

# Metformin Decreases the Adipokine Vaspin in Overweight Women With Polycystic Ovary Syndrome Concomitant With Improvement in Insulin Sensitivity and a Decrease in Insulin Resistance

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**OBJECTIVE**—Polycystic ovary syndrome (PCOS) is associated with insulin resistance and obesity. Vaspin (visceral adipose tissue–derived serine protease inhibitor) levels increase with hyperinsulinemia and obesity. Currently, no data exists on vaspin in PCOS women. We therefore assessed mRNA and protein levels of vaspin, including circulating vaspin, from subcutaneous and omental adipose tissue of PCOS women and matched control subjects. Ex vivo regulation of adipose tissue vaspin and the effects of metformin treatment on circulating vaspin levels in PCOS subjects were also studied.

**RESEARCH DESIGN AND METHODS**—Real-time RT-PCR and Western blotting were used to assess mRNA and protein expression of vaspin. Serum vaspin was quantified by enzyme-linked immunosorbent assay. The effects of D-glucose, insulin, and gonadal and adrenal steroids on adipose tissue vaspin were analyzed ex vivo.

**RESULTS**—There were significantly higher levels of circulating vaspin ( $P < 0.05$ ), vaspin mRNA ( $P < 0.05$ ), and protein ( $P < 0.05$ ) in omental adipose tissue of PCOS women. Interestingly, in omental adipose tissue explants, glucose significantly increased vaspin protein levels and secretion into conditioned media ( $P < 0.001$ ). Also, after 6 months of metformin treatment, there was a significant decrease in serum vaspin levels in PCOS women ( $P < 0.001$ ). Furthermore, multivariate regression analysis revealed that following metformin therapy, changes in circulating glucose levels were predictive of changes in serum vaspin levels ( $P = 0.014$ ).

**CONCLUSIONS**—We report, for the first time, elevated serum and omental adipose tissue levels of vaspin in overweight PCOS women and ex vivo regulation of vaspin, predominantly by glucose. More importantly, metformin treatment decreases serum vaspin levels, a novel observation. *Diabetes* 57:1501–1507, 2008

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AUC, area under the curve; DHEA-S, dehydroxyepiandrostenedione-sulfate; HOMA, homeostasis model assessment; HOMA- $\beta$ , HOMA of  $\beta$ -cell function; HOMA-IR, HOMA of insulin resistance; OGTT, oral glucose tolerance test; PCOS, polycystic ovary syndrome; vaspin, visceral adipose tissue–derived serine protease inhibitor; WHR, waist-to-hip ratio.

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Polycystic ovary syndrome (PCOS) is the most commonly encountered endocrine disorder of women, affecting 5–10% of all women in the reproductive age (1). PCOS is characterized by menstrual dysfunction and hyperandrogenism and is associated with insulin resistance, pancreatic  $\beta$ -cell dysfunction, impaired glucose tolerance, type 2 diabetes, dyslipidemia, and visceral obesity (1,2).

Adipokines play an important role in the pathogenesis of insulin resistance, diabetes, and atherosclerosis (3). It is reported that accumulation of visceral adipose tissue poses a greater cardiometabolic risk than subcutaneous adipose tissue (4,5). Recently, a novel adipokine, vaspin (visceral adipose tissue–derived serine protease inhibitor), was identified in obese, diabetic, Otsuka Long-Evans Tokushima (OLETF) rats. Vaspin levels peaked in visceral adipose tissue at 30 weeks in OLETF rats, the age at which insulin levels are maximal in these rats; however, vaspin levels decreased with worsening of diabetes at 50 weeks. Also, vaspin was found to significantly improve glucose tolerance and insulin sensitivity in diet-induced obese mice (6). Furthermore, recent studies have found a positive association between vaspin gene expression in human adipose tissue, as well as circulating vaspin levels, with obesity and type 2 diabetes (7,8). The apparent paradox, that vaspin levels fall with worsening diabetes in OLETF rats yet the contrary is observed in humans, raises the possibility of species differences in the regulation and possible role(s) of vaspin.

Because PCOS is a pro-diabetic state with a significantly higher prevalence of impaired glucose tolerance, type 2 diabetes, visceral obesity, and altered gonadal and adrenal steroids (1,2), we measured serum vaspin levels and studied the mRNA expression and protein levels of vaspin in both subcutaneous and omental adipose tissue depots in these women against age-, BMI-, and waist-to-hip ratio (WHR)-matched control subjects. We also assessed the effects of glucose, insulin, and steroid hormones on vaspin secretion in conditioned media and protein levels from human omental adipose tissue explants. Finally, we studied the effects of metformin therapy, used in the treatment of PCOS women, on circulating vaspin levels in tandem with associated changes to clinical, hormonal, and metabolic parameters in the same cohort of PCOS women.

## RESEARCH DESIGN AND METHODS

**Study 1.** All PCOS patients met all three of the revised 2003 Rotterdam European Society for Human Reproduction (ESHRE)/American Society of

TABLE 1  
Clinical, hormonal, and metabolic features of women with PCOS and control subjects

	PCOS	Control	P
<i>n</i>	12	12	
Age (years)	31.5 (29–35)	32.0 (28–36)	NS
BMI (kg/m <sup>2</sup> )	30.5 (27.8–30.9)	29.9 (28–30.5)	NS
WHR	0.85 (0.78–0.89)	0.84 (0.81–0.86)	NS
Glucose (mmol/l)	5.7 (4.8–6.0)	4.8 (4.3–5.2)	<0.01
Insulin (pmol/l)	78.9 (42.0–91.1)	57.9 (48.5–66.0)	NS
HOMA-IR	3.3 (2.0–3.5)	2.0 (1.4–2.2)	<0.05
Cholesterol (mmol/l)	4.9 (4.1–5.7)	5.1 (4.8–5.5)	NS
Triglycerides (mmol/l)	2.4 (1.5–3.2)	1.1 (0.7–1.4)	<0.01
Luteinizing hormone (IU/l)	8.9 (6.0–10.0)	6.2 (5.0–7.0)	NS
Follicular stimulating hormone (IU/l)	6.2 (6.0–7.0)	6.6 (5.0–8.0)	NS
Prolactin (mU/l)	361.0 (315.0–397.0)	295.2 (211.0–322.0)	NS
E <sub>2</sub> (pmol/l)	390.4 (287.0–471.0)	188.5 (129.0–264.0)	<0.01
Progesterone (nmol/l)	1.8 (1.3–2.1)	2.0 (1.7–2.3)	NS
17-OH-P (nmol/l)	2.6 (2.1–2.8)	2.0 (1.2–2.3)	NS
Testosterone (nmol/l)	5.8 (4.5–6.2)	2.5 (1.7–2.6)	<0.01
Androstenedione (nmol/l)	14.9 (14.2–16.8)	5.7 (5.0–8.2)	<0.01
DHEA-S (μmol/l)	5.5 (5.4–6.6)	3.3 (3.0–4.0)	<0.05
SHBG (nmol/l)	32.1 (26.7–35.2)	59.2 (47.7–66.0)	<0.01
FAI	18.1 (14.5–21.2)	4.2 (3.3–6.1)	<0.01
Vaspin (ng/ml)	1.9 (1.7–2.3)	1.2 (1.0–1.4)	<0.05
Vaspin subcutaneous mRNA (arbitrary units)	216.0 (179.8–255.6)	132.5 (63.9–182.5)	NS
Vaspin omental mRNA (arbitrary units)	647.7 (536.8–714.0)	239.7 (178.4–385.5)	<0.05
Vaspin subcutaneous protein (OD units)	3.6 (2.6–4.7)	3.4 (2.4–4.4)	NS
β-Actin subcutaneous protein (OD units)	24.9 (24.5–25.3)	24.5 (24.0–24.9)	NS
Vaspin omental protein (OD units)	9.3 (8.1–10.5)	4.3 (3.2–5.4)	<0.05
β-Actin omental protein (OD units)	25.0 (24.8–25.4)	24.7 (24.2–25.2)	NS

Data are median (interquartile range). FAI = T (nmol/liter)/sex hormone binding globulin (nmol/liter) × 100. Group comparison by Mann-Whitney *U* test. FAI, free androgen index; NS, not significant; SHBG, sex hormone-binding globulin.

Reproductive Medicine (ASRM) PCOS Consensus Workshop Group diagnostic criteria, namely, 1) oligo- and/or anovulation, 2) clinical and/or biochemical signs of hyperandrogenism, and 3) polycystic ovaries (9). Furthermore, all subjects in the control arm had normal findings on pelvic ultrasound scan, regular periods, and no hirsutism/acne. The control group had no discernible cause for infertility (unexplained infertility). None of the women were amenorrheic. All study subjects did not have endometriosis. Exclusion criteria for the study included age >40 years, known cardiovascular disease, thyroid disease, neoplasms, current smoking, diabetes, hypertension (blood pressure >140/90 mmHg), and renal impairment (serum creatinine >120 μmol/l). None of the women were on any medications for at least 6 months before the study, including oral contraceptives, glucocorticoids, ovulation induction agents, antidiabetic and antiobesity drugs, or estrogenic, antiandrogenic, or antihypertensive medication. Also, the presence of other endocrinopathies was ruled out by measuring basal serum 17-hydroxyprogesterone and prolactin and by measuring 0800- to 0900-h cortisol after 1.0-mg (2300 h) overnight dexamethasone suppression (values <30 nmol/l considered to rule out Cushing's syndrome). All subjects suppressed cortisol <30 nmol/l.

After an overnight fast, blood samples and subcutaneous and omental adipose tissues were obtained (0800–1000 h) from adult female patients undergoing elective surgery for infertility investigation. Subjects were initially seen at the infertility clinic and then scheduled for laparoscopy to assess Fallopian tube(s) patency. All subjects underwent anthropometric measurements, i.e., weight, height, and WHR. A total of 62 subjects (16 PCOS and 46 control subjects) were recruited consecutively from the infertility clinic in accordance with the inclusion/exclusion criteria. Of the 16 PCOS subjects recruited, 4 withdrew before the study could be completed. In the control group, 6 subjects did not complete the study; from the remaining 40 control subjects, 12 control subjects matched for age, BMI, and WHR were included in the final analysis (Table 1). Subcutaneous biopsies were obtained from the same site, i.e., from a 3-cm horizontal midline incision ~3 cm above the symphysis pubis. All samples were obtained during the early follicular phase (days 2–4 from the first day of spontaneous bleeding episode). Serum/plasma was immediately aliquoted on ice and stored at –80°C. The same fat pad was divided equally into two halves. Each half was either immediately frozen in liquid nitrogen and stored at –80°C or placed into a sterile container containing Medium 199 (Sigma-Aldrich, Gillingham, U.K.) for primary adipose tissue culture. All patients underwent anthropometric measurements, i.e.,

weight, height, and WHR. The local research ethics committee approved the study, and all patients involved gave informed consent, in accordance with the guidelines in The Declaration of Helsinki, 2000.

**Study 2.** Like in study 1, all PCOS patients in study 2 also met all three of the revised 2003 Rotterdam ESHRE/ASRM PCOS Consensus Workshop Group diagnostic criteria, i.e., oligo- and/or anovulation, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovaries as assessed by transvaginal ultrasound scans (9). All subjects studied were nonsmokers and otherwise healthy. Again, none of these women were on any medications for at least 6 months before the study, including oral contraceptives, glucocorticoids, ovulation induction agents, antidiabetic and antiobesity drugs, and estrogenic, antiandrogenic, or antihypertensive medication. Like study 1, all samples were obtained during the early follicular phase (days 2–4 from the first day of spontaneous bleeding episode). Serum/plasma was immediately aliquoted on ice and stored at –80°C.

A total of 83 women of Caucasian origin with PCOS were studied. Subjects were outpatients of the Department of Reproductive Medicine and Gynaecological Endocrinology of Magdeburg University and the Department of Obstetrics and Gynaecology of Martin Luther University Halle. The metabolic study was performed in the Outpatient Department of Endocrinology and Metabolism of Magdeburg University. Blood samples were collected between 0800 and 0900 h after a 3-day normal carbohydrate diet and an overnight fast. A 75-g oral glucose tolerance test (OGTT) was performed in all women, and blood samples were drawn for the determination of glucose and insulin before and at 30, 60, 90, and 120 min after glucose ingestion. Blood samples for testing of all other parameters were drawn before the OGTT. The samples were immediately cooled, and serum/plasma was prepared within 1 h and stored at –80°C until assayed.

A treatment with metformin in an “off label use” was offered to all PCOS women independently from the results of insulin sensitivity testing. In the PCOS women that agreed, therapy was initiated after basal assessment and the dose of metformin was increased to a maintenance dose of 850 mg twice daily. Women included were closely followed up for the period of the study. Although no specific diet or exercise regimen was advised for this study, in line with our clinical practice, all women were informed about the relationship between PCOS, body weight, and insulin sensitivity and given standard advice concerning the beneficial effects of lifestyle modifications. All patients underwent anthropometric measurements before and after metformin treat-

ment. The study design was approved by the local research ethics committee of the University of Magdeburg, and written informed consent was obtained from all participants, in accordance with the guidelines of the Declaration of Helsinki 2000.

**Biochemical and hormonal analysis.** Vaspin levels in sera and conditioned media from human omental adipose tissue explants were measured using a commercially available enzyme-linked immunosorbent assay (ELISA kit; AdipoGen, Seoul, Korea), according to the manufacturer's protocol, with an intra-assay CV <4%.

Assays for glucose, insulin, cholesterol, triglycerides, luteinizing hormone, follicular stimulating hormone, prolactin, 17 $\beta$ -estradiol (E<sub>2</sub>), progesterone, 17-OH-P, testosterone, androstenedione, dehydroxyepiandrosterone-sulfate (DHEA-S), and sex hormone-binding globulin were performed using an automated analyzer (Abbott Architect, Abbott Laboratories, Abbott Park, IL).

The estimate of insulin resistance by homeostasis model assessment (HOMA) score was calculated as previously described (10). In addition, insulin sensitivity was assessed using the values for glucose and insulin derived from the OGTT. For this, the area under the curve (AUC) for insulin and glucose was calculated from the values obtained during the OGTT using the trapezoid method. As a measure for postload insulin sensitivity, the ratio between the AUC for glucose and insulin was calculated as follows: AUC glucose (mg/dl)/AUC insulin (pmol/l) (11).

**Primary explant culture.** Adipose tissue organ explants were cultured using a protocol that was a modification of the method described by Fried and Moustaid-Moussa (12). Briefly, 1–3 g adipose tissue was minced into 5- to 10-mg (~1 mm<sup>3</sup>) fragments, washed with a 230- $\mu$ m mesh (filter no. 60; Sigma-Aldrich, Gillingham, U.K.), and rinsed with sterile PBS warmed to 37°C. Samples were then transferred to six-well plates (~50 mg/well) containing 3 ml Media 199 (Gibco-BRL) supplemented with 50  $\mu$ g/ml gentamicin and 1% FBS (containing insulin at a concentration of 10<sup>-14</sup> mol/l) and cultured for 24 h with or without the addition of testosterone, 17 $\beta$ -estradiol, androstenedione, DHEA-S, insulin, or D-glucose in a 37°C incubator under an atmosphere of 5% CO<sub>2</sub>/95% air.

**Total RNA extraction and cDNA synthesis.** Total RNA was extracted from adipose tissue samples using Qiagen RNeasy Lipid Tissue Mini Kit according to the manufacturer's guidelines (Qiagen). The purity of the extracted RNA was measured by a NanoDrop spectrophotometer. A set concentration of RNA was reverse transcribed into cDNA by using M-MuLV Reverse Transcriptase (Fermentas, York, U.K.) and random hexamers (Promega, Southampton, U.K.) as primers.

**RT-PCR.** Quantitative PCR of Omentin-1 was performed on a Roche Light Cycler system (Roche Molecular Biochemicals, Mannheim, Germany). PCRs were carried out in a reaction mixture consisting of 5.0  $\mu$ l reaction buffer and 2.0 mmol/l MgCl<sub>2</sub> (Biogene, Kimbolton, U.K.), 1.0  $\mu$ l of each primer (10 ng/ $\mu$ l), 2.5  $\mu$ l cDNA, and 0.5  $\mu$ l Light Cycler DNA Master SYBR Green I (Roche). Protocol conditions consisted of denaturation of 95°C for 15 s, followed by 40 cycles of 94°C for 1 s, 58°C for 10 s, and 72°C for 12 s, followed by melting curve analysis. For analysis, quantitative amounts of genes of interest were standardized against the housekeeping gene  $\beta$ -actin. The RNA levels were expressed as a ratio, using the "delta-delta method" for comparing relative expression results between treatments in real-time PCR (13). The sequences of the sense and antisense primers used were as follows: Vaspin (224 bp) 5'-AGGCAGAA-CATGGACTTAGG-3' and 5'-GTCAGCTCGTGGATGATGTA-3' and  $\beta$ -actin (216 bp) 5'-AAGAGAGGCATCCTCACCT-3' and 5'-TACATGGCTGGGGTCT-TGAA-3'.

Ten microliters of reaction mixture(s) were subsequently electrophoresed on a 1% agarose gel and visualized by ethidium bromide using a 1-kb DNA ladder (Gibco-BRL, Paisley, U.K.) in order to estimate the band sizes. As a negative control for all the reactions, preparations lacking RNA or reverse transcriptase were used in place of the cDNA. RNAs was assayed from three independent biological replicates.

**Sequence analysis.** The PCR products from the adipose tissue were purified from the 1% agarose gel using the QIAquick Gel Extraction Kit (Qiagen). PCR products were then sequenced in an automated DNA sequencer, and the sequence data were analyzed using Blast Nucleic Acid Database Searches from the National Centre for Biotechnology Information, confirming the identity of our products.

**Western blotting.** Protein lysates were prepared by homogenising adipose tissue in radioimmunoprecipitation lysis buffer (Upstate, Lake Placid, NY) according to the manufacturer's instructions. Protein samples (40  $\mu$ g/lane) containing SDS-sample buffer (5 mol/l urea, 0.17 mol/l SDS, 0.4 mol/l dithiothreitol, and 50 mmol/l Tris-HCl, pH 8.0) were subjected to SDS-polyacrylamide gel electrophoresis (10% resolving gel) and transferred to polyvinylidene difluoride membranes. The polyvinylidene difluoride membranes were incubated with primary rabbit-anti-human antibody for vaspin (Phoenix Pharmaceuticals, Burlingame, CA) (1:1,000 dilution) or primary rabbit-anti-human antibody for  $\beta$ -actin (Cell Signaling Technology, Beverly,

MA) (1:1,000 dilution) overnight at 4°C. The membranes were washed thoroughly for 60 min with TBS/0.1% Tween before incubation with the secondary anti-rabbit horseradish peroxidase-conjugated Ig (Dako, Ely, Cambridgeshire, U.K.) (1:2,000) for 1 h at room temperature. Antibody complexes were visualized using chemiluminescence (ECL+; GE Healthcare, Little Chalfont, Buckinghamshire, U.K.). Human vaspin peptide (Phoenix Pharmaceuticals, Burlingame, CA) was used as the positive control and water as the negative control (data not shown).

**Statistics.** Nonparametric tests were used. Data are presented as means  $\pm$  SEM unless otherwise indicated. Differences between two groups were assessed using the Mann-Whitney *U* test. Data involving more than two groups were assessed by Kruskal-Wallis and Friedman's ANOVA (with Dunn's test for post hoc analysis), respectively. For Western immunoblotting experiments, the densities were measured using a scanning densitometer coupled to scanning software Scion Image (Scion, Frederick, MD). Standard curves were generated to ensure linearity of signal intensity over the range of protein amounts loaded into gel lanes. Comparisons of densitometric signal intensities for vaspin and  $\beta$ -actin were made only within this linearity range. Spearman's rank correlation was used for calculation of associations between variables, and *P* < 0.05 was considered significant.

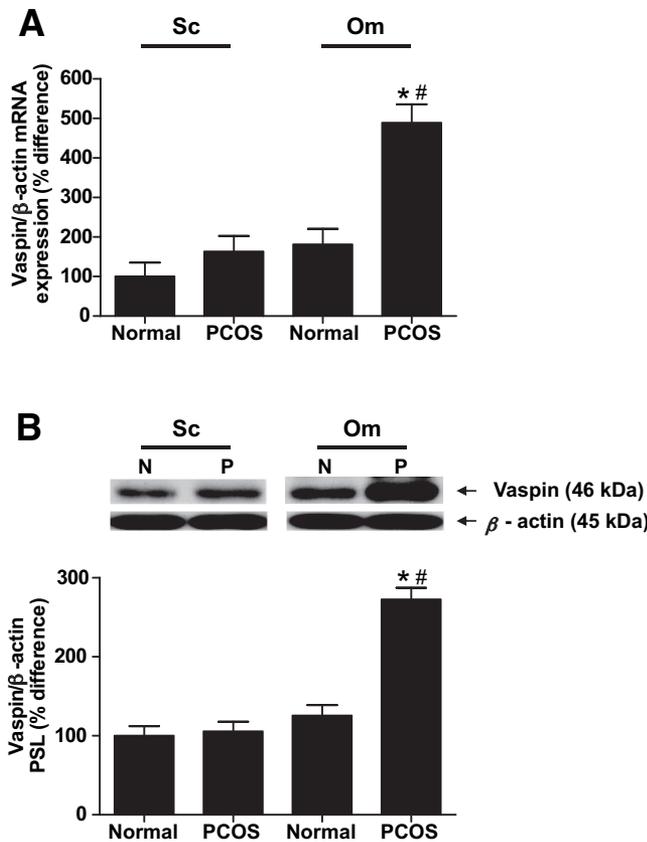
## RESULTS

**Study 1: Demographic data.** Table 1 shows the anthropometric, biochemical, and hormonal data in all subjects. Glucose, HOMA, triglycerides, 17 $\beta$ -estradiol (E<sub>2</sub>), testosterone, androstenedione, DHEA-S, and free androgen index were significantly higher, whereas sex hormone-binding globulin was significantly lower in PCOS women. Enzyme-linked immunosorbent assay analysis of serum vaspin levels revealed that PCOS patients had significantly higher levels than control subjects (1.9 [1.7–2.3] vs. 1.2 [1.0–1.4] ng/ml; *P* < 0.05) (Table 1). Serum progesterone levels in all women confirmed follicular phase of the menstrual cycle.

**mRNA expression and protein levels of vaspin in normal and PCOS women.** We detected vaspin mRNA in adipose tissue of all subjects, and subsequent sequencing of the PCR products confirmed gene identity. Real-time RT-PCR analysis corrected over  $\beta$ -actin showed a significant increase of vaspin in omental (*P* < 0.05) adipose tissues of PCOS women when compared with normal control subjects (Table 1 and Fig. 1A). Also, vaspin mRNA expression is significantly increased in human omental adipose tissue of all PCOS women compared with corresponding subcutaneous adipose tissue (*P* < 0.05). However, no significant difference in vaspin was observed in subcutaneous adipose tissues of PCOS women compared with normal control subjects (*P* > 0.05) (Table 1 and Fig. 1A). The changes noted at the mRNA level were also reflected at the protein level in PCOS women (Table 1 and Fig. 1B: *P* < 0.05 and *P* < 0.05, respectively).

**Dose-dependent effects of D-glucose, insulin, testosterone, 17 $\beta$ -estradiol, androstenedione, and DHEA-S on vaspin net protein production and secretion into conditioned media from control human omental adipose tissue explants.** Vaspin net protein production and secretion into conditioned media was significantly increased dose dependently by D-glucose in control human omental adipose tissue explants (Fig. 2A and B: *P* < 0.05, *P* < 0.01, and *P* < 0.01, respectively).

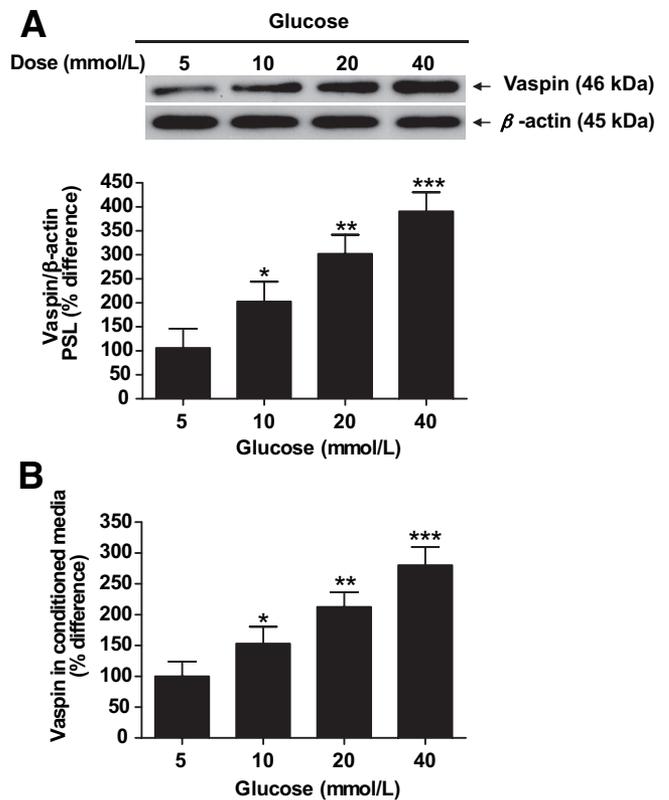
With regards to gonadal and adrenal steroids, DHEA-S showed a significant increase in vaspin net protein production at the dose of 10<sup>-7</sup> mol/l (*P* < 0.05), whereas DHEA-S effects on vaspin secretion into conditioned media failed to reach significance at the corresponding dose (*P* = 0.062). Furthermore, estradiol at the dose of 10<sup>-7</sup> mol/l increased both net vaspin protein production and secretion, although it also failed to reach statistical signif-



**FIG. 1. A:** Vaspin mRNA expression relative to  $\beta$ -actin is significantly increased in human omental (Om) and nonsignificantly increased in human subcutaneous (Sc) adipose tissue depots, respectively, when comparing all PCOS women with all normal control subjects, using real-time RT-PCR. Also, vaspin mRNA expression is significantly increased in human omental adipose tissue of all PCOS women when compared with corresponding subcutaneous adipose tissue, using real-time RT-PCR. Data are expressed as % difference to human subcutaneous adipose tissue of all normal control subjects. Group comparison was by Kruskal-Wallis ANOVA and post hoc Dunn's test.  $*P < 0.05$ ,  $\#P < 0.05$ . **B:** Western blot analysis of protein extracts from adipose tissue of all PCOS women and all normal control subjects demonstrate that the antibody against vaspin and the antibody against  $\beta$ -actin recognized bands with apparent molecular weights of 46 and 45 kDa, respectively, in human subcutaneous and omental adipose tissue depots (Fig. 1B). Densitometric analysis of vaspin complexes, having normalized to  $\beta$ -actin, revealed that protein levels of vaspin are significantly increased in human omental and nonsignificantly increased in human subcutaneous adipose tissue depots, respectively, when comparing all PCOS women with all normal control subjects. Also, vaspin protein levels are significantly increased in human omental adipose tissue of all PCOS women compared with corresponding subcutaneous adipose tissue. Data are expressed as % difference to human subcutaneous adipose tissue of all normal control subjects. Group comparison was by Kruskal-Wallis ANOVA and post hoc Dunn's test.  $*P < 0.05$ ,  $\#P < 0.05$ . PSL, phospho-stimulated light units.

icance ( $P = 0.07$  and  $P = 0.09$ , respectively). However, with regards to insulin and testosterone or androstenedione treatments, no meaningful effects on vaspin net protein production and secretion was observed (data not presented).

**Association of vaspin with covariates (study 1).** Spearman's rank analyses demonstrated that omental adipose tissue vaspin mRNA expression and protein levels were significantly positively associated with BMI, WHR, glucose, HOMA, and DHEA-S ( $P < 0.05$ ). Serum vaspin was also significantly positively associated with BMI, WHR, and glucose ( $P < 0.05$ ) but failed to reach significance with HOMA ( $P = 0.074$ ) and DHEA-S ( $P = 0.068$ ).



**FIG. 2. A:** Dose-dependent effects of D-glucose (5, 10, 20, and 40 mmol/L) on vaspin net protein production in control human omental adipose tissue explants at 24 h assessed by Western blotting compared with basal (no supplement). Western blot analysis of protein extracts from omental adipose tissues demonstrates that the antibody against vaspin and the antibody against  $\beta$ -actin recognized bands with apparent molecular weights of 46 and 45 kDa, respectively (Fig. 2A). Densitometric analysis of vaspin immune complexes, having normalized to  $\beta$ -actin, revealed that protein levels of vaspin were significantly increased by D-glucose (5, 20, and 40 mmol/L) in control human omental adipose tissue explants when compared with basal (no supplement). Data are expressed as % difference to basal human omental adipose tissue (six experiments). Each experiment was carried out with six different samples from six different subjects in three replicates. Group comparison was by Friedman's ANOVA and post hoc Dunn's test.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ . **B:** Dose-dependent effects of D-glucose on vaspin secretion into conditioned media from control human omental adipose tissue explants at 24 h were measured by enzyme-linked immunosorbent assay. Vaspin secretion was significantly increased by D-glucose (5, 20, and 40 mmol/L) from human omental adipose tissue explants. Each experiment was carried out with six different samples from six different subjects in three replicates. Group comparison was by Friedman's ANOVA and post hoc Dunn's test.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ .

**Study 2: Effects of metformin treatment on serum vaspin levels.** Metformin treatment was started in 34 women with PCOS. Only 21 women completed the study and were investigated after 6 months of metformin treatment. The anthropometric, biochemical, and hormonal data of PCOS subjects investigated in study 1 were not significantly different from those in the PCOS subjects investigated in study 2. Reasons for subjects not completing study 2 were gastrointestinal side effects, nausea ( $n = 4$ ), pregnancy ( $n = 5$ ), incomppliance ( $n = 2$ ), and loss of contact ( $n = 2$ ). The results of study 2 are presented in Table 2.

After 6 months of metformin treatment, there was a significant decrease in serum vaspin [2.1 (1.5–2.6) vs. 0.4 (0.3–0.5) ng/ml,  $P < 0.001$ ; Table 2], WHR [0.82 (0.76–0.88) vs. 0.80 (0.74–0.87);  $P < 0.05$ ; Table 2],  $E_2$  [329.8 (164.9–494.7) vs. 207.1 (103.6–310.7) pmol/l,  $P < 0.05$ ; Table 2],

TABLE 2

Clinical, hormonal, and metabolic features of women with PCOS ( $n = 21$ ) before and after metformin treatment

	Before metformin	After metformin	<i>P</i>
Age (year)	28 (26.5–31.5)	28 (27.5–32.5)	NS
BMI (kg/m <sup>2</sup> )	32.8 (29.8–36.5)	31.4 (28.2–35.1)	NS
WHR	0.82 (0.76–0.88)	0.80 (0.74–0.87)	<0.05
Glucose (mmol/l)	5.7 (4.2–5.0)	4.7 (4.4–4.9)	<0.01
Insulin (pmol/l)	75.1 (54.5–98.0)	66.9 (43.5–81.0)	NS
HOMA-IR	3.3 (2.0–3.8)	1.9 (1.3–2.2)	<0.01
HOMA-β	74.2 (37.5–91.3)	183.3 (87.7–222.5)	<0.01
Cholesterol (mmol/l)	4.7 (4.1–5.3)	4.7 (4.0–5.4)	NS
Triglycerides (mmol/l)	1.9 (0.7–2.1)	1.4 (1.0–1.7)	NS
E <sub>2</sub> (pmol/l)	329.8 (164.9–494.7)	207.1 (103.6–310.7)	<0.05
Testosterone (nmol/l)	5.6 (4.2–6.6)	4.0 (3.0–5.4)	<0.05
Androstenedione (nmol/l)	11.4 (8.0–14.0)	9.9 (7.6–12.4)	NS
DHEA-S (μmol/l)	4.3 (2.8–5.8)	5.0 (3.6–6.7)	NS
SHBG (nmol/l)	34.1 (21.0–41.0)	39.8 (21.0–47.0)	NS
FAI	19.7 (13.8–23.9)	14.5 (9.4–19.8)	<0.05
Vaspin (ng/ml)	2.1 (1.5–2.6)	0.4 (0.3–0.5)	<0.001

Data are median (interquartile range). FAI = T (nmol/liter)/sex hormone binding globulin (nmol/liter) × 100. Group comparison by Mann-Whitney *U* test. FAI, free androgen index; NS, not significant; SHBG, sex hormone-binding globulin.

testosterone [5.6 (4.2–6.6) vs. 4.0 (3.0–5.4);  $P < 0.05$ ; Table 2], FAI [19.7 (13.8–23.9) vs. 14.5 (9.4–19.8) nmol/l;  $P < 0.05$ ; Table 2] and glucose [5.7 (4.2–5.0) vs. 4.7 (4.4–4.9) mmol/l;  $P < 0.01$ ; Table 2] levels. Also, there was a concomitant improvement in insulin sensitivity as well as a decrease in insulin resistance as shown by the significant increase in HOMA of β-cell function (HOMA-β) [74.2 (37.5–91.3) vs. 183.3 (87.7–222.5);  $P < 0.01$ ; Table 2] and the significant decrease in HOMA-IR [3.3 (2.0–3.8) vs. 1.9 (1.3–2.2);  $P < 0.01$ ; Table 2] values.

More importantly, we analyzed the correlation between the change in serum vaspin levels before and after metformin therapy ( $\Delta$ vaspin) and the changes in other covariates. Interestingly,  $\Delta$ vaspin was significantly positively associated with  $\Delta$ WHR ( $P = 0.017$ ; Table 3),  $\Delta$ glucose ( $P < 0.01$ ; Table 3),  $\Delta$ HOMA-IR ( $P = 0.012$ ; Table 3), and  $\Delta$ HOMA-β ( $P = 0.013$ ; Table 3). When subjected to multiple regression analysis, only glucose remained predictive of serum vaspin levels ( $\beta = 0.572$ ,  $P = 0.014$ ; Table 3).

## DISCUSSION

We report for the first time the expression of vaspin in subcutaneous and omental human adipose tissues simultaneously at both mRNA and protein levels. Furthermore, we present novel data showing the presence and a significant increase of omental adipose tissue vaspin mRNA expression and protein levels, respectively, in overweight PCOS women. In addition, significantly higher serum vaspin levels were detected in these women. Furthermore, we describe original observations of the effect of glucose, insulin, and gonadal and adrenal steroids; interestingly, glucose caused a significant dose-dependent increase in vaspin net protein production and secretion into conditioned media from control human omental adipose tissue explants. DHEA-S also caused a significant dose-dependent increase in vaspin net protein production. Unfortunately, due to technical limitations in omental adipose tissue procurement, we were unable to obtain sufficient

TABLE 3

Linear regression analysis of variables associated with changes in serum vaspin levels (before and after metformin treatment) in PCOS subjects ( $n = 21$ )

	Simple		Multiple	
	Estimate	<i>P</i>	Estimate	<i>P</i>
$\Delta$ BMI (kg/m <sup>2</sup> )	0.291	0.201	—	—
$\Delta$ WHR	0.515	0.017	—	—
$\Delta$ Glucose (mmol/l)	0.754	<0.01	0.572	0.014
$\Delta$ Insulin (pmol/l)	-0.281	0.218	—	—
$\Delta$ HOMA-IR	0.537	0.012	—	—
$\Delta$ HOMA-β	-0.532	0.013	—	—
$\Delta$ Cholesterol (mmol/l)	-0.177	0.442	—	—
$\Delta$ Triglycerides (mmol/l)	-0.116	0.618	—	—
$\Delta$ E <sub>2</sub> (pmol/l)	0.230	0.329	—	—
$\Delta$ Testosterone (nmol/l)	-0.076	0.743	—	—
$\Delta$ Androstenedione (nmol/l)	0.152	0.511	—	—
$\Delta$ DHEA-S (μmol/l)	0.105	0.652	—	—
$\Delta$ SHBG (nmol/l)	-0.122	0.598	—	—
$\Delta$ FAI	-0.141	0.542	—	—

In multiple linear regression analysis, values included were WHR, glucose, HOMA-IR, and HOMA-β. FAI, free androgen index; SHBG, sex hormone-binding globulin.

amounts of sample per patient to perform stromal vascular separation in omental adipose tissue depots. These limitations notwithstanding, it is clear that adipose tissue from our overweight PCOS women express more vaspin. More importantly, we report for the first time that metformin (6-month treatment, 850 mg twice daily) significantly decreases serum vaspin levels in overweight PCOS subjects.

The higher serum and adipose tissue vaspin levels in women with PCOS, an insulin resistant and pro-diabetic state, is of interest given that it has recently been reported that obese insulin-resistant subjects had higher serum vaspin levels; also, in the same study, significant positive associations with BMI and insulin sensitivity were described (8). Furthermore, vaspin levels in adipose tissue had been reported to be associated with parameters of obesity and insulin resistance (7). We also detected significant positive associations between circulating vaspin as well as vaspin levels in omental adipose tissue with BMI and WHR. However, it is unlikely that either BMI or WHR are responsible for these findings, as both groups were matched for these variables.

Kloting et al. (7) reported significant associations between omental adipose tissue vaspin with 2-h OGTT plasma glucose. We found positive associations between serum and omental adipose tissue vaspin levels with glucose and HOMA-IR, respectively. Caution needs to be exercised, as these associations may be spurious, without causative significance, resulting from the simple fact that our PCOS women had significantly higher fasting glucose and HOMA-IR levels. However, we found that glucose significantly increases vaspin levels in an ex vivo adipose tissue system. Importantly, we discovered that metformin therapy given to overweight PCOS women for 6 months resulted in a significant decrease in circulating vaspin and glucose levels with a concomitant improvement in insulin sensitivity and a decrease in insulin resistance indexes. Also, although the change in serum vaspin levels was significantly positively associated with changes in WHR, glucose, HOMA-IR, and HOMA- $\beta$ , when subjected to multiple regression analysis, glucose remained the sole significant determinant of serum vaspin levels. Taken together, we hypothesize that the increased circulating as well as omental adipose tissue vaspin levels may be a compensatory mechanism for insulin resistance and/or glucose metabolism in our cohort of PCOS subjects, with glucose playing a pivotal role.

The profound effect of metformin, used in the treatment of type 2 diabetes, on serum vaspin levels is possibly through its suppressive effect on hepatic glucose production (14–16). More importantly, during the preparation of our manuscript, Youn et al. (8) described important findings of a lack of correlation between circulating vaspin and BMI in patients with type 2 diabetes. Our observations are therefore timely, as they may in part explain the findings by Youn et al., given that a proportion of their type 2 diabetic study subjects apparently were taking metformin. Our study therefore highlights metformin therapy as a confounding factor with regards to the regulation of circulating vaspin levels. This should alert investigators who are studying vaspin biology to consider this in their analyses. In addition, this point may also apply to other forms of antidiabetes therapy, and hence caution needs to be exercised appropriately.

PCOS is a state of altered steroid milieu. Recent studies have found a sexual dimorphism in circulating vaspin, with significantly elevated vaspin levels in women (8,17).

To date, there is data on the regulation and effect of gonadal and adrenal steroids on vaspin. In our cohort of PCOS subjects, levels of 17 $\beta$ -estradiol, testosterone, androstenedione, and DHEA-S were significantly higher than in the control subjects. We, like others (18,19), observed a significant decrease in levels of 17 $\beta$ -estradiol and testosterone, with no significant difference in androstenedione and DHEA-S levels, in response to metformin treatment. Could it be that a decrease in gonadal steroid levels causes a decrease in vaspin levels? Treatments of omental adipose tissue explants with different doses of testosterone, 17 $\beta$ -estradiol, androstenedione, and DHEA-S revealed that 17 $\beta$ -estradiol appeared to exhibit a dose-dependent increase in vaspin levels, albeit nonsignificantly; only DHEA-S showed a significant increase in vaspin net protein production, although vaspin secretion into conditioned media in corresponding samples was found not to be significant. It is therefore uncertain as to whether the increased levels of vaspin observed in our PCOS women is attributable to altered gonadal and adrenal steroids. Further studies are needed to elucidate the role of the effects of gonadal and adrenal steroids as well as other factors that regulate vaspin production.

A limitation of our study may relate to the number of subjects studied. However, obtaining BMI/WHR-matched and menstrual cycle-synchronized blood and adipose tissue samples from two sites impeded subject recruitment. Notwithstanding, our observations are highly consistent and significant and raise interesting questions on the mechanisms regulating vaspin production. Moreover, a sample size as in our study is only likely to detect differences that are enormous/significant.

In conclusion, we present novel data of increased circulating vaspin levels as well as increased expression of vaspin mRNA and protein levels in omental adipose tissue of overweight PCOS women and ex vivo regulation of vaspin by glucose. More importantly, we present novel data that metformin treatment, possibly via its glucose-lowering effect by suppressing hepatic glucose production (14–16), significantly decreases serum vaspin levels in overweight PCOS women. The physiologic and pathologic significance of our findings remain to be elucidated.

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