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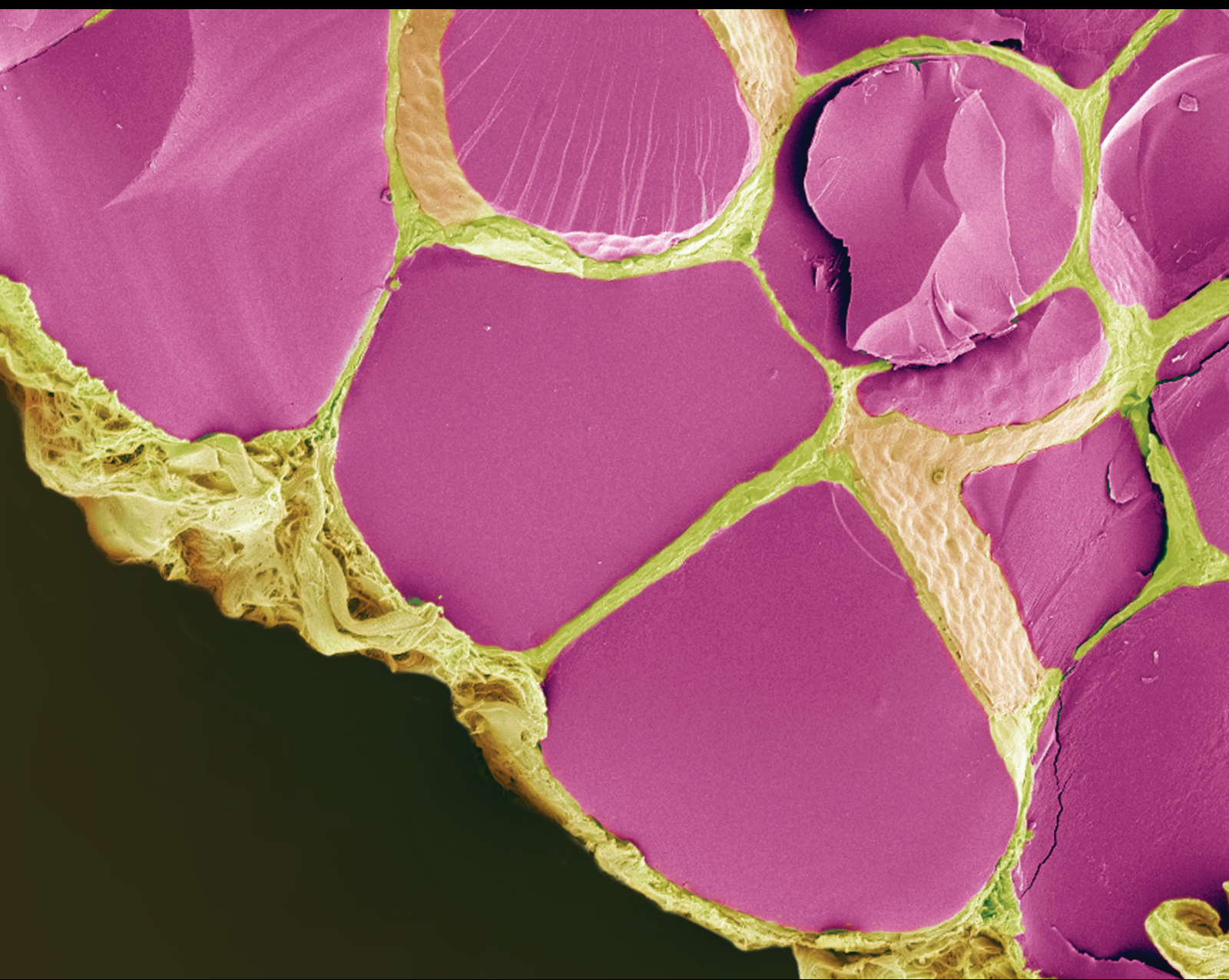
Current Management of Polycystic Ovary Syndrome: From Bench to Bedside

Special Issue Editor in Chief: Antonio Simone Laganà
Guest Editors: Salvatore Giovanni Vitale, Marco Noventa, and Amerigo Vitagliano



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Editorial Board

Contents

Hindawi
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Editorial

Current Management of Polycystic Ovary Syndrome: From Bench to Bedside

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Polycystic ovary syndrome (PCOS) affects 6–10% of women in reproductive age and is characterized by hyperandrogenism, insulin resistance, and chronic anovulation [1]. It is a heterogeneous syndrome with not completely understood etiology that is related to a complex interaction between metabolic, endocrine, genetic, and environmental factors. Increasing evidence suggests that insulin resistance and secondary hyperinsulinemia play a key synergistic role with hyperandrogenism in the development and maintenance of metabolic alterations and anovulation or irregular cycles in both obese and lean patients with PCOS [2]. On that basis, current treatment strategies aim at reducing insulin resistance in patients with PCOS and, consequently, to reach a reduction of compensatory hyperinsulinemia, improving metabolic and ovulatory features [3–5]. Insulin-sensitizer drugs are the recommended first-line therapy according to recent guidelines [6] for women with PCOS and metabolic abnormalities [7–9] with the aim at improving fertility [10–13], although physical activity and lifestyle change should be considered the first steps in overweight and obese PCOS patients to achieve weight loss [14, 15].

In this scenario, we are honored to introduce this special issue, which contains five articles that may shed new light on the topic. In particular, three articles are focused on metabolic disturbances in PCOS women: the first one ("Free Testosterone Reflects Metabolic as well as Ovarian Disturbances in Subfertile Oligomenorrheic Women") found that sex hormone-binding globulin and calculated free testosterone are associated with both ovarian ultrasound and metabolic parameters, such as the body mass index (BMI) and insulin resistance, suggesting a pivotal role for androgen excess in PCOS-related subfertility and ovulatory dysfunction; the second article ("Pericardial Fat Relates to Disturbances of Glucose Metabolism in Women with the Polycystic Ovary Syndrome, but Not in Healthy Control Subjects") found that pericardial fat measured using 1H-magnetic resonance spectroscopy and imaging is positively related to atherogenic lipid profiles, BMI, waist circumference, and liver fat in women with PCOS, suggesting it as a potential noninvasive tool to predict metabolic prognosis in this population; the third article ("Low-Dose Spironolactone-Pioglitazone-Metformin Normalizes Circulating Fetuin-A Concentrations in Adolescent Girls with Polycystic Ovary Syndrome") highlights that a low-dose combination of insulin sensitizers and an antiandrogen is able to normalize fetuin-A levels in adolescent girls with PCOS. Considering that high levels of fetuin-A have been associated with greater risks for type 2 diabetes and with features of metabolic syndrome, this treatment may significantly reduce metabolic consequences and prevent acute events.

Besides these three articles related to metabolic disturbances and their treatment, another paper ("The Place of In Vitro Maturation in PCO/PCOS") depicted a clear and

accurate summary of available evidence regarding the optimization of culture media, laboratory protocols, pregnancy rates, and neonatal outcomes following in vitro maturation (IVM) of human oocytes in PCOS women, which are known to have a variable incidence of infertility and worse outcomes following assisted reproductive technology.

Finally, the last paper ("Uterine Artery Doppler in Pregnancy: Women with PCOS Compared to Healthy Controls") investigated differences in the uterine artery pulsatility index (UtAPI) between pregnant women with PCOS and healthy controls and explored the possible effects of metformin on this parameter. Interestingly, the authors found that there was no difference in the UtAPI between women with PCOS and healthy controls in the first and second trimesters of pregnancy; in addition, metformin was not found to have an immediate effect on the UtAPI.

Overall, the manuscripts published in this special issue add significant and novel elements for the understanding of the etiology, pathophysiology, diagnosis, and treatment of this complex and multifaceted syndrome. We offer these new insights to the readers, hoping that they will stimulate further debate and address new fields of investigation in the next future.

Disclosure

The authors alone are responsible for the content and writing of the paper.

Conflicts of Interest

The authors have no proprietary, financial, professional, or other personal interests of any nature in any product, service, or company.

Antonio Simone Laganà
Salvatore Giovanni Vitale
Marco Noventa
Amerigo Vitagliano

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Research Article

Free Testosterone Reflects Metabolic as well as Ovarian Disturbances in Subfertile Oligomenorrheic Women

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Background. Diagnosing polycystic ovary syndrome (PCOS) is based on ovulatory dysfunction, ovarian ultrasound data, and androgen excess. Total testosterone is frequently used to identify androgen excess, but testosterone is mainly bound to sex hormone-binding globulin (SHBG) and albumin. Only 1-2% of nonprotein-bound testosterone (so-called free testosterone) is biologically active and responsible for androgen action. Moreover, automated immunoassays which are frequently used for female testosterone measurements are inaccurate. *Objective*. To assess the clinical usefulness of liquid chromatography-tandem mass spectrometry measured testosterone and calculated free testosterone in subfertile women attending a fertility clinic with oligomenorrhea and suspected PCOS. *Methods*. Hormonal and metabolic parameters were evaluated, and ovarian ultrasound was performed. Total testosterone was measured by liquid chromatography-tandem mass spectrometry. Free testosterone was calculated from total testosterone and SHBG. *Results*. Sixty-six women were included in the study. Total testosterone was associated with ovarian volume and antral follicle count but not with metabolic parameters. However, SHBG and calculated free testosterone were associated with both ovarian ultrasound and metabolic parameters, such as BMI and insulin resistance. *Conclusions*. Assessing SHBG and free testosterone is important in evaluating androgen excess in subfertile women with ovulatory dysfunction and suspected PCOS, as it reflects both ovarian and metabolic disturbances.

1. Introduction

Polycystic ovary syndrome (PCOS) is very common in women with subfertility and oligomenorrhea [1–3]. Diagnosing PCOS is based on the "Rotterdam criteria": the presence of at least two of the following conditions: ovulatory dysfunction, polycystic ovary morphology (PCOM) on ultrasound, or androgen excess [4]. Although very frequently used, these criteria have important limitations. Due to improved ultrasound imaging, PCOM is often present, also in normally cycling women without other PCOS features. Furthermore, these diagnostic criteria do not take into account metabolic parameters, and patients with clearly different metabolic characteristics are diagnosed under the umbrella term of PCOS [5, 6].

Diagnosing androgen excess in women can also be challenging, as it may be assessed either clinically (hirsutism or acne), biochemically, or both [2, 4]. It remains unclear which

androgen(s) should be measured to assess biochemical hyperandrogenemia in women suspected for PCOS [7]. In clinical practice, total testosterone (total T) is frequently used, but measuring total T levels in women by automated immunoassays (IA) is often inaccurate. Instead of IA, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has therefore been proposed as the method of choice for accurate measurement of low testosterone levels in women [8–11]. Also, androstenedione (A4), the steroid precursor of testosterone, has been proposed as a marker of androgen excess in PCOS patients, especially in identifying PCOS patients with a higher metabolic risk [12].

Furthermore, testosterone is mainly bound to sex hormone-binding globulin (SHBG) and albumin. Only a small fraction (1-2%) circulates as nonprotein-bound free testosterone (free T), and it is only the free T fraction that can enter the cell and exert androgen activity [13].

In a recent best practice summary, free T was proposed as the most sensitive marker for diagnosing androgen excess [10] and equilibrium dialysis as the preferred measurement method. However, this technique is only available in a limited number of reference laboratories [10, 14]. Instead of a direct free T measurement, calculated free T can be used to determine hyperandrogenemia in PCOS patients [9, 10]. Inevitably, these calculations require an accurate measurement of total T and SHBG [2]. Fortunately, when LC-MS/MS-measured total T is used, there is an excellent correlation between calculated free T and measured free T in women, and calculated free T can be used to evaluate female androgen status [9, 10, 15].

However, to date, only a limited number of studies have investigated the clinical correlates of these newly emerging LC-MS/MS measurements in the appraisal of female androgen status. Specifically, the use of LC-MS/MS-measured total T, A4, and free T (calculated from LC-MS/MS total T) is not well established in the diagnosis of PCOS. Nevertheless, accurately diagnosing hyperandrogenism is important, as women with androgen excess are at increased risk of developing type 2 diabetes and the metabolic syndrome [16].

In this study, we assessed the clinical usefulness of using state-of-the-art LC-MS/MS technology to measure sex steroids in subfertile women with oligomenorrhea and suspected PCOS. Furthermore, associations between total and free testosterone and metabolic and ovarian parameters were analyzed.

2. Methods

2.1. Subjects. 97 women with oligo- or amenorrhea (cycle length > 38 days) were recruited at the Leuven fertility center. All women were screened for pregnancy and congenital adrenal hyperplasia (21-hydroxylase deficiency). Women taking oral contraceptives were excluded. Subjects with hyperprolactinemia ($n = 1$), newly diagnosed type 2 diabetes ($n = 1$), active thyroid disease ($n = 1$), hypothalamic amenorrhea (luteinizing hormone (LH), follicle-stimulating hormone (FSH) below the lower limits of the reference intervals (<2.4 U/L for LH and <3.5 U/L for FSH), $n = 1$), or premature ovarian failure (FSH > 12 U/L) ($n = 2$) were excluded. Furthermore, 3 women were excluded because of an ovulatory (LH > 40 U/L) or luteal blood sample (progesterone > 1.5 μg/L). In 22 women, there was no serum available for additional sex steroid measurements, and these women were also excluded, leaving 66 women in the study sample (Supplementary Figure 1).

The study protocol was approved by the local ethical board of the University Hospitals Leuven. All patients gave written informed consent.

2.2. Clinical Assessments. At inclusion, weight, height, waist circumference, and blood pressure were recorded for all patients. BMI was calculated from weight and height. Hirsutism was assessed with the simplified Ferriman-Gallwey score, and patients were classified with hirsutism if this score was ≥3 [17]. Acne was self-reported. Patients with a BMI < 25 kg/m^2 were classified as having a normal BMI; patients with a BMI between 25 and 29.9 were classified as overweight, and those with a BMI ≥ 30 as obese.

2.3. Sex Steroid Measurements by Liquid Chromatography-Tandem Mass Spectrometry. Estradiol (E2) and estrone (E1) were measured by LC-MS/MS as described previously [18]. Total T and A4 were measured by a newly developed LC-MS/MS method. Method details are described in Supplementary Materials. Free testosterone was calculated with the Vermeulen formula [19]. LC-MS/MS measurements were compared with originally reported values by direct immunoassay (Diasorin Gamma Coat) for A4 and electrochemiluminescence immunoassay (ECLIA) on a Modular E platform (Roche Diagnostics) for total T for all patients if available in the medical records ($n = 57$).

2.4. Other Laboratory Measurements. LH, FSH, progesterone, SHBG, thyroid-stimulating hormone (TSH), dehydroepiandrosterone sulphate (DHEAS), and fasting insulin were measured by ECLIA (Modular E170 from Roche Diagnostics). Anti-Mullerian hormone (AMH) was measured by enzyme-linked immunosorbent assay (ELISA) (Beckman Coulter Gen II). Fasting glucose, total cholesterol, HDL cholesterol, and triglycerides were measured by a colorimetric method (Cobas c702 from Roche Diagnostics). LDL cholesterol was calculated from total cholesterol, HDL cholesterol, and triglycerides by the Friedewald formula [20]. The LH/FSH ratio was calculated by dividing the LH concentration in U/L by the FSH concentration in U/L. Insulin resistance was calculated using the updated homeostasis model assessment of insulin resistance (HOMA-IR) [21].

2.5. Ovarian Ultrasound. Two-dimensional vaginal ultrasound was performed by an experienced gynaecologist (Voluson E8, GE Healthcare). For both ovaries, the number of antral follicles (AFC) was counted, from which the mean AFC was calculated [22]. For both ovaries, ovarian volume (OV) was calculated ($0.5 \times \text{length} \times \text{width} \times \text{thickness}$). The mean volume of the left and right ovary was also calculated. Furthermore, follicle localization (random, peripheral, or both) and follicle size (uniform or nonuniform) were registered.

Table 1: Biochemistry, hyperandrogenism, metabolic and ovarian parameters, and PCOS diagnosis for 66 women with oligomenorrhea.

	Mean (SD) or n (%)	Reference interval	Limit of quantification
Age	28.3 (3.0)		
Biochemistry			
Total T (ng/dL)	46.5 (23.7)	≤41	2.5
A4 (ng/dL)	180 (90)	≤240	2.5
Free T (ng/dL)	0.57 (0.37)	≤0.49	
SHBG (nmol/L)	70.1 (32.6)	41–103	2.0
E2 (ng/L)	59.2 (50.6)	15–350	1.3
E1 (ng/L)	61.9 (33.4)	17–200	1.2
LH (U/L)	9.4 (4.8)	2.4–12.6	0.1
FSH (U/L)	5.8 (1.3)	3.5–12.5	0.1
LH/FSH	1.6 (0.9)		
DHEAS (μg/dL)	212 (91)	98.8–340	0.1
AMH (ng/mL)	8.4 (5.4)	1.0–9.5	0.03
Clinical hyperandrogenism			
Hirsutism score	2.1 (2.4)	<3	
Having hirsutism	21 (33%)		
Having acne	28 (44%)		
Metabolic parameters			
BMI	25.0 (5.2)	18.5–24.9	
% normal BMI	36 (54.6%)		
% overweight	21 (31.8%)		
% obese	9 (13.6%)		
Waist circumference (cm)	86.4 (13.3)	<80	
Glucose (mg/dL)	92.2 (15.7)	80–110	2
Insulin (pmol/L)	67.4 (40.6)	17.8–173	0.3
HOMA-IR	1.25 (0.74)		
Total cholesterol (mg/dL)	174.6 (29.6)	≤190	3.9
HDL cholesterol (mg/dL)	52.0 (14.2)	≥45	3.1
LDL cholesterol (mg/dL)	104.6 (25.0)	≤115	
Triglycerides (mg/dL)	90.1 (50.2)	≤150	8.8
Ovarian ultrasound parameters ($n = 53$)			
Mean ovarian volume (mL)	10.0 (4.1)	≤10	
Mean number of antral follicles	31.0 (14.8)	<12	
Follicle localization			
Random	29 (59%)		
Peripheral	18 (37%)		
Random and peripheral	2 (4%)		
Follicle size			
Uniform	16 (37%)		
Nonuniform	27 (63%)		
% of women meeting PCOM criteria	49 (92%)		
PCOS diagnosis ($n = 53$)			
Having PCOS	49 (92%)		
Oligomenorrhea + PCOM + high total T	24 (49%)		
Oligomenorrhea + PCOM + normal total T	25 (51%)		

For ultrasound parameters and PCOS definition: 13 patients were additionally excluded (see Methods). Total T: total testosterone; A4: androstenedione; E2: estradiol; E1: estrone; free T: calculated free testosterone; SHBG: sex hormone-binding globulin; LH: luteinizing hormone; FSH: follicle-stimulating hormone; DHEAS: dehydroepiandrosterone sulphate; AMH: anti-Mullerian hormone; BMI: body mass index; HOMA-IR: homeostasis model assessment of insulin resistance; PCOM: polycystic ovary morphology.

Thirteen patients with a dominant follicle, a corpus luteum, a hemorrhagic cyst, or a history of ovarian surgery or teratoma were additionally excluded when assessing ultrasound parameters. Patients were classified as having PCOM if they had ≥12 antral follicles (2–9 mm in diameter) in both ovaries and/or an ovarian volume > 10 mL in one or two ovaries [4, 23].

2.6. Polycystic Ovary Syndrome (PCOS) Definition. Polycystic ovary syndrome was defined by the Rotterdam criteria: the presence of at least two of the following criteria: ovulatory dysfunction, PCOM, or biochemical androgen excess [4]. PCOM was defined as discussed above. For androgen excess, total T > 41 ng/dL or free T > 0.49 ng/dL was used as cut-off [15].

2.7. Statistical Analysis. Spearman rank was used to assess correlations between hormonal measurements. Linear or logistic regression (unadjusted and adjusted for age and BMI) was used to assess associations between androgens, SHBG, and metabolic and ultrasound parameters. Pearson's r was used to assess correlations between LC-MS/MS and immunoassay results and ultrasound parameters. $P < 0.05$ was considered statistically significant. All analyses were performed using the STATA version 13 (Stata Corp).

3. Results

Age, hormonal, metabolic, and ovarian parameters of patients are reported in Table 1. Correlations between the different hormonal measurements are shown in Table 2. As expected, total T, A4, and free T were strongly correlated with each other. Furthermore, E2 was correlated with total T and A4, whereas E1 was also correlated with free T. LH and LH/FSH ratio were related to total and free T and A4. Furthermore, LC-MS/MS androgen measurements showed a better correlation with ultrasound data than immunoassay measurements (AFC and ovarian volume; $r = 0.49$ and 0.51 for LC-MS/MS total T; $r = 0.42$ and 0.41 for immunoassay total T; $r = 0.55$ and 0.58 for LC-MS/MS A4; $r = 0.44$ and 0.56 for radioimmunoassay A4, data not shown).

Associations between androgens, SHBG, and metabolic and ultrasound parameters are shown in Table 3. Total T, A4, and free T closely reflected ovarian volume and AFC in oligomenorrheic subfertile patients, independent of BMI. Neither total T nor A4 was related to BMI, insulin, or insulin resistance. In contrast, increasing free T or decreasing SHBG concentrations were associated with a higher BMI, as well as higher insulin levels and insulin resistance, but this association disappeared after adjusting for BMI. After adjusting for age and BMI, total testosterone was associated with total cholesterol and LDL cholesterol. There were no significant associations between free T and lipid measurements after adjustments for age and BMI.

Thirty-seven women (56%) had normal free T (≤0.49 ng/dL), and 29 women (44%) had high free T (>0.49 ng/dL) (Table 4). Total T, A4, LH, and LH/FSH levels were higher in women with high free T, whereas SHBG was lower. All the observed differences remained significant after adjusting for age and BMI (Table 4). Women with high free T had a higher BMI, had higher insulin levels, and were more insulin-resistant. However, the associations between free T and insulin or HOMA-IR disappeared after adjusting for age and BMI. Furthermore, patients with high free T had a higher AMH level, a higher mean ovarian volume, and an increased number of antral follicles, also after adjusting for age and BMI (Table 4). In Supplementary Table 1, the same comparisons were made between women with normal total T (≤41 ng/dL) and high total T (>41 ng/dL).

Table 2: Correlation matrix.

	Total T	A4	Free T	SHBG
Total T	1			
A4	**0.92***	1		
Free T	**0.82***	**0.83***	1	
SHBG	−0.05	−0.21	**−0.58***	1
E2	**0.45***	**0.42***	0.30	0.11
E1	**0.54***	**0.60***	**0.47***	−0.12
LH	**0.59***	**0.57***	**0.51***	−0.06
FSH	−0.10	−0.14	−0.28	0.35
LH/FSH	**0.61***	**0.61***	**0.62***	−0.21

Data are reported as Spearman's ρ. *$P < 0.05$ after the Bonferroni correction. Total T: total testosterone; A4: androstenedione; free T: calculated free testosterone; SHBG: sex hormone-binding globulin; E2: estradiol; E1: estrone; LH: luteinizing hormone; FSH: follicle-stimulating hormone.

4. Discussion

In our study, total T, A4, and free T closely reflected ovarian volume and AFC in oligomenorrheic subfertile patients with suspected PCOS. However, neither total T nor A4 was related to BMI, insulin, or insulin resistance. In contrast, increasing free T concentrations was associated with a higher BMI, as well as higher insulin levels and insulin resistance [24], but this association disappeared after adjusting for BMI.

This link between free T and metabolic parameters can, at least partly, be explained by the impact of BMI on SHBG levels. As expected, SHBG was inversely associated with BMI, and it is well known that SHBG levels decrease in obesity, both in men and women. In women, an obesity-related decrease in SHBG is accompanied by a higher free T. This is in contrast to obese men, in whom a decrease in SHBG is accompanied by a decrease in total T, whereas free T remains normal or slightly decreases [25, 26]. Furthermore, SHBG levels in women are two to three times higher than in men, as is the number of unoccupied SHBG steroid-binding sites (up to 80% in women versus 45% in men) [27, 28]. Hence, the sex steroid buffering capacity of SHBG is higher in women than in men, and the main function of SHBG in women is protection against high free androgen levels [29]. Thus, when SHBG levels decrease with increasing BMI, this buffering capacity is breached, eventually leading to increasing free T concentrations and androgen excess. It is therefore likely that even slight changes in the biological availability of androgens may have clinical consequences in women.

Table 3: Associations between androgens and SHBG and metabolic and ovarian parameters.

	Adjustments	Total T	A4	Free T	SHBG
BMI	Unadjusted	1.06 (−0.13, 2.24)	1.17 (−0.001, 2.34)	**2.09 (0.98, 3.19)*****	**−2.89 (−4.16, −1.61)*****
	Age	1.06 (−0.13, 2.25)	1.17 (−0.01, 2.35)	**2.12 (1.00, 3.23)*****	**−2.92 (−4.21, −1.64)*****
Glucose	Unadjusted	0.07 (−1.68, 1.81)	0.25 (−1.50, 1.99)	0.02 (−0.02, 0.06)	−1.50 (−3.64, 0.65)
	Age and BMI	−0.19 (−1.95, 1.57)	−0.01 (−1.78, 1.76)	0.01 (−0.03, 0.05)	−0.90 (−3.40, 1.61)
Insulin	Unadjusted	3.89 (−5.67, 13.45)	4.53 (−4.98, 14.04)	**0.01 (0.002, 0.02)***	**−19.39 (−30.20, −8.58)****
	Age and BMI	−0.79 (−8.33, 6.76)	−0.63 (−8.19, 6.94)	0.001 (−0.007, 0.01)	−5.52 (−16.16, 5.13)
HOMA-IR	Unadjusted	0.08 (−0.10, 0.25)	0.09 (−0.09, 0.27)	**0.48 (0.10, 0.85)***	**−0.36 (−0.55, −0.16)****
	Age and BMI	−0.01 (−0.14, 0.13)	0.001 (−0.14, 0.14)	0.11 (−0.38, 0.60)	−0.10 (−0.29, 0.09)
Total cholesterol	Unadjusted	**9.95 (3.56, 16.33)****	**7.22 (0.64–13.80)***	**0.01 (0.001, 0.02)***	1.92 (−6.35, 10.20)
	Age and BMI	**8.90 (2.41, 15.38)****	6.00 (−0.70, 12.71)	0.01 (−0.003, 0.01)	8.19 (−0.95, 17.31)
HDL cholesterol	Unadjusted	1.24 (−2.03, 4.52)	0.09 (−3.19, 3.37)	−0.01 (−0.03, 0.01)	**4.65 (0.84, 8.47)***
	Age and BMI	1.88 (−1.27, 5.03)	0.80 (−2.39, 3.98)	−0.004 (−0.02, 0.01)	**4.42 (0.22, 8.62)***
LDL cholesterol	Unadjusted	**7.17 (1.66, 12.67)***	**5.96 (0.39, 11.52)***	**0.01 (0.001, 0.02)***	0.08 (−6.93, 7.08)
	Age and BMI	**6.01 (0.45, 11.57)***	4.67 (−0.98, 10.32)	0.01 (−0.004, 0.02)	5.51 (−2.23, 13.25)
Triglycerides	Unadjusted	7.69 (−3.78, 19.17)	5.88 (−5.62, 17.37)	**0.01 (0.001, 0.01)***	**−14.03 (−27.68, −0.39)***
	Age and BMI	5.04 (−6.04, 16.12)	2.65 (−8.50, 13.80)	0.005 (−0.0004, 0.01)	−8.70 (−23.76, 6.35)
AMH	Unadjusted	**0.08 (0.03, 0.12)****	**0.08 (0.03, 0.12)****	**0.06 (0.02, 0.11)****	−0.01 (−0.05, 0.03)
	Age and BMI	**0.07 (0.03, 0.12)****	**0.08 (0.03, 0.12)****	**0.06 (0.02, 0.10)****	−0.01 (−0.05, 0.03)
Ovarian volume	Unadjusted	**0.15 (0.09, 0.22)*****	**0.17 (0.11, 0.23)*****	**0.16 (0.09, 0.22)*****	−0.05 (−0.11, 0.01)
	Age and BMI	**0.15 (0.08, 0.21)*****	**0.17 (0.11, 0.23)*****	**0.14 (0.08, 0.20)*****	−0.03 (−0.09, 0.02)
AFC	Unadjusted	**0.04 (0.03, 0.06)*****	**0.05 (0.03, 0.06)*****	**0.04 (0.02, 0.05)*****	−0.01 (−0.03, 0.005)
	Age and BMI	**0.04 (0.03, 0.06)*****	**0.05 (0.03, 0.06)*****	**0.04 (0.02, 0.05)*****	−0.01 (−0.02, 0.003)

Linear regression with adjustments for age and BMI. Data are reported as β coefficients with 95% confidence interval per standard deviation increase in androgen/SHBG. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. T: testosterone; A4: androstenedione; SHBG: sex hormone-binding globulin; BMI: body mass index; HOMA-IR: homeostasis model assessment of insulin resistance; AMH: anti-Mullerian hormone; AFC: antral follicle count.

Table 4: Comparison of normal versus high free T in the study sample.

	Free T ≤ 0.49 ng/dL; $n = 37$ (56%)	Free T > 0.49 ng/dL; $n = 29$ (44%)	P value unadjusted	P value (age and BMI adjusted)
Clinical parameters				
Age	28.6 (2.4)	28.0 (3.7)	0.419	
Hirsutism score	1.5 (1.9)	2.9 (2.8)	**0.029**	0.262
Having hirsutism (%)	9 (25%)	12 (42.9%)	0.131	0.515
Having acne (%)	16 (46%)	12 (41%)	0.728	0.902
Hormones				
Total T (ng/dL)	33.3 (14.7)	63.3 (22.5)	**<0.001**	**<0.001**
A4 (ng/dL)	127 (52)	247 (83)	**<0.001**	**<0.001**
Free T (ng/dL)	0.32 (0.12)	0.90 (0.33)	**<0.001**	**<0.001**
SHBG (nmol/L)	85.1 (33.0)	50.9 (19.6)	**<0.001**	**0.001**
E2 (ng/L)	56.7 (48.9)	62.3 (53.3)	0.657	0.878
E1 (ng/L)	54.7 (31.5)	71.1 (34.0)	**0.047**	0.131
LH (U/L)	7.9 (3.9)	11.3 (5.3)	**0.005**	**0.008**
LH/FSH	1.3 (0.7)	2.1 (0.9)	**<0.001**	**0.001**
AMH (ng/mL)	6.9 (4.5)	10.7 (5.9)	**0.007**	**0.005**
Metabolic parameters				
BMI	23.3 (4.3)	27.2 (5.5)	**0.003**	
% normal BMI	24 (65%)	12 (41%)		
% overweight	11 (30%)	10 (34%)		
% obese	2 (5%)	7 (24%)		
Glucose (mg/dL)	89.8 (8.0)	91.3 (6.6)	0.451	0.911
Insulin (pmol/L)	55.8 (31.6)	82.6 (46.3)	**0.010**	0.450
HOMA-IR	1.03 (0.57)	1.53 (0.85)	**0.008**	0.408
Ovarian parameters				
Mean ovarian volume (mL)	8.0 (2.8)	12.2 (4.2)	**<0.001**	**<0.001**
Mean AFC	23.9 (10.5)	39.1 (15.0)	**<0.001**	**<0.001**
Follicle localization			**0.047**	
Random	23 (74%)	10 (42%)		
Peripheral	7 (23%)	13 (54%)		
Random + peripheral	1 (3%)	1 (4%)		
Follicle size			**0.007**	
Uniform	6 (21%)	12 (60%)		
Nonuniform	23 (79%)	8 (40%)		
% of women with PCOM	27 (87%)	27 (100%)	0.053	

Data are reported as mean (standard deviation) for continuous variables or as n (percentage) for categorical variables. Linear or logistic regression was used to assess differences between groups (unadjusted, adjusted for age, and BMI). Total T: total testosterone; A4: androstenedione; free T: calculated free testosterone; SHBG: sex hormone-binding globulin; E2: estradiol; E1: estrone; LH: luteinizing hormone; FSH: follicle-stimulating hormone; HOMA-IR: homeostasis model assessment of insulin resistance; AFC: antral follicle count; PCOM: polycystic ovarian morphology.

Furthermore, free T was positively correlated with LH and LH/FSH ratio, and women with high free T have higher LH and a higher LH/FSH ratio compared to women with normal free T, independent of BMI. Androgen excess can dysregulate hypothalamic-pituitary-ovarian axis function by disrupting normal GnRH pulse frequency. As a result, pituitary LH production increases, while FSH remains inadequately low, eventually hampering cyclic ovarian estradiol and progesterone production [30]. Oligomenorrhea and ovulatory dysfunction may thus be early clinical signs of androgen excess. Moreover, women with high free T levels had a higher AMH, higher ovarian volume, and higher AFC, independent of BMI. Thus, women with high free T have numerous characteristics of PCOS: ovarian aspects (an increase in AMH as well as ultrasound features), hormonal aspects (a higher LH/FSH ratio), and also metabolic aspects (BMI, insulin levels, and insulin resistance). A similar analysis for women with high total T showed nonsignificant unadjusted P values for metabolic parameters (BMI, insulin levels, and insulin resistance; $P = 0.382$, 0.639, and 0.632, resp.).

Our study has several strengths. All sex steroids were measured by a sensitive LC-MS/MS method, suitable for precise measurement of low testosterone concentrations in women. In concordance with literature [11, 15], direct

immunoassay results misclassified almost 1 out of 4 patients as compared to LC-MS/MS measurements, reflecting the non-ideal correlation for total T as well as A4 (Pearson's $r = 0.77$ and 0.68, resp.). This further supports the use of validated LC-MS/MS methods in evaluating women for biochemical hyperandrogenemia [15, 31]. In addition, extensive sample pretreatment is not needed and only 200 μL of serum is required; therefore, our LC-MS/MS method is applicable for measuring total T and A4 in routine clinical practice. Furthermore, in all patients, ovarian ultrasound was rigorously performed, and observations were recorded in a standardized manner. Additionally, a broad range of hormonal and metabolic parameters was registered.

However, some limitations need to be considered. Our study sample is relatively small, and only oligomenorrheic patients consulting a university fertility center were included. Although 45% of patients are overweight or obese, most patients have a favorable metabolic profile. Our findings therefore need validation in other patient groups. Due to the cross-sectional and observational design of the study, we cannot discriminate between cause and effect.

In conclusion, assessing SHBG and free testosterone is important in evaluating androgen excess in subfertile women with ovulatory dysfunction and suspected PCOS, as it reflects both ovarian and metabolic disturbances.

Conflicts of Interest

The authors declare that they have no conflict of interest. Laurent M. R. is a fellow of the Research Foundation Flanders (FWO). Vermeersch P. is a senior clinical investigator of the Research Foundation Flanders (FWO).

Authors' Contributions

Antonio L. and Pauwels S. contributed equally to this work, and also, Vermeersch P. and Vanderschueren D. contributed equally to this work.

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Supplementary Materials

Supplementary Figure 1: flow chart of participants. Supplementary text: detailed description of liquid chromatography-tandem mass spectrometry method for serum total testosterone and androstenedione. Supplementary Table 1: comparison of normal versus high total testosterone. *(Supplementary Materials)*

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Research Article

Uterine Artery Doppler in Pregnancy: Women with PCOS Compared to Healthy Controls

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The objective of this study was to investigate possible differences in uterine artery pulsatility index (UtAPI) between pregnant women with PCOS and healthy controls and to explore possible effects of metformin on UtAPI. *Material and Methods*. The study was conducted in a tertiary center. Forty-eight pregnant women diagnosed with PCOS before pregnancy and 124 healthy pregnant women were included. Women with PCOS were randomly assigned to metformin 2000 mg daily or a placebo. UtAPI was measured five times during 1st and 2nd trimesters of pregnancy in women with PCOS and four times in healthy controls. *Results*. There was no difference in UtAPI between PCOS women and healthy controls at any point in time ($p = 0.34 - 0.77$). In women with PCOS, randomly assigned to metformin 2000 mg or placebo, UtAPI was unaffected by metformin two hours after intake of the first dose of study medication ($p = 0.34$). All PCOS women, regardless of randomization, had higher UtAPI two hours after intake of study medication and a meal compared to before a meal ($p = 0.02$). *Conclusions*. In the first and second trimesters of pregnancy, there was no difference in UtAPI between women with PCOS and healthy controls. Metformin had no immediate effect on the UtAPI. Interestingly, blood flow decreased after a meal, suggesting that time since last meal should be taken into consideration when interpreting the results of UtAPI measurements in pregnancy. This trial is registered with ClinicalTrials.gov (NCT00466622) Metformin in Pregnant PCOS women (PregMet) (NCT00159536).

1. Introduction

Over the last twenty years Doppler ultrasound has become a reliable and frequently used method to monitor the feto-placental unit of risk pregnancies [1–6]. Increased resistance in the uterine artery measured by the pulsatility index indicates a decreased blood flow to the placenta and may be an early sign of placenta pathology and/or hypertensive disorder in pregnancy [1, 6, 7]. PCOS is linked to pregnancy complications, such as gestational diabetes mellitus, preterm delivery, and preeclampsia [8–10]. Studies of UtAPI in women with PCOS are sparse, but some have reported decreased blood flow in the uterine artery in both nonpregnant and pregnant women with PCOS [11–14]. One study reported a significantly higher rate of PCOS women with abnormal UtAPI measurements during first and mid-second trimesters compared to controls [15]. Metformin is an old antidiabetic drug and is known to reduce fasting insulin and testosterone levels in nonobese, nonpregnant women with PCOS [16]. Metformin has also been shown to have a possible vessel-relaxing effect, with increased blood flow in both nonpregnant and pregnant women with PCOS. Results supporting this have been published both before and after we initiated this study [17–19]. We were not able to demonstrate a vessel-relaxing effect in a former study addressing this issue [20]. No studies have accounted for how soon after drug intake this effect can occur.

We hypothesized that unfavourable hemodynamic adaptations in early pregnancy explain why pregnancy complications are more common in women with PCOS. We aimed to study possible differences in UtAPI between women with PCOS and healthy controls and possible immediate effects of metformin on UtAPI.

2. Materials and Methods

The present study comprises a subgroup of women with PCOS who participated in the PregMet study, which was an RCT comparing metformin 2 g daily to placebo on the effect of pregnancy complications [21]. Women included in the present substudy underwent extended ultrasound examinations, that is, Doppler of the uterine artery, in addition to following the protocol of the main study [21]. Women were included from February 2005 to September 2008. As controls for this substudy, we recruited healthy women in a prospective observational study. Based on the healthy controls, we constructed a reference curve for UtaPI. The reference curve has been published elsewhere [22]. Healthy control women were included from July 2008 to May 2010. Participants, both women with PCOS and healthy controls, were recruited from general practitioners, the gynecological outpatient clinic of the hospital, and on the basis of "word of mouth." All women were recruited during first trimester of pregnancy. Biometric variables, including height, weight, and blood pressure were recorded.

The Committee for Medical Research Ethics of Health Region IV, Norway, approved all of the studies ((1) controls 4.2008.841, (2) PregMet 145.04, and (3) the substudy FlowMet 4.2007.97). The substudy was registered separately at http://www.clinicaltrials.gov (NCT00466622) (PregMet NCT00159536). The study on healthy controls was an observational study and not registered in any trial register. Written informed consent was obtained from each patient before inclusion, and the Declaration of Helsinki was followed throughout the studies.

The PregMet study was a multicenter randomized controlled trial (RCT) in which women received metformin or placebo. There was no difference at baseline between the groups during 1st and 2nd trimesters [21]. Inclusion criteria for the PregMet study were (1) PCOS diagnosed according to the Rotterdam criteria [23], (2) age 18–45 years, (3) gestational age between 5 and 12 weeks, and (4) a single viable fetus shown on ultrasonography. The exclusion criteria were alanine aminotransferase (ALAT) > 90 IU/L, serum creatinine concentration > 130 μmol/L, known alcohol abuse, previously diagnosed diabetes mellitus or fasting s-glucose > 7.0 mmol/L at the time of inclusion, treatment with oral glucocorticoids, or use of drugs known to interfere with metformin. PCOS women were followed up during the entire pregnancy and after delivery. A detailed description is published elsewhere [21].

Out of 273 women participating in the PregMet study, 48 were asked and agreed to participate in a substudy called the FlowMet study (Figure 1). Women were asked to participate if they lived close to the hospital, thus making the extra visits feasible. Women were not compensated for participation but were offered a free meal between or after examinations. Women included in the FlowMet study underwent four additional ultrasound examinations during the study period, and otherwise, they followed the PregMet study protocol [21]. These women participating in the substudy were comparable to the whole group of women with PCOS regarding baseline characteristics.

The first UtAPI examination was performed in the morning after an overnight fast. Immediately after the examination, women were instructed to take the first dose of study medication, metformin 500 mg or an oral placebo, and were also given a meal (a sandwich and an optional drink). Two hours after tablet intake, women returned for a second examination. We intended to investigate if metformin had an immediate effect on the uterine arteries and the placental circulation expressed as an altered UtAPI. This second nonfasting measurement was the one used in the analyses comparing PCOS women with controls (as the controls were not fasting). The third examination was performed two weeks after inclusion when the women reached the full dose of study medication (metformin 1000 mg × 2 or placebo). This examination was not done in a fasting state, and we did not record time since last meal or time of day. Examination 4 was done in the morning after an overnight fast at gestational week 18, and examination 5 was done at gestational week 24 in a nonfasting state. Time since last meal or time of day was not recorded.

As controls, we included 124 healthy pregnant women in a prospective observational study. Inclusion criteria in this study were (1) healthy women with an ongoing first trimester pregnancy, (2) viable, single fetus, (3) age 18–38 years, (4) no previous pregnancy complications (e.g., preeclampsia, intrauterine fetal death, gestational diabetes, and preterm delivery), (5) no somatic or mental diseases (e.g., diabetes, kidney or cardiovascular diseases, and PCOS), and (6) no missed abortions or severe congenital anomalies.

Five women were excluded during the pregnancy: one had PCOS, three developed preeclampsia, and one experienced intrauterine fetal death at gestational week 35. The present study includes the remaining 119 healthy controls (Figure 1).

Healthy controls were examined according to the same protocol and at the same gestational weeks as PCOS women. One important difference was that healthy controls did not fast, did not take study medications, and were therefore not examined two hours after inclusion. They were also possibly examined at a later time of the day.

2.1. Ultrasound Measurements. All study participants were examined with Siemens ACUSON Antares™ (Siemens AG, Germany), Hitachi EUB-8500 (Hitachi Medisys, France), or Voluson 730 Expert (GE, Zipf, Austria) ultrasound devices. During Doppler ultrasound measurements, care was taken to avoid insonation of the embryo/fetus. Three experienced midwives specialized in obstetric ultrasonography and three experienced doctors (all working in the same unit) carried out all scans. The thermal (TI) and the mechanical indices (MI) were kept within the recommended thresholds, and the ISUOG guidelines for the safe use of Doppler ultrasound were followed [24].

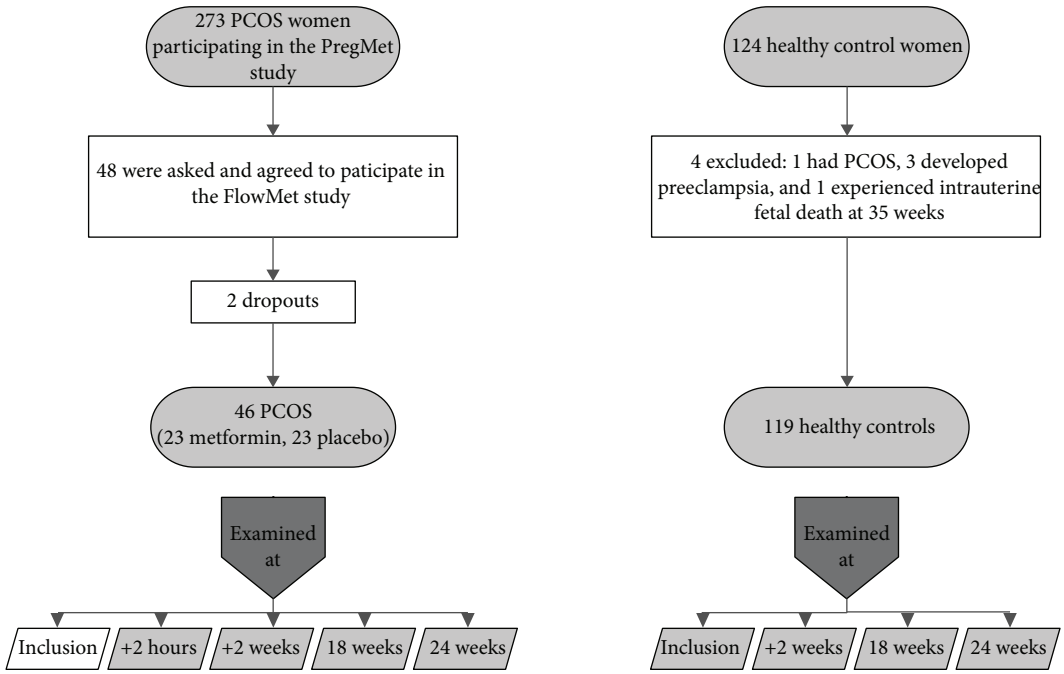

Figure 1: Participants and examinations. "+2 hours" is two hours after the first examination, and "+2 weeks" is 2 weeks after the first examination.

The first visit was scheduled based on the last menstrual period. Crown rump length (CRL) was used to estimate gestational age at the time of the first examination. Estimated date of delivery and timing of the examination in week 24 were based on a second trimester routine ultrasound examination at approximately gestational week 18.

At the first three examinations for the PCOS women and the first two examinations for the healthy controls, the uterine artery pulsatility index (UtAPI) was measured with transvaginal ultrasound according to the following method: the uterine artery was identified at the point closest to the internal cervical os with the use of colour flow imaging. The sample gate was set at 2 mm and placed to cover the whole vessel, including the vessel walls. The angle was kept below 30 degrees. Three consecutive uniform waveforms were recorded, and the mean of the three was used. At the study visits at 19-20 weeks and 23-24 weeks, the UtAPI was measured with transabdominal ultrasound using the method described by Hernandez-Andrade et al. [25].

PI was measured three times on each side in order to reduce the effect of intraobserver variability, and the mean of all six measurements from the right and left uterine arteries was used in the final analyses.

2.2. Statistical Analysis. Baseline characteristics were analysed using the two sample t-test. Confidence intervals for mean PI were t-based while comparisons of mean PI between the control group and the PCOS group were done using a linear model with adjustment for gestational age. This was done because of the rapid change in UtAPI in early pregnancy and because gestational age at inclusion varied. We consider p values < 0.05 as statistically significant. We decided not to adjust for BMI or blood pressure, as these factors may be inherent components of PCOS. (We initially adjusted for maternal age and blood pressure, but this did not significantly change the result and we chose to keep the results as unadjusted as possible.) The statistical analysis was performed using R version 2.13.1 using the package lme4 and SPSS version 20 (IBM SPSS, Armonk, NY, USA).

3. Results

3.1. PCOS versus Controls. Women with PCOS and healthy control women had comparable sociometric parameters such as civil status, occupation, educational level, ethnicity, and parity (data not shown). The PCOS women were older and had higher BMI and blood pressure compared to the healthy controls (Table 1).

Women with PCOS and healthy controls were examined with UtAPI at the same point in time during first and second trimesters of pregnancy. We found no difference in mean UtAPI between the groups at any point in time that was investigated (Figure 2) (Table 2). Women with PCOS were randomly assigned to either metformin 2000 mg or placebo. We compared PCOS (metformin) and PCOS (placebo) groups separately to controls and found that there was no difference (Table 3).

3.2. PCOS. There was no difference in UtAPI between placebo and metformin groups 2 hours after intake of study medication ($p = 0.34$). UtAPI in the PCOS women was measured in the morning after an overnight fast and again 2 hours after intake of the first dose of study medication and a meal. After 2 hours, UtAPI was significantly increased ($p = 0.018$) in the PCOS group (both metformin and placebo groups). Analysing the PCOS (metformin) and PCOS

Table 1: Baseline characteristics of the study participants.

	PCOS	Controls	p values
Age (years)	29.9 (4.6)	27.9 (4.2)	0.01
BMI (kg/m^2)	29.0 (7.6)	24.1 (4.2)	<0.001
Systolic BP (mmHg)	120 (10.6)	114 (11.6)	0.001
Diastolic BP (mmHg)	78 (9.3)	68 (10.0)	<0.001

Values are given as means and standard deviation (SD).

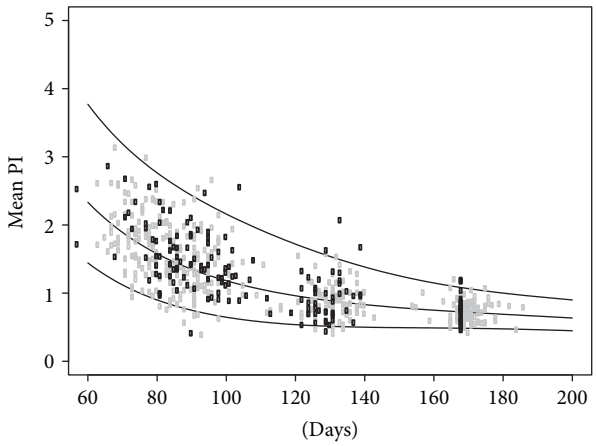

Figure 2: UtAPI percentile curve with 2.5, 50, and 97.5 percentiles. The lines are statistically calculated based on UtAPI measurements of the controls (gray rectangles) and PCOS women (black rectangles).

Table 2: Number and time of UtAPI measurements.

PCOS women	Healthy controls
(1a) Inclusion (1st trimester)	(1) Inclusion
(1b) Inclusion + 2 hours	
(2) Inclusion + 2 weeks	(2) Inclusion + 2 weeks
(3) Week 18	(3) Week 18
(4) Week 24	(4) Week 24

(placebo) groups separately, mean UtAPI increased 0.13 in the placebo group ($p = 0.18$) and 0.18 in the metformin group ($p = 0.05$) (Table 4).

4. Discussion

We observed that women with PCOS and healthy controls had similar blood flow (UtAPI) in the uterine artery in the first and second trimesters and that food intake seems to impact UtAPI in pregnant women with PCOS.

Contrary to another comparable publication [15], we found no difference in UtAPI in the first and second trimesters of pregnancy between PCOS and healthy controls. Examinations were undertaken in a similar manner and by the same experienced personnel in both groups. One difference was that healthy controls did not fast before the 18-week examination and were scheduled for examination at random times during the day. PCOS women were examined in the morning and were fasting because of the PregMet study protocol. Before the study, we had no indication that fasting would affect blood flow [26, 27], and as far as we know, there are no other publications addressing this issue.

In a previous publication, we reported no long-term difference in UtAPI between placebo and metformin groups in the PregMet study ($N = 270$) [20]. When planning the present study, we read previous reports showing an increased uterine blood flow in PCOS women who received metformin, indicating that metformin could have a vessel-relaxing/dilating effect [18, 19, 28]. We found no reports on how quickly this could occur. Accordingly, in the present substudy, we aimed to investigate a possible immediate effect of metformin on the blood flow in women with PCOS. They received 500 mg metformin (a common start dose to avoid nausea), but the dose may have been too low to observe a cardiovascular effect. We found no difference in UtAPI two hours after intake of study medication between the placebo and metformin groups.

Surprisingly, we found higher mean UtAPI in both PCOS (metformin) and PCOS (placebo) groups at the examination done after 2 hours. Women were served a meal while waiting for the second examination. Studies in healthy pregnant women show no adverse effects of fasting on UtAPI [26, 27, 29]. These studies were conducted on women fasting for Ramadan and addressed long-term effects of fasting. Two groups (fasting and nonfasting) were compared at the beginning and the end of Ramadan, and one study was randomized. They found no difference in UtAPI between the two groups after one month of daytime fasting. None of the studies described when during the day, UtAPI was measured, that is, if the women were in a fasting state when the examination was done. We anticipate that they were not in a state of fast at the time of examination. This is because you are allowed to eat before sunrise and after sunset during Ramadan, and it is likely that women were examined during daytime and had ingested a meal before sunrise.

We were not able to find any studies on the effect of a meal or glucose load on UtAPI in pregnant women. We have previously demonstrated that high fasting blood glucose correlated inversely to UtAPI in the larger group of pregnant PCOS women from which the present subgroup was collected [20]. That is, the higher the fasting blood glucose, the lower the blood flow is. We can only speculate whether the expectedly higher blood glucose after a meal contributed to the higher UtAPI. In the nonpregnant state, food intake leads to vascular redistribution and shunting of blood to the gastrointestinal tract to promote digestion and absorption of nutrients. This leads to reduced blood flow and compensatory vasoconstriction in other areas of the body. This could also be the case in pregnant women and could be reflected in increased UtAPI as a measure of reduced blood flow. Other possible physiologic explanations could be diurnal variation in blood pressure and vasoconstriction induced by caffeine intake (coffee, tea, or coke).

PCOS is closely linked to metabolic syndrome and to increased prevalence of type 2 diabetes mellitus, gestational diabetes, and obesity [30]. Whole body metabolism including

Table 3: Mean UtAPI values and number of examinations at each given study point. In the analysis, we adjusted for gestational age.

	Controls (n)	PCOS All (n)	PCOS Placebo (n)	PCOS Metformin (n)	p value
Mean UtAPI inclusion	1.79 (118) CI (1.70–1.87)	1.73 (46) CI (1.58–1.88)	1.80 (24) CI (1.58–2.03)	1.66 (22) CI (1.44–1.88)	PCOS all $p = 0.34$ PCOS placebo $p = 0.18$ PCOS metformin $p = 0.93$
Mean UtAPI inclusion + 2 weeks	1.41 (114) CI (1.33–1.49)	1.37 (43) CI (1.25–1.49)	1.41 (23) CI (1.23–1.60)	1.31 (20) CI (1.13–1.49)	PCOS all $p = 0.77$ PCOS placebo $p = 0.46$ PCOS metformin $p = 0.62$
Mean UtAPI week 18	0.89 (109) CI (0.85–0.94)	0.93 (45) CI (0.83–1.03)	1.00 (24) CI (0.84–1.17)	0.83 (21) CI (0.73–0.93)	PCOS all $p = 0.47$ PCOS placebo $p = 0.08$ PCOS metformin $p = 0.28$
Mean UtAPI week 24	0.73 (108) CI (0.71–0.76)	0.75 (33) CI (0.68–0.82)	0.81 (17) CI (0.69–0.92)	0.70 (16) CI (0.62–0.77)	PCOS all $p = 0.68$ PCOS placebo $p = 0.12$ PCOS metformin $p = 0.28$

Table 4: At inclusion (first trimester) UtAPI was measured after an overnight fasting period, then 2 hours later after ingesting the study medication and a meal.

	Mean UtAPI PCOS (SD) Fasting	Mean UtAPI PCOS (SD) After meal	p value
PCOS all	1.57 (0.50)	1.73 (0.51)	$p = 0.02$
PCOS placebo	1.66 (0.57)	1.80 (0.54)	$p = 0.18$
PCOS metformin	1.47 (0.42)	1.66 (0.50)	$p = 0.05$

glucose metabolism seems to be important in the pathogenesis of PCOS. Exact mechanisms have yet to be clarified.

5. Conclusion

Blood flow in the uterine artery does not seem to differ between women with PCOS and healthy controls. Metformin does not have any immediate effect on UtAPI, but we observed that UtAPI was significantly higher two hours after a meal in pregnant women with PCOS. Standardization of food intake should be considered in future studies measuring UtAPI.

Disclosure

The paper has been presented as an abstract in the 99th Endo Annual Meeting 2017.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Clinical Study

Pericardial Fat Relates to Disturbances of Glucose Metabolism in Women with the Polycystic Ovary Syndrome, but Not in Healthy Control Subjects

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Objective. The objective of the present study is to investigate the relationship of cardiac fat depots with disturbances of the carbohydrate metabolism in women with PCOS. *Methods*. An oral glucose tolerance test (OGTT) was realized, and metabolic parameters were collected in 48 women with PCOS and in 20 controls. Intramyocardial fat (MYCL) and pericardial fat (PERI) were measured using ^{1}H-magnetic resonance spectroscopy and imaging. *Results*. Only in PCOS women, PERI was positively and independently related to parameters of glucose metabolism (HbA1c: $p = 0.001$, fasting plasma glucose: $p < 0.001$, stimulated glucose at 30 and 60 minutes in the OGTT). Thus, the disposition index, insulin sensitivity, and adiponectin also declined with the increase of PERI in women with PCOS; however, these results were not independent of BMI and age. In addition, PERI was positively related to atherogenic lipid profiles, BMI, waist circumference, CRP, and liver fat in women with PCOS. A negative relation of PERI with triglycerides and a positive relation with BMI and waist circumference could be observed in the controls. No relationship of MYCL with diabetes-specific parameters could be found in the study population. *Conclusion*. PERI is related to metabolic disturbances in women with PCOS, but not in metabolically healthy lean subjects. This clinical trial was registered at ClinicalTrials.gov and has the registration number NCT03204461.

1. Introduction

The polycystic ovary syndrome (PCOS) is a common endocrine disorder [1] and has been shown to affect 5–10% of women at childbearing age [2]. Common disturbances of carbohydrate metabolism in PCOS are insulin resistance and hyperinsulinemia [3]. Compared to healthy women in the reproductive age, women with PCOS are at high risk for developing type 2 diabetes [1]. It has been shown that women with PCOS in the United States have a tenfold higher risk for developing type 2 diabetes compared to healthy women at the same age. In addition, they are characterised as having a two to three times higher prevalence rate of the metabolic syndrome when compared to healthy control subjects [2]. Insulin resistance plays a major role in the occurrence of an anovulatory state in women with PCOS [2]. Therefore, it has been shown that metformin has significant positive effects on this relationship [4]. However, the underlying

pathophysiological mechanisms of the increased risk for type 2 diabetes and therefore especially for insulin resistance are not yet fully understood in women with PCOS [2]. Several study groups investigated these mechanisms and could show that there is a close relationship of the amount of liver fat with insulin resistance in women with PCOS [5, 6]; however, a recent study could not find a difference in the amount of liver fat between women with PCOS and control subjects [7]. Several study groups investigated the impact of cardiac fat depots and demonstrated that there is a relationship to disturbances of glucose metabolism [8–10]. However, especially data about the role of cardiac fat depots in glucose metabolism in women with PCOS is still controversial [11–15], although it has been shown that there is a relationship of epicardial fat (EPI) with visceral abdominal adipose tissue [11]. Further, earlier studies could show that EPI is an endocrine active organ that expresses antidiabetogenic factors such as adiponectin [16] and that the fat content surrounding the heart is related to metabolic disturbances [10, 17, 18]. However, the fact that the pathophysiological mechanisms of an increased metabolic risk in women with PCOS is not clear so far and that the fat content around the heart has not yet been investigated thoroughly in this specific population led to the following aim: to investigate the relationship of PERI and MYCL with disturbances of glucose metabolism (i.e., insulin resistance; beta cell compensation in insulin resistance; whole body insulin sensitivity; dynamic changes of glucose, insulin, and C-peptide levels during the oral glucose tolerance test "OGTT" and insulin secretion) in women with PCOS.

2. Materials and Methods

The detailed description of the study procedures of this prospective cross-sectional study was explained previously [7]. However, in the present analysis, only participants who had a magnetic resonance spectroscopy (MRS) of the heart were included. Thus, in the analysis of [7], also participants who did not have a MRS of the heart were included. In short, 48 women who were diagnosed with PCOS and who were untreated, as well as 20 control subjects, had a cardiac magnetic resonance (MR) spectroscopy for the measurement of MYCL. PERI was analyzed in 43 women with PCOS (NIH group: $n = 26$, ROT group: $n = 17$) and in 20 control subjects based on a four-chamber orientation image. The study group of women with PCOS was built up according to the criteria of the National Institute of Health (NIH), which included 31 patients, and according to the Rotterdam criteria, which included 17 patients. Diabetes mellitus type 2, antidiabetic drugs, dyslipidemic treatment, and other diseases, which influence the reproductive system, were excluded. The control group consisted of 20 healthy women (10 women in the control group had a systemic hormonal contraceptive treatment) [7]. The local ethics committee of the Medical University of Vienna approved the study protocol, which was performed in accordance with the Declaration of Helsinki.

2.1. Laboratory Measurements. For the exact assessment of the glucose metabolism, a 75 g OGTT, with the measurement of fasting plasma glucose, fasting insulin, and fasting C-peptide at 0, 30, 60, 90, and 120 minutes, was done. In addition, lipid parameters, hormones, and anthropometric data, such as BMI and waist circumference, were assessed. Laboratory measurements were assessed after a >8-hour fasting period. The analyzation of the laboratory parameters was accomplished at the certified Department of Medical and Chemical Laboratory Diagnostics of the Medical University of Vienna (http://www.kimcl.at) [7]. Adiponectin was analyzed with a competitive enzyme immunoassay of Bio-Vendor (BioVendor Human Adiponectin ELISA).

2.2. Measurements of Ectopic Lipids. Myocardial lipid content (MYCL) was measured according to previously described standardized procedures, on a 3.0 Tesla Magnetom Trio Siemens System [19, 20].

Pericardial fat (PERI), which is the sum of epicardial and paracardial adipose tissue and which is located around the heart, was also analyzed according to previous studies [10, 19, 21, 22]. Therefore, a four-chamber orientation image was used for the analysis of pericardial adipose tissue by T1-weighted ECG-gated cine true fast imaging. Three slices (from the apex to the pulmonary trunk) were used for the drawing around the edges of the adipose tissue of the heart. At the end, the average value of pericardial fat, which was measured in the three slices, was given in cm^2 [19].

The amount of ectopic lipids in the liver was also measured on a 3.0 Tesla Magnetom Trio Siemens System, according to standardized and previously described methods [7].

2.3. Calculations. The Matsuda index for the evaluation of total body insulin sensitivity [23] and the oral glucose insulin sensitivity index (OGIS) [24] were calculated with data of the OGTT. For the assessment of hepatic insulin resistance, HOMA-IR was used [25]. The insulinogenic index was used for the assessment of insulin secretion during the OGTT [26]. The oral disposition index (assessment of beta cell function in insulin resistance) was calculated as the product of the insulinogenic index and the Matsuda index. The diagnosis of the metabolic syndrome was defined according to the criteria of the National Cholesterol Education Program (NCEP-ATP-III).

2.4. Statistical Analysis. Medians and interquartile ranges were applied for the presentation of continuous variables. Mann–Whitney-U test was used for group-based comparisons and Spearman correlation rank test for correlation analyses because normality assumptions of the variables were violated. The nonparametric Kruskal-Wallis test was used for the assessment of differences between more than two groups. Linear regression models were utilized for multivariable adjustment including BMI and age. Therefore, all results which were not independent of BMI and age were stated in the manuscript. A two-sided p value of <0.05 was considered as statistically significant. Statistical analysis was performed with IBM SPSS version 23.

Table 1: Basic characteristics of the study population.

	PCOS total ($n = 48$)	PCOS-NIH ($n = 31$)	PCOS-ROT ($n = 17$)	Controls ($n = 20$)	p value
Age (years)	25 (21–30)	25 (21–28)	24 (22–32)	23 (23–25)	0.475
BMI (kg/m^2)	24 (22–30)§	25 (23–30)§	23 (21–30)	21 (20–24)	**0.005**
Waist (cm)	84 (74–93)	88 (80–96)§*	75 (69–84)	78 (75–85)	**0.003**
RRsys (mmHg)	114 (105–120)	113 (107–121)	115 (104–122)	116 (111–126)	0.487
RRdia (mmHg)	72 (65–79)	72 (66–79)	70 (63–78)	73 (63–76)	0.739
HF (beats per minute)	73 (64–80)	74 (66–85)	72 (64–80)	74 (63–77)	0.698
Total cholesterol (mg/dl)	177 (153–201)	178 (153–203)	175 (162–198)	170 (158–180)	0.435
HDL-cholesterol (mg/dl)	58 (46–67)§	57 (44–65)§	60 (47–73)	68 (60–78)	**0.019**
LDL-cholesterol (mg/dl)	100 (74–125)	99 (73–128)	102 (76–121)	81 (70–87)	0.074
Triglycerides (mg/dl)	75 (60–87)§	72 (59–84)§	84 (63–88)	99 (78–110)	**0.030**
Matsuda index	5.5 (3.4–8.8)§	5.1 (3.6-7.1)§	6.2 (3.0–11.0)§	8.9 (5.5–13.3)	**0.004**
HOMA-IR	1.6 (1.0–2.4)§	1.6 (1.1-2.4)§	1.7 (0.8–2.4)	0.9 (0.5–1.7)	**0.024**
Insulinogenic index	1.1 (0.6–2.0)	1.1 (0.6–2.2)	1.1 (0.7–1.6)	0.9 (0.6–1.3)	0.582
Disposition index (IGI*ISI)	5.4 (3.2–9.0)	5.3 (3.2–9.8)	5.7 (2.9–8.8)	6.4 (4.7–11.5)	0.278
OGIS	494 (446–542)	481 (449–542)	503 (417–544)	537 (499–549)	0.179
MYCL (%)	0.48 (0.27–1.07)	0.49 (0.28–1.36)	0.47 (0.22–0.87)	0.50 (0.23–0.75)	0.801
PERI (cm^2)	11.22 (7.91–16.61)	11.65 (7.60–17.34)	10.67 (8.05–16.20)	8.52 (7.07–12.04)	0.231

PCOS total: sum of PCOS-NIH and PCOS-ROT; PCOS-NIH: women with PCOS defined according to the criteria of the National Institute of Health; PCOS-ROT: women with PCOS defined according to the Rotterdam criteria; BMI: body mass index: waist: waist circumference; RRsys: systolic blood pressure; RRdia: diastolic blood pressure; HF: heart frequency; HDL-cholesterol: high-density lipoprotein cholesterol; LDL-cholesterol: low-density lipoprotein cholesterol; MYCL: intramyocardial fat; PERI: pericardial fat. §Versus controls: $p < 0.05$; *PCOS-NIH versus PCOS-ROT: $p < 0.05$.

3. Results

3.1. Baseline Clinical and Metabolic Characteristics. The detailed baseline characteristics of the study population in the present study were previously published [7]. In this analysis, 31 women with PCOS, defined according to the NIH criteria, and 17 women with PCOS defined according to the ROT criteria, as well as 20 control subjects, were included.

Table 1 shows the baseline characteristics including anthropometric and clinical data, as well as metabolic and endocrine parameters. Women with PCOS showed a worse metabolic profile such as higher BMI levels, increased waist circumference measures, lower HDL cholesterol, and higher triglyceride levels and were characterised by a more pronounced insulin resistance compared to the control group (Table 1). In detail, one study participant in the PCOS-NIH group had an impaired glucose tolerance (IGT), while no participant suffered from impaired fasting glucose (IFG) in the present study. Seven participants in the PCOS-NIH group and four participants in the PCOS-ROT group were shown to be insulin resistant (defined by a HOMA-IR ≥ 2.5). The metabolic syndrome occurred in two women, one being in the PCOS-NIH group and the other in the PCOS-ROT group.

3.2. PERI. Considering the amount of PERI, no significant difference can be reported between the group of women with PCOS and the group consisting of healthy women (Table 1).

3.2.1. PERI and Glucose Metabolism. Correlation analyses showed that only in women with PCOS, a positive relation of PERI with parameters of glucose metabolism, including the following, could be observed: HbA1c (Table 2) and fasting plasma glucose (Figure 1 and Table 2), as well as dynamic glucose levels after 30, 60, 90, and 120 minutes in the OGTT (Table 2). A negative relation of PERI with the beta cell function in insulin resistance (measured with the disposition index) and with insulin sensitivity (measured with OGIS) was found in women with PCOS (Table 2). Additional statistical analyses showed that there is a negative relationship of PERI with the levels of adiponectin (rho $= -0.39$, $p = 0.009$) in the PCOS total group, which was not independent of BMI. Thus, adiponectin was negatively related to insulin resistance which was evaluated with the HOMA-IR (rho $= -0.33$, $p = 0.032$) in the PCOS total group. However, this relationship was not independent of the BMI. Linear regression models showed that all the observed significant relationships of PERI with parameters affecting glucose metabolism still remained significant after the correction for BMI and age, except glucose levels at 90 and 120 minutes in the OGTT, the disposition index, OGIS (Supplementary Table 1), and dynamic insulin levels at 60 minutes. By splitting the study cohort of women with PCOS, according to the NIH and the ROT criteria, all significant relationships of PERI with parameters of glucose metabolism were independent of BMI and age, except glucose levels at 30 and 120 minutes in the NIH group. As shown in Table 2 and in contrast to the PCOS group, there was no correlation of PERI with parameters and indices of glucose metabolism in the control group. These missing relationships were also observed in a sensitivity analysis, after excluding 10 women with systemic contraceptive agents.

Table 2: Correlation analyses of pericardial fat (PERI) with parameters and indices of glucose metabolism in women with PCOS and healthy control subjects.

PERI	PCOS total		PCOS-NIH		PCOS-ROT		Controls	
	Rho	p	Rho	p	Rho	p	Rho	p
Disposition index (IGI∗ISI)	**−0.346**	**0.027**	−0.295	0.162	−0.453	0.068	0.403	0.087
OGIS	**−0.389**	**0.012**	−0.359	0.085	−0.471	0.057	−0.192	0.432
Matsuda index	−0.242	0.132	−0.124	0.574	−0.360	0.155	0.264	0.274
HOMA-IR	0.273	0.076	0.197	0.334	0.311	0.224	−0.268	0.254
Insulinogenic index	−0.129	0.409	−0.138	0.502	−0.108	0.680	0.010	0.967
HbA1c	**0.486**	**0.001**	**0.573**	**0.003**	0.442	0.076	−0.384	0.095
Fasting plasma glucose	**0.682**	**<0.001**	**0.737**	**<0.001**	**0.630**	**0.007**	0.128	0.591
Glucose 30′	**0.449**	**0.003**	**0.441**	**0.024**	0.469	0.058	−0.238	0.313
Glucose 60′	**0.505**	**0.001**	**0.596**	**0.001**	0.363	0.152	0.052	0.828
Glucose 90′	**0.389**	**0.012**	**0.448**	**0.028**	0.305	0.234	−0.187	0.444
Glucose 120′	**0.377**	**0.013**	**0.389**	**0.050**	0.241	0.352	−0.094	0.693
Insulin 0′	0.193	0.214	0.112	0.587	0.262	0.309	−0.269	0.252
Insulin 60′	**0.306**	**0.046**	0.260	0.199	0.365	0.149	0.182	0.442
C-peptide 0′	0.206	0.185	0.021	0.919	0.395	0.117	−0.223	0.345

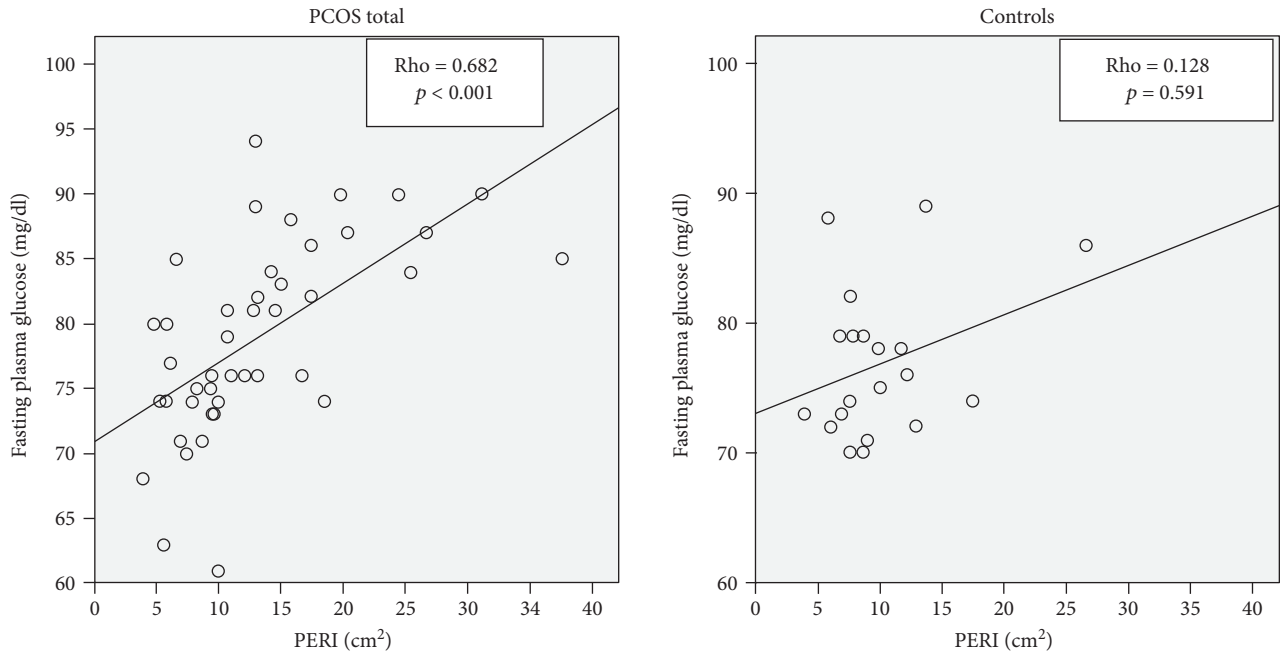

Figure 1: Correlation analyses of PERI with fasting plasma glucose in PCOS women and control subjects.

3.3. PERI and Ectopic Lipids. As displayed by Table 3, there was an association of PERI with the lipid content in the liver in women with PCOS.

3.4. PERI and the Relation to Lipid Parameters and to Parameters of Body Composition. In women with PCOS, a positive relation of PERI with LDL cholesterol and a negative one with HDL cholesterol could be observed, yet these relations were not independent of BMI and age (Table 3). In control subjects, there was a negative association of PERI with triglyceride levels (Table 3). PERI was significantly related to parameters of body composition, such as BMI and waist circumference in control subjects, as well as in women with PCOS (Table 3).

3.5. PERI and Hormones. Control subjects showed a significant and positive correlation of PERI with prolactin levels (Table 3). Therefore, the correlation of PERI with prolactin

Table 3: Correlation analyses of pericardial fat (PERI) with lipid parameters, ectopic lipids, hormones, and anthropometric data in women with PCOS and healthy control subjects.

PERI	PCOS total		PCOS-NIH		PCOS-ROT		Controls	
	Rho	p	Rho	p	Rho	p	Rho	p
TG	0.156	0.318	0.195	0.339	0.054	0.837	**−0.512**	**0.021**
HDL	**−0.343**	**0.024**	**−0.460**	**0.018**	0.015	0.955	0.266	0.257
LDL	**0.381**	**0.012**	**0.530**	**0.005**	0.086	0.743	0.135	0.569
BMI	**0.343**	**0.024**	**0.475**	**0.014**	0.164	0.529	**0.506**	**0.023**
Waist	**0.377**	**0.014**	**0.578**	**0.002**	0.084	0.750	**0.509**	**0.022**
Liver fat	**0.464**	**0.002**	**0.598**	**0.002**	0.309	0.228	0.422	0.072
CRP	**0.328**	**0.032**	**0.517**	**0.007**	−0.006	0.981	−0.440	0.052
hsCRP	**0.337**	**0.031**	**0.489**	**0.015**	0.108	0.679	−0.407	0.075
Cortisol	−0.173	0.293	−0.326	0.112	0.205	0.483	−0.410	0.081
Prolactin	−0.117	0.462	−0.026	0.900	−0.356	0.176	**0.577**	**0.010**
Bioestradiol	−0.133	0.408	−0.337	0.100	0.108	0.692	0.228	0.395
Biotestosterone	0.073	0.645	−0.063	0.761	0.380	0.147	0.100	0.675
Progesterone	−0.091	0.570	−0.115	0.577	−0.064	0.820	0.081	0.734

TG: triglycerides; HDL: high-density lipoprotein cholesterol; LDL: low-density lipoprotein cholesterol; BMI: body mass index; waist: waist circumference; hsCRP: high sensitive C-reactive protein; Bioestradiol: bioavailable estradiol; Biotestosterone: bioavailable testosterone.

levels in the control group remained significant in a sensitivity analysis, after the exclusion of 10 women with systemic contraceptive agents. However, neither in the control group nor in the PCOS group, a relationship of PERI with estradiol, testosterone, progesterone (Table 3), or the free androgen index (FAI) could be observed.

3.6. MYCL. As shown in Table 1, no difference in the amount of MYCL between women with PCOS and control subjects was observed in this study. Neither in women with PCOS nor in the control subjects, a relation of MYCL to parameters of glucose metabolism (HbA1c, insulin, fasting glucose and stimulated glucose, C-peptide, Matsuda index, HOMA-IR, insulinogenic index, and OGIS), lipid parameters (triglycerides, LDL cholesterol, HDL cholesterol, and total cholesterol), blood pressure, heart frequency, inflammatory parameter CRP and us-CRP, cortisol, hormones, and ectopic lipids could be reported.

4. Discussion

In the present study, we investigated the impact of PERI and MYCL on basal and dynamic levels of parameters of carbohydrate metabolism in women with PCOS. Women with PCOS, especially when defined by the NIH criteria, are characterised by having an inauspicious body composition, as well as having worse levels of diabetes specific parameters when compared to healthy control subjects. The present study shows that only in PCOS women, PERI is related to parameters of glucose metabolism. Women with PCOS also feature a decreased beta cell compensation in insulin resistance, as well as a decreased insulin sensitivity, alongside an increase of PERI. However, we could not find a relationship of the intramyocardial lipid content with diabetes-specific parameters. According to these results, one can hypothesize that PERI, but not MYCL, is involved in the pathophysiological mechanisms of disturbances in glucose metabolism in PCOS and may have positive effects under physiological conditions.

Women with PCOS are at an increased risk of developing metabolic diseases; however, the exact pathophysiological mechanisms of a pathological glucose metabolism are up to date not entirely known in this specific population [7]. There is some evidence that the amount of ectopic lipids, such as liver fat, may be significantly influential in the development of insulin resistance in women with PCOS [5, 6, 27]. Therefore, especially the role of EPI, which is an endocrine active organ and which is localized around the heart, is interesting in this specific population. Thus, data showed that EPI strongly expresses factors, which are related to disturbances of glucose metabolism (e.g., adiponectin), yet depending on physiological and pathological conditions [10, 16, 28]. The data about the role of the lipid content around the heart is sparse in women with PCOS. Studies showed that women with PCOS, diagnosed according to the Rotterdam criteria, are characterised by a higher accumulation of fat around the heart when compared to control groups [14, 15]. In the present study, we analyzed PERI, which is the sum of EPI and paracardial adipose tissue, as previously realized in earlier studies [10, 19, 22], while the separation of these two fat depots was especially in lean subjects not possible. Thus, we could not find significant differences in the amount of PERI between women with PCOS, defined by the NIH and the ROT criteria and the control subjects. An analysis of the whole PCOS population presented a 24% higher amount of PERI, when compared to the controls. Therefore, the lack of significance in the present study could have occurred due to the inequality and because of the lower number in the PCOS group and the control group and the differences in the mean BMI. An additional reason for the differences in the results compared to the studies of Aydogdu et al. [15] and Sahin et al. [14] could be explained by the

implementation of different techniques in the measurement of EPI/PERI. The other studies measured the amount of EPI with echocardiography [14, 15], while we used the gold standard, the cardiac magnetic resonance imaging, for the analyzation of PERI [29]. Although we could not find significant differences in the amount of cardiac fat depots, PERI crystallized to be an interesting aspect in the carbohydrate metabolism especially in women with PCOS in the present study. We observed with a detailed assessment of glucose metabolism that only in PCOS women, who were characterised by a more pronounced insulin resistance when compared to the control group, PERI is positively related to parameters of glucose metabolism. These results are in line with prior reports showing that the increase of adipose tissue around the heart is related to disturbances of glucose metabolism [10, 12, 17, 18, 30], which concluded their results based upon mixed study populations. Controversial data about the relationship of EPI with parameters of glucose metabolism in the specific population of women with PCOS exist so far [11, 13–15]. This could possibly be the case because the assessment of glucose metabolism in the existing studies in women with PCOS was done with blood samples under fasting conditions and not with a detailed examination of glucose metabolism, such as the OGTT. A study group, investigating women with PCOS (mean BMI of 32 kg/m^2) with blood samples under fasting conditions, demonstrated that EPI is related to fasting insulin and to HOMA-IR but not to glucose levels in women with PCOS [14]. These results are not at all in line with the results of the present study, demonstrating that there is a relationship of PERI with fasting glucose but not fasting insulin levels. However, the missing association of HOMA-IR with PERI in the present study could be caused by the differences in the mean BMI, when compared to the study group of Sahin et al. [14]. Nevertheless, the implementation of the OGTT in the present study allowed us to investigate the glucose metabolism in more detail, and we could additionally show that the increase of PERI in PCOS women is related to dynamic glucose and insulin levels and HbA1c levels and to inauspicious changes in beta cell function and insulin sensitivity. Thus, by splitting the PCOS cohort according to the NIH and ROT criteria, especially the relationship of PERI with fasting and dynamic glucose levels in the NIH group remained significant. However, in the ROT group, only the relationship of PERI with fasting plasma glucose levels could be observed. Therefore, these differences could be explained not only by the lower sample size in the ROT group but also by the differences in the waist circumference between the two study groups. In addition, it has to be mentioned that the different diagnostic criteria for PCOS of the NIH and the ROT may describe different phenotypes of PCOS. Thus, the fact that EPI is an endocrine active organ that expresses antidiabetogenic factors, such as adiponectin yet only under physiological conditions [16, 28], could play a major role. Studies showed that there is an inverse relationship of PERI with the levels of adiponectin [10, 31]. This could also be observed in the present study in the total group of women with PCOS. Thus, we also found a negative relationship of adiponectin with insulin resistance. So, the insufficient expression of antidiabetogenic factors by PERI under pathological conditions, such as in women with PCOS, seems to be related to the regulation of glucose metabolism in this specific population. The impression of a relationship of factors expressed by the adipose tissue around the heart and metabolic disturbances in PCOS could be justified by the fact that women with PCOS have lower levels of adiponectin compared to control subjects [15, 32]. Therefore, in addition to a control group, it is important to categorize patient groups according to various metabolic diseases, which enable clear assumptions according to the underlying pathophysiological mechanisms of PERI. The antidiabetogenic effect of PERI under physiological conditions is an interesting topic and not only the expression of adipokines but also the expression of other factors could be related to the regulation of glucose metabolism in PCOS. We observed a significant positive relation of PERI with prolactin levels in the control group of the present study. There is a link between the levels of prolactin and disturbances of glucose metabolism. Studies have shown that prolactin receptors are expressed by adipose tissue in humans [33] and that lower levels of prolactin are related to disturbances of glucose metabolism [34] whereas physiologically increased levels of prolactin have shown to have beneficial effects on glucose metabolism [35]. Further studies would therefore be necessary in order to investigate if there is a relation of the expression of prolactin by PERI with protective effects on glucose metabolism in healthy control subjects. Interestingly, the present study demonstrated that the amount of fat depots surrounding the heart is related to disturbances of glucose metabolism in women with PCOS; however, intramyocardial fat content is not. MYCL, the amount of fat in the heart, has been investigated in detail previously. Study groups demonstrated in different patient cohorts, including patients possibly having or not having diabetes [18] or patients with the metabolic syndrome [36] and also metabolically healthy subjects [19], that there is not a relationship of the amount of MYCL with parameters of glucose metabolism. In the present study, neither the control subjects nor the high-risk population of women with PCOS present a relation of MYCL with parameters and indices of the glucose metabolism. Furthermore, the entire study population was additionally analyzed, yet no relationship could be evaluated. Victor et al. hypothesized that insulin resistance in PCOS is related to the inflammatory status [37] and it has been demonstrated that PERI expresses proinflammatory markers [28]. In line with the reported results of Cakir et al. [13], we also observed a positive relation of PERI with the proinflammatory hs-CRP in women with PCOS, portraying that PERI is also related to the cardiovascular risk in women with PCOS. Besides the already discussed effects of PERI amongst pathological conditions, the fat content around the heart could be accompanied by dyslipidemia. Marchington et al. observed that the expression of free fatty acids was two times higher in EPI compared to other fat depots [38]. This allows to assume that there is an increased action of lipolytic activity [31]. Therefore, our observation that an increased amount of PERI is related to inauspicious concentrations of lipid parameters, such as LDL cholesterol or HDL cholesterol in women with PCOS, is in line with prior reports [31]. On the contrary, we observed a significant inverse

relationship of PERI with triglyceride levels in the control group. Conclusively, physiologically increased levels of PERI in metabolically healthy subjects could have a positive influence on lipid parameters. Our results, which showed that PERI is related to the parameters of body composition (BMI and waist circumference) in the specific population of women with PCOS as well as in control subjects and in the whole study population, are in line with earlier studies [8, 19]. We also observed a positive relation of PERI with adipose tissue in the liver similar to prior studies [39].

5. Limitations

Our study has limitations and strengths. A limitation of the present study is the fact that the number of participants in the PCOS and the control group is unequal. A further limitation occurred, due to differences in the mean BMI, between women with PCOS and the healthy control group. In addition, 10 women of the control group were taking hormonal contraceptives, which could influence the results. Thus, we can also report about strengths of the present study. Therefore, the pericardial fat content was analyzed with the gold standard, the magnetic resonance spectroscopy. A further strength of the study is the assessment of glucose metabolism by using a dynamic test, the OGTT, which allowed us to analyze the glucose metabolism in more detail.

6. Conclusion

We can conclude that PERI, but not MYCL, is related to metabolic disturbances in women with PCOS and that it is important to categorize different patient groups in order to get conclusive results about the influence of PERI on carbohydrate metabolism. However, larger and longitudinal studies would be necessary to prove the results of the present study.

Conflicts of Interest

The authors have no competing financial interests.

Authors' Contributions

Michael Leutner, Helmut Steinbrecher, Christian Göbl, Latife Bozkurt, and Ivica Just-Kukurova researched the data. Michael Leutner did the statistical analysis and wrote the manuscript. Christian Göbl, Peter Wolf, Katharina Maruszczak, Johannes Ott, Christian Egarter, Siegfried Trattnig, and Alexandra Kautzky-Willer reviewed the manuscript.

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Supplementary Materials

Table 1: linear regression models in the PCOS total group. *(Supplementary Materials)*

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Review Article

The Place of In Vitro Maturation in PCO/PCOS

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In vitro maturation (IVM) of human oocytes is an emerging treatment option for women with polycystic ovary/polycystic ovary syndrome (PCO/PCOS) in addition to the standard in vitro fertilization (IVF) treatment. There has been significant improvements in pregnancy rates with IVM over the last two decades. This article reviews the place of IVM for women with PCO/PCOS, placing an emphasis on the predictors of successful pregnancy, optimization of culture media, IVM protocols, pregnancy rates, and neonatal outcomes following IVM treatment.

1. Introduction

Polycystic ovary syndrome (PCOS) is considered the most common endocrine disorder of women in their reproductive years and may lead to anovulation and infertility. It affects up to 4–12% of women generally [1, 2]. Various treatment modalities are used for treatment of PCOS-related infertility, including lifestyle modification as a first-line treatment for obese and overweight women with anovulation, ovulation induction with either oral agents or gonadotrophins and laparoscopic ovarian drilling as second-line therapy [1]. However, a subset of these patients will either be resistant to treatment or will fail to conceive despite ovulation induction treatment and will eventually need controlled ovarian stimulation (COS) and in vitro fertilization (IVF) [3]. Additionally, they may have compromised fallopian tube function or male factor infertility and require IVF from the start. However, when undergoing IVF treatment, women with PCOS are predisposed to developing ovarian hyperstimulation syndrome (OHSS) due to their high antral follicle count; this facet also make them ideal for in vitro maturation (IVM) treatment [3, 4]. OHSS is a significant cause of discomfort, distress, hospitalisation, and even mortality for women undergoing IVF treatment, due to the extravasation of fluid out of the vascular system leading to the development of ascites and potentially pleural effusion and thromboembolic phenomena [1, 5].

In vitro maturation of oocytes has been suggested as an alternative approach to conventional IVF as it completely avoids the risk of OHSS [6]. IVM treatment typically involves a relatively short duration of gonadotrophin stimulation and the retrieval of oocytes from follicles at a much smaller diameter than with conventional IVF treatment, often without the use of a trigger injection and oocyte maturation occurs in vitro [4]. The process of IVM involves the collection of immature oocytes at the germinal vesicle (GV) or metaphase I (MI) stages of meiosis, retrieved from small ovarian follicles, by transvaginal oocyte retrieval. Subsequently, these oocytes undergo resumption of meiosis and maturation to metaphase II (MII) oocytes in the laboratory.

The *in vivo* preparation for IVM treatment is a source of contention, and it has been suggested that cycles involving both gonadotrophin and an ovulation trigger should instead be referred to as "truncated" or "minimal stimulation" IVF [7] and not IVM, and the definition of true IVM has recently been debated in the literature by De Vos et al. [7]. By the administration of a human chorionic gonadotrophin (hCG) trigger prior to oocyte collection,

"hCG priming," the resumption of meiosis begins and subsequently oocytes are collected that may be at varying stages of the maturation process; GV, MI, or MII oocytes. In turn, this makes *in vitro* culture, fertilization, embryo culture timing, and embryo transfer logistically difficult, as the oocytes need to be treated individually according to their stage of development. In agreement with De Vos, it is our view that the true classification of IVM should be restricted to cycles without the use of a hCG trigger, with the process of germinal vesicle breakdown and resumption of meiosis completed "*in vitro*." Hence, true IVM involves the culture of germinal vesicle (GV) oocyte in vitro culture.

2. History of IVM

The technique of IVM has been used in veterinary practice for a long time [8, 9]. However, the first pregnancy resulting from IVM in humans was reported in 1991 using donor oocytes from unstimulated ovaries from women undergoing gynaecological surgery [10]. In 1994, Trounson et al. reported a pregnancy in an anovulatory woman with PCOS after IVM of her own oocytes with an abbreviated steroid replacement protocol after embryo transfer [11]. Following these early reports, and likely due to the widespread uptake of ovarian stimulation, research progressed slowly for IVM. Initial reports focused on the development of specific culture conditions [12], variations in stimulation and priming protocols [13, 14], and patient selection [15, 16], as well as fertilization techniques [17]. Traditionally, cycles of IVM are performed using intracytoplasmic sperm injection (ICSI) for fertilization, although similar fertilization rates with IVM-IVF have been reported by Walls et al. making IVM-IVF an acceptable option, which is a cost-effective and potentially less invasive treatment than traditional IVF [18]. More recently, research has progressed to include assessments of IVM outcomes using the advanced technologies of time-lapse incubation [19] and preimplantation genetic screening [20]. Together with the introduction of freeze-all protocols to reduce the incidence of miscarriage and allow success rates closer to standard IVF [4], these advances have generated a renewed interest in IVM research, particularly for PCOS patients. Thus, we believe that despite the use of strategies to minimise the risk of OHSS, such as the use of gonadotrophin-releasing hormone (GnRH) antagonists for pituitary suppression [21], IVM should still be viewed as an alternative treatment option for women with PCOS.

3. Indications for IVM

The use of IVM for infertility treatment has several perceived advantages over conventional IVF for women with a high antral follicle count, such as women with PCOS. These include a shorter duration of stimulation and the use of less gonadotrophins. Additionally, there is the avoidance of the supraphysiologic levels of oestradiol, with its symptomatic benefits, and the opportunity to minimise exposure to high oestradiol concentrations for a woman undergoing ovarian stimulation for fertility preservation with breast cancer, or a woman with a thrombophilia, and the elimination of the risk of OHSS. However, the initial interest and enthusiasm for IVM has waned, due to the perceived lower pregnancy rates achieved with IVM treatment and the relatively recent introduction of easily accessible strategies to reduce the risk of OHSS. Such modifications in the stimulation protocols for women with PCOS, undergoing IVF treatment perceived to be at a significant risk of OHSS, include the use of GnRH antagonist protocols [22], with the use of a GnRH agonist as a trigger injection prior to oocyte retrieval, the concurrent use of metformin during stimulation [21, 23], and the use of dopamine agonists [5]. However, despite these strategies, OHSS still occurs, albeit with less frequency [3]. Further perceived benefits of an IVM treatment cycle include a lower treatment burden for the patient, a lower cost, greater patient safety, and an alternative to standard IVF treatment [4].

In addition, IVM can be used in patients with ovarian resistance to follicular-stimulating hormone (FSH) [24], fertility preservation of cancer patients (particularly women with leukemia and oestrogen-sensitive tumours), and endometriosis patients undergoing extensive endometrioma excision [24]. It can also be used as a fertility-preserving option for women at risk of premature ovarian failure [25]. It has also been used in normal responders with history of poor oocyte/embryo quality as well as for oocyte donation cycles to avoid the discomfort of the stimulation for a donor. Furthermore, the laboratory processes of IVM employed on immature oocytes derived from ovarian tissue enable clinicians to offer another option to preserve fertility for women who may be undergoing oophorectomy [26]. Segers et al. have reported a successful pregnancy after ex vivo method of oocyte cryopreservation after oophorectomy followed by IVM [27], and our group have performed oophorectomy after a few days of ovarian stimulation, without a trigger and we derived 18 mature oocytes after IVM [26].

Many couples drop out from IVF treatment due to the physical and psychological burden of conventional ovarian stimulation, and therefore, IVM can provide for some couples a less intense option that avoids the bloating discomfort of conventional treatment. Furthermore, in countries where the patient is required to pay for her medication, IVM offers a shorter, minimal stimulation approach at a lower cost. In addition, IVM may be used for patients who live in a rural or remote environment who are at risk of OHSS after COS, requiring intense post egg collection monitoring and risk a cycle cancellation where the requirement for frequent observation poses logistical problems, such as in our rural environment in Western Australia.

4. Improving the Success of IVM

The major reason why IVM has not been adopted more widely to treat women with PCO or PCOS is due to the perceived reduced likelihood of successful treatment. Hence, this led to the adoption of treatment protocols involving the transfer of multiple embryos in a fresh treatment cycle [28]. Earlier studies that compared the outcomes of IVM to conventional IVF reported significantly worse pregnancy rates with IVM, as the majority of these treatment protocols

involved hCG priming. This led to the early resumption of meiosis, and due to the short duration of the follicular phase of all IVM protocols, a poor luteal phase endometrium developed. Hence, the adoption of treatment protocols using a short period of ovarian stimulation, the avoidance of hCG priming, and the adoption of a "freeze-all" [6, 29] approach with the transfer of a single blastocyst in a subsequent frozen embryo transfer cycle have led to live birth rates that approximate those of traditional IVF cycles, with the avoidance of OHSS. Good patient selection, optimization of IVM protocols, oocyte retrieval procedure, and potentially improving culture media may offer future potential to improve treatment outcomes.

5. Optimization of IVM Protocol

Various IVM protocols have been described, with oocyte aspiration performed in unstimulated cycles or stimulated cycles with FSH priming and with or without HCG trigger [6, 30–32]. Although success rates were low in initial IVM studies, with improved regimes and protocols, the rates of oocyte maturation, fertilization, and implantation have been significantly improved [4, 6].

The effect of various IVM protocols using no priming, FSH only, hCG only, and FSH with hCG, had been studied by Fadini et al. in normoovulatory women [31] and reviewed by Siristatidis et al. [33]. Their data demonstrates the use of FSH with hCG improved clinical pregnancy rates and implantation rates in a randomized trial [31]. The effects of FSH priming in the follicular phase are due to the recruitment of greater number of follicles, whereas hCG priming causes maturation of some follicles in vivo leading to recruitment of oocytes at different stages [6, 32]. Hence, in IVM cycles with hCG priming, it is possible to collect oocytes in various stages of maturity from follicles from 2–13 mm in size [14, 34]. In a sibling oocyte study, Son et al. reported that after hCG priming, the embryo development was similar irrespective of the size of the follicle the oocyte was aspirated from, whether larger or smaller than 10 mm in diameter [35]. Hence, it would appear that the timing of oocyte retrieval is not so critical when hCG priming is used; however, it is critical when no trigger is used. Both our group and the Belgian group (De Vos et al. and Ortega-Hrepich et al.) have found improved clinical outcomes with transfer of single vitrified-warmed embryos in non-hCG-primed IVM cycle in PCOS patients, as compared to fresh embryo transfer [6, 29]. These effects are mainly attributed to poor endometrial receptivity in fresh embryo transfer cycles. With the opportunity to introduce adjuvants to the culture media such as C-Type natriuretic peptide (CNP) and amphiregulin, the optimal follicular size at the time of retrieval in non-hCG-primed cycles may reduce to 8 mm [36].

With regard to the follicle aspiration technique employed in an IVM cycle, most centres use a small gauge needle (16 or 17 gauge) with suction pressures ranging from 52 mm to 200 mm Hg, with either a single or double lumen needle; in our unit, we use a double lumen needle to enable follicular flushing [6, 37–42]. When Junk and Yeap published their optimized IVM protocol from our clinic in 2012 by the use of IVM in combination with FSH priming, the collection of oocytes when the leading follicle was 10–12 mm in diameter and the transfer of a single blastocyst-stage embryo with modified hormone therapy to assist endometrial development, they demonstrated excellent implantation and pregnancy rates [6]. With ongoing evaluation of our IVM cycle results, we now just perform an embryo transfer in a subsequent vitrified-warmed cycle, as our clinical pregnancy rates are the same as our IVF cycle results for women with PCOS [4].

Many studies have described excellent pregnancy rates using FSH or/and hCG priming [4, 6, 31]. A Cochrane review reported that hCG priming for IVM treatment had no effect on pregnancy, live birth, or miscarriage rates; however, the evidence was low, due to the limited amount of studies available for review [43]. Regardless, this evidence, coupled with the logistical difficulties encountered following hCG priming and the more recently reported success rates following IVM treatment without hCG priming, demonstrates that hCG priming is not an advisable methodology in IVM treatment.

6. Predictive Markers of Success of IVM

A recent study by Tannus et al. found that the most significant predictors for live birth after IVM in PCOS patients are a short duration of infertility, a higher oocyte retrieval number, a higher number of blastomeres within the embryo, and a better embryo grade. Potentially, these predictive factors can be used when planning treatment or counselling patients [3]. In addition, the paper by Walls et al. demonstrated very poor IVM treatment outcomes for women over 36 years of age [4].

The serum anti-Mullerian hormone (AMH) concentration and the antral follicle count (AFC) are useful factors for the prediction of pregnancy outcomes for women with PCOS prior to the commencement of an IVM cycle [44, 45]. Seok *et al.* reported in a retrospective case-control study of patients with PCOS that women with serum AMH concentrations above 8.5 ng/mL had IVM pregnancy outcomes comparable to women undergoing conventional IVF treatment [44]. Furthermore, the serum AMH and the AFC appear to be independent predictors of cumulus oocyte complex (COC) yield, with the cumulative, ongoing clinical pregnancy rate being greater for women who had more than eight COC retrieved. Guzman et al. described a predictive model of IVM success incorporating the serum AMH and AFC [45]. As would be expected, the presence of an abundance of antral follicles, which predispose a woman with PCOS susceptible to OHSS when undergoing IVF treatment, in fact makes IVM treatment ideal for such women.

However, the pregnancy rates in unstimulated hCG-primed IVM cycles appear to be impaired in women with PCOS with insulin resistance, as hyperinsulinemia appears to have a negative effect on endometrial function and the implantation process rather than embryo quality [46]. In addition, the ratio of the serum gonadotrophins has reportedly had no difference on pregnancy rates in woman

with PCOS undergoing IVF with GnRH agonist, GnRH antagonist, and IVM cycles [47].

7. Optimization of Culture Media

Until recently, maturation media formulations and culture protocols did not differ significantly from one another, except for more than 24-hour variations in culture timing (generally reported between 24 h and 48 h) and occasional variation in culture media additives. At a basic level, IVM culture media consists of a base culture media, hormonal additives, and a source of protein. Reported base media consist of either commercially available IVM media [48] or blastocyst defined media [6] with no reported differences in success rates between the two [49]. For successful resumption of meiosis, the addition of either FSH and either hCG or LH to the culture media is necessary to promote the proliferation and expansion of the coronal cells and aid in the final stages of oocyte maturation in vitro. Interestingly, one study demonstrated that after oocyte retrieval without hCG priming, the larger GV oocytes have the greater potential for meiotic resumption [50]. Most clinical protocols reported have included either autologous maternal serum, human serum albumin (HSA), or human follicular fluid (HFF) as a source of protein for use in culture with comparable efficacy [51]. Preference may be given to HSA, as HFF and maternal serum have the potential to introduce contaminants and other elements which may impact negatively on oocyte or embryo developmental competence, as well as contributing to the lack of heterogeneity across cases, as they do not allow for adequate quality control.

Other culture additives have been suggested to improve IVM success rates over the years; however, their reports are sporadic and rarely used in everyday culture. Insulin-like growth factor (IGF-I) has shown promise in animal models and early human studies, promoting cumulus cell expansion [52], and recombinant epidermal growth factor has been added with success to some culture systems [53] as well as its family members amphiregulin and epiregulin showing promise in terms of maturation rates and embryo developmental capacity to the day two to three stage [54]. In recent years, the discovery of other factors which may promote oocyte maturation such as oocyte-secreted factors BMP-15 and GDF9 and their heterodimer "cumulin" has shown promise in animal models [55], and we have seen the emergence of dynamic *in vitro* systems to improve embryo quality and quantity, the so-called prematuration or pre-IVM systems [56]. One of the important aspects is to maintain optimal concentration of cyclic adenosine monophosphate/cyclic guanosine monophosphate (cAMP/cGMP) levels after removal from the follicle, as they play an important role in oocyte meiosis resumption/arrest [56]. Pre-IVM with cAMP modulators have been shown to improve IVM outcomes in bovine oocytes [57], and a recent study demonstrated a strategy involving prematuration culture (PMC) in the presence of CNP followed by IVM using FSH and amphiregulin, which increased oocyte maturation potential, leading to a higher availability of day three embryos and good-quality blastocysts for single embryo transfer [36]. Like most research in the IVM field, this will need to be validated by further large-scale trials.

8. Safety of IVM

One of the primary concerns regarding IVM treatment are the neonatal outcomes and any adverse effects on the growth and development of children born following the procedure. Increased rates of congenital malformations have been reported in children born following conventional IVF treatment compared with the general population [58], as well as a potential increase in metabolic disorders [59]. While there is currently limited evidence of the long-term outcomes of children born following IVM, early research has demonstrated that outcomes are comparable to conventional IVF controls [60].

With respect to embryonic development, our group have reported an increase in early embryo arrest in women with PCOS after IVM as compared to women with PCOS undergoing standard ICSI using time-lapse analysis, although no difference was recorded in the morphokinetic development of the useable embryos between the groups [19]. We have also noted that PCOS-IVM oocytes were significantly larger as compared to the oocytes of women from PCOS-ICSI and control-ICSI groups [61]. These differences had been attributed to the in vitro maturation process with inadequate completion through the stages of cytoplasmic maturation. These changes may be associated with a decrease in the rate of fertilization and impaired blastocyst development for PCOS patients undergoing IVM. A similar finding was recorded in a recent study by Roesner et al. using time-lapse analysis, where significant differences were noted in embryo development between PCOS-IVM as compared to PCOS-ICSI and control-ICSI groups, with similar pregnancy and live birth rates resulting in these groups [62]. The rates of embryo development differed between these two studies, and this is attributed to the difference in IVM protocols used (e.g., FSH priming or FSH and hCG priming, or potentially the duration of FSH use), differences in IVM culture media, or possibly patient demographics.

There have been concerns regarding the association of epigenetic defects with IVM treatment. Recent gene studies have shown reassuring results, although the small sample size is a limiting factor of these studies. Pliushch et al. studied 15 developmentally important genes and two repetitive elements for methylation levels in 11 patients undergoing IVM treatment and 19 patients undergoing standard IVF/ICSI. They analysed tissues from chorionic villous sampling and cord blood sampling and demonstrated minimal effects of IVM treatment on the methylation patterns of the sampled tissue [63]. Using the same technique of bisulphite pyrosequencing for analysis of gene methylation patterns, Kuhtz et al. studied three maternally methylated and one paternally methylated gene for imprinting errors and found no differences in the methylation patterns in these genes after IVM treatment as compared to in vivo-developed oocytes [64]. Thus, these studies provide some reassuring

data regarding any potential epigenetic effects resulting from IVM treatment.

Junk and Yeap reported no congenital defects in 28 patients who had live birth in their study [6]. In the review of IVM strategies by Mikkelsen in 2005, of the 46 patients who delivered a baby, none of the children conceived after IVM had chromosomal abnormalities, one baby had a soft cleft palate, and there was one stillbirth that was not attributable to IVM [30].

With regard to the obstetric outcomes after IVM treatment, the preterm birth rates and the infant birth weights, both important predictors of health outcomes, are comparable after IVM and standard IVF conception, with a possible lower preterm birth rate after IVM treatment [4]. In a French study, the authors reported two-year follow-up of children born after IVM treatment in comparison to those born after standard ICSI treatment. In their study, the mean weight and height of boys were similar amongst the two groups, although girls were significantly heavier in the IVM group [65]. Fadini et al. also reported higher birth weight in singleton children born after IVM [66]. The mean birth weight in IVM infants was higher than spontaneously conceived infants, potentially due to the higher risk of gestational diabetes in women with PCOS. Another study reported that in comparison to the general population, the mean gestational age at delivery and birth weight, for both singletons and twins, was comparable to the general population [67]. However, as concerns have been raised regarding the possibility of epigenetic changes resulting from IVM treatment, larger studies are required [68].

With regard to childhood development, a recent prospective controlled study comparing the embryonic, neonatal, and two-year developmental outcomes in children born after IVM, IVF, and ICSI treatments demonstrated no difference in Bayley' developmental scores between the groups [60]. In another two-year follow-up study of children born from IVM treatment, recording the growth and development using Bayley's scales, the authors reported normal scores for 34 out of 35 children and a mild development delay in one child. Their neuropsychological scores at two years of age were normal in this study. Furthermore, in another study, a cohort of children born after IVM in women with PCOS patients matched with spontaneously conceived children, when they underwent developmental assessment between 6 and 24 months of age using Bayley's scales, there were no differences in their mental or psychomotor development and no concerns regarding their neonatal or early infancy development [69]. Thus, the outcomes of IVM have been reassuring so far; however, the sample size in these studies is small, warranting interpretation of results with caution and emphasizing the need for further study.

9. Conclusions

Tannus et al. have reported clinical pregnancy rates of 44.7% and live birth rate of 34.6%, for women undergoing IVM treatment, with the majority of transfers being single [3]. Furthermore, our group compared the cumulative live births obtained after IVM treatment and conventional IVF/ICSI treatment, for patients with PCOS in Western Australia, and reported similar per frozen embryo transfer cycle pregnancy rates across both groups. However, we recorded a higher cumulative live birth rate achieved after standard IVF in comparison to IVM treatment (55% versus 41%) [4]. The authors attribute this finding to the lower number of MII oocytes obtained in the IVM group in comparison to the IVF/ICSI group, where roughly half the number of oocytes are retrieved as follicles are aspirated at an IVM collection. Importantly, embryo development per MII oocyte was similar, and the embryo implantation potential was also similar when examined in freeze-thaw cycles [4]. Consequently, the improvement of the MII oocyte rate is the key to further optimize the potential of IVM as a technique. Importantly, there were no cases of OHSS in the IVM group, whereas seven patients in the IVF/ICSI group developed OHSS; consequently, the elimination of OHSS is a significant advantage of IVM making it a safer option and potentially a more "patient friendly" approach. There were no multiple pregnancy or births in the IVM group and only two sets of twins in the IVF group, attributed to the predominantly single blastocyst transfer approach [4].

The adoption of a "freeze-all" approach has led to the avoidance of the difficulty in overcoming the poor luteal phase in a fresh IVM cycle and has been adopted now as routine in our practice [4]. Also, other groups have demonstrated that the implementation of hormone therapy regimens including high-dose oestrogen therapy commenced earlier in the treatment cycle may lead to an improved endometrial environment for embryo implantation, in comparison to other regimes [6].

A recent meta-analysis of IVM protocols, with and without the use of FSH and with and without hCG priming, has provided evidence demonstrating that IVM seems to be the preferable approach in treating women with PCOS during an IVF cycle as compared to those without PCOS [33]. This meta-analysis included 11 trials with 268 PCOS, 100 PCO patients, and 440 women with other causes of subfertility; they concluded that IVM appears to be a more efficient treatment option in terms of clinical pregnancy, implantation and cycle cancellation rates for women with PCOS when compared to the non-PCOS group. They also observed a borderline, but meaningful, trend in live birth rates in the PCOS group, favouring IVM [33]. Oocyte maturation and miscarriage rates did not differ between the groups, while a borderline trend towards lower fertilization rates among PCOS patients was observed. Previously, the same group in 2013 were unable to find any randomized control trials with the intention to perform IVM before IVF or ICSI in PCOS patients. They state that it is imperative that large multicentre studies are required in the field of IVM to answer the question whether IVM should be done prior to standard IVF/ICSI in PCOS women [70]. However, before such a study were to commence, a standardized IVM protocol must be agreed upon; with or without the use of FSH stimulation, either with or and without hCG priming, and whether to include a fresh or just the frozen transfers of a single embryo.

The IVM approach offers an excellent treatment option for women with PCOS, who are required to undergo assisted reproduction, as many subfertile women with PCOS will conceive with ovulation induction therapy alone. IVM offers several advantages over standard IVF, particularly the elimination of the risk of OHSS, it is cheaper and with a lower side effect profile than IVF, and offers a "patient friendly" approach to assisted reproduction.

Conflicts of Interest

Dr. Shital Julania declares that there is no conflict of interest regarding the publication of this paper. Dr. Melanie L Walls has previously received educational support from Cook Medical. Professor Roger Hart is the Medical Director of Fertility Specialists of Western Australia and a shareholder in Western IVF and has received educational support from Ferring Pharmaceuticals, MSD, and Merck.

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Research Article

Low-Dose Spironolactone-Pioglitazone-Metformin Normalizes Circulating Fetuin-A Concentrations in Adolescent Girls with Polycystic Ovary Syndrome

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Background. Fetuin-A is a glycoprotein produced in the liver and related to metabolic syndrome; fetuin-A secretion is divergently regulated in different pathological conditions. In girls with polycystic ovary syndrome (PCOS), insulin sensitization results in a more favorable endocrine-metabolic outcome than oral contraception; we assessed whether those differences are underscored by changes in circulating fetuin-A. *Methods*. Fetuin-A concentration endocrine-metabolic markers and hepatovisceral fat were measured longitudinally in 35 PCOS girls [age, 16 yr; body mass index (BMI), 23 kg/m^2] randomized to receive either oral contraception [ethinylestradiol-levonorgestrel ($n = 18$)] or a low-dose combination of spironolactone, pioglitazone, and metformin (SPIOMET, $n = 17$) over 12 months. Healthy adolescent girls (age- and BMI-matched) were used as controls ($n = 25$). *Results*. Pretreatment fetuin-A serum levels in PCOS girls were lower than those in controls. After 12 months on treatment, fetuin-A raised to control levels only in the SPIOMET subgroup ($P = 0.009$, versus oral contraception); this increase was paralleled by a healthier metabolic profile with less hepatic fat (by MRI); baseline serum fetuin-A as well as the changes over 12 months was inversely related to hepatic adiposity. *Conclusions*. A low-dose combination of insulin sensitizers and an antiandrogen—but not oral contraception—normalizes fetuin-A levels in adolescent girls with PCOS. This trial is registered with ISRCTN29234515.

1. Introduction

Fetuin-A (α2-HS glycoprotein, AHSG) is a glycoprotein produced primarily in the liver and secreted into circulation in high concentrations in humans with fatty liver disease [1]; it binds the insulin receptor and inhibits hepatic and muscle insulin signaling resulting in insulin resistance [2]. In humans, high levels of fetuin-A have been associated with greater risks for type 2 diabetes (T2D) and with features of the metabolic syndrome [3]; paradoxically, increased fetuin-A concentrations prevent vascular calcification and exert a protective role in systemic inflammation, suggesting that fetuin-A secretion can be divergently regulated in different pathological conditions [4].

Polycystic ovary syndrome (PCOS) is the most common cause of hirsutism, acne, and menstrual irregularity in girls and young women and associates to comorbidities in adulthood, including subfertility and T2D. We have previously shown that in adolescent girls with PCOS, oral contraception (OC)—as compared to combined low-dose insulin

sensitization plus antiandrogen therapy—is linked to a less favorable endocrine-metabolic profile [5, 6]. To date, the available studies reporting fetuin-A in PCOS have been performed in adult women, include heterogeneous populations, and have a cross-sectional nature [7–9]. Here, we assessed longitudinally whether the divergent effects of oral contraception and low-dose combined insulin sensitization plus antiandrogen therapy in adolescent PCOS are underscored by changes in circulating fetuin-A.

2. Subjects and Methods

2.1. Study Population and Design. The study population consisted of 35 nonobese adolescent girls with PCOS [mean age, 16 yr; body mass index (BMI), 23 kg/m^2]; all of them were at least 2 yr beyond menarche. The inclusion criteria were as described [10] (1) hirsutism (score > 8 on Ferriman and Gallwey scale); (2) oligomenorrhea (menstrual intervals > 45 days); and (3) absence of sexual activity throughout the study duration (and thus, no need for contraception). The girls were recruited in the Adolescent Endocrinology Unit of Sant Joan de Déu University Hospital, Barcelona, Spain [10]. Recruitment was biased against overweight/obesity because, in our setting, those girls are primarily referred to the adolescent obesity unit. Exclusion criteria were thyroid dysfunction, anemia, bleeding disorder, hyperprolactinemia, Cushing syndrome, adrenal hyperplasia, liver or kidney dysfunction, and use of drugs affecting gonadal or renal function or carbohydrate or lipid metabolism. Patients were included in a randomized, open-label study comparing the effects of OC with ethinylestradiol-levonorgestrel [EE-LNG; 20 μg of EE plus 100 mg of LNG for 21 of 28 days, placebo for 7 of 28 days; Loette Diario, Pfizer, Madrid, Spain] with those of a low-dose combination of spironolactone 50 mg/d, pioglitazone 7.5 mg/d, and metformin 850 mg/d (SPIOMET). The study was registered as ISRCTN29234515 and lasted for 24 months (12 months on treatment; then 12 months off treatment) [10]. The present report specifically included those girls with complete longitudinal data in whom the remaining serum sample was sufficiently abundant to measure fetuin-A at baseline and after 12 months on treatment (~90% of the initial study population, $n = 18$ and $n = 17$ in the EE-LNG and SPIOMET subgroups, resp.). Twenty-five age- and BMI-matched healthy girls recruited in nearby schools served as controls. All had regular menstrual cycles, and none was hirsute or was on OC or other medications affecting ovarian function or carbohydrate or lipid metabolism.

2.2. Clinical and Endocrine-Metabolic Assessments. One investigator (unblinded to treatment) measured weight and height (Harpenden Stadiometer) and scored hirsutism (Ferriman-Gallwey). Systolic and diastolic blood pressures were recorded after a 5-minute rest with the girl supine, using an electronic sphygmomanometer (767 series, Welch Allyn, Spain).

Endocrine-metabolic assessments were performed in the early morning, in the follicular phase (days 3–7) of the cycle or after 2 months of amenorrhea, as described [5]. Briefly, circulating insulin and SHBG were assayed by immunochemiluminescence (IMMULITE 2000, Diagnostic Products, Los Angeles, CA). HOMA-insulin resistance (HOMA-IR) was calculated as [fasting insulin in mU/L] × [fasting glucose in mg/dL]/405. Serum C-reactive protein (CRP) was analyzed by immunochemiluminescence (ARCHITECT i2000SR, Abbott Diagnostics, Abbot Park, IL, USA); intra- and interassay coefficients of variation (CVs) were <10%. HMW adiponectin was assessed by ELISA (R&D Systems, Minneapolis, MN, USA); intra- and interassay CVs were <9%. Circulating fetuin-A was assessed with a specific ELISA (fetuin-A, R&D systems, Minneapolis, MN, USA); the intra- and interassay CVs were 4.2% and 7.4%, respectively.

2.3. Abdominal Fat Partitioning. Subcutaneous, visceral, and hepatic fat was assessed by magnetic resonance imaging (MRI) using a multiple-slice MRI 1.5 Tesla scan (Signa LX Echo Speed Plus Excite, General Electric, Milwaukee, WI) [10].

2.4. Statistical Analyses and Ethics. Statistical analyses were performed with SPSS 23.0 (SPSS Inc. Chicago, IL). Results are expressed as mean ± SEM. Comparisons within and between groups at each time point were performed using general linear model. Correlation analysis was used to study the associations between fetuin-A levels and auxological and endocrine-metabolic parameters. Two-way analysis of variance (ANOVA) was performed to assess the influence of treatment and time on fetuin-A levels. $P < 0.05$ was considered statistically significant.

The study was conducted after approval by the Institutional Review Board of Sant Joan de Déu University Hospital, after written consent by parents and after assent by each of the participants, including the healthy controls who allowed to obtain indicative values.

3. Results

Both treatments reduced androgen excess comparably, but SPIOMET was followed by a more favorable endocrine-metabolic profile, as expected (see Table 1 for differences in selected variables between subgroups).

Pretreatment serum concentrations of fetuin-A in PCOS girls were lower than those in controls. After 12 months on treatment, fetuin-A levels increased only in the SPIOMET subgroup ($P = 0.009$ versus the OC subgroup), reaching control levels (Figure 1, Table 1).

At baseline, circulating fetuin-A correlated negatively with hepatic fat in both controls and PCOS girls ($r = -0.739$; $P = 0.03$ and $r = -0.446$; $P = 0.006$, resp.). After treatment, fetuin-A negatively associated with visceral fat in the SPIOMET subgroup ($r = -0.583$, $P = 0.004$) and with diastolic blood pressure in both the SPIOMET and OC subgroups ($r = -0.729$, $P = 0.002$, and $r = -0.584$, $P = 0.03$, resp.). The change in serum fetuin-A concentrations 0–12 months correlated negatively with diastolic blood pressure ($r = -0.442$; $P = 0.039$), hepatic fat ($r = -0.647$; $P = 0.002$), and C-reactive protein (CRP; $r = -0 - 617$; $P = 0.003$), only in the SPIOMET subgroup. Two-way ANOVA showed that both time and treatment have an effect on fetuin-A levels ($P = 0.02$ and $P = 0.006$, resp.).

Table 1: Data from adolescent girls with polycystic ovary syndrome (PCOS) who were randomized to receive either ethinylestradiol-levonorgestrel (EE-LNG; $n = 18$) or low-dose spironolactone-pioglitazone-metformin (SPIOMET; $n = 17$) for 12 months.

	Controls ($n = 25$)	All PCOS ($n = 35$)	EE-LNG ($n = 18$)			SPIOMET ($n = 17$)		
		Baseline[a]	Baseline	12 months	Δ 0–12 months	Baseline	12 months	Δ 0–12 months
Age (year)	15.6 ± 0.2	15.8 ± 0.2	15.9 ± 0.3	—	—	15.7 ± 0.3	—	—
BMI (kg/m^2)	22.2 ± 0.5	23.6 ± 0.5	23.9 ± 0.8	24.0 ± 0.8	0.04 ± 0.27	23.1 ± 0.7	23.0 ± 0.7	−0.31 ± 0.20
SBP (mmHg)	112 ± 2	114 ± 1	115 ± 2	112 ± 3	−3.2 ± 2.9	113 ± 2	109 ± 1[b]	−4.9 ± 2.2
DBP (mmHg)	70 ± 1	71 ± 1	72 ± 2	75 ± 2	3.7 ± 2.9	70 ± 1	70 ± 1	−0.5 ± 1.6
AST (UI/L)	17.8 ± 0.7	16.0 ± 0.5	15.7 ± 0.7	16.5 ± 1.1	0.8 ± 0.7	16.3 ± 0.9	17.3 ± 1.0	0.8 ± 0.9
ALT (UI/L)	15.0 ± 1.1	14.2 ± 0.8	15.1 ± 1.3	18.9 ± 2.3[b]	3.9 ± 1.7	13.2 ± 0.6	15.4 ± 1.5	1.7 ± 1.2
GGT (UI/L)	13.9 ± 1.4	12.3 ± 0.4	12.3 ± 0.7	16.4 ± 1.0[d,i]	4.1 ± 0.9	12.2 ± 0.6	10.9 ± 0.4	−1.1 ± 0.6[g]
CRP (nmol/L)	7.5 ± 1.5	15.3 ± 2.3**	14.5 ± 2.3	28.5 ± 5.9[b,i]	14 ± 6.6	16.1 ± 4.2	5.5 ± 0.8[c]	−10.6 ± 3.7[f]
N/L ratio	1.6 ± 0.1	1.8 ± 0.1	1.9 ± 0.2	1.8 ± 0.3	−0.1 ± 0.4	1.8 ± 0.1	1.6 ± 0.2	−0.2 ± 0.1
Glucose (mmol/L)	5.1 ± 0.1	4.7 ± 0.1***	4.7 ± 0.1	4.5 ± 0.1[b]	−0.2 ± 0.1	4.6 ± 0.1	4.3 ± 0.1[c]	−0.3 ± 0.1
Insulin (pmol/L)	56 ± 6	80 ± 7*	92 ± 12	110 ± 18[h]	18 ± 13	70 ± 7	44 ± 7[c]	−27 ± 7[g]
HOMA-IR	1.9 ± 0.2	2.3 ± 0.1	2.5 ± 0.2	2.5 ± 0.3[i]	0.2 ± 0.4	2.1 ± 0.2	1.2 ± 0.2[c]	−0.8 ± 0.2[e]
HDL-C (mmol/L)	1.44 ± 0.04	1.31 ± 0.04*	1.34 ± 0.05	1.35 ± 0.06	0.02 ± 0.05	1.28 ± 0.05	1.40 ± 0.07	0.12 ± 0.06
LDL-C (mmol/L)	2.26 ± 0.11	2.31 ± 0.09	2.31 ± 0.13	2.62 ± 0.19	0.31 ± 0.15	2.30 ± 0.12	2.32 ± 0.10	0.01 ± 0.07
Triglycerides (mmol/L)	0.63 ± 0.05	0.63 ± 0.04	0.60 ± 0.06	0.62 ± 0.04	0.02 ± 0.05	0.66 ± 0.07	0.56 ± 0.05	−0.10 ± 0.05
Testosterone (nmol/L)	0.97 ± 0.05	2.01 ± 0.10***	2.14 ± 0.19	0.09 ± 0.08[d]	−1.23 ± 0.17	1.89 ± 0.08	0.96 ± 0.08[d]	−0.93 ± 0.09
SHBG (nmol/L)	58 ± 4	29 ± 2***	30 ± 3	63 ± 7[d,i]	33 ± 6	28 ± 2	32 ± 3	3 ± 3[g]
Fetuin-A (g/L)	1.18 ± 0.05	0.92 ± 0.03***	0.93 ± 0.04	0.94 ± 0.04[h]	0.01 ± 0.07	0.92 ± 0.05	1.13 ± 0.05[c]	0.23 ± 0.07[e]
Subcutaneus fat (cm^2)[†]	98 ± 12	150 ± 13*	149 ± 18	142 ± 18	−7.8 ± 9.9	150 ± 19	142 ± 18	−7.8 ± 7.6
Visceral fat (cm^2)[†]	32 ± 2	46 ± 3*	43 ± 4	42 ± 5	−1.3 ± 3.6	49 ± 5	33 ± 2[b]	−16 ± 5.4[e]
Hepatic fat (%)[†]	12.6 ± 1.4	16.7 ± 1.0*	17.0 ± 1.4	19.8 ± 1.4[i]	2.8 ± 1.4	16.5 ± 1.4	10.1 ± 0.9[c]	−6.4 ± 1.0[g]

Values are mean ± SEM. BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; CRP: c-reactive protein; N/L: neutrophile-to-lymphocyte ratio; HOMA-IR: homeostatic model assessment-insulin resistance; HMW: adiponectin, high molecular weight; TC: total cholesterol; TG, triglycerides; SHBG: sex hormone binding globulin; D4-A: 4-androstenedione; DHEAS: dehydroisoandrosterone sulphate. [†]By MRI; $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ between controls and PCOS girls at baseline. [a]No significant differences between randomized PCOS subgroups at baseline; [b]$p < 0.05$, [c]$p < 0.01$, and [d]$p < 0.001$ within subgroups for 0-to-12-month changes (Δ); [e]$p < 0.05$, [f]$p < 0.01$, and [g]$p < 0.001$ between subgroups for 0-to-12-month changes (Δ); [h]$p < 0.01$ and [i]$p < 0.001$ between subgroups at 12 months.

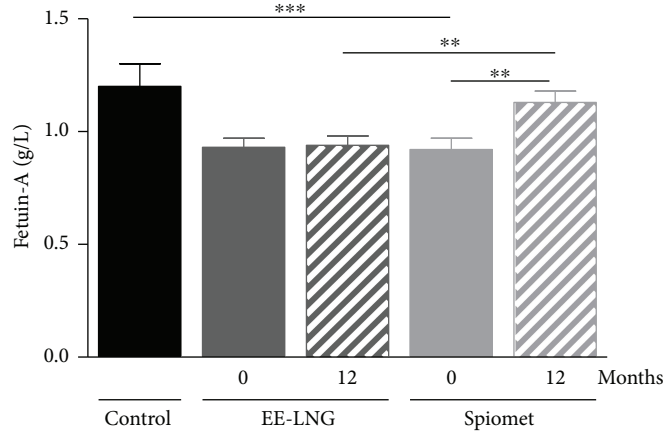

Figure 1: Longitudinal fetuin-A concentrations (mean ± SEM) in adolescent girls with polycystic ovary syndrome who were randomized to receive either an oral contraceptive [ethinylestradiol-levonorgestrel (EE-LNG) $n = 18$] or low-dose spironolactone (50 mg/d), pioglitazone (7.5 mg/d) plus metformin (850 mg/d) (SPIOMET, $n = 17$), for 12 months. Control girls ($n = 25$) matched for age and body mass index were assessed for comparison. $^{***}P < 0.001$ for baseline differences between patients and controls; $^{**}P < 0.01$ within patient subgroups for 0 to 12 months and between groups at 12 months.

4. Discussion

Here, we characterize for the first time the longitudinal outcome of circulating fetuin-A concentrations in nonobese adolescents with PCOS and show that fetuin-A levels normalize after treatment with a low-dose combination of insulin sensitizers and an antiandrogen, but not after OC.

Growing evidence supports the relationship between fetuin-A and hepatic fat depots in obesity [11]. Also, strong associations between fetuin-A and insulin resistance have been reported in subjects without diabetes [12]; indeed, fetuin-A promotes insulin resistance through inhibition of insulin receptor tyrosine kinase in hepatocytes and skeletal muscle and activation of Toll-like receptor 4 in response to free fatty acids, stimulating an inflammatory signaling pathway [2, 13]. In contrast, other studies report no associations between fetuin-A and insulin resistance in T2D patients or suggest that fetuin-A is not causally related to diabetes risk [14, 15]. Moreover, the impact of fetuin-A on cardiovascular disease is controversial depending on the presence or absence of diabetes. For example, nondiabetic subjects—but not T2D patients—with higher fetuin-A levels have a lesser risk for cardiovascular disease and related mortality [14]. Besides, fetuin-A would prevent liver and vascular fibrosis through the inhibition of transforming growth factor-β1 signaling [16].

Our apparently discordant data could be partially explained bearing in mind that fetuin-A comprises a mixture of circulating isoforms regulating specific biological processes and that the available ELISA assays may differ in their specificity for different glycosylated forms [17]. Moreover, defects of glycosylation/sialylation of fetuin-A leading to protein inactivation have also been reported, indicating that the overall glycosylation status of fetuin-A would also be relevant in the regulation of fetuin-A actions [18].

Fetuin-A also has an anti-inflammatory role, acting as endogenous inhibitor of zinc metalloproteases [19]. This feature could explain the association between fetuin-A concentrations and the change in CRP levels over 12 months in the girls receiving SPIOMET, suggesting that fetuin-A levels within the normal range are required to maintain inflammation homeostasis.

The dual functionality of fetuin-A in diabetes risk, inflammation, and cardiovascular disease raises the question as to whether decreasing fetuin-A concentrations should be customarily recommended [20], especially taking into account the heterogeneity of fetuin-A levels reported in different studies using the same method in similar age groups [21].

The finding of lower levels of fetuin-A in PCOS girls was unexpected and could be derived—at least in part—from the status of low-grade inflammation associated with this entity, since it is known that proinflammatory cytokines and proteins such as CRP—which are increased in PCOS—downregulate fetuin-A expression in the liver [4, 22]. This would also explain the negative association between fetuin-A concentrations and hepatic fat. SPIOMET treatment was followed by a healthier endocrine-metabolic profile, as judged by the reduction of insulin levels and the drop in ectopic fat; in addition, SPIOMET but not OC raised fetuin-A levels towards normal. This finding may be perceived as contradictory, because pioglitazone—but not metformin—has been reported to reduce fetuin-A levels in patients with T2D [23]. However, in those studies, pioglitazone was given in monotherapy, and at doses, at least fourfold higher [23].

The main study limitations include the small sample size and the lack of obese PCOS and obese control girls, precluding to discern the separate effects of obesity and PCOS on fetuin-A levels. The strengths include the longitudinal design, the homogeneous study population, and the assessment of the impact of two interventions with divergent effects on the endocrine-metabolic status and, potentially, on cardiometabolic risk. The cross-sectional nature and population heterogeneity of the so far available studies in PCOS women may explain the discrepancies among populations [7–9].

5. Conclusions

In conclusion, we report for the first time that fetuin-A levels are reduced in nonobese girls with PCOS and that a low-dose combination of insulin sensitizers and an antiandrogen—but not an OC—increases towards normal fetuin-A concentrations, together with an improvement of the endocrine-metabolic status. The divergent regulation and thus potential duality of fetuin-A effects in diverse pathological conditions deserve further investigation.

Conflicts of Interest

Marta Díaz, José Miguel Gallego-Escuredo, Abel López-Bermejo, Francis de Zegher, Francesc Villarroya, and Lourdes Ibáñez have no conflicts of interest to disclose. Marta Díaz and Lourdes Ibáñez are clinical investigators of CIBERDEM (Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas, ISCIII, Madrid, Spain). Francesc Villarroya and José Miguel Gallego-Escuredo are clinical investigators of CIBEROBN (Centro de Investigación Biomédica en Red Fisiopatología de la Obesidad y Nutrición, ISCIII, Madrid, Spain). José Miguel Gallego-Escuredo is a "Sara Borrell" fellow by Instituto de Salud Carlos III, Madrid, Spain. Abel López-Bermejo is a clinical investigator of the I3 Fund for Scientific Research (Ministry of Science and Innovation, Spain). Francis de Zegher is a clinical investigator (Clinical Research Council of Leuven University Hospitals).

Authors' Contributions

Marta Díaz contributed to study design, researched data, wrote, reviewed, and edited the manuscript. José Miguel Gallego-Escuredo researched the data. Abel López-Bermejo reviewed the manuscript. Francesc Villarroya, Francis de Zegher, and Lourdes Ibáñez contributed to study design and reviewed and edited the manuscript.

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