOS women without severe obesity. The benefit of improvement of IS appears to be more important than that in changes of BMI after treatment even the BMI is one of the most crucial factors for IS. And IS improvement also benefits PCOS women without IR at baseline. Metformin and other related drugs, and lifestyle modification, may also be capable of improving IS. It appears exenatide had a better effect than metformin at improving IS and increasing pregnancy rate. Although additional large, well-designed RCTs are necessary to confirm the benefits, the review emphasized the importance of achieving IS improvement in infertile PCOS women treatment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

YL provided the conception, searched the literature, participated in planning how to do the work, abstracting study details and writing the manuscript. JL participated in screening and abstracting study details, discussing and organized the main text and writing the manuscript. ZY provided the conception, set the inclusion/exclusion criteria, participated in discussing and organizing the main text and revised the manuscript. DL provided clinical advice during the whole work in the view of gynecology and obstetrics. JM screened the studies. NT planed the whole work and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Early Exposure to High-Sucrose Diet Leads to Deteriorated Ovarian Health

Giuliane Barros de Melo[1†], *Jéssica Furtado Soares*[1,2], *Thamyres Cristhina Lima Costa*[1,2], *Renata Ohana Alves Benevides*[1,2], *Caroline Castro Vale*[1,2], *Antonio Marcus de Andrade Paes*[1,2] *and Renato Simões Gaspar*[1,3*†]

[1] Laboratory of Experimental Physiology, Department of Physiological Sciences, Biological and Health Sciences Centre, Federal University of Maranhão, São Luís, Brazil, [2] Health Sciences Graduate Program, Biological and Health Sciences Centre, Federal University of Maranhão, São Luís, Brazil, [3] Institute for Cardiovascular and Metabolic Research, School of Biological Sciences, University of Reading, Reading, United Kingdom

Background: The metabolic syndrome (MetS) is correlated with disorders of the reproductive system, such as the polycystic ovary syndrome (PCOS). While consumption of a diet rich in carbohydrates is linked to the development of MetS, it is still unclear if this diet leads to ovarian dysfunction and PCOS.

Objectives: We investigated the influence of a high-sucrose diet (HSD) on the ovarian milieu of Wistar rats and studied the correlation between high consumption of sugary drinks and the prevalence of PCOS in women.

Methods: Wistar rats were given a standard laboratory diet (CTR, 10% sucrose, n = 8) or HSD (HSD, 25% sucrose, n = 8) from postnatal day 21 to 120. Animals were evaluated weekly to calculate food intake, feed efficiency and weight gain. Both onset of puberty and estrous cycle were monitored. Metabolic serum biochemistry, organ morphometry and ovarian histology were performed upon euthanasia. In parallel, a fixed-effects multiple linear regression analysis was performed using data from Brazilian states (459 state-year observations) to test the correlation between the consumption of sugar-sweetened beverages (surrogate for HSD intake) and the prevalence of PCOS (surrogate for ovarian dysfunction).

Results: HSD animals showed increased adipose tissue accumulation, hyperglycaemia and insulin resistance when compared to CTR. Interestingly HSD rats also entered puberty earlier than CTR. Moreover, ovaries from HSD animals had an increased number of atretic antral follicles and cystic follicles, which were correlated with the hypertrophy of periovarian adipocytes. Finally, there was a positive correlation between the intake of sugary drinks and prevalence of PCOS in women of reproductive age.

Conclusions: HSD ingestion leads to ovarian dysfunction in rats and could be correlated with PCOS in women, suggesting these alterations could lead to public health issues. Therefore, we reinforce the deleterious impact of HSD to the ovarian system and suggest that the reduction of added sugars intake could be beneficial to ovarian health.

Keywords: metabolic syndrome, diet rich in sucrose, hyperglycaemia, ovarian dysfunction, polycystic ovary syndrome

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Elisabetta Baldi,
University of Florence, Italy

Reviewed by:
Alessandro Conforti,
University of Naples Federico II, Italy
Gonzalo Cruz,
Universidad de Valparaiso, Chile

***Correspondence:**
Renato Simões Gaspar
renatosgaspar@gmail.com

[†]*These authors have contributed equally to this work*

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INTRODUCTION

Metabolic syndrome (MetS) is defined as the presence of at least three of the following: obesity, insulin resistance (IR), dyslipidaemia, hypertension and increased fasting glycaemia (1). MetS prevalence ranges from 10 to 40% worldwide, posing it as an alarming epidemic (2, 3). Of importance, comorbidities associated with MetS have also increased globally in children (4), being linked to higher consumption of sugar-enriched foods, which correspond to nearly 25% of the total energy intake in childhood (5, 6). Indeed, in developing countries, such as Brazil, over 60% of the population was reported to consume excessive amounts of added sugars (7). This same study showed that women consumed more sugar when compared to men. In parallel, it is estimated that 16.8% of men and 24.4% of women are obese in Brazil (8), suggesting that high prevalence of obesity is associated with excessive intake of dietary sugars. This is of particular importance, since MetS and obesity generate a high burden to both the individual and the health care system because of their association with increased risk of developing cardiovascular diseases, non-alcoholic fatty liver disease and reproductive disorders (9).

The link between MetS and reproductive disorders is multifaceted due to hormonal imbalances. For instance, it has been reported that hyperinsulinemia and IR are involved with hyperandrogenism, since insulin stimulates the production of ovarian androgens in synergy with luteinizing hormone (10). Both hyperandrogenism and IR are intrinsically related to ovarian disorders, such as the polycystic ovary syndrome (PCOS), which is characterized by the presence of at least two of the following: irregular menstrual cycles, hyperandrogenism and polycystic ovaries (11). In addition, female obesity is associated with increased concentrations of leptin in serum and follicular fluid and decreased serum levels of adiponectin, which consequently causes or worsens infertility (12). Since the prevalence of obesity among women of reproductive age has been increasing in recent years (13), more efforts are needed to understand the consequences of obesity on ovarian function.

In this regard, the effects of high-fat diet (HFD) consumption have been studied in rodents. HFD ingestion is reported to cause irregular estrous cycle, decreased fertility rates and impaired follicular development, while increasing ovarian inflammation and leptin levels (14). Moreover, it has been proposed that both the quantity and quality of total carbohydrate consumption are also related to infertility in women, reiterating that insulin and glucose metabolism are important factors in female fertility (15). Recently, it has been reported that among women attempting to conceive in Denmark and North America, consumption of diets high in glycaemic load, carbohydrate-to-fiber ratio, and added sugars were negatively associated with reduced fecundability (16). However, there are still very few basic, clinical or epidemiological studies exploring the association of high-sugar dietary intake and reproductive disorders in females.

Therefore, taking into account the high prevalence of obesity amongst women of reproductive age and the scarcity of studies in rodents and humans that explore the effects of high-sucrose diet (HSD) intake to ovarian function, we hypothesized that HSD ingestion leads to ovarian dysfunction. Of note, the HSD used in this study mimics dietary intake of sucrose found in Latin American countries (7). Our main objective was to determine if HSD could lead to ovarian dysfunction. To address this question, we have measured biochemical, morphometric and histological parameters in female rats after exposure to HSD from weaning to 120 days-old. In addition, we sought to correlate the ingestion of HSD with ovarian dysfunction in an ecologic study, to assess the epidemiologic relevance of data found in rodents. Our data shows that HSD intake was deleterious to the ovarian function of rats, while there was a positive correlation between high-sugar intake and prevalence of PCOS in women of reproductive age.

MATERIALS AND METHODS

Animals

Female Wistar rats (Rattus norvegicus) with 21 days of age and approximately 45 g were used, supplied by the Animal Facility House of the Federal University of Maranhão (UFMA) and kept in the Bioresources Unit of the Experimental Physiology Laboratory. Animals were kept at constant temperature (23 ± 2°C), 12h light/dark cycle and *ad libitum* access to water and food. All experimental procedures were performed in accordance with the rules of Brazilian Council for the Control of Animal Experimentation and approved by the Ethical Committee on Animal Use and Welfare at UFMA, ruling number 23115.007440/2016-71.

Preparation of High-Sucrose Diet

HSD was manufactured with powdered standard chow (40%), condensed milk (40%), refined sugar (8.5%) and filtered water *qsp*, as previously described (17) for a final mixture containing 65% total carbohydrates (25% sucrose), 12.3% proteins and 4.3% total lipids, totalling 3.48 kcal/g. The standard laboratory diet (Nuvital, Nuvilab, Brazil) contained 55.4% total carbohydrates (10% sucrose), 21% proteins and 5.2% total lipids, totalling 3.52 kcal/g.

Study Design

Weaned female Wistar rats were provided by the Animal Facility House of UFMA and randomized into two groups: standard laboratory diet (CTR, n = 8) and HSD (n = 8) groups. Dietary intervention started at postnatal day (pnd) 21 and continued until euthanasia, at pnd 120. Animals were weekly monitored for: weight gain, food intake and feed efficiency. In order to verify the onset of puberty, the vaginal opening was observed daily, starting at pnd 21. From the pnd 90 until euthanasia, the phases of the estrous cycle were determined as described below. During the last week of dietary intervention, animals were submitted to glucose and insulin tolerance tests. The Lee Index ((body weight (g)1/3 ÷ naso-anal length (cm)) × 1000) was used to assess body mass index (18) at pnd 120. Between pnd 118 and 122, rats in the oestrous phase were fasted for 8-h and anesthetized (70 mg/kg ketamine and 10 mg/kg xylazine, i.p.) for blood and tissue collection. Blood samples were processed for serum separation,

which were stored for later biochemical analyses. Morphometric analysis of organs such as liver, pancreas, ovaries and retroperitoneal and visceral fat depots were also performed. In addition, ovaries and attached fat pads (periovarian fat) were collected, fixed in 4% v/v paraformaldehyde and kept on 70% v/v ethanol for histological analysis as described previously (19).

Assessment of Vaginal Opening and Estrous Cyclicity

In order to determine the beginning of sexual maturity, the vaginal opening was observed daily, beginning at pnd 21. The estrous cycle was evaluated daily starting at pnd 90 until pnd 120, between 4:00 pm and 6:00 pm, according to Marcondes et al. (20) with minor adaptations. Briefly, vaginal smears were obtained using a plastic pipette containing 10 µL of saline solution (0.9% NaCl v/v). The collected material was placed on fresh glass slides and observed with an optical microscope with 10x and 40x lenses. The proportion of nucleated epithelial cells, cornified cells or leukocytes was used to determine the different stages of the estrous cycle (proestrus, oestrus, metestrus and diestrus) as described by Hubscher et al. (21).

Oral Glucose Tolerance (OGTT) and Insulin Tolerance (ITT) Tests

For the OGTT, animals were fasted for 8 hours and subsequently a glucose bolus (4 g/kg body weight) was administered by oral gavage. Blood was collected through puncture of the caudal vein and glycaemia assessed using a glucometer (Accu-Chek Active®, Roche, USA) at 0 (before glucose administration), 15, 30, 60 and 120 minutes after glucose administration. For ITT, fed animals received recombinant human insulin (2 IU/kg preheated to 36 °C). Glycaemia was measured at 0 (before insulin administration), 3, 5, 10, 15 and 20 minutes after insulin administration. The constant rate of glucose disappearance (kITT) was calculated from the regression slope obtained from the log-transformed glucose values between 3 and 20 minutes after insulin injection (22).

Biochemical Analyses

Upon euthanasia, blood aliquots were collected and centrifuged (1372 × *g* for 10 min) to obtain serum. Fasting triglyceride and cholesterol levels were detected in serum using spectrophotometric test kits following the manufacturer's guidelines (Labtest, Lagoa Nova, MG, Brasil). Fasting and fed blood glucose levels were defined as time 0 of GTT and ITT assays, respectively. The TyG index was used to infer insulin resistance using the formula: Ln [glucose (mg/dL) x triglycerides (mg/dL)/2] (23).

Ovarian Histology

Rats at the oestrous phase were euthanized and the right ovary and surrounding fat pads were removed, cleaned, fixed in 4% paraformaldehyde for 24 hours and kept in 70% ethanol. The right ovary was immersed in paraffin, cut into 6 µm slices and stained with hematoxylin-eosin (HE). Since an oocyte has a diameter of 20 to 30 µm, we analysed one section every six cuts, therefore ensuring a 36 µm distance to prevent us from double-counting the same follicles. On average, nine slices in three sections were analysed per ovary. Full details on the classification of follicles, definition of atresia and follicular cysts can be found in previous reports of our lab (19, 24). Representative images of primordial, primary, secondary, antral and cystic follicles are presented in **Supplementary Figure 1**. A single-blinded competent researcher performed the histological analyses and the follicle count was divided by the number of sections analysed to obtain an average number of follicles per section.

Periovarian Adipose Tissue Histology

The fat pads surrounding the right ovary were cut into 6 µm sections and stained with HE. Histology of adipocytes was performed as described previously (25). At least 100 adipocytes were analysed per animal. Representative photos were taken using an AxionVision (AxioVs40x64 V 4.9.1.0, Carl Zeiss GmBH Microscopy) and frequency distribution calculated according to adipocyte area.

Ecologic Study

Data were collected from the Global Health Data Exchange, the Institute for Health Metrics and Evaluation, the Brazilian Institute of Geography and Statistics (IBGE) and the Brazilian Ministry of Health. Data were acquired for the 26 states and the Federal District from 2000 to 2017, totalling 459 state-year observations. The dependent variable was the prevalence of PCOS per 100,000 habitants, while the main independent variable was the summary exposure value (SEV) for diet high in sugar-sweetened beverages, as a surrogate for high sucrose intake. Control variables consisted of gross domestic product (GDP) in Brazilian Reais (R$) per capita, SEV for high LDL-cholesterol and SEV for high fasting glycaemia. Prevalence and SEV data were collected for women at reproductive age (15 to 49 years) and were defined and calculated by the global burden of diseases (GBD) study group (26). Detailed information with regards to data sources and definition of variables can be found in **Supplementary Table 1** and **Supplementary Table 2**. In addition, state- and year- fixed effects models were used to control for unobserved state-invariant and time-invariant heterogeneity, while increasing the robustness of the model.

Therefore, the fixed effects model used can be written as:

$$PCOS_{lt} = \alpha + \beta_1 HighSugar_{lt} + \beta_2 LDL_{lt} + \beta_3 HG_{lt} + \beta_4 GDP_{lt} + L_t + T_l + \epsilon_{lt}$$

Where $PCOS_{lt}$ is the prevalence of PCOS in state l year t, $HighSugar_{lt}$ is the SEV of diet high in sugar-sweetened beverages in state l year t, LDL_{lt} is the SEV of high LDL-cholesterol in state l year t, HG_{lt} is the SEV of hyperglycaemia in state l year t, GDP_{lt} is the GDP per capita in state l year t, L is state fixed effect and T is time fixed effect. All variables were log-transformed to facilitate interpretation of estimates and ensure normal distribution.

A lag analysis was performed to test if the consumption of sugar-sweetened beverages in a given state was associated with the prevalence of PCOS in that same state 10 years later. This was performed using prevalence of PCOS in women aged 35-39 years and 40-44 years. The 10-year lag and age brackets were used due to restrictions on data collected from the GBD database.

The lagged fixed effects model can be written as:

$$PCOS_{lt} = \alpha + \beta_1 HighSugar_{l-10t} + \beta_2 LDL_{l-10t} + \beta_3 HG_{l-10t} + \beta_4 GDP_{lt} + L_t + T_l + \epsilon_{lt}$$

Where l–10t denotes the SEV of risk factors for state t in year l minus 10.

Statistical Analysis

The results are expressed as mean ± SEM for rodent data and coefficient ± 95% confidence interval (CI) for the ecologic study. For the rodent study data were analysed using unpaired Student's t test or two-way ANOVA, with Tukey's post-test and n=6-8, except for ITT (n=5) and the histology of periovarian fat (n=4). The sample size for the animal study was calculated using the free software for sample calculation: G*Power 3.1

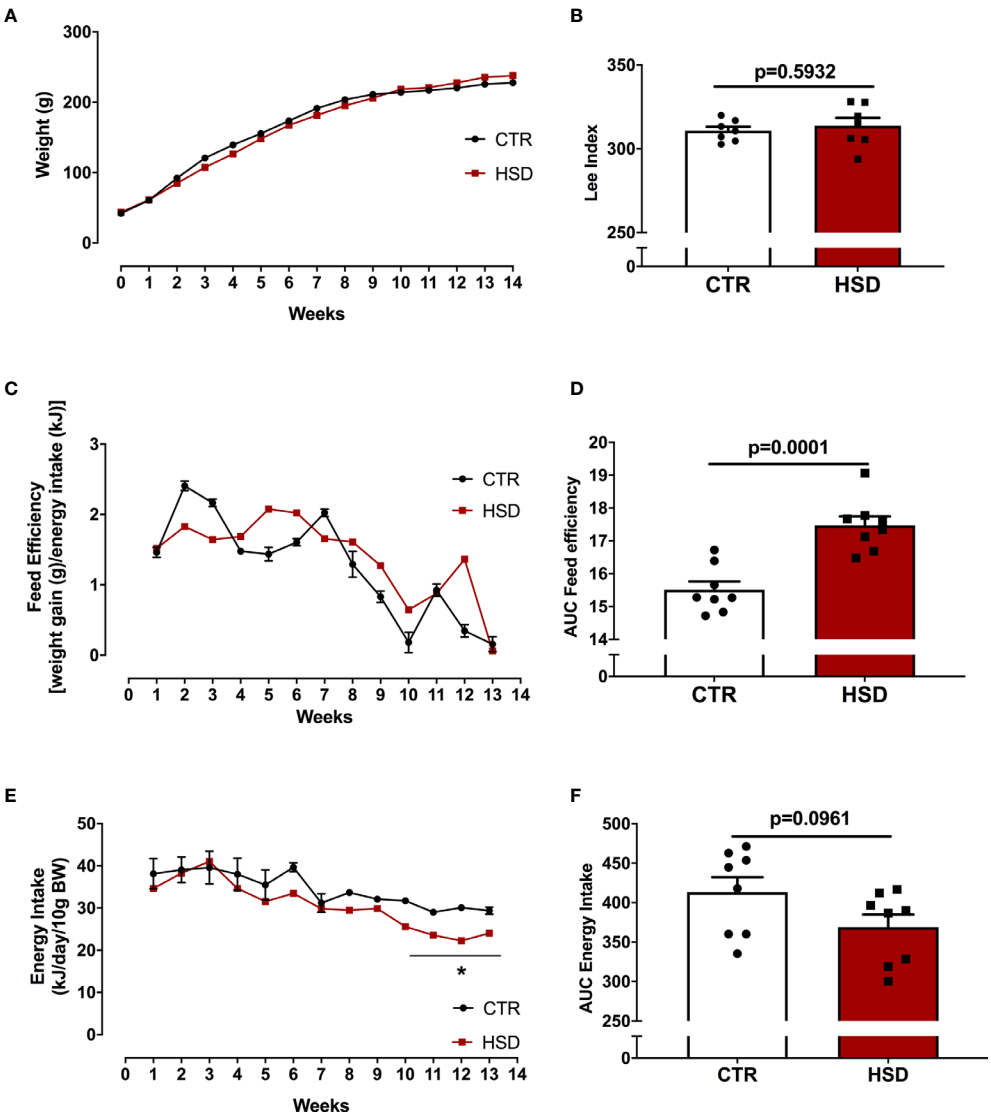

FIGURE 1 | HSD ingestion results in no weight gain in spite of higher feed efficiency. **(A)** All animals were weighed weekly from pnd 21 (week 0) until pnd 120 (week 14). **(B)** Lee Index was calculated as described in Materials and Methods. **(C)** Feed efficiency was also monitored throughout dietary intervention. **(D)** Area under the curve (AUC) of feed efficiency. **(E)** Energy intake calculated as described in *Materials and Methods*. **(F)** AUC of energy intake. n=8 animals in each group. Graphs express mean ± SEM and bar graphs show individual values. *p < 0.05 *vs* CTR, assessed by two-way ANOVA with Tukey's post-test. P-value presented for bar graphs and calculated through unpaired Student t-test.

(Heinrich-Heine University Düsseldorf, Düsseldorf (NRW), Germany). The sample size of eight animals per group was found by inserting in the software sample power of 80% ($1 - \beta = 0{\cdot}8$), significant level of 5% ($\alpha = 0{\cdot}05$), and parameters from previous studies on the effects of MSG on the number of ovarian cysts, antral follicles, and adipocytes area (19, 24). For the ecologic study, least squares multiple linear regression models were run and n=459 state-year observations. The differences were significant when p <0.05. The analyses were performed using the Graphpad Prism 7.0 and 9.0 software (GraphPad, San Diego, USA).

RESULTS

Early Exposure of HSD to Female Rats Induced Fat Accumulation With No Increase in Body Weight

To assess the impacts of HSD on the development of obesity, animals received HSD or CTR diet for a period of 14 weeks, from pnd 21 to 120. Body weight was assessed twice a week and fat pads were isolated at the end of the dietary intervention. As shown in **Figure 1**, HSD did not increase body weight or Lee's Index over time (**Figures 1A, B**), despite a 17% increase in feed efficiency when compared to CTR (**Figures 1C, D**). There was no difference in energy intake (**Figures 1E, F**). Although there were no changes in body weight, HSD animals showed heavier retroperitoneal, visceral and periovarian fat depots, as shown in **Table 1**. Interestingly, HSD rats displayed lighter kidneys than did CTR rats, which should be addressed in the future. Together, these results suggest that HSD ingestion since weaning led to increased fat accumulation, in spite of no detectable changes in body weight and body mass index.

HSD Alters Glucose Metabolism and Causes Insulin Resistance in Female Rats

In order to determine the effects of HSD on the glucose-insulin axis of female rats, blood glucose (fasting and fed), cholesterol and triglycerides were measured. In addition, OGTT and ITT were performed to assess glucose tolerance and insulin sensitivity, respectively. As shown in **Figure 2**, the HSD group presented increased levels of both fasting (CTR 86.13 ± 5.16 *vs* HSD 102.30 ± 4.43, $p = 0.03$) and fed (CTR 106.0 ± 2.26 *vs* HSD 121.40 ± 2.30, $p = 0.0003$) blood glucose when compared to CTR rats (**Figures 2A, B**), however, there were no differences in cholesterol or triglyceride levels across groups (**Figures 2C, D**). HSD rats displayed increased TyG Index (CTR 7.36 ± 0.18 *vs* HSD 8.05 ± 0.18, $p = 0.01$), suggesting these animals presented IR (**Figure 2E**) in spite of similar tolerance to both glucose (**Figures 2F, G**) and insulin (**Figures 2H, I**) between groups. Therefore, the ingestion of HSD by female rats caused a mild metabolic dysfunction, characterized by increased fat accumulation, hyperglycaemia and IR. This could potentially damage the reproductive function of these animals.

TABLE 1 | Morphometric and hepatic lipid profile of CTR and HSD rats.

	CTR (mean ± SEM)	HSD (mean ± SEM)
Morphometric features		
Ovary (g/100 g)	0.098 ± 0.006	0.097 ± 0.008
Uterus (g/100 g)	0.229 ± 0.020	0.195 ± 0.019
Liver (g/100 g)	3.109 ± 0.074	3.064 ± 0.166
Visceral fat pads (g/100 g)	2.669 ± 0.265	4.088 ± 0.413*
Retroperitoneal fat pads (g/100 g)	1.30 ± 0.077	1.672 ± 0.110*
Periovarian fat pads (g/100 g)	353.30 ± 56.650	565.20 ± 46.320*
Heart (g/100 g)	0.296 ± 0.006	0.299 ± 0.005
Kidneys (g/100 g)	0.840 ± 0.032	0.719 ± 0.034*
Pancreas (g/100 g)	0.322 ± 0.025	0.340 ± 0.031
Hepatic lipid profile		
Triglycerides (mg/g Liver)	54.380 ± 2.437	54.940 ± 2.798
Total Cholesterol (mg/g Liver)	77.210 ± 4.478	82.280 ± 2.848

*Results expressed as mean ± S.E.M. *p < 0.05 vs CTR compared with unpaired Student t-test. n=8 for CTR and HSD.*

HSD Exposure Anticipates Puberty and Impairs Ovarian Follicular Development in Female Rats

Taking into account the influence of metabolic changes on the reproductive system, vaginal opening (indicative of the beginning of sexual maturity) and oestrous cycle were monitored (**Figure 3**). As shown in **Figure 3A**, the HSD group entered puberty 2.5 days earlier than CTR animals (CTR 42.75 ± 0.31 *vs* HSD 40.25 ± 0.16, $p < 0.0001$), suggesting that HSD ingestion led to early puberty. In spite of earlier vaginal opening, HSD rats ovulated and cycled normally, similar to CTR animals (**Figures 3B–F**).

The ovarian histology was analysed to assess if the mild metabolic dysfunction observed in HSD rats were correlated with impairments in the development of ovarian follicles (**Figure 4**). Representative micrographs are presented in **Figures 4A, B**. There were no differences on the number of ovarian follicles between groups (**Figure 4C**). However, the HSD group showed a greater number of atretic antral follicles (**Figure 4D**) (CTR 0.19 ± 0.06 *vs* HSD 1.22 ± 0.28, $p = 0.002$) and cystic follicles (**Figure 4E**) (CTR 0.28 ± 0.11 *vs* HSD 1.04 ± 0.31, $p = 0.04$) when compared to ovaries from CTR rats. The number of corpora lutea was also unaltered in HSD rats when compared to those of CTR animals (**Figure 4F**). In spite of unaltered ovulation, these data suggest ovarian dysfunction similar to a PCOS-like phenotype in HSD rats, considering the 1) earlier vaginal opening, 2) increased atretic antral follicles and 3) increased number of ovarian cysts. These ovarian dysfunctions were associated with increased fat deposition and impaired glucose-insulin axis.

PCOS-Like Features of HSD Rats Are Associated With Hypertrophy of Periovarian Adipocytes

Previous studies of our group have shown that the periovarian fat pad is correlated with PCOS-like ovarian dysfunctions (19, 24), such as the ones presented by HSD rats. Therefore, we sought to

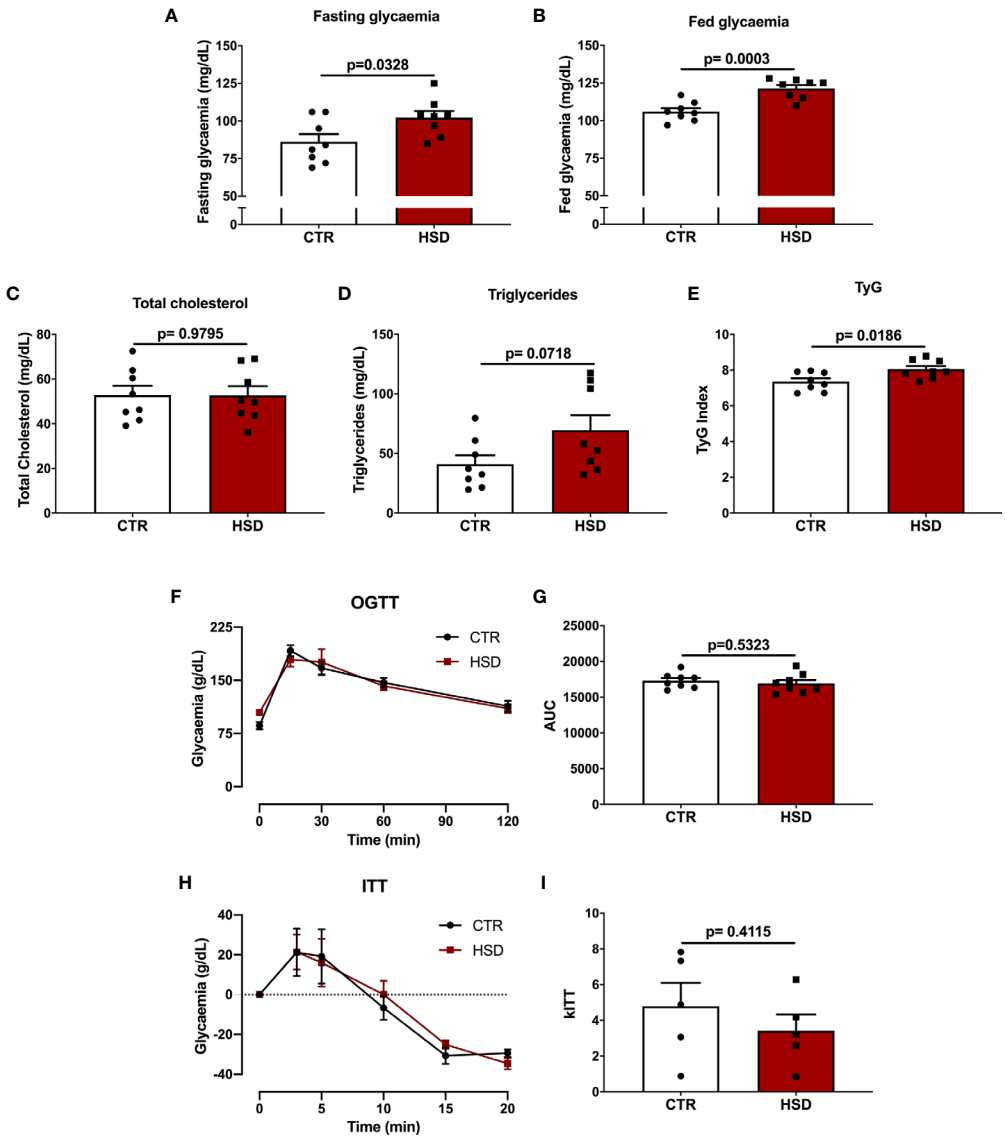

FIGURE 2 | High-sucrose diet (HSD) alters glucose metabolism and causes insulin resistance in female rats. **(A)** Fasting glycaemia was calculated as time 0 of OGTT. **(B)** Fed glycaemia was calculated as time 0 of ITT. **(C)** Total cholesterol and **(D)** triglycerides were measured in fasting rats upon euthanasia (pnd 120). **(E)** TyG Index was calculated as described in Materials and Methods. **(F)** Glucose tolerance test (OGTT) was assessed in 8-hour fasted rats at times 0, 15, 30, 60 and 120 min. **(G)** Area under the curve (AUC) of OGTT. **(H)** Insulin tolerance test (ITT) assessed in fed animals at times 0, 3, 5, 10, 15 and 20 min. **(I)** kITT calculated as described in Materials and Methods. n=5-8 animals in each group. Graphs express mean ± SEM and bar graphs show individual values. P-values are shown in bar graphs and were calculated using unpaired Student t-test.

analyse if a similar phenotype was present in HSD animals (**Figure 5**). According to the histological analysis of periovarian fat, it was observed that HSD contributed to a larger average area of adipocytes, increasing 59% when compared to CTR (CTR 353.30 ± 56.65 *vs* HSD 565.20 ± 46.32 μm^2, $p = 0.275$) (**Figure 5C**). This is reinforced by the frequency distribution graph showing the periovarian fat of HSD animals presented a higher frequency of larger adipocytes (**Figure 5D**). Collectively, these data indicate that the mild metabolic dysfunction caused by HSD was associated with PCOS-like features, which were also related to the hypertrophy of periovarian fat.

Consumption of Sugar-Sweetened Beverages Is Correlated With Prevalence of PCOS

To further explore the interaction between consumption of HSD and ovarian function, we performed a fixed-effect regression model using data from Brazilian states and tested the correlation between diet high in sugar-sweetened beverages

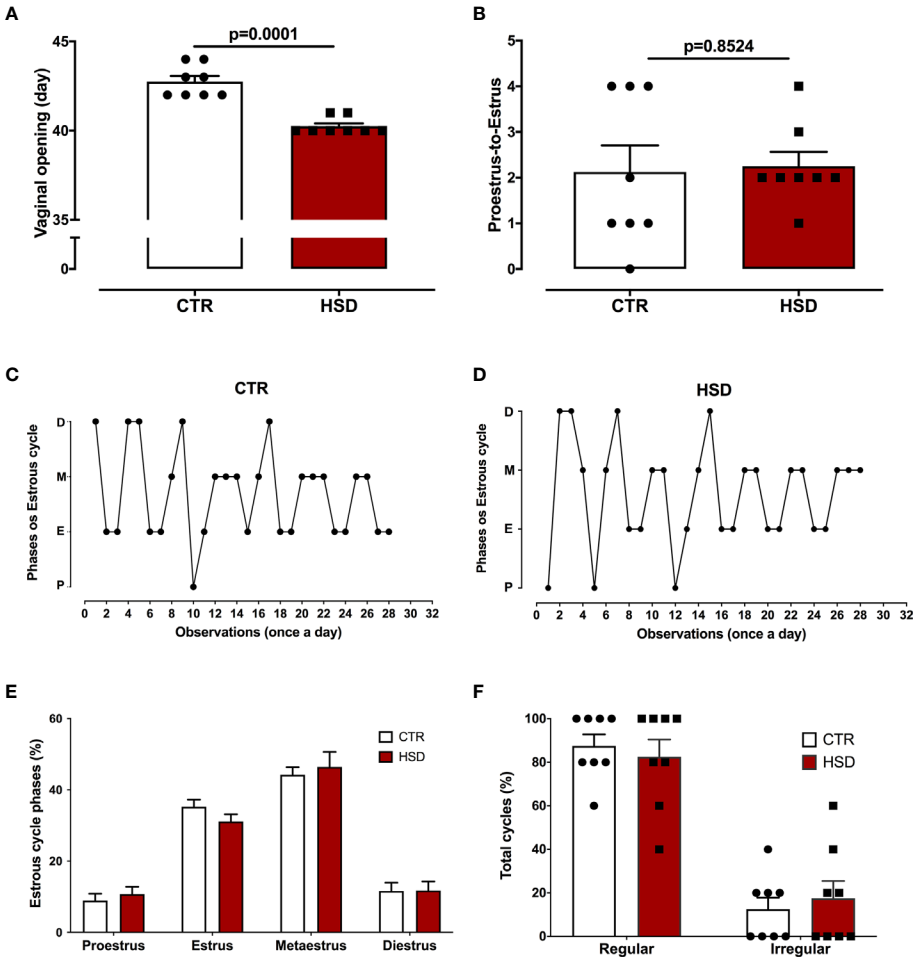

FIGURE 3 | High-sucrose diet (HSD) exposure anticipates puberty. **(A)** Vaginal opening assessed daily, beginning at pnd 21. **(B)** Proestrus-to-estrus change as a surrogate for ovulation. **(C)** Oestrous cycles from a representative CTR rat, where proestrus (P), oestrus **(E)**, metestrus (M) and diestrus **(D)**. **(D)** Oestrous cycle from a representative HSD rat. **(E)** Percentage of each oestrous phase. **(F)** Percentage of regular or irregular cycles, as defined in Materials and Methods. n=8 animals in each group. Graphs express mean ± SEM and bar graphs show individual values. P-values are shown in bar graphs when significant ($p < 0.05$) and were calculated using unpaired Student t-test in **(A)** and two-way ANOVA in **(D, E)**.

(surrogate for HSD intake) and the prevalence of PCOS (surrogate for ovarian dysfunction) in women at reproductive age. Controls for income and other risk factors, namely hyperglycaemia and dyslipidaemia were used, since similar alterations were seen in HSD rats. The prevalence of PCOS in women at reproductive age was positively correlated with the consumption of sugar-sweetened beverages (**Figure 6A**) (0.011, 0.006 – 0.022 95%CI), while there were no associations for other risk factors. Interestingly, there was a positive correlation between the consumption of sugar-sweetened beverages in women aged 25-29 and the prevalence of PCOS in women after a 10-year lag period, aged 35-39 (**Figure 6B**) (0.03, 0.02 – 0.04). The magnitude of this association was three times higher than that of the non-lagged analysis. Moreover, the lag effect was lost when an older population was used (**Figure 6C**). There were no associations for other risk factors and the prevalence of PCOS in the lagged analyses. Altogether, these data suggest a positive association between the consumption of sugar-sweetened beverages and deteriorated ovarian function in the Brazilian population. Although unable to infer causality, this reinforces the need to explore the effects of HSD consumption in women.

DISCUSSION

Here we hypothesized that consumption of HSD, at a proportion similar to that consumed in Latin American countries (7), would lead to ovarian dysfunction in rats. This issue was addressed in two ways: first by giving HSD to female rats and measuring the ovarian repercussions of such intervention, and second by analysing the correlation between consumption of sugar-sweetened beverages and prevalence of PCOS in a Latin American country, i.e. Brazil. We show that HSD led to mild

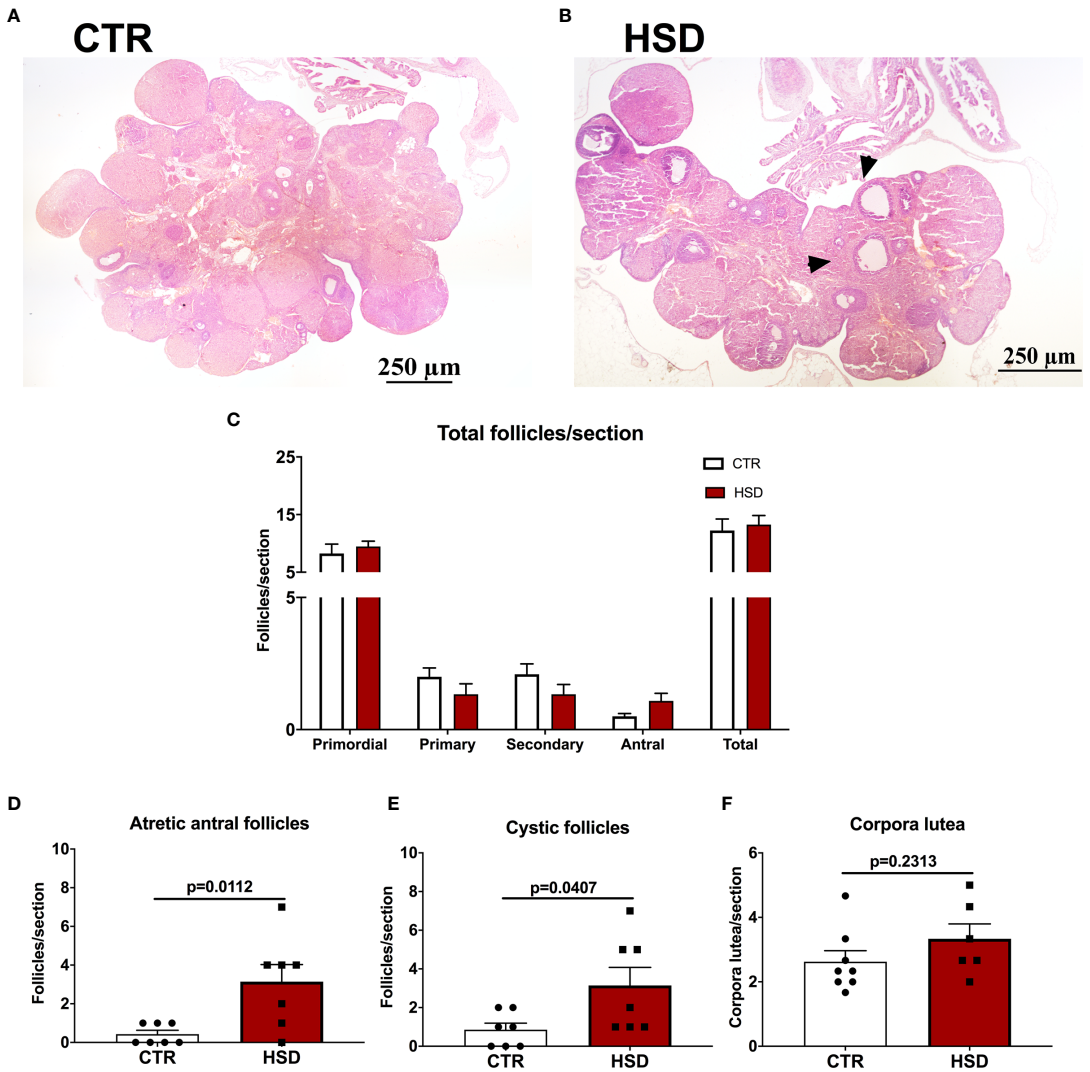

FIGURE 4 | High-sucrose diet (HSD) ingestion causes ovarian dysfunction in female rats. Ovaries were analysed and follicles classified according to *Materials and Methods*. **(A)** Representative micrograph of an ovary from a CTR rat. **(B)** Representative micrograph of an ovary from an HSD rat, depicting cystic follicles (arrowheads). **(C)** Number of primordial, primary, secondary, antral and sum of all follicles (total) per section. **(D)** Number of atretic antral follicles per section. **(E)** Number of cystic follicles per section. n=7-8 animals in each group. **(F)** Number of corpora lutea per section. Graphs express mean ± SEM and bar graphs show individual values. P-values are shown in bar graphs when significant ($p < 0.05$) and were calculated using two-way ANOVA in **(C)** and unpaired Student t-test in **(D–F)**.

metabolic dysfunctions in female rats, while there were earlier vaginal opening, increased presence of ovarian cysts and hypertrophy of periovarian adipocytes, suggesting PCOS-like features. However, we could not ascertain all features of this syndrome in our experimental model. In the ecologic study, there was a positive correlation between the prevalence of PCOS (surrogate for ovarian dysfunction) and consumption of diet high in sugar-sweetened beverages (a surrogate for HSD intake). Altogether, the consumption of HSD resulted in deleterious impacts to the ovarian milieu, which could be associated with an increased prevalence of ovarian dysfunction in the population.

Despite not influencing body weight gain, HSD rats displayed greater food efficiency compared to CTR. These findings are in agreement with previous studies that demonstrated the association between HSD and rises in circulating leptin levels, a hormone responsible for satiety and, consequently, decreased food intake (27). Moreover, fasting and postprandial hyperglycemia, coupled with IR found in the HSD group, may have contributed to the reduction of energy consumption, given that higher glucose levels lead to the production of malonyl-CoA. This peptide, in turn, negatively modulates the energy balance through the anorexigenic orexigenic neuropeptide system (28), a mechanism activated by high-sucrose but not high-fat diets (29).

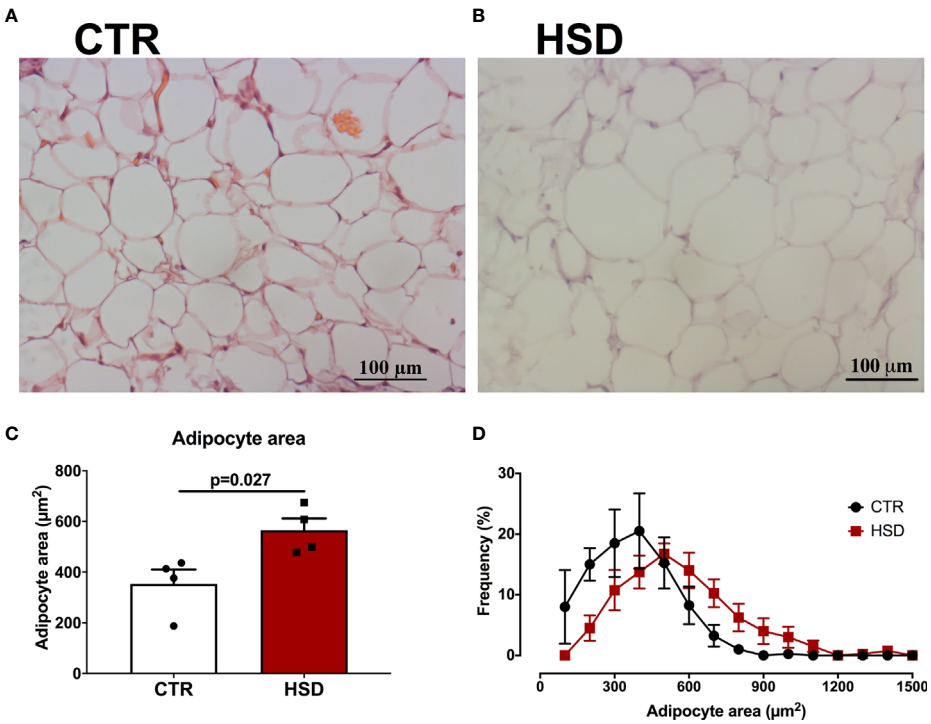

FIGURE 5 | HSD rats present hypertrophy of periovarian adipocytes. Periovarian fat pads were analysed as described in Materials and Methods. **(A)** Representative micrograph of periovarian fat from a CTR rat. **(B)** Representative micrograph of periovarian fat from an HSD rat. **(C)** Mean adipocyte area of periovarian fat. **(D)** Frequency distribution per adipocyte area. n=4 animals in each group. Graphs express mean ± SEM and bar graphs show individual values. P-values are shown in bar graphs when significant ($p < 0.05$) and were calculated using Student t-test in **(C)**.

Notwithstanding, adipose tissue accumulation in the HSD group was increased in all the assessed fat pads, which could be a consequence of excess circulating levels of sucrose-derived fructose. Indeed, fructose is massively up-taken by adipocytes, where it is metabolised into triglycerides and contributes to adipocyte hypertrophy (30). Collectively, this set of data demonstrates that post-weaning exposure of female rats to HSD leads to consistent metabolic disturbances, although at a lesser extent than those observed in age-matched male rats (31).

In addition to metabolic changes, our study showed reproductive disorders in HSD animals. The first striking observation was that HSD rats entered puberty earlier than CTR, despite being exposed to the diet for a short period of time, which could be due to increased periovarian adipose tissue accumulation. In immature rats, the periovarian fat has been shown to synthesize oestrogen at a greater capacity than the ovary, serving as a primary source of this hormone in the prepubertal period, thus leading to the onset of puberty (32). Notwithstanding, Connor et al. (33) suggested that the mechanism by which fat accumulation influences early puberty is mainly due to excessive energy supply. Although we have not assessed periovarian fat pad on HSD rats at the time of puberty onset, a previous report from our lab showed that short-term exposure to HSD significantly augmented the periepididymal fat accumulation in age-matched male mice (34). In women, it has been shown that precocious puberty is associated with MetS risk factors, such as IR and excessive body fat (35). Moreover, the consumption of sugary drinks has been associated with early menarche (36). These data collectively show that early introduction of HSD hastens feminine sexual maturation.

In line with early puberty onset, HSD rats displayed increased cystic and atretic antral ovarian follicles. Likewise, Nino et al. (37) found that a high-carbohydrate diet was able to increase the number of atretic follicles in rats. Furthermore, Roberts et al. (38) have shown that animals fed a high-fat, high-sugar diet had higher frequency of cystic follicles when compared to control animals. It is yet unclear how the ingestion of HSD or other types of hypercaloric diets lead to ovarian dysfunction, however feasible underlying mechanisms include: hyperinsulinemia culminating in higher production of ovarian androgens in synergy with the luteinizing hormone (10), increased expression of anti-mullerian hormone in granulosa cells (19) and increased production of reactive oxygen species (39). The identification of underlying mechanisms linking increased dietary intake of sugars and ovarian dysfunction will lead to novel targets to treat these conditions.

Clinically, the main endocrinopathy linked to impaired follicular health is PCOS, a condition that affects 5% to 15% of women at reproductive age (40). Previous studies conducted by our lab have shown that exposure to monosodium L-glutamate

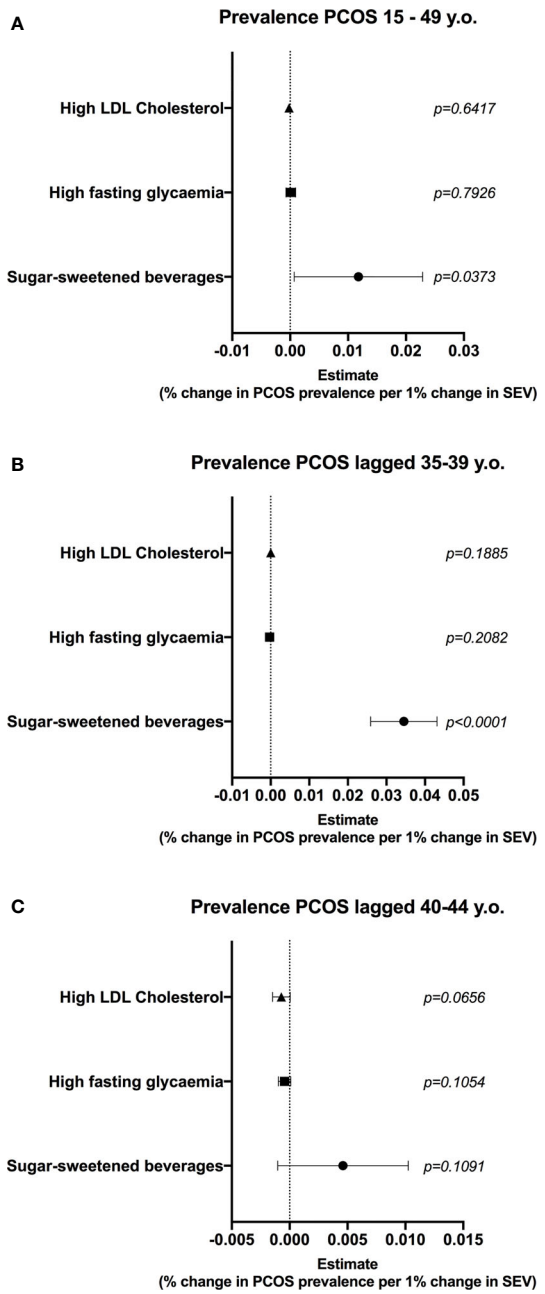

FIGURE 6 | Consumption of diet high in sugar-sweetened beverages is correlated with prevalence of PCOS in women at reproductive age. A multiple linear regression model (459 state-year observations) was used to test the correlation between diet high in sugar-sweetened beverages and prevalence of polycystic ovary syndrome (PCOS). State and year fixed effects were included in the model. Gross domestic product (GDP) per capita was used to control for income. **(A)** Correlation between summary exposure value (SEV) of risk factors and prevalence of PCOS in women at reproductive age. **(B)** Correlation between SEV of risk factors and prevalence of PCOS 10 years later, in women aged 35-39. **(C)** Correlation between SEV of risk factors and prevalence of PCOS 10 years later, in women aged 40-45. Graphs express mean ± 95% confidence interval and *p* is shown for each SEV.

(MSG) was able to induce a PCOS-like phenotype (19). This was thought to be due to hyperinsulinemia found in MSG rats, since ovaries remain sensitive to insulin (41). The ovarian dysfunction phenotype observed in HSD rats, as well as in other models using high-carbohydrate diets may help identify risk factors and underlying mechanisms for PCOS in women.

To translate findings in rodents to a broader context, we have tested if there was a correlation between the consumption of sugar-sweetened beverages (as a proxy for HSD) and the prevalence of PCOS (proxy for ovarian dysfunction) in the Brazilian population. It was evident that there was a positive correlation between consumption of sugar-sweetened beverages and prevalence of PCOS. Interestingly, there was a lag effect between the consumption of sugar-sweetened beverages at 25-29 years and the prevalence of PCOS at 35-39 years that was lost when an older bracket of the population was analysed. This is in line with the programming effect of HSD consumption at younger ages, such as demonstrated for HSD rats. In parallel, it has been reported that adolescents with PCOS tend to consume a large amount of foods rich in sugar (42), while, in developing countries, such as Brazil, over 60% of the population was reported to consume excessive amounts of added sugars (7). Indeed, a relationship was identified between women with PCOS and the consumption of foods with a high glycemic index (43). These results reinforce the positive correlation between the consumption of sugary drinks and the prevalence of PCOS found in our study.

Some limitations of the current study need to be addressed in the future. First, while we identified early puberty in HSD rats, we were unable to explore mechanisms that led to this event. A feasible hypothesis is that HSD could cause the hypertrophy of periovarian fat in prepubertal rats, as discussed above. Future studies should assess if periovarian fat pads are altered in prepubertal rodents exposed to HSD. Second, further studies are needed to extend our observations *in vivo* using a larger sample size. Third, we were unable to ascertain if HSD rats developed PCOS due to lack of hormonal levels and we suggest this to be explored in the future. Finally, the ecologic study does not allow causal inference between the consumption of sugary drinks and the prevalence of PCOS. While other factors could influence the prevalence of PCOS, controls for income and fixed effects models were employed to account for some externalities that could influence the prevalence of PCOS. Nonetheless, it is imperative to address in the future if HSD can lead to PCOS in women.

In conclusion, data herein presented show that early exposure to HSD causes metabolic and ovarian dysfunctions in rats, which could have implications to the prevalence of PCOS in women. More studies are needed to address the link between HSD consumption and PCOS development. Moreover, our study suggests that early introduction of added sugars in the diet, particularly following breastfeeding cessation, is detrimental to female reproductive health. Therefore, sucrose-limiting policies could potentially hinder the deleterious impacts described above.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval were not required for the study on human participants, because data were collected from a public database, in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The animal study was reviewed and approved by the Ethical Committee on Animal Use and Welfare at UFMA, ruling number 23115.007440/2016-71.

AUTHOR CONTRIBUTIONS

GM, TC, RB, and CV have performed experiments and analysed data. JS has analyzed data and written the manuscript. AP has supervised experiments, conceived the study, analyzed data, and reviewed the manuscript. RG has performed experiments, conceived the study, analyzed data, and written the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Three-Dimensional Genome Interactions Identify Potential Adipocyte Metabolism-Associated Gene *STON1* and Immune-Correlated Gene *FSHR* at the rs13405728 Locus in Polycystic Ovary Syndrome

Can-hui Cao[1,2], Ye Wei[2], Rang Liu[1], Xin-ran Lin[1], Jia-qi Luo[1], Qiu-ju Zhang[1], Shou-ren Lin[1], Lan Geng[1], Si-kang Ye[3], Yu Shi[4] and Xi Xia[1]**

[1] Center for Reproductive Medicine, Department of Obstetrics and Gynecology, Peking University Shenzhen Hospital, Shenzhen Peking University-The Hong Kong University of Science and Technology Medical Center, Shenzhen, China, [2] Department of Gynecologic Oncology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [3] Department of Critical Care Medicine, The Eighth Affiliated Hospital, Sun Yat-sen University, Shenzhen, China, [4] Department of Ultrasonography, Peking University Shenzhen Hospital, Shenzhen Peking University-The Hong Kong University of Science and Technology Medical Center, Shenzhen, China

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***Correspondence:**
Xi Xia
xixia1126@hotmail.com
Yu Shi
shiyu@pkuszh.com

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Background: rs13405728 was identified as one of the most prevalent susceptibility loci for polycystic ovary syndrome (PCOS) in Han Chinese and Caucasian women. However, the target genes and potential mechanisms of the rs13405728 locus remain to be determined.

Methods: Three-dimensional (3D) genome interactions from the ovary tissue were characterized *via* high-through chromosome conformation capture (Hi-C) and Capture Hi-C technologies to identify putative targets at the rs13405728 locus. Combined analyses of eQTL, RNA-Seq, DNase-Seq, ChIP-Seq, and sing-cell sequencing were performed to explore the molecular roles of these target genes in PCOS. PCOS-like mice were applied to verify the expression patterns.

Results: Generally, *STON1* and *FSHR* were identified as potential targets of the rs13405728 locus in 3D genomic interactions with epigenomic regulatory peaks, with *STON1* (P=0.0423) and *FSHR* (P=0.0013) being highly expressed in PCOS patients. *STON1* co-expressed genes were associated with metabolic processes (P=0.0008) in adipocytes (P=0.0001), which was validated in the fat tissue (P<0.0001) and ovary (P=0.0035) from fat-diet mice. The immune system process (GO:0002376) was enriched in *FSHR* co-expressed genes (P=0.0002) and PCOS patients (P=0.0002), with *CD4* high expression in PCOS patients (P=0.0316) and PCOS-like models (P=0.0079). Meanwhile, *FSHR* expression was positively correlated with *CD4* expression in PCOS patients (P=0.0252) and PCOS-like models (P=0.0178). Furthermore, androgen receptor (*AR*) was identified as the common transcription factor

for *STON1* and *FSHR* and positively correlated with the expression of *STON1* (*P*=0.039) and *FSHR* (*P*=4e-06) in ovary tissues and PCOS-like mice.

Conclusion: Overall, we identified *STON1* and *FSHR* as potential targets for the rs13405728 locus and their roles in the processes of adipocyte metabolism and *CD4* immune expression in PCOS, which provides 3D genomic insight into the pathogenesis of PCOS.

Keywords: PCOS, three-dimensional genome analysis, rs13405728 locus, *STON1*, *FSHR*, *AR*

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a gynecological endocrine disorder that has been one of the leading causes in female infertility (1, 2). It is characterized by hormonal imbalance and ovarian dysfunction, with symptoms of hyperandrogenism, anovulation, and polycystic ovarian morphology (3). PCOS occurs in 4-8% of women worldwide and affects 6-12% (approximately 5 million) of reproductive age women in the United States (4). Moreover, women with PCOS have been reported to be at higher risk for hypertension, insulin resistance (IR), diabetes, psychiatric disorders, dyslipidemia, and cancers (4, 5).

The high heritability of PCOS as a genetic trait has been reported to account for 70% of the incidence of the disorder (2). The application of genome-wide association studies (GWAS) in large case-control cohorts has successfully supported the discovery and characterization of PCOS susceptibility loci. Some loci are close to genes that play a role in reproductive processes or metabolic dysfunction, such as rs13405728, rs2268361, and rs2349415 to follicle stimulating hormone receptor (*FSHR*), rs11031006 to *FSHB*, rs2059807 to *INSR*, and rs2272046 to *HMGA2* (6). The growing list of PCOS risk loci contributes to the understanding of the etiological pathways and processes of the syndrome and reveals the relative homology genetic basis of PCOS (6, 7). However, over 95% of GWAS-associated risk loci were found to be localized in the non-coding regions (8), while long distances exist between risk loci and target genes (9), making their pathological roles in PCOS unclear. rs13405728 has been identified as the most susceptibility locus for PCOS on 2p16.3 in Han Chinese women (7, 10) and Caucasian women (11). However, the target genes of rs13405728 and the roles of such risk locus in the development of PCOS remain to be determined.

Comprehensive and direct long-range mapping of regulatory elements and target genes is crucial to systematically understand the transcriptional regulation of human diseases (12, 13). Since researchers have provided insight into the three-dimensional (3D) structural genome of disease by mapping the interactions between baits and target genes using high-throughput and long-range approaches, such as high-throughput chromosome conformation capture (Hi-C), or Capture Hi-C (14, 15), it is increasingly evident that alternative chromatin interactions are responsible for the gene dysregulation and biological phenotype in human disease or complex traits (13). For example, SNPs in intron 19 of *CLEC16A* are associated with the expression of *DEXI* (16), rs6927172 in region 6q23 in autoimmune diseases is associated with the increased expression of *IL20RA* (17), and rs9349379 in vascular diseases is associated with the expression of *EDN1* (18).

In this study, 3D structural genomic analysis from Hi-C and Capture Hi-C, expression profiling of PCOS patients and PCOS-like models, ChIP-Seq analysis of androgen receptor (*AR*) in *STON1* and *FSHR*, and single-cell sequencing of ovary tissue were used to synthesize the 3D interactions, adipocyte metabolism association with *STON1*, and *CD4* immune association with *FSHR* at the rs13405728 locus in PCOS.

MATERIAL AND METHODS

PCOS-Like Models and Mouse Tissue Acquisition

The animal study (C57BL/6) was performed with the approval of the Ethics Committee of the Peking University Shenzhen Hospital (PKUSH) and performed in Shenzhen Peking University-The Hong Kong University of Science (PKU-HKUST) and Technology Medical Center. The PCOS-like models (testosterone-treated and high-fat diet) of research were followed by the previous studies (19–21). Fat mice were treated with a fat diet (with 60% fat), while control mice were treated with a normal diet. Testosterone-treated mice used dihydrotestosterone release pellet (Dow Corning, Midland, MI, USA, 10 mg, S4757, Selleck) with a hypodermic way for 90 days. All performances were conducted under the Animal Welfare Act (AWA) and the Administrative Procedure Act (APA) Guidelines. Hematoxylin and eosin (H&E) staining of representative ovaries and quantitative analysis of cystic follicles were shown in **Supplementary Figures 1A, B**.

Immunohistochemistry (IHC)

Mouse tissue was prepared as formaldehyde-fixed and paraffin-embedded (FFPE) after collection and rinse. 4 μm sections were obtained from FFPE tissue with a microtome and then de-paraffinization and antigen retrieval were completed. To prevent background staining and false-positive results, endogenous peroxidase was inactivated by 3% H_2O_2 and any non-specific binding proteins were quenched by bovine serum albumin (BSA, 5%, Servicebio). Primary antibody against FHSR (A3172, ABclonal, 1:100), *STON1* (PA5-75314, Invitrogen, 1:50)

and AR (A19611, ABclonal, 1:100) was applied at 4°C overnight. After rinsing, the samples were treated with biotinylated secondary anti-rabbit immunoglobins and peroxidase-conjugated streptavidin, incubating at room temperature for 1 hour. The score of results was evaluated *via* Image-Pro Plus.

Hi-C Maps and Virtual 4C Analysis

Hi-C experiments of ovary tissue were downloaded from GSM2322546 (22), which were performed by HindIII restriction enzyme using the Hi-C "dilution" protocol (9). NHEK Hi-C data were downloaded from GSE63525 (23), which were performed by MboI restriction enzyme using the *in situ* Hi-C Protocol. Comparative Hi-C map between ovary tissue and GM12878 (as control) was generated by 3DIV tool (http://kobic.kr/3div), an online interaction viewer for Hi-C interactions (24). Interaction genes of rs13405728 were shown in **Supplementary Table 1**. rs13405728 was used as the bait with a 500Kb interaction range on chromosome 2 (chr2: 48478159-49478159). Virtual 4C map was generated from the ovary Hi-C data with the viewpoint of chr2:47978158-49978158 *via* 3D Genome Browser (http://3dgenome.org) (25), rs13405728 was used as the bait with 500Kb interaction range. All data was processed by a custom pipeline with the hg19 reference genomes.

Capture Hi-C and DHS Linkage Analysis

Capture Hi-C data of the ovary tissue were downloaded from GSM2322546 (22). Capture Hi-C analysis was performed by 3D Genome Browser (http://3dgenome.org) with the default settings (25). rs13405728 was used as the bait with a 500Kb interaction range on chromosome 2. DNase hypersensitive site (DHS)-linkage profiling was performed as described previously (26), which was performed by 3D Genome Browser (http://3dgenome.org) with the default settings (25). DHS-linkage computed the Pearson correlation coefficients for all distal DHSs with gene proximal DHS, which was based on the tissue-specificity (25).

Compartment A/B Analysis

A/B compartment of cells (Normal cervical cells and cervical carcinoma) was downloaded from Genome Sequence Archive (GSA, http://bigd.big.ac.cn/gsa/), with the link number CRA001401. A Compartment matrix was performed as described previously (27). A/B compartment matrix was constructed using Integrative Genomics Viewer (v2.5.0), with region chr2: 47978158-49978158 (GRCh37/hg19).

Chromatin Immunoprecipitation Sequencing (ChIP-Seq) Analysis

ChIP-Seq (H3K36me3, H3K4me1, H3K9me3, and H3K27ac) of the ovary was explored in Roadmap Epigenomics Project (http://www.roadmapepigenomics.org/data/), an online public resource of epigenomic maps for primary *ex vivo* tissues (release 9). Peak annotation of H3K36me3, H3K4me1, H3K9me3, and H3K27ac were integrated from adult human ovaries. Genome region were chr2:48478158-49478158 (GRCh37/hg19). ChIP-Seq data AR of primary tissues were downloaded from androgen receptor programming of human tissue (GSE56288 and GSE70079). The ChIP-Seq results were viewed using the UCSC Genome Browser.

Data for Single-Cell Sequencing

Single-cell sequencing data of mouse ovary was downloaded from Mouse Cell Atlas (MCA) (28), which were performed by following the Microwell-seq protocol. The pooled data of mouse tissue included embryo, brain, heart, intestine, kidney, liver, lung, pancreas, stomach, testis, uterus, bladder, spleen, thymus, and prostate, and the cells were mapped into 99 clusters in tSNE plot. 4363 cells of adult mouse ovary were sequenced to analyze the expression of different cells. All these cells were clustered into 14 types with the tSNE dimension reduction method. The heatmap of cell types was conducted by Mouse Cell Atlas (MCA2.0, http://bis.zju.edu.cn/MCA/index.html). The expression of *Fshr* was explored in different cell types, with the mean expression of the cluster. The results were read by transcripts per kilobase of exon model per million mapped reads (TPM, **Supplementary Table 1**).

Expression Data Acquisition and Correlation Analysis

Expression data (GSE156895, GSE145461, GSE114419, GSE138518, GSE8157, GSE124707, GSE135917, and GSE43322 profiling data, **Supplementary Table 2**) were downloaded from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo). RNA-seq data of ovary tissue was downloaded from the Roadmap Epigenomics Project (http://www.roadmapepigenomics.org/data/). In GSE135917 and GSE43322, *STON1* probe expressions were extracted and analyzed for Pearson's correlation with BMI of samples in different groups. In GSE8157 and GSE124707, *STON1* expression signals were extracted and performed for Pearson's correlation with *CD4* expression signals in different groups. Correlation analyses of *STON1* and *AR*, *FSHR* and *AR* were performed in Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/index.html) using the ovary tissue data from Genotype-Tissue Expression (GTEx) project. Data of PCOS patients were from Chinese infertility women (GSE145461, GSE114419), Han Chinese infertility women (GSE138518), and Caucasian infertility women (GSE8157). The race of PCOS patients used to explore phenotype was consistent with the population of rs13405728 locus. In addition, the main phenotypes of the four data were listed in **Supplementary Table 3**, including age, BMI (Kg/m2), LH(IU/L), FSH(IU/L), testosterone (ng/dL), and so on.

Expression Quantitative Trait Loci (eQTL) Analysis

eQTL analysis was performed in QTLbase (http://mulinlab.org/qtlbase/index.html) and GTExPortal (https://gtexportal.org/home/). To investigate the effect of rs13405728 locus candidate on target gene expression, eQTL analysis was performed *via* GTEx project for single-tissue eQTL and QTLbase for Cis-eQTL, which were used to evaluate the expression changes and understand the biological function of genetic polymorphism.

Co-Expression Networks

Co-expression networks of *STON1* and *FSHR* were performed in GeneMANIA (29) (http://genemania.org), an online tool including 2277 associated networks mapped to 163599 genes from 9 organisms. *STON1* (ENSG00000243244) and *FSHR* (ENSG00000170820) were used as input genes. Co-expression networks of *STON1* and *FSHR* were explored in humans (*Homo sapiens*) with the default settings. Co-expression networks included physical interactions, co-expression, predicted interactions, co-localization, pathway interactions, genetic interactions, and shared protein domains.

Gene Set Enrichment Analysis and Enrichment Analysis in PaGenBase

Gene Ontology (GO) analysis was performed to annotate the gene function and biological characteristics in interaction networks using Gene Ontology consortium (http://www.geneontology.org/). GO analysis of co-expression genes and differentially expressed genes (DEGs) in PCOS was performed by Metascape (30) (https://metascape.org). Gene lists of co-expressed genes of *STON1* and *FSHR* were analyzed in Metascape (https://metascape.org) *via* the PaGenBase tool, which was a pattern gene database for understanding the gene function (31).

Transcription Factor (TF) Analysis

An online pipeline for TF analysis, Toolkit for Cistrome Data Browser (32) (http://dbtoolkit.cistrome.org/), was used to construct the hierarchical TFs of *STON1* and *FSHR*. Genome used the human/hg38, the half-decay distance to the transcription start site was 10kb, and data type in Cistrome was transcription factor and chromatin regulator. *STON1* (chr2:48530168-48598514, NM_001198595) and *FSHR* (chr2:48953160-49154526, XM_011532734) were used as the input genes. The top 20 factors are shown in the plot. Regulatory potential (RP) represented the score to estimate how possible the TF could regulate the gene. Y-axis is the RP score, X-axis is different factors. Dots in the X-axis represent the same factors.

Statistical Analysis

Data were presented as mean ± standard deviation (SD). All statistical analyses were performed on the statistical package of GraphPad Prism 6 (v6.02). Pearson's correlation coefficient was used for the evaluation of the correlation between *FSHR* and *CD4*, *STON1* and BMI, *AR* and *STON1*, *AR* and *FSHR*. The Student's t-test was used for the assessment of the difference among different groups. All the parameters would be considered statistically significant with a *P*-value<0.05.

RESULTS

Hi-C Maps and Epigenomic Peaks in the Region of the rs13405728 Locus

We explored Hi-C interactions of ovary tissue (**Supplementary Table 3**) and performed a comparative Hi-C map between ovary tissue and GM12878 (as control) (**Figure 1A**), finding that *FOXN2*, *STON1-GTF2A1L*, *STON1*, *GTF2A1L*, and *FSHR* were the putative targets of the rs13405728 (Chr2:48978158) locus with interaction arcs in ovary tissue (**Figure 1B**). We then explored Hi-C maps and TADs of ovary tissue and NHEK cells (normal epithelium) in the region of rs13405728 (**Supplementary Figures 2A, B**) and found that rs13405728, *STON1*, *LHCGR*, *STON1-GTF2A1L*, and *FSHR* tended to be in the same TAD. In addition, such domain of rs13405728 was identified as B compartment in Hi-C data of different cells (**Supplementary Figure 2C**).

Virtual 4C signal of ovary tissue was used to analyze the interactions between rs13405728 and target genes (**Figure 1C**). DNase-Seq, ChIP-Seq of H3K36me3, H3K4me1, H3K9me3, and

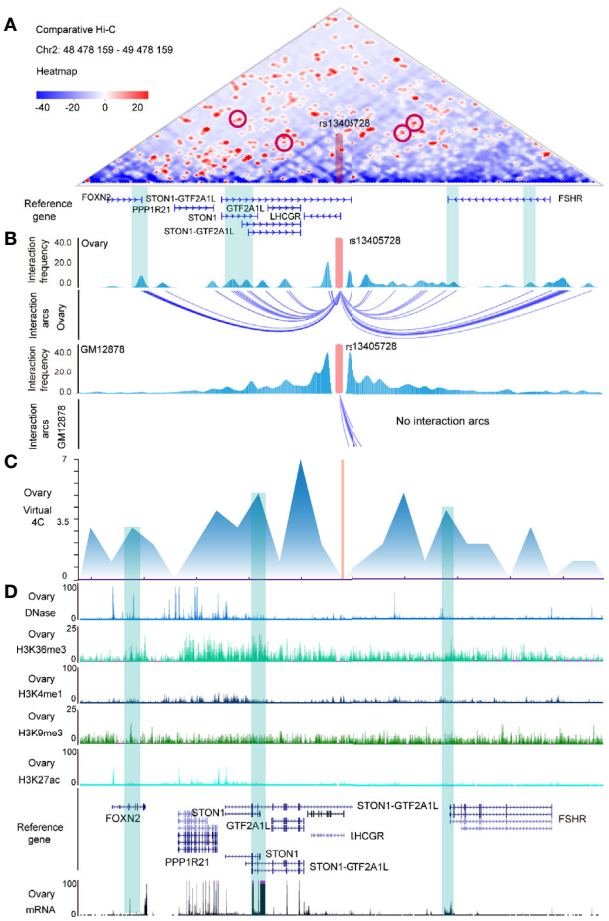

FIGURE 1 | Hi-C maps and epigenomic peaks in the region of the rs13405728 locus. **(A)** Comparative Hi-C map between ovary tissue and GM12878 in the Chr2:48478159-49478159 region with the rs13405728 locus labeled. **(B)** Interaction frequency and interaction arcs of the rs13405728 locus from ovary tissue and GM12878. **(C)** Virtual 4C interactions of the ovary in the Chr2:48478159-49478159 region. **(D)** DNase-Seq, ChIP-Seq (H3K36me3, H3K4me1, H3K9me3, and H3K27ac), and RNA-Seq peaks of the ovary. Reference genes and the rs13405728 locus are shown. Hi-C high-through chromosome conformation capture.

H3K27ac and mRNA data were characterized to identify the epigenomic modulation of ovary tissue. We found the epigenomic peaks and expression peaks in *FOXN2*, *STON1*, *STON1-GTF2A1L*, *GTF2A1L*, and *FSHR* (**Figure 1D**).

Expression Analysis Between the rs13405728 Locus and Potential Target Genes

To further explore the molecular patterns between rs13405728 and target genes, we conducted Cis-eQTL analysis around the rs13405728 locus region (+/- 10Mb region) from 12 tissues (**Figure 2A**). We further investigated whether these genes were changed in PCOS patients from GEO datasets, comparing with normal patients. *PPP1R21*, *STON1*, and *LHCGR* were found to be associated with SNP of rs13405728. In PCOS from the GSE145461 dataset, only *STON1* (P = 0.0423) was elevated in PCOS patients, with non-significance in *PPP1R21*, *LHCGR*, *FOXN2*, *STON1-GTF2A1L*, and *GTF2A1L* (**Figure 2B** and **Supplementary Figure 3A**). Although *LHCGR* was reputed as target gene for rs13405728 locus, there was no expression differences in PCOS patients. Additionally, *STON1* was identified as the target gene in Capture Hi-C interactions in ovary tissue, which was not found in blood control cells (CD4+ T cells and CD8+ T cells, B cells as control cells, **Supplementary Figure 3B**).

When comparing the expression of *FSHR* in human tissues (**Supplementary Figure 3C**), *FSHR* expression was specific to the ovary and testis. Single-cell sequencing data of ovary tissue was then mapped and found that *FSHR* highly expressed in Cumulus cell_Ube2c high cluster (P = 2.3293e-19), Granulosa cell_Inhba high cluster, and Cumulus cell_Car14 high cluster (**Figure 2C**). Such expression patterns were validated in mouse ovary tissues (**Figure 2D**). In ovarian granulosa cells of patients, the expression of *FSHR* was found to be higher in PCOS patients than normal patients, with GSE114419 (P = 0.0232) and GSE138518 (P = 0.0013) datasets (**Figure 2E**). In addition, we performed single-tissue eQTL analysis of *STON1*, *FSHR*, and rs13405728 SNP in ovary tissue (**Figure 2F**).

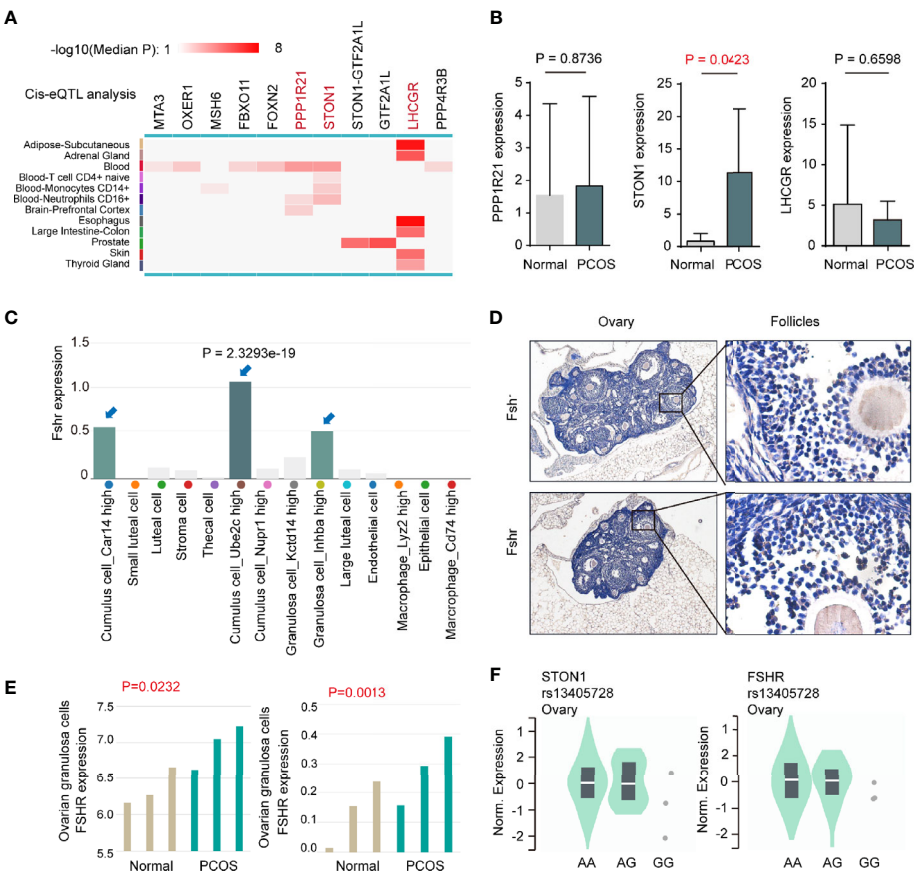

FIGURE 2 | Expression analysis between the rs13405728 locus and potential target genes. **(A)** Cis-eQTL analysis of potential target genes around the rs13405728 locus region (+/- 10Mb region) from 12 tissues, the color bar is shown. **(B)** Gene expression in PCOS patients and normal patients from the GSE145461 dataset. **(C)** Fshr expression in ovarian cell clusters from ovary single-cell sequencing data. 14 clusters are shown. **(D)** IHC staining of Fshr in C57BL/6 ovary tissue. **(E)** Fshr expression of ovarian granulosa cells in PCOS patients and normal patients from GSE114419 and GSE138518 datasets. **(F)** Single-tissue eQTL analysis of *STON1* and *FSHR* at the rs13405728 locus from normal ovary tissue. PCOS, polycystic ovary syndrome; eQTL, expression quantitative trait loci.

STON1 Was Associated With Metabolic Processes in Adipocytes and Highly Expressed in Mouse Fat and Ovary Tissue From Fat-Diet Mice

Since the biological roles of *STON1* in PCOS were unclear, we performed co-expression networks of *STON1* in the public dataset to analyze the molecular function of *STON1* in cells (**Figure 3A**). GO biological process analysis demonstrated that co-expressed genes of *STON1* were associated with metabolic processes (GO:0008152, P = 0.0008), cellular component organization or biogenesis (P = 0.001), and localization (P = 0.007) (**Figure 3B**). Enrichment analysis in PaGenBase showed

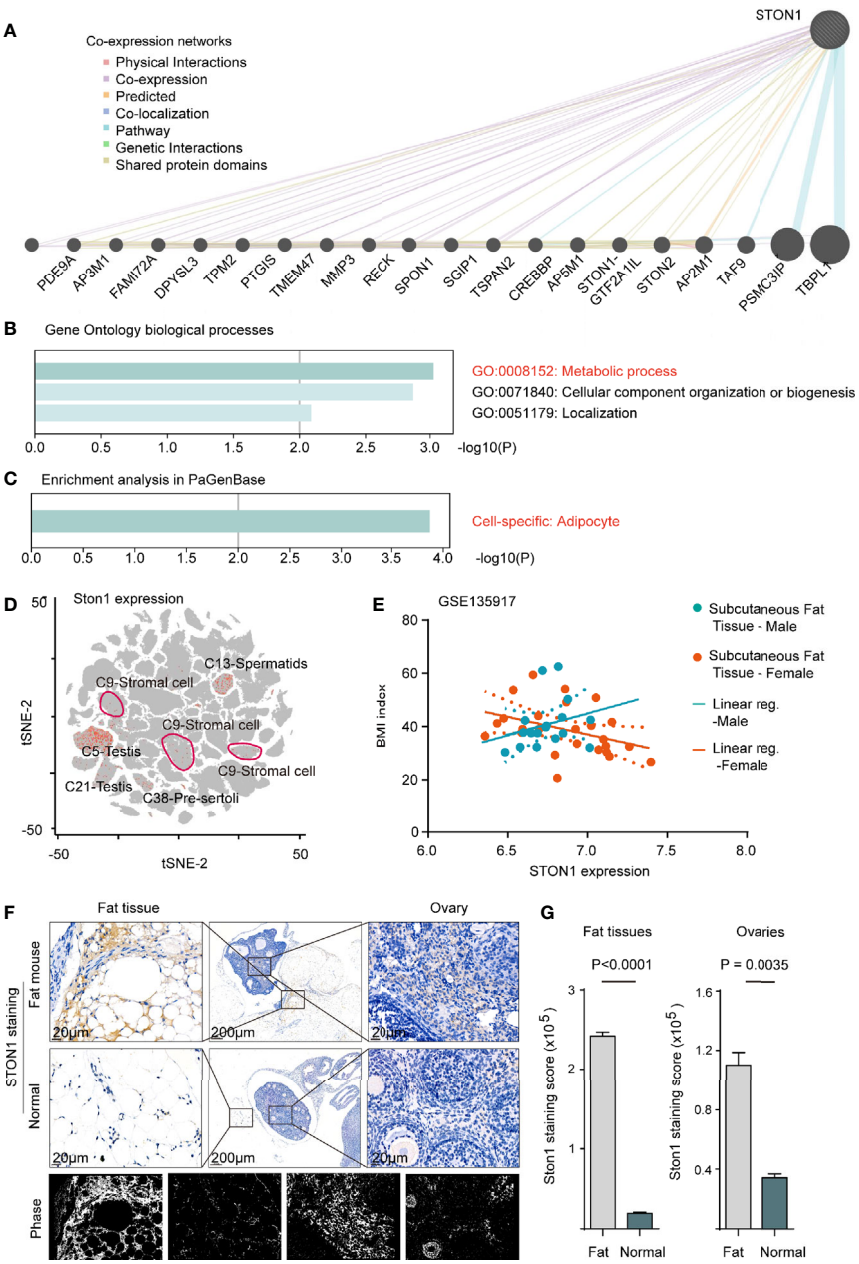

FIGURE 3 | *STON1* was associated with metabolic processes in adipocytes and highly expressed in mouse fat and ovary tissue from fat-diet mice. **(A)** Co-expression networks of *STON1*, co-expressed genes and interactions are indicated. **(B)** GO enrichment analysis of *STON1* co-expressed genes. **(C)** Cell-specific analysis of *STON1* co-expressed genes from PaGenBase. **(D)** tSNE map of *STON1* in pooled mouse tissues from mouse single-cell sequencing data (Han et al., 2018), *STON1* expression is shown with cluster labeled. **(E)** Correlation analysis between *STON1* expression and BMI in males and females from GSE135917 dataset. IHC staining **(F)** and staining score **(G)** of *STON1* in fat tissue and ovary tissue from fat-diet mice. GO, Gene Ontology; tSNE, t-distributed stochastic neighbor embedding; BMI, body mass index; IHC, immunohistochemistry.

that cell-specific enrichment of the networks was in adipocytes (P = 0.0001, **Figure 3C**).

To further explore the expression of *STON1* in the single-cell pattern, we performed the single-cell transcriptional analysis in the pooled data with 15 mouse tissues (see Methods). *STON1* was found in the reproductive gland (Cluster5, Cluster6, Cluster13, Cluster21, Cluster38) and stromal cells (Cluster9) (**Figure 3D** and **Supplementary Figures 4A, B**). In the GSE135917 dataset, the correlation tendency between *STON1* expression of subcutaneous fat and body mass index (BMI, kg/m^2) was contrary between males and females (**Figure 3E**). In GSE155489, the expression of *STON1* was higher in PCOS than in normal control (**Supplementary Figure 4C**, P = 0.0391). Importantly, IHC staining of *STON1* was higher in fat diet mice, both in fat tissue ($P < 0.0001$) and ovary tissue (P = 0.0035), than normal diet mice (**Figures 3F, G**).

FSHR Was Associated With The Immune System Processes And Positively Correlated With *CD4* Expression in PCOS Patients and PCOS-Like Models

To evaluate the molecular basis of *FSHR* in PCOS, we firstly performed co-expression networks of *FSHR* (**Figure 4A**). GO enrichment analysis demonstrated that co-expressed genes of *FSHR* were associated with the reproductive process (GO:0022414, $P = 0.0002$) and the immune system process (GO:0002376, $P = 0.0002$) (**Figure 4B**). The immune system process (GO:0002376, $P = 0.0002$) was found to be enriched in the differential expressed genes (DEGs) of follicular fluid between PCOS and normal patients (**Figures 4C, D**).

The hyperandrogenic phenotype was reported to be an important molecular mechanism of PCOS (33), thus prenatally androgenized (PNA) mice were conducted to analyze the DEGs between PCOS-like mice and normal control (**Figure 4E**). High expression of CD4 et al. was found in both PCOS patients ($P = 0.0316$) and PCOS-like mice ($P = 0.0079$) (**Figures 4F–H**). Furthermore, *FSHR* was found to be positively correlated ($P = 0.0252$, r = 0.6967) with *CD4* expression in PCOS patients (**Figure 4I**), and in PCOS-like (*Macaca mulatta*) model ($P = 0.0178$, r = 0.8889, **Figure 4J**).

AR Was Identified as the Common Transcription Factor of *STON1* and *FSHR* and Positively Correlated With *STON1* and *FSHR* Expression in Ovary Tissues

Given the increased expression of *STON1* and *FSHR* in PCOS patients and PCOS-like models, we hypothesized a potential role of *STON1* and *FSHR* in PCOS and explored the high expression mechanism underlying PCOS. We then performed transcription factors (TFs) analysis in the region of the rs13405728 locus (Chr2: 48478159-49478159) and gene regions of *STON1* and *FSHR* (**Figures 5A–C** and **Supplementary Tables 4–6**). AR was the only TF among them, *AR* and *FOXA1* were found to be the same TFs of *STON1* and *FSHR* (**Supplementary Figure 5A**), Further ChIP-Seq analysis of *STON1* and *FSHR* showed the modulation peaks of *AR* in primary tissues (**Figures 5D, E**). Moreover, the expression of *AR* was found to be positively correlated with the expression of *STON1* ($P = 0.039$, r = 0.22) and *FSHR* ($P = 4e\text{-}06$, r = 0.47) in normal ovary tissues (**Figure 5F**). However, the correlations were not found in *FOXA1* (**Supplementary Figure 5B**). Meanwhile, the expression of Ar, Fshr, and *STON1* were elevated in testosterone-treated and high-fat diet mice than normal mice (**Supplementary Figure 5C**), and their expression showed positive correlations (**Figure 5G**).

DISCUSSION

Since the rs13405728 locus has been identified as a common risk locus of PCOS (**Supplementary Figure 6**) in Han Chinese women (7, 10) and Caucasian women (11), it was necessary to identify target genes at the rs13405728 locus based on alternative genome conformation in the development of PCOS. We mapped Hi-C interactions, Capture Hi-C interactions, and virtual 4C interactions from the ovary tissue, and identified the potential targets at the rs13405728 locus. In addition, we explored the changes in expression of potential targets in PCOS patients and PCOS-like models, comparing with the normal patients and normal control, and identified *STON1* and *FSHR* as the most functional targets at the rs13405728 locus in PCOS. The Hi-C approach holds the advantages of capturing long-range interactions across the whole human genome (9), which is entirely useful for understanding the genetic trait with high heritability in the development of PCOS (2).

STON1 has been reported to be involved in spermatogenesis of the mouse models (34), in accordance with our findings that *STON1* expressed in the reproductive gland and stromal cells in single-cell sequencing patterns, which was validated in adipocytes and ovaries of the high-fat diet mouse models. Our results found an opposite tendency of correlations between *STON1* and BMI in male and female adipocytes. In male adipocytes, BMI was positively correlated with *STON1* expression. In PCOS, high BMI is a common characteristic and was a predictor of hyperandrogenism (35), consisting of the findings that *STON1* was highly expressed in PCOS and PCOS-like models. These results suggested that high *STON1* expression may be responsible for the hyperandrogenic phenotype in PCOS patients with dysregulated metabolic phenotypes.

Currently, PCOS is also reputed as an autoimmune disorder with high autoantibodies recorded in long-term clinical management (36). In our findings, the immune system processes were enriched in PCOS patients, with a high *CD4* expression phenotype in PCOS patients and PCOS-like models. In addition, *FSHR*, a receptor for *FSH*, plays a role in the development of follicles, maturation of the oocyte, and regulation of steroidogenesis and may be an important candidate gene for PCOS (37). However, the role of *FSHR* in the development of PCOS is unclear. Here, we showed an enrichment of the immune system processes and reproductive processes in *FSHR* co-expressed genes, following a positive correlation between *CD4* and *FSHR* both in PCOS patients and PCOS-like models. These results are supported by the findings

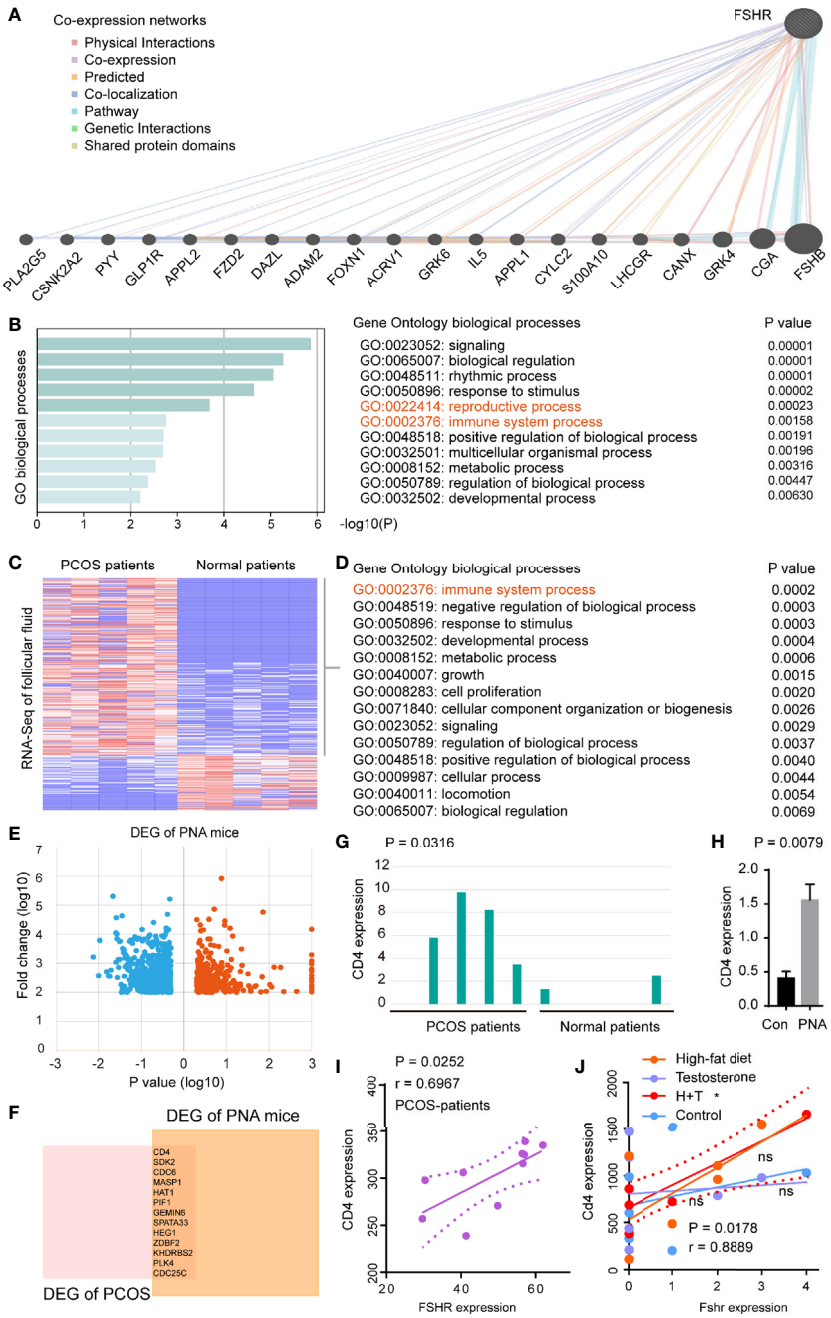

FIGURE 4 | FSHR was associated with the immune system processes and positively correlated with CD4 expression in PCOS patients and PCOS-like models. **(A)** Co-expression networks of FSHR, co-expressed genes and interactions are indicated. **(B)** GO enrichment analysis of FSHR co-expressed genes. Heatmaps of DEGs **(C)** and GO enrichment analysis of upregulation DEGs **(D)** in the GSE145461 dataset. **(E)** Volcano plot of DEGs between the two groups of samples in GSE156895 dataset. Red spots indicate the up-regulated genes, blue spots indicate the down-regulated genes. **(F)** Venn diagram of upregulation DEGs between GSE145461 and GSE156895 datasets. Bar chart of CD4 expression between the two sets of samples in GSE145461 **(G)** and GSE156895 **(H)**. Correlation analysis of FSHR expression with CD4 expression in GSE8157 **(I)** and GSE124707 **(J)**. DEGs, differentially expressed genes; PNA mice, prenatally androgenized mice; H, high-fat diet; T, testosterone-treated. * < 0.05; ns, no significance.

that PCOS had lower global DNA methylation in monocytes, T helper cells, T cytotoxic cells, and B cells (38).

The biochemical and clinical changes of hyperandrogenism (high levels of androgen) are important phenotypes of PCOS, which was associated with anovulation and menstrual dysfunction (3). Therefore. prenatally androgenized (PNA) models (39) or testosterone-treated models (20) were used as PCOS-like models for hyperandrogenism basis, which would

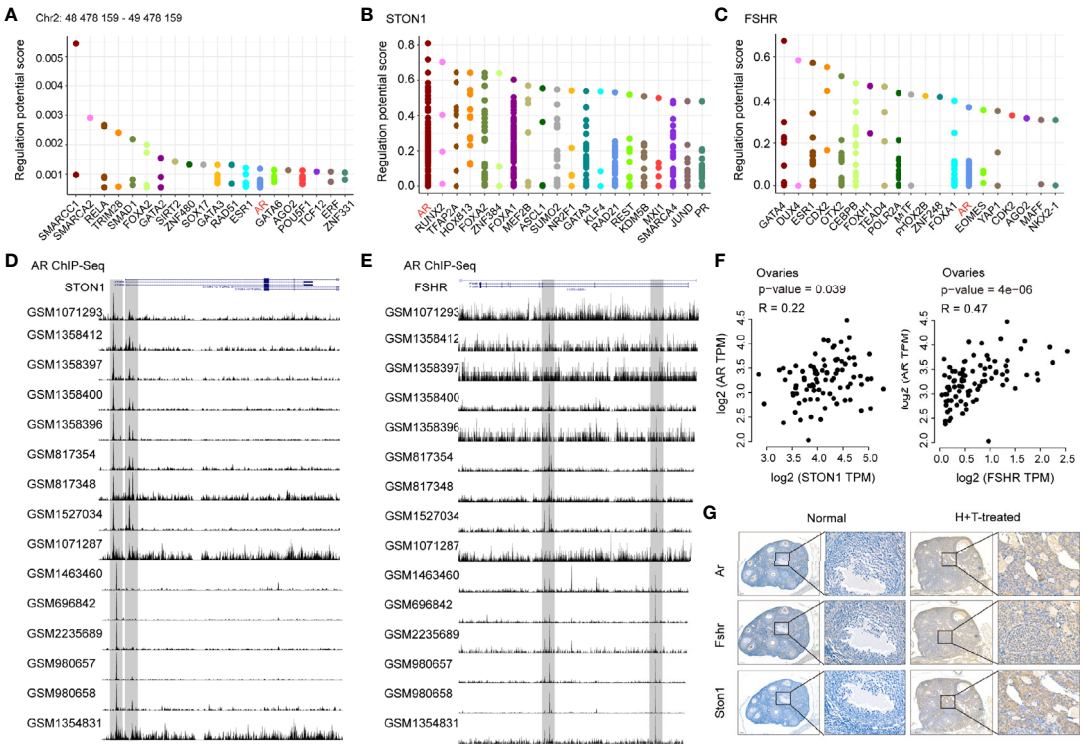

FIGURE 5 | AR was identified as the common transcription factor of *STON1* and FSHR and positively correlated with *STON1* and FSHR expression in ovary tissues. The top 20 transcription factors for the region of the rs13405728 locus (Chr2: 48478159-49478159) **(A)** *STON1* **(B)** and FSHR **(C)**, regulatory potential (RP) represented the score to estimate how likely the TF could regulate the gene. Y-axis is the RP score, X-axis is different factors. Dots in the X-axis represent the same factors. ChIP-Seq peaks of AR in *STON1* **(D)** and FSHR **(E)** from androgen receptor programming of human tissues (GSE56288 and GSE70079). Correlation analysis between the expression of AR and *STON1*, AR and FSHR **(F)** in ovary tissues from GTEx dataset. **(G)** IHC staining of Ar, Fshr, and *STON1* in normal mice and high-fat diet and testosterone-treated mice. AR, androgen receptor; GTEx, Genotype-Tissue Expression; H+T, high-fat diet and testosterone-treated.

highly increase the expression of *AR* (40). In our findings, AR was identified as the common TF of *STON1* and *FSHR* and positively correlated with their expression in ovary tissues. These results suggested the underlying interactions of hyperandrogenism, *AR*, *STON1*, and *FSHR* in the development of PCOS.

Insulin resistance is reputed as a key element contributing to the pathogenesis of PCOS patients (41). Recent studies have identified some candidate genes related to PCOS susceptibilities, such as the processes of insulin secretion and action in cells (42, 43). Therefore, we analyzed the expression levels of candidate genes in PCOS patients and PCOS-like models compared with normal patients and control. *IGF1* and *IGF1R* showed differential expression between PCOS and normal patients (**Supplementary Figure 7A**). *Igf1*, *Igfbp1*, *Pparg*, and *Shbg* were down-regulated in PCOS-like mouse models (**Supplementary Figure 7B**). In addition, we analyzed the inter-chromosomal interactions between candidate genes and rs13405728 locus with Hi-C data. Although these candidate genes have been shown the association with PCOS, no single candidate gene showed inter-chromosomal interactions with rs13405728 locus (**Supplementary Figure 7C**). This may be attributed to the disease heterogeneity observed in PCOS (44). Since fat tissue is the target of insulin resistance and metabolic disorder in PCOS (45), we explored the expression levels of these candidate genes in adipose tissue of Macaca mulatta (macaque) among normal diet, testosterone treatment, western-style diet, and the combination of testosterone treatment and western-style diet groups from GSE124707. *IRS1* was up-regulated after testosterone treatment. Although these candidate genes showed slight expression differences compared to the normal diet group, no single gene showed statistical significance (**Supplementary Figure 8**).

The current data did not show an eQTL correlation at rs13405728 locus for *STON1* and *FSHR*. It is possible that the effects of the risk variants for *STON1* and *FSHR* were not validated in the PCOS cohort and hence not detected in this study. Although the data presented herein provided statistical differences between PCOS and normal controls, and the gene nearby the locus may be the potential candidates for PCOS, particularly concerning adipocyte metabolic and *CD4* immunological processes, further studies should be performed to determine the roles of the rs13405728 locus, *STON1*, and *FSHR* in the pathogenesis of PCOS.

In summary, 3D genomic interactions in primary ovary tissue identified the interaction genes at the rs13405728 locus as *STON1* and *FSHR*, which were highly expressed in PCOS patients. Further analysis showed the adipocyte metabolism roles of *STON1*, which was validated in the adipose tissue and ovaries of the fat-diet mice. In addition, immune system processes were enriched in PCOS, with *CD4* high expression in PCOS patients and PCOS-like models, which was consistent with the *CD4* immunological correlation of *FHSR* in PCOS patients and PCOS-like models. Furthermore, we found that *AR* was the common transcription factor for *STON1* and *FSHR* and positively correlated with *STON1* and *FSHR* expression in ovary tissues. Overall, we identified *STON1* and *FSHR* as potential targets of rs13405728 locus in adipocyte metabolism and immune processes in the pathogenesis of PCOS.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of Shenzhen Peking University-The Hong Kong University of Science and Technology Medical Center.

AUTHOR CONTRIBUTIONS

C-hC performed the analysis of data, C-hC, YW, and X-rL performed the animal experiments, C-hC performed IHC of tissue. C-hC wrote the manuscript. J-qL, Q-jZ, S-rL, LG, and S-kY provided feedback. YS and XX designed the study and funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.686054/full#supplementary-material

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Obstructive Sleep Apnea Is Associated With Low Testosterone Levels in Severely Obese Men

Milina Tančić-Gajić[1], Miodrag Vukčević[2], Miomira Ivović[1], Ljiljana V. Marina[1], Zorana Arizanović[1], Ivan Soldatović[3], Miloš Stojanović[1], Aleksandar Đogo[4], Aleksandra Kendereški[1] and Svetlana Vujović[1]*

[1] Department for Obesity, Reproductive and Metabolic Disorders, Clinic for Endocrinology, Diabetes and Metabolic Diseases, University Clinical Centre of Serbia, Faculty of Medicine, University of Belgrade, Belgrade, Serbia, [2] Department of Pulmonology, Clinical Hospital Centre Zemun, Faculty of Medicine, University of Belgrade, Belgrade, Serbia, [3] Institute of Medical Statistics and Informatics, Faculty of Medicine, University of Belgrade, Belgrade, Serbia, [4] Department of Endocrinology, Clinical Center of Montenegro, Podgorica, Montenegro

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Elisabetta Baldi, University of Florence, Italy

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****Correspondence:***
Ljiljana V. Marina
ljikka@yahoo.com;
ljilja.marina@gmail.com

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Background: Disrupted sleep affects cardio-metabolic and reproductive health. Obstructive sleep apnea syndrome represents a major complication of obesity and has been associated with gonadal axis activity changes and lower serum testosterone concentration in men. However, there is no consistent opinion on the effect of obstructive sleep apnea on testosterone levels in men.

Objective: The aim of this study was to determine the influence of obstructive sleep apnea on total and free testosterone levels in severely obese men.

Materials and methods: The study included 104 severely obese (Body Mass Index (BMI) ≥ 35 kg/m^2) men, aged 20 to 60, who underwent anthropometric, blood pressure, fasting plasma glucose, lipid profile, and sex hormone measurements. All participants were subjected to polysomnography. According to apnea-hypopnea index (AHI) patients were divided into 3 groups: <15 (n = 20), 15 - 29.9 (n = 17) and ≥ 30 (n = 67).

Results: There was a significant difference between AHI groups in age (29.1 ± 7.2, 43.2 ± 13.2, 45.2 ± 10.2 years; p < 0.001), BMI (42.8 ± 5.9, 43.2 ± 5.9, 47.1 ± 7.8 kg/m^2; p = 0.023), the prevalence of metabolic syndrome (MetS) (55%, 82.4%, 83.6%, p = 0.017), continuous metabolic syndrome score (siMS) (4.01 ± 1.21, 3.42 ± 0.80, 3.94 ± 1.81, 4.20 ± 1.07; p = 0.038), total testosterone (TT) (16.6 ± 6.1, 15.2 ± 5.3, 11.3 ± 4.44 nmol/l; p < 0.001) and free testosterone (FT) levels (440.4 ± 160.8, 389.6 ± 162.5, 294.5 ± 107.0 pmol/l; p < 0.001). TT level was in a significant negative correlation with AHI, oxygen desaturation index (ODI), BMI, MetS and siMS. Also, FT was in a significant negative correlation with AHI, ODI, BMI, age, MetS and siMS. The multiple regression analysis revealed that both AHI and ODI were in significant correlation with TT and FT after adjustment for age, BMI, siMS score and MetS components.

Conclusion: Obstructive sleep apnea is associated with low TT and FT levels in severely obese men.

Keywords: obesity, metabolic syndrome, sleep apnea, testosterone, male

INTRODUCTION

Obesity is a complex metabolic disorder with a markedly increased prevalence in both the developed and underdeveloped countries. Over the past four decades, the percentage of obese people has doubled among females and quadrupled among males (1, 2).

Excess body weight is a crucial risk factor for mortality and morbidity, especially in obese men with body mass index (BMI) over 35 kg/m^2 (1, 3). The risk of developing male infertility increases with obesity severity. Alterations in sex steroid hormones contribute to infertility in obese men (4). Obesity in men is associated with low testosterone and low measured or calculated free and bioavailable testosterone (1). Men with a BMI of 35–40 kg/m^2 can have up to 50% less free and total testosterone when compared to age-matched peers with a normal BMI (5). Low testosterone levels in obese men are considered a consequence of reduced sex hormone binding globulin (SHBG) synthesis, increased androgens aromatization to estradiol, and central gonadal axis suppression. Complex metabolic disorders, increased pro-inflammatory adipocytokines, impaired insulin signaling in the central nervous system, dysregulated leptin signaling, and increased estrogen may lead to hypothalamic suppression *via* effects on kisspeptin neurons in obese men (6, 7).

Furthermore, metabolic syndrome, as an adverse health consequence of obesity, is associated with lower testosterone levels independent of age and BMI (8, 9). Disrupted sleep affects cardio-metabolic and reproductive health. The most important clinical cause of disrupted sleep is obstructive sleep apnea syndrome (OSAS) (10). OSAS is emerging as a new area of interest for andrological issues (4). It is characterized by repetitive episodes of upper airway obstruction that occur during sleep and is associated with a complete (apnea) or incomplete (hypopnea) cessation of airflow. This is commonly accompanied by loud snoring and reduction in blood oxygen saturation, followed by arousal, fragmented sleep, and daytime sleepiness (11, 12). The most important epidemiological risk factor for sleep apnea is obesity, preceding age, and male gender (13). The prevalence of OSAS in obese individuals is over 30%, and 50-98% in morbidly obese patients (14). There is growing evidence to support an independent association of OSAS with cardiovascular, neuropsychiatric, pulmonary, and renal disorders as well as with metabolic and endocrine co-morbidities. There is convincing evidence that OSAS is also an independent risk factor for metabolic syndrome (15). It is clinically noticeable that obese men with sleep apnea have lower than expected concentrations of total testosterone (TT) and free testosterone (FT) (3, 9, 10). However, the independent effect of OSAS on blood testosterone concentrations has been shown in some (9, 16–20) but not in all cross-sectional studies (21, 22).

Considering the inconclusive data, the purpose of this study was to determine the influence of obstructive sleep apnea on TT and FT levels in severely obese men.

Abbreviations: BMI, body mass index; SHBG, sex hormone-binding globulin; OSAS, obstructive sleep apnea syndrome; Tg, triglyceride; HDL, high-density lipoprotein; LH, luteinizing hormone; FSH, follicle-stimulating hormone; AHI, apnea-hypopnea index; ODI, oxygen desaturation index; MetS, metabolic syndrome; siMS, continuous metabolic syndrome score.

MATERIALS AND METHODS

Subjects

This was an observational, cross-sectional study. We have evaluated 165 severely obese men (BMI ≥ 35kg/m^2), aged 20 to 60, admitted to the Department for Obesity, Metabolic and Reproductive Disorders at the Clinic for Endocrinology, Diabetes and Metabolic Diseases University, Clinical Centre of Serbia between 2006 and 2016.

Detailed personal history, the biochemical and endocrinological evaluation was conducted in order to detect and exclude patients with: hypercortisolism (0) and/or hypothyroidism (5), history of alcohol consumption (≥ 2 units per day) or substance abuse (2), hormonal therapy (3), hypogonadism due to the pituitary (3) or testicular diseases (3), craniofacial abnormalities (2), liver or kidney diseases (2), neuromuscular diseases (4), chronic obstructive pulmonary disease (4), asthma (3), manifest cardiologic diseases (13), malignancies (2), psychiatric diseases (3) and missing data (12). In total, sixty-one patients were excluded from the study as provided in the STROBE flowchart (**Figure 1**).

This study was designed in agreement with the Declaration of Helsinki and was approved by the local Ethical Committee. The patients gave their informed consent.

Study Protocol

All study patients underwent anthropometric and blood pressure measurements, biochemical and hormonal analysis, as well as the overnight polysomnography study.

Anthropometry and Measurement of Blood Pressure

Weight was measured in the morning, without shoes, in light clothing, using a medical scale with an accuracy of 0.1 kg. Height was measured without shoes, using a stadiometer with an accuracy of 0.1 cm. Waist, hip, and neck circumferences expressed in centimeters were measured using a non-elastic band in the standing position. Waist circumference was measured at the end of the expiration at the midline between

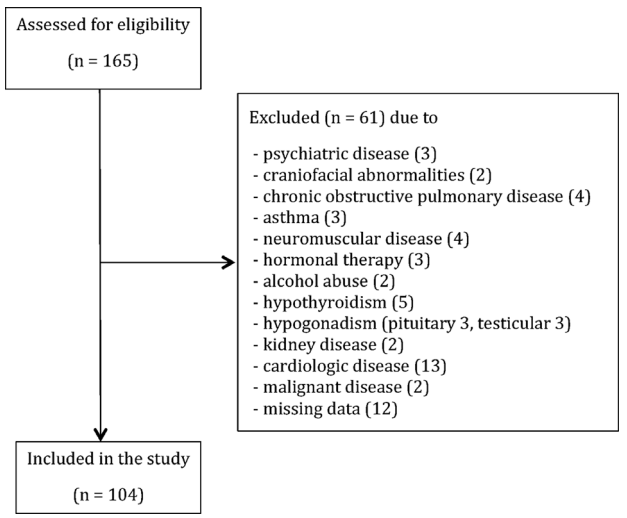

FIGURE 1 | STROBE flowchart of the present study.

the lowest point of the costal arch and the highest point of the iliac crest. Hip circumference was measured at the level of the large trochanter of the femur (23). Neck circumference was measured at the level of the cricothyroid membrane (24). The systolic (SBP) and diastolic (DBP) blood pressure was measured using a standard sphygmomanometer in the sitting position: three values were obtained with a 2-minute time-lapse in between, and the average was recorded (23).

Blood Samples

Fasting blood samples were taken to measure glucose, triglyceride (Tg), high-density lipoprotein (HDL), albumin, luteinizing hormone (LH), follicle-stimulating hormones (FSH), estradiol, testosterone, and SHBG in the morning following polysomnography.

Sleep Study

The apnea-hypopnea index (AHI) and the oxygen desaturation index (ODI) were derived from nocturnal sleep studies using a seven-channel portable sleep recorder (Stardust II, Respironics, Inc., USA). The system detected apneas and hypopneas by measuring: pressure-based airflow with snoring detection, pulse rate, arterial oxyhemoglobin saturation, chest or abdominal effort, and body position changes. The sleep studies were scored at the hospital by a certified sleep specialist. Apneas were defined as a total cessation of airflow for at least 10s if a respiratory effort was present. Hypopneas were defined as a decrease in nasal pressure signal of ≥ 30% of baseline, which was associated with a ≥ 3% desaturation. The apnea-hypopnea index (AHI) was calculated as the total number of obstructive apneas and hypopneas per hour of sleep. OSAS was classified based on the AHI as follows: mild (≥ 5 and < 15 events/h), moderate (≥ 15 and < 30 events/h), or severe (≥ 30 events/h). The oxygen desaturation index (ODI) was defined as the number of oxygen desaturations ≥3% per hour of sleep (25).

Biochemical and Hormonal Assays

The fasting plasma glucose (FPG) levels were measured by the glucose-oxidase method (Beckman). Fasting serum lipid levels (HDL and Tg) were analyzed enzymatically using a commercial kit (Bushranger Mannheim GmbH Diagnostica). The serum LH (The ImmuChem hLH IRMA kit, ICN Biomedicals, Inc., CA, USA, CV 2.4%), FSH (The ImmuChem FSH- CT IRMA kit, ICN Biomedicals, Inc., CV 2.6%), estradiol (ESTR- US- CT Cisbio, Bioassays, CV 2.8%), testosterone (TESTO-CT2, Cisbio International, CV 3.1%), and SHBG (SHBG-RIACT, Cisbio International, France, CV 3.6%) were measured by radioimmunoassay.

Calculations

Metabolic syndrome (MetS) was defined by three of five criteria: FPG ≥ 5.6mmol/l or antidiabetic therapy, increased waist circumference equal to or greater than 94 cm, TG ≥ 1.7mmol/l, HDL < 1.0mmol/l or antilipidemic therapy, and blood pressure ≥ 130/85 mmHg or therapy (26).

To evaluate the metabolic syndrome, we used siMS score - continuous metabolic syndrome score for quantification of patients' metabolic status. siMS score (siMS) was calculated using the following formula: siMS score = 2*Waist/Height + FPG/5.6 + Tg/1.7 + SBP/130—HDL/1.02 (27).

FT was calculated based on TT, SHBG, and albumin with the formula as reported by Vermeulen et al. (28). TT < 11 nmol/l and FT < 220 pmol/l were deemed low (29).

Statistical Analysis

Results are presented as count (%), mean ± standard deviation, or median (25th-75th percentile) depending on the data type and distribution. Groups were compared with parametric (ANOVA) and nonparametric (Kruskal-Wallis test, Mantel-Haenszel chi-square test for trend) tests. To test the correlation between the variables, Pearson and Spearman's correlation were used. Multiple linear regression analysis was performed to evaluate the relationship between the dependent variable and independent variables. All p values below 0.05 were considered significant. All data were analyzed using SPSS 20.0 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.).

RESULTS

The general characteristics of the study subjects are summarized in **Table 1**. The mean age was 41.8 ± 11.9 years, the mean BMI was 45.7 ± 7.4 kg/m^2, and the mean weight was 144.9 ± 24.2 kg. 77.9% of patients had MetS, 48.1% had low concentrations of the TT, and 29.8% had low concentrations of FT. The prevalence of sleep apnea was 96.2%, out of which 15.4% had mild, 16.3% moderate, and 64.5% had severe obstructive sleep apnea.

Patients were divided into groups according to AHI levels in order to present values of clinical parameters in an easy-to-understand fashion. Taking into account that the group with no OSAS was too small (only 4 patients), we classified patients into

TABLE 1 | General characteristics of the total cohort and AHI groups.

	Total	AHI			p value
		<15 (n=20)	15-29.9 (n=17)	≥30 (n=67)	
Anthropometry					
Age (yrs.)	41.8 ± 11.9	29.1 ± 7.2	43.2 ± 13.2	45.2 ± 10.2	<0.001[a]
BMI (kg/m^2)	45.7 ± 7.4	42.8 ± 5.9	43.2 ± 5.9	47.1 ± 7.8	0.023[a]
Weight (kg)	144.9 ± 24.2	139.3 ± 15.5	136.2 ± 24.2	148.8 ± 28.5	0.116[a]
Waist circumference (cm)	139.1 ± 15.8	130.2 ± 11.0	133.9 ± 12.7	143.0 ± 16.4	0.002[a]
Hip circumference (cm)	134.9 ± 17.4	133.0 ± 11.6	130.7 ± 14.1	136.6 ± 19.4	0.395[a]
Neck circumference (cm)	48.3 ± 3.9	45.1 ± 2.9	47.7 ± 3.3	49.4 ± 3.7	<0.001[a]
SBP (mmHg)	136.8 ± 15.7	130.0 ± 13.6	129.7 ± 11.5	140.6 ± 16.0	0.003[a]
DBP (mmHg)	88.2 ± 12.2	81.2 ± 11.2	86.0 ± 11.7	90.8 ± 11.9	0.005[a]
Hypertension	85 (81.7%)	11 (55.0%)	14 (82.4%)	60 (89.6%)	0.001[c]
Habits					
Smoking	47 (45.2%)	11 (55.0%)	6 (35.3%)	30 (44.8%)	0.483[c]
Biochemistry					
HDL (mmol/L)	0.99 ± 0.23	0.93 ± 0.22	1.11 ± 0.31	0.99 ± 0.21	0.049[a]
Tg (mmol/L)	2.34 ± 1.55	1.86 ± 1.11	2.47 ± 2.09	2.46 ± 1.51	0.035[b]
FPG (mmol/L)	4.9 (4.5-5.8)	4.5 (3.9-4.9)	4.9 (4.4-5.9)	5.2 (4.6-6.2)	<0.002[b]
T2DM	28 (26.9%)	2 (10.0%)	7 (41.2%)	19 (28.4%)	0.229[c]
Metabolic syndrome					
MetS	81 (77.9%)	11 (55.0%)	14 (82.4%)	56 (83.6%)	0.013[c]
MetS No of comp.					
1	2 (1.9%)	1 (5%)	1 (5.9%)	0	0.029[b]
2	21 (20.2%)	8 (40%)	2 (11.8%)	11 (16.4%)	
3	26 (25.0%)	4 (20%)	8 (47.1%)	14 (20.9%)	
4	36 (34.6%)	5 (25%)	3 (17.6%)	28 (41.8%)	
5	19 (18.3%)	2 (10%)	3 (17.6%)	14 (20.9%)	
siMS	4.01 ± 1.21	3.42 ± 0.80	3.94 ± 1.81	4.20 ± 1.07	0.038[a]
Sex hormones					
FSH (IU/l)	4.9 (3.0-7.8)	4.6 (2.4-7.0)	6.2 (3.9-7.4)	4.9 (3.2-8.3)	0.400[b]
Estradiol (pmol/l)	121.1 ± 51.5	108.6 ± 65.9	104.4 ± 49.9	129.2 ± 45.8	0.114[a]
LH (IU/l)	3.62 ± 1.88	3.57 ± 1.29	4.49 ± 1.67	3.41 ± 2.03	0.105[a]
SHBG (nmol/l)	18.4 (11.9-26.3)	19.3 (12.8-27.6)	16.1 (11.4-32.8)	18.5 (11.9-24.7)	0.860[b]
T (nmol/l)	12.9 ± 5.4	16.6 ± 6.1	15.2 ± 5.3	11.3 ± 4.4	<0.001[a]
FT (pmol/l)	338.1 ± 141.2	440.4 ± 160.8	389.6 ± 162.5	294.5 ± 107.0	<0.001[a]
TT < 11 (nmol/l)	44 (42.3%)	6 (30.0%)	2 (11.8%)	36 (53.7%)	0.012[c]
FT < 220 (pmol/l)	21 (20.2%)	0	2 (11.8%)	19 (28.4%)	0.004[c]
T T< 11(nmol/l) & FT<220 (pmol/l)	20 (19.2%)	0	1 (5.9%)	19 (28.4%)	0.002[c]

AHI, apnea-hypopnea index; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HDL, high density lipoprotein; Tg, triglycerides; T2DM, type 2 diabetes mellitus; MetS, metabolic syndrome; siMS, continuous metabolic syndrome score; FSH, follicle -stimulating hormone; LH, luteinizing hormone; SHBG, sex hormone-binding globulin; TT, Total Testosterone; FT, Free Testosterone; [a]ANOVA, [b]Kruskal-Wallis, [c]Mantel Haenszel chi-square test for trend.

3 AHI groups: AHI < 15 (20 patients), AHI 15 - 29.9 (17 patients), and AHI ≥ 30 (67 patients) as presented in **Table 1**. Our results showed a clear significant difference between AHI groups in TT and FT levels (p<0.001). There was also a significant difference between AHI groups in terms of age, BMI, waist and neck circumference, blood pressure levels, hypertension prevalence, triglyceride, high-density lipoprotein and FPG levels, metabolic syndrome prevalence, and siMS score (**Table 1**). There was no significant difference in TT (12.8 ± 5.3 *vs.* 13.1 ± 5.5 nmol/l; p = 0.734) or FT level (346.1 ± 124.9 *vs.* 331.6 ± 154.1 pmol/l; p = 0.605), AHI (46.4 ± 29.7 *vs.* 44.7 ± 27.4; p = 0.762) and ODI (44.5 ± 31.8 *vs.* 42.7 ± 29.9; p = 0.767) between smokers and non-smokers.

There was a significant negative correlation between TT and AHI (r = -0.409, p < 0.001) and ODI (r = -0.458, p < 0.001) levels. There was also a significant negative correlation between FT and AHI (r = -0.389, p < 0.001) and ODI (r = -0.438, p <0.001) levels (**Figure 2**). Both TT and FT levels were in a significant negative correlation with BMI (r = -0.269, p = 0.006 and r = -0.311, p = 0.001, respectively), weight (r = -0.203, p = 0.039 and r = -0.227, p = 0.02, respectively), hip circumference (r = -0. 234, p = 0.017 and r = -0.243, p = 0.013, respectively), some metabolic parameters such as waist circumference (r = -0.374, p < 0.001 and r = -0.398, p < 0.001, respectively) DBP (r = -0.264, p = 0.007 and r = -0.294, p = 0.002, respectively), FPG (r = -0.274, p = 0.005 and r = -0.296, p = 0.002, respectively), and also with MetS prevalence r = -0.193, p = 0.049 and r = -0.195, p = 0.048, respectively) and siMS score levels (r = -0.321, p = 0.001 and r = -0.283, p = 0.004, respectively) (**Table 2**). FT levels were in a significant negative correlation with age (r = -0.346, p<0.001) (**Table 2**).

Furthermore, there was a significant positive correlation between AHI and ODI levels with age (r = 0.320, p = 0.001 and r = 0.339, p < 0.001, respectively), BMI (r = 0.382, p < 0.001

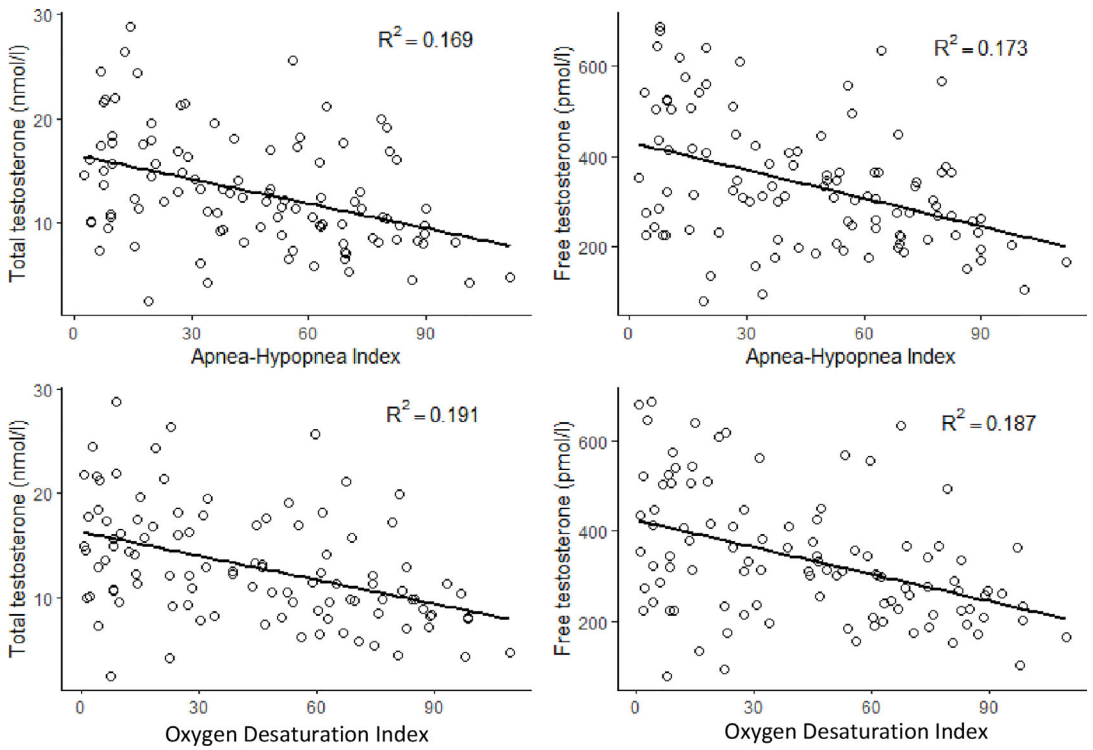

FIGURE 2 | Correlations between total testosterone and free testosterone with apnea-hypopnea index and oxygen desaturation index.

TABLE 2 | Correlation matrix between testosterone, free testosterone, AHI, ODI and general characteristics of patients.

	TT[a]	FT[a]	AHI[b]	ODI[b]
TT	1	.847**	-.409**	-.458**
FT	.847**	1	-.389**	-.438**
AHI	-.412**	-.416**	1.000	.916**
ODI	-.437**	-.433**	.916**	1.000
Age	-.170	-.346**	.320**	.339**
BMI	-.269**	-.311**	.382**	.391**
Waist	-.374**	-.398**	.413**	.429**
Weight	-.203*	-.227*	.300**	.298**
Neck	-.174	-.225*	.490**	.469**
Hip	-.234*	-.243*	.230	.246
SBP	-.104	-.166	.280**	.324**
DBP	-.264**	-.294**	.314**	.322**
HDL	.119	.062	.015	-.040
Tg	-.195*	-.134	.151	.169
FPG	-.274**	-.296**	.275**	.250*
MetS	-.193*	-.195*	.163	.224*
siMS	-.321**	-.283**	.283**	.312**
FSH	.060	-.095	.063	.116
LH	.230*	.181	-.151	-.093
E	-.070	.016	.210*	.174
SHBG	.366**	-.122	-.080	-.098

*TT, total testosterone; FT, free testosterone; AHI, apnea-hypopnea index ;ODI, oxygen desaturation index; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high density lipoprotein; Tg, triglycerides; FPG, fasting plasma glucose; MetS, metabolic syndrome; siMS, continuous metabolic syndrome score; FSH, follicle -stimulating hormone; LH, luteinizing hormone; Results are presented as Pearson correlation coefficient or Spearman's rank correlation coefficient, [a]Pearson correlation; [b]Spearman correlation; *p < 0.05 **p < 0.01*

and r = 0.391, p < 0.001, respectively), weight (r = 0.300, p = 0.002 and r = 0.298, p = 0.002, respectively) waist (r = 0.413, p < 0.001 and r = 0.429, p < 0.001, respectively), hip (r = 0.230, p = 0.019 and r = 0.246, p = 0.012) and neck circumference (r = 0.490, p < 0.001 and r = 0.469, p < 0.001, respectively), SBP (r = 0.280, p = 0.004 and r = 0.324, p = 0.001, respectively), DBP (r = 0.314, p = 0.001 and r = 0.322, p = 0.001, respectively), FPG (r = 0.275, p = 0.005 and r = 0.250, p = 0.001, respectively), and siMS score levels (r = 0.283, p = 0.004 and r = 0.312, p< 0.001, respectively) (**Table 2**).

In multiple regression analysis, after adjustment for age, BMI, siMS score and MetS components, both AHI and ODI were in significant correlation with TT and FT (p < 0.05) (**Table 3**). The results of multiple linear regression analysis did not change in subgroup of patients with BMI ≥ 40kg/m^2 (80 patients in total) (**Table 4**). The calculated variance inflation factors showed no multicollinearity in regression models.

In order to assess if TT and FT can be used as discriminative variables for the assessment of sleep apnea severity (using 15 and 30 level cut-off for AHI) we have performed receiver-operating characteristic (ROC) curves analysis. AUC for AHI ≥ 15 for TT was AUC_{TT} = 0.714 (95% CI 0.587 - 0.841; p = 0.003) with cut off = 14.5 (Sn = 0.726; Sp = 0.650), and for FT AUC_{FT} = 0.719 (95% CI 0.588 - 0.851; p = 0.002) with cut off = 412 (Sn = 0.821; Sp = 0.600) (**Figure 3A**).

AUC for AHI ≥ 30 for TT was AUC_{TT} = 0.748 (95% CI 0.648 - 0.849; p < 0.001) with cut off = 14.3 (Sn = 0.806; Sp = 0.649) and

TABLE 3 | Multiple linear regression model for prediction of total testosterone (TT) and free testosterone (FT).

	TT		FT	
	B (95% CI)	**Adj R^2**	**B (95% CI)**	**Adj R^2**
AHI	-0.078 (-0.112 to -0.044)	0.162	-2.071 (-2.961 to -1.182)	0.165
AHI adjusted for age, BMI, siMS score	-0.060 (-0.098 to -0.022)	0.216	-1.088 (-2.057 to -0.120)	0.273
AHI adjusted for age, waist circumference, SBP, FPG, Tg, HDL	-0.059 (-0.096 to -0.021)	0.232	-1.185 (-2.145 to -0.226)	0.266
ODI	-0.077 (-0.108 to -0.046)	0.183	-1.991 (-2.804 to -1.177)	0.180
ODI adjusted for age, BMI, siMS score	-0.060 (-0.096 to -0.025)	0.229	-1.070 (-1.970 to -0.170)	0.277
ODI adjusted for age, waist circumference, SBP, FPG, Tg, HDL	-0.061 (-0.096 to -0.027)	0.251	-1.208 (-2.104 to -0.313)	0.274

p values in all models are < 0.05, AHI, apnea-hypopnea index; BMI, body mass index; siMS, continuous metabolic syndrome score; SBP, systolic blood pressure; FPG, fasting plasma glucose; Tg, triglycerides; HDL, high density lipoprotein; ODI, oxygen desaturation index.

TABLE 4 | Multiple linear regression model for prediction of total testosterone (TT) and free testosterone (FT) for patients with BMI ≥ 40 kg/m^2.

	TT		FT	
	B (95% CI)	**Adj R^2**	**B (95% CI)**	**Adj R^2**
AHI	-0.070 (-0.109 to -0.031)	0.129	-1.995 (-2.971 to -1.019)	0.164
AHI adjusted for age, BMI, siMS score	-0.071 (-0.112 to -0.029)	0.267	-1.511 (-2.572 to -0.450)	0.257
ODI	-0.068 (-0.103 to -0.033)	0.148	-1.832 (-2.722 to -1.941)	0.166
ODI adjusted for age, BMI, siMS score	-0.069 (-0.106 to -0.032)	0.284	-1.379 (-2.341 to -0.417)	0.258

p values in all models are < 0.05, AHI, apnea-hypopnea index; BMI, body mass index; siMS, continuous metabolic syndrome score; ODI, oxygen desaturation index.

for FT AUC_{FT} = 0.728 (95% CI 0.620 - 0.836; p < 0.001) with cut off = 396 (Sn = 0.866; Sp = 0.568) (**Figure 3B**).

DISCUSSION

Obesity is considered to be the main cause of hypogonadism in men. Hypogonadism is a clinical entity characterized by low serum TT concentration and/or FT concentration and associated symptoms and signs of testosterone deficiency (30). Obesity-related hypogonadism is functional, and it can be reverted by substantial weight loss achieved with non-surgical or surgical interventions (31). Hypoandrogenemia is a term referring to the finding of subnormal testosterone concentrations in men without taking into consideration clinical symptoms or signs of decreased serum testosterone levels. The prevalence of hypoandrogenemia is 4% to 5% in the general male population and as much as 20% to 40% in obese men (30). In our study, 48.1% of severely obese men had TT level < 348.3 ng/dl, and 29.8% had FT level < 70.0 pg/ml. Our results are consistent with the previous data that the presence of low TT and FT in men is closely related to the increased BMI with the highest hypoandrogenemia in more severe obesity (30).

Age is the most prominent predictor of most diseases in humans. Chronological aging per se and age-related changes in overall health and lifestyle are associated with natural declines in serum testosterone (32), presented in our study as a negative relationship between FT and age. Abdominal adiposity is one of five clinical risk factors used as diagnostic criteria for metabolic syndrome. Although obesity and metabolic syndrome frequently coexist, this is not always the case.

A significant proportion of obese individuals do not have metabolic syndrome, and, conversely, metabolic syndrome may be present in non-obese individuals (33). In our study, 77.9% of severely obese men had metabolic syndrome. The metabolic syndrome in obese men is associated with a further decline in testosterone level, with a negative inverse relationship between TT and/or FT levels and metabolic syndrome (8, 34, 35), also observed in our study.

The most striking result to emerge from our data is that OSAS, measured by both AHI and ODI, is an independent determinant of serum testosterone concentration in severely obese men after adjustment for BMI, age, or siMS score. Moreover, the ROC analysis showed that both TT and FT levels could be used as discriminative variables for the assessment of sleep apnea severity.

Patients with OSAS have reduced quality and quantity of sleep due to sleep fragmentation, intermittent nocturnal hypoxia, reduced deep and rapid eye movement (REM) sleep, reduced sleep duration, and sleep efficiency, all of which lead to pituitary-gonadal dysfunction and low testosterone levels in male patients (5, 36).

The association of respiratory hypoxia and low serum testosterone in men was observed as early as 40 years ago when it was shown that in patients with chronic obstructive pulmonary disease, erectile dysfunction and low serum testosterone concentrations correlated with the degree of arterial hypoxia (37, 38). A couple of years later, the same results were obtained in patients with pulmonary fibrosis (39), pulmonary heart (40), and Pickwick syndrome (41). Kouchiyama et al., in the study conducted on 24 patients, showed that greater nocturnal oxygen desaturation in men led to the disruption of the circadian rhythm of testosterone secretion (42).

In the animal models, studies focusing on the effects of continuous or intermittent hypoxia on sex hormones were

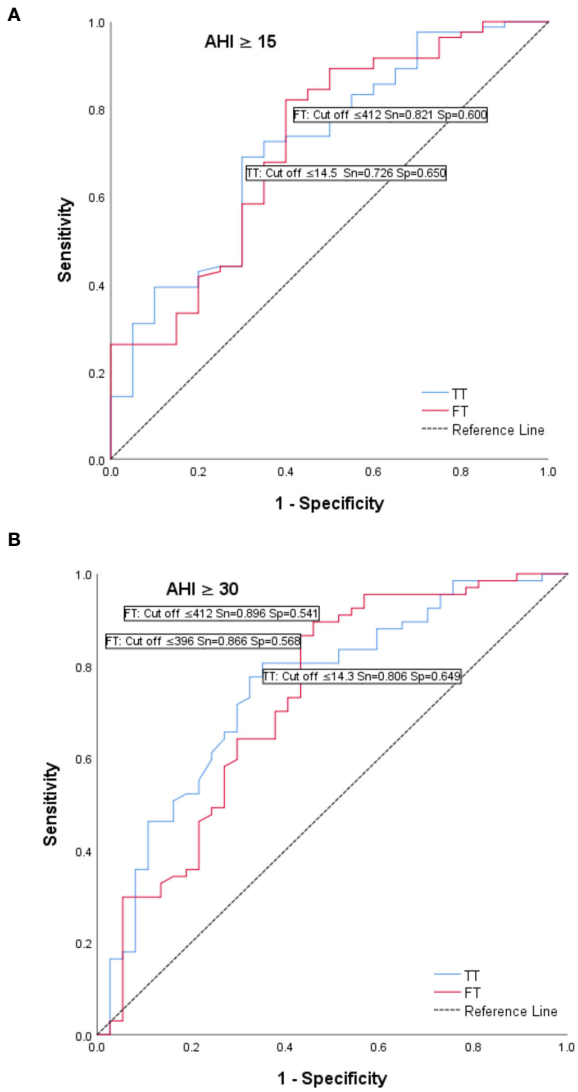

FIGURE 3 | Receiver operating characteristic (ROC) curves – total testosterone (T) and tree testosterone (FT) as discriminative variables for the assessment of sleep apnea severity. **(A)** AUC for AHI ≥ 15 for TT was AUC_T = 0.714 (95% CI 0.587 - 0.841; p = 0.003) with cut off = 14.5 (Sn = 0.726; Sp = 0.650), and for FT AUC_{FT} = 0.719 (95% CI 0.588 - 0.851; p = 0.002) with cut off = 412 (Sn = 0.821; Sp = 0.600). **(B)** AUC for AHI ≥ 30 for TT was AUC_T = 0.748 (95% CI 0.648 - 0.849; p < 0.001) with cut off = 14.3 (Sn = 0.806; Sp = 0.649) and for FT AUC_{FT} = 0.728 (95% CI 0.620 - 0.836; p < 0.001) with cut off = 396 (Sn = 0.866; Sp = 0.568).

inconclusive because - depending on the study - they showed an increase (43), decrease (44, 45), and unchanged testosterone concentrations (46).

Partial or complete upper airway obstruction in OSAS patients is the cause of not only nocturnal oxygen desaturation but also sleep fragmentation. A significant number of studies have been carried out by Luboshitzky and colleagues to investigate whether reproductive hormones are correlated with sleep patterns in men with OSAS. They showed that the patients with fragmented sleep had a blunted nocturnal rise of testosterone only if they did not show REM sleep (47). The same team suggested that OSAS in male patients is associated with reduced androgen secretion resulting from altered pituitary-gonadal function (48). This is caused by obesity and aging, with hypoxia and sleep fragmentation being additional contributing factors in decreasing pulsatile testosterone secretion in these patients (49).

Clinical evidence is contradictory, with some (9, 16–20), but not all (21, 22), studies reporting that OSAS is a factor favoring hypoandrogenemia independent of obesity. This could be due to high heterogeneity of study design, patients' characteristics such as age and BMI, exclusion or inclusion criteria, limited or absent covariates in the data analyses, and the time point of data collection (9, 16–22).

Hammoud et al. published a study similar to ours. This study included 89 severely obese men with a BMI ≥ 35 kg/m^2 to examine the effect of sleep apnea on the reproductive hormones and sexual function in obese men. They showed that increased severity of sleep apnea is associated with lower TT and FT levels independent of age and BMI, which is consistent with the results of our study (18). Their study, unlike our study, did not take into account the metabolic syndrome as a contributing factor to hypoandrogenemia in obese men.

Concerning this latter aspect, there is only one study published by Gambineri et al. that analyzed the severity of OSAS, testosterone levels, and some of the parameters of MetS (waist circumference, FPG, HDL, Tg) in severely obese men. They suggested that OSAS may contribute to causing metabolic abnormalities in men and that this relationship may be in part related to the reduced testosterone concentrations (9). Taking this into consideration, we included metabolic syndrome as a potential confounding factor for hypoandrogenemia in our obese men.

Gambineri and colleagues documented that in men with obesity and OSAS, the severity of hypoxia measured by ODI may be an additional factor in reducing testosterone levels, regardless of BMI and abdominal fatness (9). Our study shows that AHI and ODI, as pivotal markers of OSAS severity are in significant correlation with TT and FT independent of age, BMI, siMS score and MetS components in severely obese men.

The effects of OSAS treatment on testosterone levels are debatable. Continuous positive airway pressure (CPAP) is the most effective non-surgical treatment for OSAS. Furthermore, the efficacy of CPAP on hypoandrogenemia in OSAS male subjects are still controversial. Some studies have demonstrated that CPAP elevates testosterone levels (49, 50). However, the majority of other studies, including two meta-analyses, have reached a different conclusion (51, 52). As pointed out by one of the latest systematic reviews, a small number of included studies in their meta-analysis reported an adequate CPAP use (4 h per night on at least 70% of nights), and thus the results might reflect, at least in part, suboptimal CPAP therapy. Furthermore, Santamaria et al. thoroughly conducted a prospective study of uvulopalatopharyngoplasty therapy effects on testosterone levels in male subjects with moderate and severe OSAS. Interestingly,

they showed improvement in testosterone levels three months after the surgery, with correlated improvement in sleep-disordered breathing without significant changes in BMI (53). On the other hand, CPAP as the mainstay of treatment for OSAS will not cure obesity, as a cornerstone of OSAS and metabolic syndrome, as well as hypoandrogenemia in obese men. In any case, significant weight loss clearly improved OSAS, metabolic syndrome, and hypogonadism associated with obesity (1–5, 54).

Some limitations of this investigation should be acknowledged. The design of our study did not provide for monitoring the symptoms of sexual dysfunction and hypogonadism in the subjects. Thus, we can discuss sex hormones only from the perspective of hypoadrogenism and not in terms of hypogonadism. Another limitation of our study is that instead of using 'full' polysomnography, we used a seven-channel portable sleep recorder. Given the fact that there was no electroencephalography monitoring, we do not have data about the relationships between sleep stages and sex hormone levels. Also, we could not assess if the physical activity or regular alcohol consumption influenced the results of our study, as we did not have this data available.

CONCLUSION

Our data show that obstructive sleep apnea syndrome is in significant correlation with TT and FT levels in severely obese men. Further research is needed to elucidate the complex link between sleep apnea and testosterone levels in obese men for the purpose of appropriate management of these patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Faculty of Medicine, University of Belgrade, Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MT-G, MV, and SV: conceived and designed the study, collected and contributed to the data, and analyzed and interpreted data. MT-G: wrote the manuscript. MV and SV: revised the article. MI, LM, ZA, MS, and AĐ: collected and contributed to the data and revised the article. AK: analyzed and interpreted data, and revised the article. IS: analyzed and interpreted data, made tables, and revised the article. All authors contributed to the article and approved the submitted version.

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The Combined Impact of Female and Male Body Mass Index on Cumulative Pregnancy Outcomes After the First Ovarian Stimulation

Zhonghua Zhao[1,2†], *Xue Jiang*[1,2†], *Jing Li*[1,2], *Menghui Zhang*[1,2], *Jinhao Liu*[1,2], *Shanjun Dai*[1,2], *Hao Shi*[1,2], *Yuling Liang*[1,2], *Li Yang*[1,2] *and Yihong Guo*[1,2*]

[1] *Center of Reproductive Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China,* [2] *Henan Key Laboratory of Reproduction and Genetics, Zhengzhou, China*

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***Correspondence:**
Yihong Guo
13613863710@163.com

[†]*These authors have contributed equally to this work and share first authorship*

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Objectives: To evaluate the combined impact of male and female BMI on cumulative pregnancy outcomes after the first ovarian stimulation.

Design: Retrospective cohort study.

Setting: University-affiliated reproductive medicine center.

Patients: A total of 15,972 couples undergoing their first ovarian stimulations from June 2009 to June 2016 were included. During the follow-up period between June 2009 and June 2018, 14,182 couples underwent a complete ART cycle involving fresh embryo transfer and subsequent frozen embryo transfers (FETs) after their first ovarian stimulations. Patients with a BMI <24 kg/m^2 served as the reference group. Patients with a BMI ≥ 24 kg/m^2 were considered to be overweight, and those with a BMI ≥28 kg/m^2 were considered to be obese.

Intervention(s): None.

Primary Outcome Measure: The primary outcome was the cumulative live birth rate (CLBR), which defined as the delivery of at least one live birth in the fresh or in the subsequent FET cycles after the first ovarian stimulation.

Results: In the analyses of females and males separately, compared with the reference group, overweight and obese females had a reduced CLBR (aOR 0.83, 95% CI 0.7.92 and aOR 0.76, 95% CI 0.64–0.90). Similarly, overweight males had a reduced CLBR (aOR 0.91, 95% CI 0.83–0.99) compared with that of the reference group. In the analyses of couples, those in which the male was in the reference or overweight group and the female was overweight or obese had a significantly lower CLBR than those in which both the male and female had a BMI <24 kg/m^2.

Conclusions: The CLBR is negatively impacted by increased BMI in the female and overweight status in the male, both individually and together.

Keywords: body mass index, assisted reproductive technology, cumulative live birth rate, male, overweight, obesity

INTRODUCTION

Overweight and obesity are defined as abnormal or excessive fat accumulation that threatens the health of the individual. A body mass index (BMI) over 25 kg/m^2 is considered overweight, and a BMI over 30 kg/m^2 is considered obese. The BMIs of Asian populations is generally lower than those of non-Asian populations (1). The World Health Organization (WHO) has predicted that approximately 20% of adults worldwide will be obese in 2025. An elevated BMI is a crucial risk factor for noncommunicable diseases, including cardiovascular diseases, musculoskeletal disorders and certain types of cancer. An increased BMI may also place women at risk for impaired fertility and adverse pregnancy outcomes, especially among couples seeking assisted reproductive technology (ART) (2–4). The obesity state can elevate proinflammatory adipokines through adipose tissue inflammation, such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and free fatty acids (FFAs), which can induce both insulin resistance and compensatory hyperinsulinism (5). Hyperinsulinemia contributes to excess androgen, which is aromatized to estrogen in expansive adipose tissue (6). Raised estrogen levels lead to ovulatory dysfunction through a negative feedback mechanism within the HPO axis. The deleterious impact of a high female BMI on ART outcomes has been extensively studied (7) and systematically reviewed in the clinic (8). However, the couple rather than the individual is the object of interest in IVF treatment for sterile couples. Therefore, the importance of the male partner in couple fecundity should not be neglected, and assessing both male and female BMI is particularly necessary. Our previous study showed that couples with a higher female BMI had a lower live birth rate (LBR) than those with a normal BMI in IVF cycles (9). Similarly, Petersen et al. found that higher BMIs among couples negatively affect the LBR (10). Even if pregnancy is successful after ART treatment, McPherson et al. found that the combination of both maternal and paternal preconception overweight/obesity has a greater impact on infant birthweight (11). However, these studies were all limited by their lack of evaluations of the cumulative live birth rate (CLBR), an indicator of ART success that has been highly recommended in recent years (12). Furthermore, these studies have evaluated the relationship between BMI and CLBR in females only (13) while paying little attention to the relationship between BMI and CLBR in males (14). Therefore, we aimed to evaluate the combined impact of female and male BMI on cumulative pregnancy outcomes after the first ovarian stimulation.

MATERIALS AND METHODS

Patients

This was a retrospective cohort study performed at a single reproductive medicine center of a university affiliated hospital fertility center. Data were collected from the Clinical Reproductive Medicine Management System/Electronic Medical Record Cohort Database (CCRM/EMRCD) at the Reproductive Medical Center, First Affiliated Hospital of Zhengzhou University, and the Henan Province Key Laboratory for Reproduction and Genetics. Cycles were excluded if either or both of the couples had an abnormal karyotype. We also excluded cycles with donor oocytes or sperm and excluded preimplantation genetic testing for aneuploidy (PGT-A) cycles, preimplantation genetic testing for monogenic/single gene defect (PGT-M) cycles, and preimplantation genetic testing for chromosomal structural rearrangement (PGT-SR) cycles. Cycles with no viable embryos were also excluded.

From June 2009 to June 2016, a total of 15,972 couples undergoing their first ovarian stimulation (IVF/ICSI) were screened for inclusion. Our follow-up period was from June 2009 to June 2018 with a minimum of 2 years of follow-up to observe whether the patients achieved live birth in the fresh cycle or subsequent frozen embryo transfer (FET) cycle. In total, 14,182 couples underwent a complete IVF treatment cycle during the follow-up period. A complete IVF treatment cycle was defined as achieving at least one live birth in the fresh or subsequent FET cycle with or without embryos remaining afterward or as not achieving a live birth after using all viable embryos. In total, 1,790 couples with remaining frozen embryos from the first ovarian stimulation discontinued fertility treatment due to personal factors after failing to achieve a live birth. Therefore, we analyzed cumulative pregnancy outcomes among the 14,182 couples. This study was authorized by the Institutional Review Board and Ethics Committee of the First Affiliated Hospital of Zhengzhou University. All the participants signed written informed consent forms.

Dataset

BMI was calculated from information on weight and height at the initial consultation. According to the BMI guidelines for the Chinese population (1), we divided the female and male samples separately into three groups: reference group (BMI<24 kg/m^2), overweight group (BMI≥ 24 kg/m^2) and obese group (BMI≥ 28 kg/m^2). For the couples analysis, we combined the females and males according to the BMI group.

Ovarian Stimulation Schemes

The protocols were formulated according to the day of the patient's menstrual cycle when she visited the hospital. A patient who was in the follicular phase was injected with triptorelin depot (decapeptyl 3.75 mg; Ipsen Pharma, France) intramuscularly on days 2–3 of the menstrual cycle. Pituitary downregulation was achieved after 28–42 days (E2 < 50 pg/ml, LH < 3 mIU/ml and ovarian cysts less than 10 mm). Patients who were in the luteal phase were injected with triptorelin (Ferring GmbH, 0.1 mg, Switzerland; Ipsen Pharma Biotech, 0.1 mg, France) intramuscularly during the midluteal phase, and 10 days later, the dose was decreased to 0.05 mg/d until pituitary downregulation (E2 < 50 pg/ml, LH<3 mIU/ml and ovarian cysts less than 10 mm) was achieved. Follicle-stimulating hormone (FSH) was used to start ovarian hyperstimulation (Gonal-F, Serono, Puregon, Netherlands, u-FSH, Livzon). The initial dose was dependent on the patient's characteristics and antral follicle count (AFC), and the subsequent dose was adjusted according to follicle development and hormone levels.

Human menopausal gonadotropin (HMG, Livzon) was added if needed. Oocyte maturation was triggered by 2000 IU of human chorionic gonadotropin (hCG, Livzon) and recombinant human chorionic gonadotropin (Merck Serono, Italy) when the maximal follicle diameter was more than 20 mm and when more than 2/3 follicles were >16 mm in diameter. Oocyte retrieval was performed 36–37 hours after hCG administration and with transvaginal ultrasound guidance. The insemination method was chosen based on sperm parameters. Then, the patient underwent embryo transfer (ET) (day-3 cleavage-stage embryos or day-5 blastocysts); however, patients at risk for ovarian hyperstimulation syndrome (OHSS), those with progestin suppression of the LH surge and those requiring fertility preservation underwent whole-embryo cryopreservation. Luteal phase support was sustained with progesterone vaginal gel (Merck Serono, Switzerland) at a dose of 90 mg/day from the day of ovum pick-up (OPU).

Endometrial preparation schemes for FET in the current study included natural cycles and artificial (estrogen (E)-P) cycles. The detailed procedures are described in our previous report (15).

Outcomes

The primary outcome was the cumulative live birth rate, defined as the delivery of at least one live birth in the fresh or in the subsequent FET cycles, and only the first live birth event was considered in the analysis. Live birth was defined as the delivery of an infant after at least 24 weeks' gestational age. The secondary outcome was the cumulative clinical pregnancy rate (CCPR) calculated based on observations of a gestation sac by B-mode ultrasound 35 days after ET.

Statistical Analysis

SPSS (Statistical Package for Social Science, SPSS Inc., Chicago, IL, USA) version 26.0 was used for data analysis. Continuous variables are presented as the mean ± SD, and differences between groups were compared by means of one-way ANOVA. Categorical variables are presented as frequencies (percentages) and were compared using the chi-square test. All tests were two-sided, and statistical significance was defined as $P<0.05$.Pairwise comparisons between all adjacent groups were performed with the Bonferroni correction, and $P<0.05/3$ was set as a significant difference. Logistic regression was performed for the pregnancy outcomes. The results are presented as the adjusted odds ratio (aOR) and 95% confidence interval (CI).

RESULTS

In total, 15,972 couples underwent their first ovarian stimulations, 14,182 (88.8%) of these couples underwent a complete ART cycle, and 1,790 (11.2%) discontinued fertility treatment. Therefore, we analyzed cumulative pregnancy outcomes among the 14,182 couples; of these couples, 11,257 achieved at least one live birth, and 2,925 did not achieve a live birth after using all of their frozen embryos from the first ovarian stimulation (**Figure 1**).

Table 1 shows the clinical characteristics of the study population according to sex and BMI. The average age of the males was significantly higher in the overweight group than in the other groups ($P<0.05$). The average age of the females was significantly lower in the reference group ($P<0.05$), but there was no difference in age between the overweight group and obese group ($P>0.05$). Reference group showed higher baseline serum FSH and LH levels and lower AFC than the other groups ($P<0.05$). Regarding the cause of infertility, polycystic ovary syndrome (PCOS) was significantly more common among females in the overweight and obese groups than in the reference group ($P<0.05$).

Table 2 shows the treatment and pregnancy outcomes according to sex and BMI. Among females, the three groups were comparable in terms of the numbers of oocytes retrieved, insemination method and CCPR ($P>0.05$). The Gn dose differed between the groups, and the highest dose was administered in the obese group ($P<0.05$). The CLBRs of the overweight group and obese group were comparable (77.6% *vs.* 77.2%, $P>0.05$) and significantly lower than that in the reference group (80.1%, $P<0.05$). Among males, although the difference in terms of the CCPR and CLBR between the three groups was statistically significant per the chi-square test, there was no statistically significant difference between any two groups after the Bonferroni correction (reference *vs.* overweight *vs.* obese, CCPR: 84.0% *vs.* 82.8% *vs.* 84.8%, CLBR: 79.9% *vs.* 78.4% *vs.* 80.3%, $P<0.05$).

Table 3 shows the results of the multilevel analysis according to female and male BMI. After adjustments were made for confounders, an increased female BMI was associated with worse pregnancy outcomes after the first ovarian stimulation. The obese group had worse results than the overweight group. Compared with the reference group, the overweight and obesity groups had 17% (95% CI 0.75–0.92) and 24% (95% CI 0.64–0.90) reductions in CLBR and 12% (95% CI 0.79–0.98) and 25% (95% CI 0.62–0.90) reductions in CCPR, respectively. Similar tendencies were seen among males in the overweight group compared with males in the reference group, with a 9% (95% CI 0.83–0.99) reduction in CLBR. The effect of male obesity on the CLBR was not statistically significant ($P>0.05$).

Table 4 shows the results of the multilevel analysis of pregnancy outcomes based on the combined female and male BMI. The association persisted after adjustments for confounding factors. The reference group (couples with BMI<24 kg/m^2) accounted for the largest proportion (31.3%). As shown in the findings from the separate analysis of females and males in **Table 3**, couples with an overweight or obese female (with any male BMI status) had a significantly lower CLBR than couples in which both the male and female had a BMI <24 kg/m^2, but the reductions in aORs were not statistically significant for couples with male obesity. Among couples with a male BMI <24 kg/m^2, those with female obesity had a significantly lower CLBR than couples with an overweight female [aOR (95% CI): 0.69 (0.52–0.92) *vs.* 0.83 (0.70–0.97), $P<0.05$]. Similarly, among couples with an overweight male,

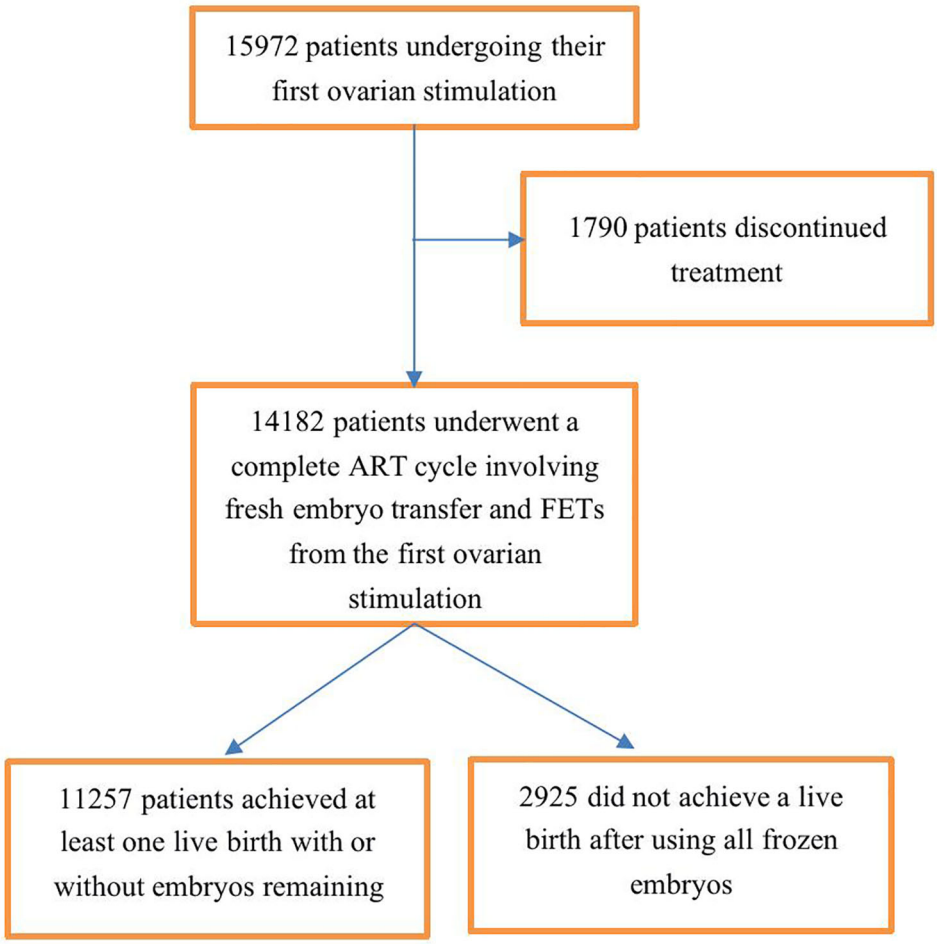

FIGURE 1 | Flowchart of patient selection. A complete ART treatment cycle is defined as achieving at least one live birth in the fresh cycle or subsequent frozen embryo transfer (FET) cycles with or without embryos remaining afterward or is defined as not achieving a live birth after using all fresh and frozen embryos.

those with female obesity had a lower CLBR than those with an overweight female [aOR (95% CI): 0.73 (0.55–0.95) *vs.* 0.78 (0.67–0.90), $P<0.05$]. Couples consisting of an obese male and an overweight or obese female had a decreased CLBR compared with those consisting of a female from reference group [aOR (95% CI): 0.84 (0.68–1.04) *vs.* 0.84 (0.59–1.19) *vs.* 1.04 (0.90–1.21), respectively], though the P value was >0.05. The results were worse in the group in which both members of the couple were overweight, rather than only than female [aOR (95% CI): 0.78 (0.67–0.90) *vs.* 0.83 (0.70–0.97), $P<0.05$].

DISCUSSION

In summary, the major finding of this study was that both in the separate and combined analyses, increased female BMI and overweight in males adversely affected the cumulative pregnancy outcomes after the first ovarian stimulation, leading to decreases in the CLBR.

Effects of Female Overweight/Obesity on the CLBR

In our study, females with an increased BMI had a significantly lower CCPR and CLBR, which showed a downward trend when the female was obese compared with when she was overweight. The negative effects of increased female BMI on pregnancy outcomes have been well established in prior work. For example, a 2019 systematic review showed that compared with normal-weight women, overweight women had a lower probability of giving birth following IVF [RR: 0.94; 95% CI: (0.91–0.97)], and women with obesity had a significantly lower LBR [RR: 0.85; 95% CI: (0.84–0.87)] (8). A similar result has been reported in other observational studies; specifically, Kawwass et al. (4) showed that in a retrospective cohort study (494,097 fresh autologous IVF cycles), compared with normal-weight women, women with obesity had a significantly lower probability of intrauterine pregnancy and live birth. In another retrospective analysis of 239,127 fresh IVF cycles, Provost et al.

TABLE 1 | Clinical characteristics of the study population according to sex and body mass index.

		Reference	Overweight	Obese	*P* value
Female	N (total n=14,182)	10,268 (72.4)	3113 (22.0)	801 (5.6)	
	Age at start of 1st cycle (years)				
	Mean ± SD	30 ± 4.8	31.1 ± 5.1	31.0 ± 4.9	<0.001[ab]
	<30 years	5201 (50.7)	1266 (40.7)	327 (40.8)	<0.001[ab]
	≥30 years	5067 (49.3)	1847 (59.3)	474 (59.2)	
	Infertility cause, n (%)				<0.001[abc]
	Tubal factor	4887 (47.6)	1387 (44.6)	336 (41.9)	
	Male	2483 (24.2)	667 (21.4)	167 (20.8)	
	PCOS	575 (5.6)	342 (11.0)	132 (16.5)	
	Uterine anomalies	277 (2.7)	112 (3.6)	26 (3.2)	
	Endometriosis	221 (2.2)	51 (1.6)	7 (0.9)	
	Ovarian disease	95 (0.9)	35 (1.1)	6 (0.7)	
	Missing observations	1730 (16.8)	519 (16.7)	127 (15.9)	
	Basal FSH (IU/L)	7.3 ± 2.5	6.9 ± 2.4	6.5 ± 2.0	<0.001[abc]
	Basal LH (IU/L)	5.8 ± 3.5	5.4 ± 3.5	5.5 ± 4.1	<0.001[ab]
	Antral Follicle Count(AFC)	12.1 ± 0.1	12.7 ± 0.1	14.1 ± 0.2	<0.001[abc]
Male	N (total n=14,182)	5845 (41.2)	5949 (41.9)	2388 (16.8)	
	Age at start of 1st cycle (years)				
	Mean ± SD	30.8 ± 5.4	32.4 ± 5.8	31.7 ± 5.3	<0.001[abc]
	<30 years	2689 (46.0)	2023 (34.0)	891 (37.3)	<0.001[abc]
	≥30 years	3156 (54.0)	3926 (66.0)	1497 (62.7)	
	Infertility cause, n (%)				<0.001[ab]
	Male	1510 (25.8)	1289 (21.7)	518 (21.7)	
	Female	3402 (58.2)	3617 (60.8)	1470 (61.6)	
	Missing observations	933 (16.0)	1043 (17.5)	400 (16.8)	

Data are presented as the mean ± SD or frequency (percentage).
The differences between groups (Bonferroni correction, P < 0.05/3) are indicated by the following superscripts:
[a]P: Comparison of reference and overweight groups.
[b]P: Comparison of reference and obese groups.
[c]P: Comparison of overweight and obese groups.

TABLE 2 | Treatment and pregnancy outcomes according to sex and body mass index.

		Reference	Overweight	Obese	P
Female	Total gonadotropin dose (IU)	2032.9 ± 889.1	2276.0 ± 937.1	2507.8 ± 974.4	<0.001[abc]
	No. of oocytes retrieved	12.4 ± 6.6	12.6 ± 6.9	12.8 ± 6.8	0.314
	Insemination method, n (%)				0.051
	IVF	7206 (70.2)	2257 (72.5)	590 (73.7)	
	ICSI	2897 (28.2)	811 (26.1)	200 (25.0)	
	IVF+ICSI	165 (1.6)	45 (1.4)	11 (1.4)	
	Cumulative clinical pregnancies per woman	8632 (84.1)	2579 (82.8)	653 (81.5)	0.066
	Cumulative live births per woman	8224 (80.1)	2415 (77.6)	618 (77.2)	0.003[ab]
Male	Insemination method, n (%)				<0.001[ab]
	IVF	4032 (69.0)	4322 (72.7)	1699 (71.1)	
	ICSI	1737 (29.7)	1525 (25.6)	646 (27.1)	
	IVF+ICSI	76 (1.3)	102 (1.7)	43 (1.8)	
	Cumulative clinical pregnancies per woman	4695 (84.0)	4924 (82.8)	2027 (84.8)	0.035
	Cumulative live births per woman	4467 (79.9)	4662 (78.4)	1919 (80.3)	0.039

Data are presented as the mean ± SD or frequency (percentage).
The differences between groups (Bonferroni correction, P < 0.05/3) are indicated by the following superscripts:
[a]P: Comparison of reference and overweight groups.
[b]P: Comparison of reference and obese groups.
[c]P: Comparison of overweight and obese groups.

(7) reported that there was a significant decrease in CPR and LBR as BMI increased.

The aforementioned studies did not evaluate the association between female BMI and CLBR.A recent study about the correlation between female BMI and CLBR showed that the CLBR in overweight and obese patients decreased significantly compared with normal weight patients (13). In our previous study, we also found that overweight and obesity were associated with a decreased CCPR and CLBR in both women with PCOS and women with tubal factor infertility (16). Consistent with our

TABLE 3 | Results from logistic regression analyses of pregnancy outcomes in IVF/ICSI cycles according to sex and stratified by body mass index (BMI).

		CCPR		CLBR	
		Crude OR (95%CI) *P*-value	**Adjust OR (95%CI) *P*-value**	**Crude OR (95% CI) *P*-value**	**Adjust OR (95%CI) *P*-value**
Female					
	Reference	1 (ref)	1 (ref)	1 (ref)	1 (ref)
	Overweight	0.92 (0.82-1.02) 0.106	0.88 (0.79-0.98) 0.021	0.86 (0.78-0.95) 0.002	0.83 (0.75-0.92) <0.001
	Obese	0.84 (0.69-1.01) 0.060	0.75 (0.62-0.90) 0.003	0.84 (0.71-1.00) 0.046	0.76 (0.64-0.90) 0.002
Male					
	Reference	1 (ref)	1 (ref)	1 (ref)	1 (ref)
	Overweight	0.91 (0.83-1.00) 0.061	0.92 (0.83-1.01) 0.075	0.91 (0.83-0.99) 0.029	0.91 (0.83-0.99) 0.037
	Obese	1.07 (0.93-1.22) 0.349	1.07 (0.94-1.22) 0.331	1.02 (0.91-1.15) 0.710	1.03 (0.91-1.16) 0.682

Female analyses adjusted for age, baseline serum FSH level,baseline serum LH level.
AFC and infertility cause.
Male analyses adjusted for age.

TABLE 4 | Results from logistic regression analysis of joint couple BMI on pregnancy outcomes in IVF/ICSI cycles.

Combination of BMI (kg/m^2)			CCPR				CLBR			
Female	**Male**	**n (%)**	**n (%)**	**OR**	**95% CI**	***P* value**	**n (%)**	**OR**	**95% CI**	***P* value**
Reference	Reference	4432 (31.3)	3745 (84.5)	1 (ref)	–		3577 (80.7)	1 (ref)	–	
Reference	Overweight	4251 (30.0)	3531 (83.1)	0.93	(0.82–1.04)	0.191	3360 (79.0)	0.93	(0.83–1.03)	0.160
Reference	Obese	1585 (11.2)	1356 (85.6)	1.10	(0.93–1.29)	0.264	1287 (81.2)	1.04	(0.90–1.21)	0.572
Overweight	Reference	1133 (8.0)	942 (83.1)	0.88	(0.73–1.05)	0.144	885 (78.1)	0.83	(0.70–0.97)	0.022
Overweight	Overweight	1383 (9.8)	1136 (82.1)	0.83	(0.71–0.98)	0.027	1061 (76.7)	0.78	(0.67–0.90)	0.001
Overweight	Obese	597 (4.2)	501 (83.9)	0.92	(0.72–1.16)	0.465	469 (78.6)	0.84	(0.68–1.04)	0.108
Obese	Reference	280 (2.0)	226 (80.7)	0.67	(0.49–0.92)	0.013	214 (76.4)	0.69	(0.52–0.92)	0.012
Obese	Overweight	315 (2.2)	257 (81.6)	0.75	(0.56–1.01)	0.060	241 (76.5)	0.73	(0.55–0.95)	0.022
Obese	Obese	206 (1.5)	170 (82.5)	0.80	(0.55–1.16)	0.231	163 (79.1)	0.84	(0.59–1.19)	0.325

Data are presented as ORs with 95% CIs.
Confounding factors included female age, male age, baseline serum FSH level, baseline serum LH level,AFC and infertility cause.

findings, an American study conducted by Goldman et al. (17) showed that women with overweight, class III obesity or superobesity had progressively lower CLBRs [HR (CI): 0.96 (0.93–0.99), 0.76 (0.68–0.85), and 0.41 (0.26–0.63), respectively]. The same results were also reported by Toftager et al. (18) and Hu et al. (14) found that females with obesity had a lower CCPR and CLBR than females who were overweight.

Effects of Male Overweight/Obesity on CLBR

Regarding males in this study, CLBR was significantly negatively influenced only by overweight. To our knowledge, the effect of male BMI on ART outcomes is contradictory, especially for CLBR. For example, some studies have reported the negative effect of a higher male BMI on pregnancy outcomes (9, 19), while Umul et al. (20) and Merhi et al. (21) have reported no effect. Hu et al. (14) found no significant correlation between paternal BMI and CLBR in a multiple regression model, whereas paternal overweight had a negative impact on the CLBR in women over 35 years old. Nevertheless, we failed to observe a significant difference in the effect of male obesity on cumulative pregnancy outcomes. It may be that the number of obese males was relatively small (41.2% in the reference group, 41.9% in the overweight group, and 16.8% in the obese group), which led to a statistically undetectable difference. Next, because of the inherent limitations of retrospective data, we were unable to set an exclusion criterion that was strictly standard for males, which may contribute to selection bias. In addition, we did not have information about sperm, which is a potential confounder affecting the CLBR (22).

Effects of Combined BMI on CLBR

To date, studies of the synergistic effects of male and female BMI on CLBR are scarce. We extended the field and found that an increased female BMI had a negative impact on the CLBR regardless of whether the male was in the reference or overweight group. Additionally, the results worsen when both members of the couple are overweight rather than when only the female is. Ramlau-Hansen et al. (23) found that couples have a high risk of infertility if they are both obese. Setti et al. (24) observed that couples with a normal BMI had a significantly higher fertilization rate, high-quality embryo rate on day 2, blastocyst development rate, and implantation rate than couples in which at least one partner had an abnormal BMI (>24.9 kg/m^2) in ICSI cycles. Similarly, an animal study based on diet-induced obese mice also showed that combined parental obesity led to a lower blastocyst rate and slower embryo development speed than single parental obesity (25). Regarding

time-to-pregnancy (TTP) in couples, Sundaram et al. observed that couples whose BMIs were within obese class II (≥35 kg/m^2) had a longer TTP than couples whose BMIs were <25 kg/m^2 (26). Consistent with our study, a retrospective study showed that couples with a higher female BMI had a lower LBR than couples with normal weight after the IVF cycle, and no association was found in ICSI cycles (9), which was similar to the findings of Petersen et al. (10). They found that higher BMIs among members of the couple negatively affect the LBR. On the basis of the aforementioned studies, we further observed a cumulative negative effect of female BMI on the CLBR, namely, that female obesity had more negative effects than female overweight among couples with a male BMI in the reference or overweight group. The same cumulative negative effect of male BMI was achieved among couples with female overweight and obesity.

Strengths and Limitations

The major strengths of our study are its ability to fill a gap in the existing literature by examining the joint effect of male and female BMI on cumulative pregnancy outcomes after the first ovarian stimulation. Second, our study had a large sample size, which allows for more exact estimates of outcomes. Nevertheless, there are some limitations in our study. First, this was a retrospective design that included a single medical center. Second, the data for smoking status, alcohol intake, metabolic health of the patients and whether diabetes was present was not recorded and their influence cannot be eliminated, which weakens the generalizability of the findings. Additionally, the lack of detailed data of male also weakens the universality of conclusions. Therefore, we urge caution in interpreting the study results.

CONCLUSION

In conclusion, the results of our study indicate that an increased BMI in females and overweight in males, both independently and combined, negatively impact the cumulative pregnancy outcomes after the first ovarian stimulation, leading to a lower CLBR. Therefore, effective management of the couple's BMI, such as weight loss and lifestyle changes, might help to improve pregnancy outcomes. With the joint action of the members of the couple, it will be easier to implement these changes and more effectively reach an ideal BMI.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board and Ethics Committee of the First Affiliated Hospital of Zhengzhou University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZZ, XJ, and YG contributed to the conception and design of the study. JL, MZ, and JHL were responsible for the data collection and checking. ZZ and XJ performed the data analysis, interpretation and manuscript drafting. SD and HS assisted in the data analysis. YL, LY, and YG supervised the project administration and assisted in writing the paper. All authors contributed to the article and approved the submitted version.

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