

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

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Running Title: Metformin Decreases Vaspin

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Received for publication 29 January 2008 and accepted in revised form 21 March 2008.

ABSTRACT

Polycystic ovary syndrome (PCOS) is associated with insulin resistance and obesity. Vaspin levels increase with hyperinsulinemia and obesity. Currently, no data exists on vaspin in PCOS women.

Objectives: To assess mRNA and protein levels of vaspin from subcutaneous (sc) and omental (om) adipose tissue (AT) of PCOS women and matched controls, including circulating vaspin. *Ex vivo* regulation of AT 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 ELISA. The effects of D-glucose, insulin, gonadal and adrenal steroids on AT vaspin were analysed *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 om AT of PCOS women. Interestingly, in om AT 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 was predictive of changes in serum vaspin levels ($P = 0.014$).

Conclusions: We report for the first time elevated serum and om AT levels of vaspin in overweight PCOS women, and its *ex vivo* regulation mainly by glucose. More importantly, metformin treatment decreases serum vaspin levels, a novel observation.

KEYWORDS: Metformin, vaspin, PCOS, adipose tissue, adipocyte, adipokine, insulin resistance, metabolic syndrome, glucose

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 characterised by menstrual dysfunction, hyperandrogenism and associated with insulin resistance (IR), pancreatic β -cell dysfunction, impaired glucose tolerance (IGT), type 2 diabetes mellitus (T2DM), 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 cardio-metabolic risk than subcutaneous adipose tissue (4, 5). Recently, a novel adipokine named ‘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, the age when insulin levels were 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 T2DM (7, 8). The apparent paradox that vaspin levels fall with worsening diabetes in rats (OLETF), yet the contrary is observed in humans, raises the possibility of species differences in the regulation and possible role(s) of vaspin.

As PCOS is a pro-diabetic state with a significantly higher prevalence of IGT, T2DM, 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 (sc) and omental (om)

adipose tissue depots in these women against age, body mass index (BMI) and waist to hip circumference ratio (WHR) matched controls. We also assessed the effects of glucose, insulin and steroid hormones on vaspin secretion into conditioned media and protein levels from human om 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.

SUBJECTS AND METHODS

Subjects. Study 1 All PCOS patients met all 3 criteria of the revised 2003 Rotterdam ESHRE/ASRM PCOS Consensus Workshop Group diagnostic criteria. The 3 criteria are: 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 US scan, regular periods and no hirsutism/acne. The control group had no discernible cause for infertility (unexplained infertility). No women were amenorrhic. All subjects that were studied did not have endometriosis. Exclusion criteria for the study included age over 40 years, known cardiovascular disease, thyroid disease, neoplasms, current smoking, diabetes mellitus, hypertension (blood pressure, >140/90 mmHg), renal impairment (serum creatinine, >120 μ mol/L). None of these women were on any medications for at least 6 months prior to the study, including oral contraceptives, glucocorticoids, ovulation induction agents, anti-diabetic and anti-obesity drugs, oestrogenic, anti-androgenic or anti-hypertensive medication. Also, the presence of other endocrinopathies were ruled out by measuring basal serum 17-hydroxyprogesterone, prolactin, and by measuring 0800-0900 h cortisol after 1.0 mg

(2300 h) overnight dexamethasone suppression (value below 30nmol/l was considered to rule out Cushing's syndrome). All subjects suppressed cortisol below 30nmol/l.

After an overnight fast, blood samples, sc and om 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 in order to assess Fallopian tube(s) patency. All subjects underwent anthropometric measurements i.e. weight, height and waist to hip circumference ratio (WHR). A total of 62 subjects were recruited consecutively from the infertility clinic in accordance with the inclusion/exclusion criteria (PCOS: n = 16; Controls: n = 46). 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). Sc 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 (day 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, UK) 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 their 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 3 criteria 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 non-smokers and otherwise healthy. Again, none of these women were on any medications for at least 6 months prior to the study, including oral contraceptives, glucocorticoids, ovulation induction agents, anti-diabetic and anti-obesity drugs, oestrogenic, anti-androgenic or anti-hypertensive medication. Like *Study 1*, all samples were obtained during the early follicular phase (day 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 hours, after a 3-day normal carbohydrate diet and an overnight fast. A 75g 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 hour 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 those PCOS women that agreed, therapy was initiated after basal assessment, and the dose of metformin was increased to a maintenance dose of 850mg 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 standard advice concerning the beneficial effects of lifestyle modifications was given. All patients underwent anthropometric measurements before and after metformin treatment. 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 in The Declaration of Helsinki 2000.

Biochemical and hormonal analysis. Vaspin levels in sera and conditioned media from human om adipose tissue explants were measured using a commercially available ELISA (ELISA kit: AdipoGen, Seoul, Korea), according to manufacturer's protocol, with an intra-assay coefficient of variation of less than 4%.

Assays for glucose, insulin, cholesterol, triglycerides, luteinizing hormone (LH), follicular stimulating hormone (FSH), prolactin, 17 β -estradiol (E₂), progesterone, 17-OH-P, testosterone, androstenedione, dehydroxyepiandrosterone-sulphate (DHEA-S) and sex hormone binding globulin (SHBG) were performed using an automated analyzer (Abbott Architect, Abbott Laboratories, Abbott Park, IL).

The estimate of IR 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 post-load insulin sensitivity, the ratio between the AUC for glucose and insulin was calculated as follows: AUC glucose (mg/dl)/AUC insulin (pmol/liter) (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 to 3 g of adipose tissue was minced into 5-10 mg (~1 mm³) fragments, washed with a 230 μ m mesh (Filter no. 60, Sigma-Aldrich, Gillingham, UK) and rinsed with sterile PBS warmed to 37°C. Samples were then transferred to six well plates (~50mg/well) containing 3 ml of Media 199 (Gibco-BRL) supplemented with 50 μ g/ml gentamicin and 1 % FBS (containing insulin at a concentration of 10⁻¹⁴ M) and cultured for 24 hours with or without the addition of testosterone, 17 β -estradiol, androstenedione, DHEA-S, insulin or D-glucose in a 37°C incubator under an atmosphere of 5% CO₂/95% 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, UK). 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, UK) and random hexamers (Promega, Southampton, UK) as primers.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Quantitative PCR of Omentin-1 was performed on a Roche Light Cycler™ system (Roche Molecular Biochemicals, Mannheim, Germany). PCR reactions were carried out in a reaction mixture consisting of 5.0 μ l reaction buffer and 2.0mM MgCl₂ (Biogene, Kimbolton, U.K.), 1.0 μ l of each primer (10ng/ μ l), 2.5 μ l of

cDNA and 0.5 μ l of Light Cycler DNA Master SYBR[®] Green I (Roche, Mannheim, Germany). Protocol conditions consisted of denaturation of 95°C for 15 seconds, followed by 40 cycles of 94°C for 1 sec, 58°C for 10 seconds and 72°C for 12 seconds, followed by melting curve analysis. For analysis, quantitative amounts of genes of interest were standardised against the housekeeping gene β -actin. The RNA levels were expressed as a ratio, using “Delta-delta method” for comparing relative expression results between treatments in real-time PCR (13). The sequences of the sense and anti-sense primers used were: Vaspin (224bp) 5'-AGGCAGAACATGGACTTAGG-3' and 5'-GTCAGCTCGTGGATGATGTA-3'; β -actin (216bp) 5'-AAGAGAGGCATCCTCACCCCT-3' and 5'-TACATGGCTGGGGTCTTGAA-3'. 10 μ l of the reaction mixture(s) were subsequently electrophoresed on a 1% agarose gel and visualised by ethidium bromide, using a 1 kb DNA ladder (Gibco-BRL, Paisley, UK) 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, UK). 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 (RIPA) lysis buffer (Upstate, Lake Placid, NY, USA) according to manufacturer's instructions. Protein samples (40 μ g/lane) containing SDS-sample

buffer (5 M urea, 0.17 M SDS, 0.4 M dithiothreitol, and 50mM Tris-HCl, pH 8.0) were subjected to SDS-polyacrylamide gel electrophoresis (10% resolving gel) and transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were incubated with primary rabbit-anti-human antibody for vaspin (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) [1:1000 dilution] or primary rabbit-anti-human antibody for beta-actin (Cell Signalling Technology Inc., Beverly, MA, USA) [1:1000 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, UK) [1:2000] for one hour at room temperature. Antibody complexes were visualized using chemiluminescence (ECL+; GE Healthcare, Little Chalfont, Buckinghamshire, UK). Human vaspin peptide (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) was used as the positive control and water as the negative control (data not shown).

STATISTICS

Non-parametric tests were used. Data are presented as means \pm SEM unless indicated otherwise. 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 Corporation, Frederick, MD, USA). 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 β -actin were made only within this linearity range. Spearman

Rank correlation was used for calculation of associations between variables, $P < 0.05$ was considered significant.

RESULTS

Study 1 .Demographic Data. Table 1 shows the anthropometric, biochemical and hormonal data in all subjects. Glucose, HOMA, triglycerides, 17β -estradiol (E_2), testosterone, androstenedione, DHEA-S levels and free androgen index (FAI) were significantly higher whereas sex hormone binding globulin (SHBG) was significantly lower in PCOS women. ELISA analysis of serum vaspin levels revealed that PCOS patients had significantly higher levels when compared to controls (1.9 ± 0.2 vs. 1.2 ± 0.4 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 β -actin showed a significant increase of vaspin in om ($*P < 0.05$) adipose tissues of PCOS women, when compared to normal controls (Table 1 & Figure 1A). Also, vaspin mRNA expression is significantly increased in human om adipose tissue of all PCOS women when compared to corresponding sc adipose tissue ($^{\#}P < 0.05$). However, no significant difference in vaspin was observed in sc adipose tissues of PCOS women, when compared to normal controls ($P > 0.05$) [Table 1 & Figure 1A]. The changes noted at mRNA level were also reflected at protein level in PCOS women (Table 1 & Figure 1B; $*P < 0.05$, $^{\#}P < 0.05$).

Dose dependent effects of D-glucose, insulin, testosterone, 17β -estradiol, androstenedione and DHEA-S on vaspin net protein production and secretion into conditioned media from control human om adipose tissue explants. Vaspin net protein production

and secretion into conditioned media was significantly increased dose dependently by D-glucose in control human om adipose tissue explants (Figure 2A-B: $*P < 0.05$, $**P < 0.01$, $***P < 0.01$, respectively).

With regards to gonadal and adrenal steroids, DHEA-S showed a significant increase in vaspin net protein production at the dose of 10^{-7} M ($P < 0.05$), whereas DHEA-S effects on vaspin secretion into conditioned media just failed to reach significance at the corresponding dose ($P = 0.062$). Furthermore, estradiol at the dose of 10^{-7} M increased both net vaspin protein production and secretion, albeit just failing to reach statistical significance ($P = 0.07$, $P = 0.09$, respectively). However, with regards to insulin, 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 Rank analyses demonstrated that om 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, glucose ($P < 0.05$) but failed to reach significance with HOMA ($P = 0.074$) and DHEA-S ($P = 0.068$).

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 compared with the PCOS subjects investigated in *Study 2*. Reasons for subjects not completing *Study 2* were nausea and gastrointestinal side effects and nausea ($n = 4$), pregnancies ($n = 5$), 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 ± 0.4 vs. 0.4 ± 0.1 ng/ml; $P < 0.001$: Table 2), WHR (0.82 ± 0.1 vs. 0.80 ± 0.07 ; $P < 0.05$: Table 2), E_2 (329.8 ± 102.5 vs. 207.1 ± 83.7 pmol/L; $P < 0.05$: Table 2), testosterone (5.6 ± 0.5 vs. 4.0 ± 0.3 nmol/L; $P < 0.05$: Table 2), FAI (19.7 ± 5.1 vs. 14.5 ± 3.6 nmol/L; $P < 0.05$: Table 2) and glucose (5.7 ± 0.5 vs. 4.7 ± 0.2 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- β -cell (74.2 ± 27.8 vs. 183.3 ± 73.0 ; $P < 0.01$: Table 2) and the significant decrease in HOMA-IR (3.3 ± 0.8 vs. 1.9 ± 0.4 ; $P < 0.01$: Table 2) values, respectively.

More importantly, we analysed the correlation between the change in serum vaspin levels before and after metformin therapy (Δ vaspin) and the changes in other covariates. Interestingly, Δ vaspin was significantly positively associated with Δ WHR ($P = 0.017$: Table 3), Δ glucose ($P < 0.01$: Table 3), Δ HOMA-IR ($P = 0.012$: Table 3) and Δ HOMA β -cell ($P = 0.013$: 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 sc and om human adipose tissues simultaneously at both mRNA and protein levels. Furthermore, we present novel data showing the presence and a significant increase of om 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, 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 om adipose tissue explants; DHEA-S also caused a significant dose-dependent increase in vaspin net protein production. Unfortunately, due to technical limitations in om adipose tissue procurement, we were unable to obtain sufficient amounts of sample/patient to perform stromal vascular separation in om 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 months treatment; 850mg twice daily) significantly decreases serum vaspin levels in overweight PCOS subjects.

The higher serum and adipose tissue vaspin levels in women with PCOS, an IR 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 IR (7). We also detected significant positive associations between circulating vaspin as well as vaspin levels in om 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* reported significant associations between om adipose tissue vaspin with 2 h OGTT plasma glucose (7). We found positive associations between serum and om 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 indices. Also, although the change in serum vaspin levels was significantly positively associated with changes in WHR, glucose, HOMA-IR and HOMA β -cell, 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 om adipose tissue vaspin levels may be a compensatory mechanism to 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 mellitus, 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) describe important findings of a lack of correlation between circulating vaspin and BMI in patients with type 2 diabetes mellitus. 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 anti-diabetic 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 17β -estradiol, testosterone, androstenedione and DHEA-S were significantly higher compared to control subjects. We, like others (18, 19), observed a significant decrease in levels of 17β -estradiol and testosterone, with no significant difference in androstenedione and DHEA-S levels, in response to metformin treatment. Therefore, could it be that a decrease in gonadal steroid levels causes a decrease in vaspin levels? However, treatments of om adipose tissue explants with different doses of testosterone, 17β -estradiol, androstenedione and DHEA-S revealed that 17β -estradiol appeared to exhibit a dose dependent increase in vaspin levels, albeit non-significantly; only DHEA-S showed a significant increase in vaspin net protein production although vaspin secretion into conditioned media in corresponding samples were found not to be significant. It is therefore uncertain as to whether or not 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 synchronised 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 om adipose tissue of overweight PCOS women and its *ex vivo* regulation 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.

ACKNOWLEDGEMENTS

The General Charities of the City of Coventry in part funded this study. HSR would like to acknowledge S. Waheguru, University of Warwick for his continual support.

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TABLE 1. Clinical, hormonal and metabolic features of women with PCOS and controls.

Variable	PCOS (<i>n</i> = 12)	Controls (<i>n</i> = 12)	Significance
Age (year)	31.5 (29 - 35)	32.0 (28 - 36)	NS
BMI (kg/m ²)	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)	<i>P</i> < 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)	<i>P</i> < 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)	<i>P</i> < 0.01
LH (IU/L)	8.9 (6.0 - 10.0)	6.2 (5.0 - 7.0)	NS
FSH (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 ₂ (pmol/L)	390.4 (287.0 - 471.0)	188.5 (129.0 - 264.0)	<i>P</i> < 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)	<i>P</i> < 0.01
Androstenedione (nmol/L)	14.9 (14.2 - 16.8)	5.7 (5.0 - 8.2)	<i>P</i> < 0.01
DHEA-S (μmol/L)	5.5 (5.4 - 6.6)	3.3 (3.0 - 4.0)	<i>P</i> < 0.05
SHBG (nmol/L)	32.1 (26.7 - 35.2)	59.2 (47.7 - 66.0)	<i>P</i> < 0.01
FAI	18.1 (14.5 - 21.2)	4.2 (3.3 - 6.1)	<i>P</i> < 0.01
Vaspin (ng/ml)	1.9 (1.7 - 2.3)	1.2 (1.0 - 1.4)	<i>P</i> < 0.05
Vaspin Sc mRNA (arbitrary units)	216.0 (179.8 - 255.6)	132.5 (63.9 - 182.5)	NS
Vaspin Om mRNA (arbitrary units)	647.7 (536.8 - 714.0)	239.7 (178.4 - 385.5)	<i>P</i> < 0.05
Vaspin Sc protein (OD units)	3.6 (2.6 - 4.7)	3.4 (2.4 - 4.4)	NS
β-actin Sc protein (OD units)	24.9 (24.5 - 25.3)	24.5 (24.0 - 24.9)	NS
Vaspin Om protein (OD units)	9.3 (8.1 - 10.5)	4.3 (3.2 - 5.4)	<i>P</i> < 0.05
β-actin Om protein (OD units)	25.0 (24.8 - 25.4)	24.7 (24.2 - 25.2)	NS

Free Androgen Index (FAI) = T (nmol/liter)/SHBG (nmol/liter) x 100

Data are medians (interquartile range). Group comparison by Mann-Whitney *U* test.

Sc = subcutaneous adipose tissue; Om = omental adipose tissue

NS = not significant

TABLE 2. Clinical, hormonal and metabolic features of women with PCOS ($n = 21$) before and after metformin treatment.

Variable	Before metformin	After metformin	Significance
Age (year)	28 (26.5 - 31.5)	28 (27.5 - 32.5)	NS
BMI (kg/m ²)	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)	$P < 0.05$
Glucose (mmol/L)	5.7(4.2 - 5.0)	4.7 (4.4 - 4.9)	$P < 0.01$
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)	$P < 0.01$
HOMA β -cell	74.2 (37.5 - 91.3)	183.3 (87.7 - 222.5)	$P < 0.01$
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 ₂ (pmol/L)	329.8 (164.9 - 494.7)	207.1 (103.6 - 310.7)	$P < 0.05$
Testosterone (nmol/L)	5.6 (4.2 - 6.6)	4.0 (3.0 - 5.4)	$P < 0.05$
Androstenedione (nmol/L)	11.4 (8.0 - 14.0)	9.9 (7.6 - 12.4)	NS
DHEA-S (μ mol/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)	$P < 0.05$
Vaspin (ng/ml)	2.1 (1.5 - 2.6)	0.4 (0.3 - 0.5)	$P < 0.001$

Free Androgen Index (FAI) = T (nmol/liter)/SHBG (nmol/liter) x 100

Data are medians (interquartile range). Group comparison by Mann-Whitney U test.

NS = not significant

TABLE 3. Linear regression analysis of variables associated with changes in serum vaspin levels (before and after metformin treatment), Δ vaspin, in PCOS subjects ($n = 21$).

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

In multiple linear regression analysis, values included were WHR, glucose, HOMA-IR and HOMA β -cell.

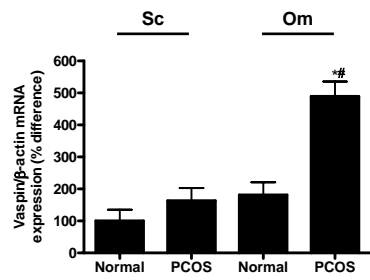
FIGURE LEGENDS

Figure 1. (A) vaspin mRNA expression relative to β -actin is significantly increased in human om and non-significantly increased in human sc adipose tissue depots, respectively, when comparing all PCOS women to all normal controls, using real-time RT-PCR. Also, vaspin mRNA expression is significantly increased in human om adipose tissue of all PCOS women when compared to corresponding sc adipose tissue, using real-time RT-PCR. Data are expressed as % difference to human sc adipose tissue of all normal controls. Group comparison 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 controls demonstrate that the antibody against vaspin and the antibody against β -actin recognised bands with apparent molecular weights of 46kDa and 45kDa respectively in human sc and om adipose tissue depots (**Figure 1B-inserts**). Densitometric analysis of vaspin complexes having normalized to β -actin respectively revealed that protein levels of vaspin are significantly increased in human om and non-significantly increased in human sc adipose tissue depots, respectively, when comparing all PCOS women to all normal controls. Also, vaspin protein levels are significantly increased in human om adipose tissue of all PCOS women when compared to corresponding sc adipose tissue. Data are expressed as % difference to human sc adipose tissue of all normal controls. Group comparison by Kruskal-Wallis ANOVA and post hoc Dunn's test. $*P < 0.05$, $^{\#}P < 0.05$. PSL, phospho-stimulated light units.

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

Figure 1

A



B

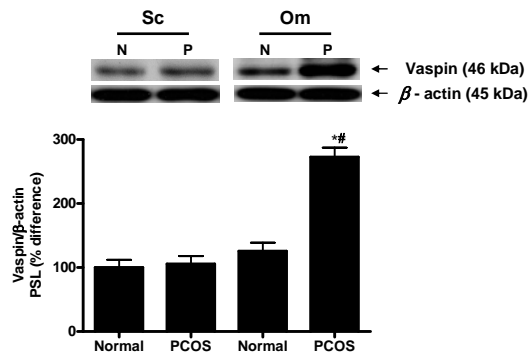
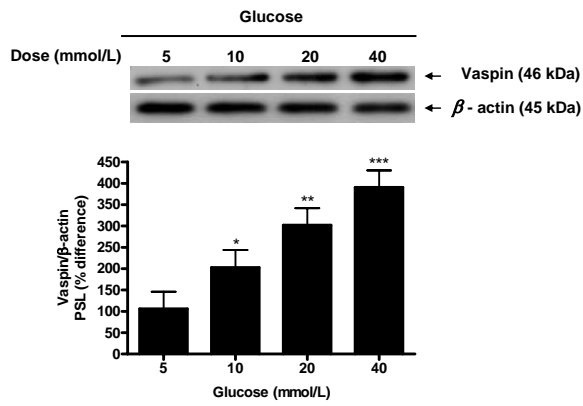


Figure 2

A



B

