Metformin Treatment May Increase Omentin-1 Levels in Women with Polycystic Ovary Syndrome

**Short Title:** Omentin-1 and Angiogenesis

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**Objectives:** Polycystic ovary syndrome (PCOS) is associated with the metabolic syndrome. Decreased omentin-1 levels are associated with obesity and diabetes. To study the effects of metformin treatment on omentin-1 levels in PCOS subjects and effects of omentin-1 on *in vitro* migration and angiogenesis.

**Research Design and Methods:** Serum omentin-1 was measured by ELISA. Angiogenesis was assessed by studying capillary tube formation in Human Microvascular Endothelial Cells (HMEC-1) on growth factor reduced Matrigel. Endothelial cell migration assay was performed in a modified Boyden chamber. Nuclear Factor-κB (NF-κB) was studied by stably transfecting HMEC-1 cells with a cis-reporter plasmid containing luciferase reporter gene linked to five repeats of NF-κB binding sites. Akt phosphorylation was assessed by western blotting.

**Results:** Serum omentin-1 was significantly lower in PCOS women (*P* < 0.05). After 6 months of metformin treatment, there was a significant increase in serum omentin-1 (*P* < 0.01). Importantly, changes in hs-CRP were significantly negatively correlated with changes in serum omentin-1 (*P* = 0.036). *In vitro* migration and angiogenesis were significantly increased in serum from PCOS women (*P* < 0.01) compared to matched controls; these effects were significantly attenuated by metformin treatment (*P* < 0.01) plausibly through the regulation of omentin-1 levels via NF-κB and Akt pathways. CRP and VEGF induced *in vitro* migration and angiogenesis was significantly decreased by omentin-1.

**Conclusions:** Increases in omentin-1 levels may play a role but are not sufficient to explain the decreased inflammatory and angiogenic effects of sera from metformin treated PCOS women.

Polycystic ovary syndrome (PCOS) is a pro-inflammatory state associated with type 2 diabetes, visceral obesity and cardiovascular complications; features of the metabolic syndrome (1-5). The metabolic syndrome is associated with accumulation of visceral adipose tissue (AT). Visceral AT produces cytokines termed ‘adipokines’ implicated in the pathogenesis of diabetes and atherosclerosis (6-9). Omentin-1 has been described as a novel adipokine preferentially produced by visceral adipose tissue. *In vitro* experiments revealed that treatment with recombinant omentin-1 enhances insulin stimulated glucose uptake in human adipocytes. Also, omentin-1 was shown to trigger Akt signalling both in the absence and presence of insulin (10, 11). Additionally, omentin plasma levels and omentin gene expression in visceral AT are decreased in obesity (12). Recently, we have reported novel data showing a significant decrease of adipose tissue and circulating omentin-1 levels in overweight PCOS women (13). Thus, given the above functions of omentin-1, increasing omentin-1 levels in PCOS women may alleviate cardio-metabolic dysfunction in these women.

We studied the effects of metformin treatment on omentin-1 levels in PCOS subjects and effects of omentin-1 and serum on *in vitro* migration and angiogenesis. Researchers have utilized serum to perform functional experiments in endothelial, cardiac and neural cells (14-16). Finally, given the link between inflammation and angiogenesis (17), we explored Nuclear Factor-kappaB (NF-κB), Erk1/2 and Akt pathways; important regulators of inflammation and angiogenesis (18, 19).
SUBJECTS AND METHODS

Subjects. All PCOS patients met all 3 criteria of the revised 2003 Rotterdam European Society of Human Reproduction and Embryology (ESHRE)/American Society of Reproductive Medicine (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 (20). 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). No women were amenorrheic. 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 µmol/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, estrogenic, anti-androgenic or anti-hypertensive medication. All patients underwent anthropometric measurements. Blood pressure was measured in a sitting position within a quiet and calm environment after a rest of at least 5 minutes. The average of three measurements was obtained. 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. Blood samples were drawn into serum separator tubes that contain no additives or anticoagulants, allowed to clot, and centrifuged (3000 rpm for 10 minutes) to separate sera, and stored at -80°C.

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. All patients underwent anthropometric measurements before and after metformin treatment. Carotid intima media thickness (IMT) was measured in PCOS subjects, before and after metformin (see below). A total of 83 women of Caucasian origin with PCOS were recruited, 49 of which did not participate in the metformin arm of the study. The baseline characteristics of the 49 PCOS women were comparable to the 34 PCOS women who participated in the metformin arm of the study. Furthermore, the 21 women who completed the metformin arm of the study were comparable to the 13 PCOS women who did not complete the metformin arm of the study. For the purposes of elucidating the effects of metformin in PCOS women, the 21 PCOS subjects were studied. 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.

Intima Media Thickness. Measurement of carotid IMT is a widely accepted tool to provide information about preclinical vascular disease. B-mode sonography of the proximal part of the carotid bulb was conducted on both sides, and the segments of the common carotid arteries 1.0 cm proximal were scanned longitudinally with Hitachi EUB-5000 plus-G (Hitachi Ltd., Tokyo, Japan) using a 10-MHz
linear-array transducer. All women were investigated in a supine position with the head slightly hyper-extended and turned away from the side being scanned. The image was focused on the posterior wall, and the resolution function was used to magnify the arterial far wall. When an optimal image was obtained, it was frozen, and the distance from the junction of the lumen and intima and the junction of the media and adventitia was measured by electronic calipers in end-diastolic phase, to minimize variability during the cardiac cycle. All images were photographed. Five measurements were conducted on each side, and the mean IMT was calculated as the average of these measurements. All measurements were performed by an experienced ultrasound sonographer (intra-observer coefficient of variation was 6.8%) who was blinded to the diagnosis of subjects.

**Biochemical and hormonal analysis.** Assays for glucose, insulin, cholesterol, triglycerides, luteinizing hormone (LH), follicular stimulating hormone (FSH), testosterone, androstenedione, DHEA-S and SHBG were performed using an automated analyzer (Abbott Architect, Abbott Laboratories, Abbott Park, USA). The estimate of IR by Homeostasis Model Assessment (HOMA-IR) score was calculated as previously described (21). High sensitivity C-reactive protein (hs-CRP) was determined immunoturbidimetrically using a modular system random-access analyzer (Roche Diagnostics).

Omentin-1 levels in sera were measured using a commercially available ELISA kit (AXXORA, Nottingham, UK), according to manufacturer’s protocol, with an intra-assay coefficient of variation of less than 6%.

**Immunodepletion of Serum Omentin-1 and CRP.** Immunodepletion of serum omentin-1 and CRP were performed using the immunoprecipitation protocol provided by Dr Kazuyuki Itoh and Montecucco et al., respectively (14, 22). 1 ml of human serum were incubated with mouse-anti-human omentin-1 IgG-protein G-Sepharose conjugate (1-2µg/100-500µg of total protein) or control mouse IgG-protein G-Sepharose conjugate; goat-anti-human CRP IgA-protein A-Sepharose conjugate (10µg/100-500µg of total protein) or control goat IgA-protein A-Sepharose conjugate, mixed overnight at 4°C and centrifuged to sediment the conjugated Sepharose beads. The supernatants were collected and similar procedures were repeated eight times. Supernatants and sera were subjected to immunoblotting for omentin-1 as previously described (13). Immunoblotting for CRP is described below. Immunoblotting confirmed that these protocols decreased serum omentin-1 and CRP by approximately 80% (data not shown), respectively.

**Endothelial Cell culture.** Human Microvascular Endothelial Cells (HMEC-1) were obtained from the Centre for Disease Control, Atlanta, USA. HMEC-1 cells were cultured in MCDB medium (Sigma-Aldrich, Gillingham, UK) supplemented with 10% Fetal Calf Serum (FCS) [Sigma-Aldrich, Gillingham, UK], 100IU/ml penicillin (Sigma-Aldrich, Gillingham, UK), 100µg/ml streptomycin (Sigma-Aldrich, Gillingham, UK), 5ml of 200mM L-glutamine/500ml of media, hydrocortisone 2µM, epidermal growth factor 2ng/ml (Invitrogen, Paisley, UK) at 37°C in 5% CO$_2$/95% air. Prior to each experiment, cells were fed with MCDB with addition of 1% FCS for 16 hours. For endothelial cell signaling experiments, HMECs were pre-incubated with or without omentin-1 (Kamiya Biomedical Company, Seattle, USA) for 30 minutes followed by treatment with or without 1% of human serum from normal ($n=39$; individually tested) and PCOS women [before ($n=21$; individually tested) and after ($n=21$; individually tested) 6 months of metformin treatment] at various time points (0 minutes, 2 minutes, 5 minutes,
In Vitro Angiogenesis Assay. Angiogenesis was assessed by studying the formation of capillary tube-like structures by culturing HMEC-1 cells on growth factor reduced Matrigel (BD Biosciences, San Jose, USA). Matrigel was coated onto the culture plates as per manufacturer's instructions. HMEC-1 cells were pre-incubated with or without omentin-1 (Kamiya Biomedical Company, Seattle, USA) and/or with or without PI3K/Akt inhibitor (LY294002) [Calbiochem, San Diego, USA] for 30 minutes and 1 hour, respectively, followed by treatment with or without 1% of human serum from normal (n = 39; individually tested) and PCOS women [before (n = 21; individually tested) and after (n = 21; individually tested) 6 months of metformin treatment] or CRP or omentin-1 [PCOS after metformin (n = 21; individually tested)] and CRP [PCOS before metformin (n = 21; individually tested)] immunodepleted sera. Vascular endothelial growth factor (VEGF) served as positive control. Dose and time dependent experiments were performed to determine the optimum concentration and time point. Capillary tube formation images were captured with a digital microscope camera system (Olympus, Tokyo, Japan). Image Pro Plus software was used to quantify tube length formation; the lengths of tubes in 3-4 randomly selected fields in each of the wells were measured (23, 24).

In Vitro Migration assay. Endothelial cell migration assay was performed in a modified Boyden chamber using a protocol obtained from BD BioCoat Angiogenesis System (BD Biosciences, San Jose, USA). Endothelial cells were trypsinized; a cell suspension of 4 x 10^5cells/ml was prepared and 250µl of which was added to each of the trans-well insert. 750µl of starvation media was added to the lower chamber and incubated overnight. Following this, the cells were labelled by incubating with Hank's Balanced Salt Solution medium (Sigma-Aldrich, Gillingham, UK) containing 4µg/ml Calcein-AM (BD Biosciences, San Jose, USA) for 90 minutes. The cells were pre-incubated with or without omentin-1 (Kamiya Biomedical Company, Seattle, USA) and/or with or without PI3K/Akt inhibitor (LY294002) [Calbiochem, San Diego, USA] for 30 minutes and 1 hour, respectively, followed by treatment with or without 1% of human serum from normal (n = 39; individually tested) and PCOS women [before (n = 21; individually tested) and after (n = 21; individually tested) 6 months of metformin treatment] or CRP or omentin-1 [PCOS after metformin (n = 21; individually tested)] and CRP [PCOS before metformin (n = 21; individually tested)] immunodepleted sera. VEGF served as positive control. Dose and time dependent experiments were performed to determine the optimum concentration and time point. Cells were fixed with 2% formaldehyde. Fluorescence of migrated cells was read in a fluorescence plate reader with bottom reading capabilities at excitation/emission wavelengths of 494/517 nm. Only those labelled cells that have migrated through the pores of the membrane will be detected (23, 24).

NF-κB activation. We studied Nuclear Factor-κB (NF-κB) activation by stably transfecting HMEC-1 cells with a cis-reporter plasmid containing luciferase reporter gene linked to five repeats of NF-κB binding sites (pNF-κB-Luc; Stratagene, La Jolla, USA). Multiple clones were selected for the analysis of NF-κB activation. HMEC-1 cells were pre-incubated with or without omentin-1 (Kamiya Biomedical Company, Seattle, USA) for 30 minutes followed by treatment with or without 1% of human serum from normal (n =
39; individually tested) and PCOS women [before \((n = 21; \text{individually tested})\) and after \((n = 21; \text{individually tested})\) 6 months of metformin treatment] or CRP or omentin-1 [PCOS after metformin \((n = 21; \text{individually tested})\)] and CRP [PCOS before metformin \((n = 21; \text{individually tested})\)] immunodepleted sera for 2 hours. Cells were lysed and luciferase activities were measured. Experiments were also performed with TK plasmid (Promega, Southampton, UK) as negative control and TNF-\(\alpha\) as positive control. Dose and time dependent experiments were performed to determine the optimum concentration and time point.

**Western Blotting.** Endothelial cells were lysed with SDS-sample buffer (5 M urea, 0.17 M SDS, 0.4 M dithiothreititol, and 50mM Tris-HCl, pH 8.0), mixed, sonicated, boiled, centrifuged (5000 rpm for 2 minutes) and then stored at -80\(^\circ\)C until use. 80\(\mu\)g of each sample, supernatants and sera were subjected to SDS-polyacrylamide gel electrophoresis (8\% resolving gel) and transferred to PVDF membranes. PVDF membranes were blocked in TBS containing 0.1% Tween-20 and 5\% BSA for two hours. The PVDF membranes were then incubated with polyclonal primary rabbit-anti-human antibody for phospho-Erk1/2 (Cell Signaling Technology Inc., Beverly, USA) [1:1000 dilution] or polyclonal primary rabbit-anti-human antibody for phospho-Akt (Ser473) (Cell Signaling Technology Inc., Beverly, USA) [1:1000 dilution] or monoclonal primary goat-anti-human antibody for CRP (Sigma-Aldrich, Gillingham, UK) [1:1000 dilution] overnight at 4 \(^\circ\)C. The membranes were washed thoroughly for 60 min with TBS-0.1% Tween before incubation with the secondary anti-rabbit horseradish peroxidase-conjugated immunoglobulin (Dako, Ely, UK) [1:2000] or secondary anti-goat horseradish peroxidase-conjugated immunoglobulin (Dako, Ely, UK) [1:2000] for one hour at room temperature. Antibody complexes were visualized using chemiluminescence (ECL+; GE Healthcare, Little Chalfont, UK). For standardization, the same membranes were stripped and reprobed using polyclonal primary rabbit-anti-human antibodies for total Erk1/2 (Cell Signaling Technology Inc., Beverly, USA) [1:1000 dilution] or total Akt (Cell Signaling Technology Inc., Beverly, USA) [1:1000 dilution]. The densities were measured using a scanning densitometer coupled to scanning software Scion Image\textsuperscript{TM} (Scion Corporation, Frederick, 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 phospho-Erk1/2, phospho-Akt, total Erk1/2 and total Akt were made only within this linearity range.

**Statistics.** Data were analysed by Mann-Whitney \(U\) test or Kruskal-Wallis ANOVA (post hoc analysis: Dunn’s test) according to the number of groups compared. Data are medians (interquartile range). Spearman Rank correlation was used for calculation of associations between variables. If individual bivariate correlations achieved statistical significance, multiple regression analysis with omentin-1 as dependent variable was performed to test the joint effect of these parameters on omentin-1. \(P < 0.05\) was considered significant.

**RESULTS**
Table 1 shows the anthropometric, biochemical and hormonal data in all subjects. Insulin, HOMA-IR, cholesterol, triglycerides, luteinizing hormone (LH), testosterone, free androgen index (FAI), systolic blood pressure (SBP), diastolic blood pressure (DBP), IMT and hs-CRP were significantly higher whereas dehydroepiandrosterone-sulfate (DHEA-S) and sex hormone binding globulin (SHBG) were significantly lower in PCOS women.
Serum omentin-1 levels were significantly lower in PCOS subjects compared to controls [23.7 (20.0 - 27.9) vs. 27.6 (25.6 - 29.4) ng/ml; \( P < 0.05 \); Table 1]. Serum progesterone levels in all women confirmed follicular phase of the menstrual cycle.

Table 2 shows the effects of metformin treatment on serum omentin-1 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. Reasons for subjects not completing Study 2 were nausea and gastrointestinal side effects \((n = 4)\), pregnancies \((n = 5)\), incompliance \((n = 2)\), and loss of contact \((n = 2)\).

After 6 months of metformin treatment, there was a significant increase in serum omentin-1 [23.7 (20.0 - 27.9) vs. 59.2 (52.1 - 60.6) ng/ml; \( P < 0.01 \); Table 2]. Also, there were significant decreases in WHR, testosterone, glucose, HOMA-IR and IMT.

**Correlation of omentin-1 with covariates.** Spearman Rank analyses demonstrated that serum omentin-1 levels were significantly negatively correlated with BMI, WHR, glucose, HOMA-IR and hs-CRP \((P < 0.05)\). However, when subjected to multiple regression analysis, none of these variables were significantly negatively correlated with serum omentin-1 levels \((P > 0.05)\).

Furthermore, we analyzed the correlation between the change in serum omentin-1 levels before and after metformin therapy \((\Delta \text{Omentin-1})\) and the changes \((\Delta)\) in other covariates (Table 3). We found that \(\Delta \text{Omentin-1} \) was significantly negatively associated with \(\Delta \text{WHR}, \Delta \text{Glucose}, \Delta \text{HOMA-IR} \) and \(\Delta \text{hs-CRP}\). When subjected to multiple regression analysis, only \(\Delta \text{hs-CRP} \) was significantly negatively correlated with \(\Delta \text{Omentin-1} \) \((\beta = -0.526; P = 0.036)\).

**Effects of omentin-1 on in vitro migration and angiogenesis.** Given the above, we studied the effects of omentin-1 on in vitro migration and angiogenesis. Capillary tube formation was optimal at 24 hours, after which, capillary tubes begin to disintegrate. In vitro migration and angiogenesis at 24 hours was significantly increased when comparing sera of PCOS women to normal controls (Figure 1 & 2; \(* P < 0.01\) ). Also, in vitro migration and angiogenesis were significantly decreased in PCOS women following 6 months of metformin treatment (Figure 1 & 2; \(** P < 0.01\) ) and when omentin-1 (200ng/ml) or the PI3K/Akt inhibitor (LY294002; 10\( \mu \)M) were added to sera (Figure 1 & 2; \(* P < 0.05, ** P < 0.01\) ). Furthermore, CRP (1ug/ml) and VEGF (10ng/ml) induced in vitro migration and angiogenesis was significantly decreased by omentin-1 (Figure 1 & 2; \(* P < 0.05\) ).

Moreover, in vitro migration and angiogenesis were significantly decreased in CRP (PCOS before metformin) but not significantly altered by omentin-1 (PCOS after metformin) immunodepleted sera (Figure 1 & 2; \(P > 0.05, * P < 0.05\) ).

**Effects of omentin-1 on NF-\(\kappa\)B activation in Human Microvascular Endothelial Cells.** In HMEC-1 cells, NF-\(\kappa\)B was significantly activated by sera from normal and PCOS women compared to controls (Figure 3; \(* P < 0.05, ** P < 0.01\) ). NF-\(\kappa\)B was significantly activated by sera from PCOS women compared to normal women (Figure 3; **\(P < 0.01\) ). Also, NF-\(\kappa\)B activation was significantly decreased in PCOS women following 6 months of metformin treatment (Figure 3; **\(P < 0.01\) ) and when omentin-1 (200ng/ml) was added to sera (Figure 3; \(* P < 0.05\) ). Furthermore, CRP (1ug/ml) and TNF-\(\alpha\) (10ng/ml) induced NF-\(\kappa\)B activation was significantly decreased by omentin-1 (Figure 3; \(* P < 0.05\) ).

Moreover, NF-\(\kappa\)B activation were significantly decreased in CRP (PCOS before metformin) but not significantly altered by omentin-1 (PCOS after metformin) immunodepleted sera (Figure 3; \(P > 0.05, * P < 0.05\) ).
Effects of omentin-1 on Erk1/2 and Akt signaling pathways in Human Microvascular Endothelial Cells. In HMEC-1 cells, Erk1/2 and Akt pathways were significantly activated by sera from normal and PCOS women compared to controls (Figure 3A & 3B; *P < 0.05, **P < 0.01). Erk1/2 and Akt phosphorylation were significantly increased by sera from PCOS women compared to normal women (Figure 3A & 3B; **P < 0.01). Also, Erk1/2 and Akt activation were significantly decreased in PCOS women following 6 months of metformin treatment (Figure 3A & 3B; *P < 0.05, **P < 0.01). Furthermore, Akt activation was significantly decreased when omentin-1 (200ng/ml) was added to sera from PCOS women before metformin treatment (Figure 3B; *P < 0.05). However, there was no significant difference in Erk1/2 activation (Figure 3A). The detected proteins for phosphorylated Erk1/2, total Erk1/2, phosphorylated Akt and total Akt had apparent molecular weights of 44/42kDa, 44/42kDa, 60kDa and 60kDa, respectively (Figure 3A & 3B-inserts).

DISCUSSION
We confirm our previous data of decreased circulating omentin-1 in PCOS women. Also, serum omentin-1 levels were significantly negatively correlated with BMI, WHR, glucose, HOMA-IR and hs-CRP. We report that metformin (6 months treatment; 850mg twice daily) significantly increases serum omentin-1 levels with concomitant decreases in insulin resistance and carotid IMT in PCOS subjects. Although the change in serum omentin-1 levels were significantly negatively associated with changes in WHR, glucose, HOMA-IR and hs-CRP; when subjected to multiple regression analysis, only the change of hs-CRP was significantly negatively correlated with the change of serum omentin-1 levels. The possible implication is that changes in omentin-1 levels associated with metformin treatment observed in our PCOS subjects are more robustly linked to changes in inflammatory rather than metabolic parameters. Of relevance, there is evidence that inflammatory markers other than CRP are elevated in PCOS subjects (3-5); also, studies have indicated metformin’s potential anti-inflammatory effects via modulation of TNF-α, IL-6, IL-18 and IL-1-β levels (27-29).

In PCOS women, metformin corrects the associated endocrine and metabolic abnormalities (30); metformin counters IR by inhibiting hepatic glucose production (31-33). Therefore, glucose may regulate circulating omentin-1 levels; however, it is possible that the effect of metformin on omentin-1 levels could be via modulation of other circulating factors. Further studies are needed to clarify this point.

PCOS is a state of altered steroid milieu. We, like others (34), observed a significant decrease in testosterone levels following metformin treatment. However, treatments of omental adipose tissue explants with gonadal and adrenal steroids revealed no meaningful effects on omentin-1 levels (13). It is therefore unlikely that the effects of metformin on serum omentin-1 levels observed in this study are attributable to altered gonadal and adrenal steroids.

Furthermore, functional assays revealed that in vitro migration and angiogenesis were significantly increased by sera from both normal and PCOS women; in PCOS women, in vitro migration and angiogenesis was attenuated after 6 months of metformin treatment. Addition of omentin-1 to sera from PCOS women before metformin treatment significantly decreased in vitro migration and angiogenesis. Omentin-1 also decreased the effects of CRP and VEGF induced in vitro migration and angiogenesis. However, in vitro migration and angiogenesis were significantly decreased in CRP but not significantly altered by omentin-1 immunodepleted sera. It is
important to consider that omentin-1 levels in tissues are unknown; the levels are likely to be significantly higher than in sera as sequestration of omentin-1 in vascular tissue, in particular, within stromal-vascular cells, have been shown (10, 35). All of these effects appear to be associated with NF-κB and Akt signalling pathways; both well established mediators of angiogenesis (36-38). Taken together, increases in omentin-1 levels may play a role but are not sufficient to explain the decreased inflammatory and angiogenic effects of sera from metformin treated PCOS women. It is important to bear in mind that metformin may also modulate angiogenesis via the VEGF system. Of relevance, metformin decreases VEGF levels in type 2 diabetics (39).

The link between angiogenesis and the pathogenesis of atherosclerosis has been well described (17). Given our data of an increase in circulating omentin-1 levels with concomitant decrease in IMT, omentin-1 may have a potential role in the pathogenesis of atherosclerosis.

Omentin-1, by promoting activation of the Akt signalling pathway and in turn modulating the endothelial nitric oxide synthase in endothelial cells may impact upon endothelial dysfunction, a step that is crucial in the pathogenesis of atherosclerosis (40, 41). Our novel data of omentin-1 on angiogenesis in relation to the Akt signaling pathway thus clarifies omentin-1’s potentially important role in the pathogenesis of cardiovascular disorders.

A limitation of our study may relate to the lack of a lean PCOS comparator group. Only 3 of the 21 PCOS subjects and 6 of the 39 control subjects had BMI < 25 kg/m²; thus, no meaningful result could be obtained. Future studies should address this point. Furthermore, given ethical constraints i.e. blood samples could and were only obtained at baseline and after 6 months of metformin treatment; it is therefore difficult to be certain as to whether changes in serum omentin-1 levels precede or follow changes in clinical and hormonal indices as a consequence of metformin treatment. Also, as diet and lifestyle modifications were only subjectively assessed, the changes in serum omentin-1 levels may be partially attributable to diet and lifestyle modifications. Further studies are needed to elucidate this point. Additionally, like others (42), we employed WHR, a universally accepted, simple and cost-effective measure of adiposity as a surrogate marker for central fat mass. However, Cascella et al. has reported that WHR does not distinguish between visceral and subcutaneous fat mass in PCOS women (43). Therefore, more accurate and equally cost-effective methods of assessing central obesity are needed. Finally, given that PCOS is a pro-inflammatory state associated with clustering of cardiovascular risk factors, it would be of importance to study the effect of metformin therapy in the context of omentin-1 biology on reactive oxygen species (ROS) in PCOS women as ROS not only induces tissue damage and inflammation, but is also increased in dysmetabolic states including PCOS (44).

In conclusion, we provide evidence that increases in omentin-1 levels may play a role but are not sufficient to explain the decreased inflammatory and angiogenic effects of sera from metformin treated PCOS women. Our findings provide novel insights into the relationship between obesity, IR and metformin therapy with dysregulated angiogenesis in PCOS women in the context of omentin-1 biology.

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**Table 1:** Clinical, hormonal and metabolic features of women with PCOS and controls.

| Variable               | PCOS (n = 21)    | Controls (n = 39) | Significance |
|------------------------|------------------|-------------------|--------------|
| Age (year)             | 28 (26.5 – 31.5) | 28 (23 - 32)      | NS           |
| BMI (kg/m²)            | 32.8 (29.8 - 36.5) | 29.1 (27.3 - 34.1) | NS           |
| WHR                    | 0.82 (0.76 - 0.88) | 0.81 (0.78 - 0.86) | NS           |
| Glucose (mmol/L)       | 5.1 (4.7 - 5.5)   | 4.8 (4.4 - 5.1)   | NS           |
| Insulin (pmol/L)       | 70.0 (54.5 - 94.5) | 41.0 (32.0 - 55.0) | *P* < 0.01   |
| HOMA-IR                | 2.2 (2.0 - 3.0)   | 1.2 (1.0 - 1.7)   | *P* < 0.01   |
| Cholesterol (mmol/L)   | 4.9 (4.1 - 5.3)   | 4.3 (3.8 - 4.8)   | *P* < 0.05   |
| Triglycerides (mmol/L) | 1.0 (0.8 - 1.7)   | 0.8 (0.6 - 1.1)   | *P* < 0.05   |
| LH (IU/L)              | 7.8 (4.7 - 11.0)  | 3.8 (2.7 - 5.4)   | *P* < 0.01   |
| FSH (IU/L)             | 5.2 (4.0 - 6.2)   | 5.7 (4.3 - 7.5)   | NS           |
| Testosterone (nmol/L)  | 1.8 (1.5 - 2.5)   | 1.2 (1.0 - 1.6)   | *P* < 0.01   |
| Androstenedione (nmol/L)| 3.0 (2.3 - 3.8)  | 2.6 (1.9 - 3.6)   | NS           |
| DHEA-S (µmol/L)        | 4.3 (2.7 - 5.6)   | 6.1 (3.8 - 7.5)   | *P* < 0.01   |
| SHBG (nmol/L)          | 27.0 (20.5 - 41.0) | 45.0 (34.0 - 59.0) | *P* < 0.01   |
| FAI                    | 6.6 (4.6 - 9.7)   | 3.0 (1.9 - 4.4)   | *P* < 0.01   |
| SBP (mm Hg)            | 125.0 (120.0 - 130.0) | 120.0 (110.0 - 120.0) | *P* < 0.01   |
| DBP (mm Hg)            | 80.0 (75.0 - 80.0) | 75.0 (70.0 - 80.0) | *P* < 0.01   |
| IMT (mm)               | 0.53 (0.48 - 0.58) | 0.42 (0.40 - 0.44) | *P* < 0.01   |
| hs-CRP (mg/L)          | 3.3 (2.4 - 5.5)   | 1.3 (0.5 - 2.5)   | *P* < 0.01   |
| Omentin-1 (ng/ml)      | 23.7 (20.0 - 27.9) | 27.6 (25.6 - 29.4) | *P* < 0.05   |

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

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

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/m²)               | 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)          | 5.1 (4.7 - 5.5)  | 4.8 (4.4 - 4.9)  | P < 0.05     |
| Insulin (pmol/L)          | 70.0 (54.5 - 94.5)| 61.0 (43.5 - 83.0)| NS           |
| HOMA-IR                   | 2.2 (2.0 - 3.0)  | 1.6 (1.3 - 2.2)  | P < 0.05     |
| Cholesterol (mmol/L)      | 4.9 (4.1 - 5.3)  | 5.0 (4.1 - 5.4)  | NS           |
| Triglycerides (mmol/L)    | 1.0 (0.8 - 1.7)  | 1.2 (1.0 - 1.9)  | NS           |
| Testosterone (nmol/L)     | 1.8 (1.5 - 2.5)  | 1.3 (1.1 - 1.8)  | P < 0.05     |
| Androstenedione (nmol/L)  | 3.0 (2.3 - 3.8)  | 2.8 (2.2 - 3.6)  | NS           |
| DHEA-S (µmol/L)           | 4.3 (2.7 - 5.6)  | 5.4 (3.6 - 6.7)  | NS           |
| SHBG (nmol/L)             | 27.0 (20.5 - 41.0)| 29.0 (21.5 - 46.5)| NS           |
| FAI                       | 6.6 (4.6 - 9.7)  | 5.3 (3.1 - 6.6)  | NS           |
| SBP (mm Hg)               | 125.0 (120.0 - 130.0)| 120.0 (120.0 - 127.5)| NS           |
| DBP (mm Hg)               | 80.0 (75.0 - 80.0)| 75.0 (75.0 - 80.0)| NS           |
| IMT (mm)                  | 0.53 (0.48 - 0.58)| 0.47 (0.41 - 0.53)| P < 0.05     |
| hs-CRP (mg/L)             | 3.3 (2.4 - 5.5)  | 3.1 (2.0 - 6.2)  | NS           |
| Omentin-1 (ng/ml)         | 23.7 (20.0 - 27.9)| 59.2 (52.1 - 60.6)| P < 0.01     |

Table 3: Linear regression analysis of variables associated with changes in serum omentin-1 levels (before and after metformin treatment), ΔOmentin-1, in PCOS subjects (n = 21).

| (A) Simple Multiple | Variable          | r   | P    | β    | P    |
|---------------------|-------------------|-----|------|------|------|
| BMI (kg/m²)         | -0.400            | 0.072|
| WHR                 | -0.445            | **0.043**|
| Glucose (mmol/L)    | -0.471            | **0.031**|
| Insulin (pmol/L)    | -0.014            | 0.953|
| HOMA-IR             | -0.461            | **0.035**|
| Cholesterol (mmol/L)| 0.100             | 0.667|
| Triglycerides (mmol/L)| 0.390           | 0.080|
| Testosterone (nmol/L)| -0.126           | 0.586|
| Androstenedione (nmol/L)| 0.139          | 0.548|
| DHEA-S (µmol/L)     | -0.232            | 0.311|
| SHBG (nmol/L)       | 0.131             | 0.570|
| FAI                 | -0.200            | 0.385|
| SBP (mm Hg)         | -0.222            | 0.333|
| DBP (mm Hg)         | 0.032             | 0.889|
| IMT (mm)            | -0.032            | 0.892|
| hs-CRP (mg/L)       | -0.665            | < **0.010**|

Spearman Rank correlation was used for calculation of associations between variables. If individual bivariate correlations achieved statistical significance, multiple regression analysis with omentin-1 as dependent variable was performed to test the joint effect of these parameters on omentin-1. Multiple regression analysis contained WHR, glucose, HOMA-IR and hs-CRP.
FIGURE LEGENDS

Figure 1. *In vitro* tube formation by human serum at 24 hours from normal controls \((n = 39)\) with or without omentin-1 \([200 \text{ng/ml}]\), PCOS women \((n = 21)\) with or without omentin-1 \([200 \text{ng/ml}]\) or PI3K/Akt inhibitor \((\text{LY294002; } 10 \mu\text{M})\) or CRP immunodepletion \((\text{CRP_IP})\), PCOS women after 6 months of metformin treatment \((n = 21)\) with or without omentin-1 immunodepletion \((\text{Omentin-1_IP})\), CRP \((1 \mu\text{g/ml})\) with or without addition of omentin-1 \([200 \text{ng/ml}]\) \((n = 6)\) and VEGF \((10 \text{ng/ml})\) with or without addition of omentin-1 \([200 \text{ng/ml}]\) \((n = 6)\); respectively. Data are expressed as % difference of median of normal controls [medians (interquartile range)]. Each experiment was performed in three replicates. Group comparison by Kruskal-Wallis ANOVA (post hoc analysis: Dunn’s test) and Mann-Whitney \(U\) test, respectively. \(*P < 0.05, **P < 0.01; *P < 0.05, **P < 0.01.\)

Figure 2. Endothelial cell migration by human serum at 24 hours from normal controls \((n = 39)\) with or without omentin-1 \([200 \text{ng/ml}]\), PCOS women \((n = 21)\) with or without omentin-1 \([200 \text{ng/ml}]\) or PI3K/Akt inhibitor \((\text{LY294002; } 10 \mu\text{M})\) or CRP immunodepletion \((\text{CRP_IP})\), PCOS women after 6 months of metformin treatment \((n = 21)\) with or without omentin-1 immunodepletion \((\text{Omentin-1_IP})\), CRP \((1 \mu\text{g/ml})\) with or without addition of omentin-1 \([200 \text{ng/ml}]\) \((n = 6)\) and VEGF \((10 \text{ng/ml})\) with or without addition of omentin-1 \([200 \text{ng/ml}]\) \((n = 6)\), respectively. Data are expressed as % difference of median of normal controls [medians (interquartile range)]. Each experiment was performed in three replicates. Group comparison by Kruskal-Wallis ANOVA (post hoc analysis: Dunn’s test) and Mann-Whitney \(U\) test, respectively. \(*P < 0.05, **P < 0.01; *P < 0.05, **P < 0.01.\)

Figure 3. Effects of serum from normal controls \((n = 39)\) with or without omentin-1 \([200 \text{ng/ml}]\), PCOS women \((n = 21)\) with or without omentin-1 \([200 \text{ng/ml}]\) or CRP immunodepletion \((\text{CRP_IP})\), PCOS women after 6 months of metformin treatment \((n = 21)\) with or without omentin-1 immunodepletion \((\text{Omentin-1_IP})\), CRP \((1 \mu\text{g/ml})\) with or without addition of omentin-1 \([200 \text{ng/ml}]\) \((n = 6)\) and TNF-\(\alpha\) \((10 \text{ng/ml})\) with or without addition of omentin-1 \([200 \text{ng/ml}]\) \((n = 6)\), respectively, on NF-\(\kappa\)B activation in HMEC-1 cells stably transfected with pNF-\(\kappa\)B-luciferase at 2 hours. Cells were lysed and luciferase activities (RLU) were measured. Data are expressed as % difference of median of basal [medians (interquartile range)]. Each experiment was performed in three replicates. Group comparison by Kruskal-Wallis ANOVA (post hoc analysis: Dunn’s test) and Mann-Whitney \(U\) test, respectively. \(*P < 0.05, **P < 0.01; *P < 0.05, **P < 0.01.\)

Figure 4. Effects of serum from normal controls \((n = 39)\), PCOS women \((n = 21)\), PCOS women after 6 months of metformin treatment with or without addition of omentin-1 \([200 \text{ng/ml}]\) \((n = 21)\) on Erk1/2 and Akt phosphorylation in HMEC-1 cells at 2 minutes and 5 minutes, respectively. Data are expressed as % difference of median of basal [medians (interquartile range)]. Each experiment was performed in three replicates. Group comparison by Kruskal-Wallis ANOVA (post hoc analysis: Dunn’s test) and Mann-Whitney \(U\) test, respectively. \(*P < 0.05, **P < 0.01; *P < 0.05, **P < 0.01.\)
Figure 1
Figure 2
Figure 3

[Graph showing NF-κB activity (RLU) with various conditions and annotations for statistical significance (e.g., * P < 0.05, ** P < 0.01, *** P < 0.001).]

| Condition                  | NF-κB Activity (RLU) |
|----------------------------|----------------------|
| Control                    | -                    |
| TK plasmid                 | -                    |
| Normal Women               | -                    |
| PCOS Women                 | -                    |
| Before Metformin           | -                    |
| After Metformin            | -                    |
| CRP_IP                     | -                    |
| Omentin-1_IP               | -                    |
| Omentin-1                  | -                    |
| CRP                        | -                    |
| TNF-α                      | -                    |
Figure 4

A

P-Erk1/2
Erk1/2

Phos/Total Erk1/2
PSL (% difference)

Control
Normal Woman
PCOS Women
Before Metformin
After Metformin
Omentin-1

** P < 0.01
*** P < 0.01
#

B

P-Akt
Akt

Phos/Total Akt
PSL (% difference)

Control
Normal Woman
PCOS Women
Before Metformin
After Metformin
Omentin-1

** P < 0.01
** P < 0.05
#