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ORIGINAL ARTICLE

Increased levels of serum advanced glycation end-products in women with polycystic ovary syndrome

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Summary

Objective Women with polycystic ovary syndrome (PCOS) carry a number of cardiovascular risk factors and are considered to be at increased risk for atherosclerosis. Elevated concentrations of advanced glycation end-products (AGE), which exert their effects through interaction with specific receptors (RAGE), have been implicated in the cellular and tissue damage during atherosclerotic processes.

Design/patients We investigated serum AGE levels in 29 young women with PCOS as well as the expression of their receptor, RAGE, in circulating monocytes and compared them levels with 22 healthy control women.

Measurements/results Women with PCOS had higher levels of serum AGE proteins compared to healthy individuals ($9{\cdot}81 \pm 0{\cdot}16$ *vs.* $5{\cdot}11 \pm 0{\cdot}16$, $P < 0{\cdot}0001$), and increased RAGE expression was observed in monocytes of PCOS women compared to controls ($30{\cdot}91 \pm 10{\cdot}11$ *vs.* $7{\cdot}97 \pm 2{\cdot}61$, $P < 0{\cdot}02$). A positive correlation was observed between AGE proteins and testosterone (T) levels ($r = 0{\cdot}73$, $P < 0{\cdot}0001$). The correlation between AGE proteins and T levels remained high (partial correlation coefficient $= 0{\cdot}61$, $P = 0{\cdot}0001$) after controlling for body mass index (BMI), insulin levels and the area under the curve for glucose (AUCGLU) during an oral glucose tolerance test (OGTT). A positive correlation was also observed between AGE proteins and the free androgen index (FAI) ($r = 0{\cdot}58$, $P < 0{\cdot}0001$), waist-to-hip ratio (WHR) ($r = 0{\cdot}31$, $P < 0{\cdot}02$), insulin ($r = 0{\cdot}46$, $P < 0{\cdot}001$), homeostasis model assessment (HOMA) ($r = 0{\cdot}47$, $P < 0{\cdot}0001$), AUCGLU ($r = 0{\cdot}52$, $P < 0{\cdot}002$) and RAGE ($r = 0{\cdot}59$, $P < 0{\cdot}01$). A negative correlation was observed between AGE proteins and glucose/insulin ratio (GLU/INS) ($r = -0{\cdot}35$, $P < 0{\cdot}01$), and the quantitative insulin sensitivity check index (QUICKI) ($r = -0{\cdot}50$, $P < 0{\cdot}01$). In multiple regression analysis T was the only independent predictor of AGE levels ($P < 0{\cdot}0001$, $b = 0{\cdot}044$) between BMI, insulin, SHBG and AUCGLU (adjusted $R^2 = 0{\cdot}59$, $F = 44{\cdot}41$, $P < 0{\cdot}0001$).

Conclusion These data clearly demonstrate, for the first time, that PCOS women without overt hyperglycaemia have increased AGE levels and elevated RAGE expression when compared with controls.

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Polycystic ovary syndrome (PCOS) is a common and heterogeneous reproductive endocrine disorder that affects 4–6% of premenopausal women.[1,2] Its aetiology is unknown and it is diagnosed by the presence of hyperandrogenism and chronic anovulation with the exclusion of the adrenal, ovary and pituitary disorders.[3,4] PCOS is also characterized by several metabolic aberrations, including high incidence of impaired glucose tolerance,[5] hyperinsulinaemia and insulin resistance,[6,7] which in turn have been shown to increase the risk of cardiovascular disease and diabetes mellitus (DM).[8–12] The mechanism by which the vascular bed is affected under the influence of various metabolic and hormonal abnormalities is not clear. Several hypotheses have been formulated, and several factors appear to have a synergistic role in the process. Insulin resistance, a common finding in PCOS, seems to play a key role in the development of endothelial damage.[13]

Furthermore, advanced glycation end-products (AGE), which are protein products of nonenzymatic glycation, have been proposed to be among the main mediators of molecular damage to cellular and tissue structures in the vascular bed of DM patients.[14–16] These compounds have unique properties, such as fluorescence, browning and cross-linking, and their accumulation has been shown in several tissues in association with ageing and age-enhanced disease states, including atherosclerosis, diabetic complications, haemodialysis-related amyloidosis and Alzheimer's disease.[17–19] Although the main mechanism of AGE formation is glycosylation, recent studies have shown involvement of the insulin receptor pathway and especially phosphatidyl inositol-3 kinase (PI-3K) in the endocytotic uptake of AGE by their receptor (RAGE) regulating their rapid degradation and elimination processes.[20]

Previous studies have shown that insulin receptor-mediated signal transduction is defective in women with PCOS.[21,22] Specifically in this condition, there is an increased serine phosphorylation of the

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insulin receptor and a significant decrease in insulin-stimulated receptor tyrosine autophosphorylation. Furthermore, recent studies have demonstrated a defective PI-3K activity and decreased insulin-mediated glucose uptake in skeletal muscle tissue from PCOS women.[23] Interestingly, one of the proposed mechanisms in the elimination process of AGE is mediated via the insulin receptor pathway.

In order to investigate whether, in PCOS, AGE levels were altered; we examined normoglycaemic insulin-resistant PCOS young women and compared them to normal controls. AGE levels were therefore estimated in serum samples of the two groups along with RAGE expression in circulating monocytes.

Research design and methods

Subjects

The study consisted of 51 women who were all recruited from the Outpatient Department of Endocrinology, of Laiko University Hospital in Athens. The study protocol was approved by the local ethics board and informed consent was obtained from all participants. All subjects were selected to be nonsmokers, and none was taking any medication known to affect carbohydrate or sex hormone metabolism = 1 month before study, except for oral contraceptive agents, which were stopped 3 months before the study. Twenty-nine women [mean age 25·79 ± 4·98 years; body mass index (BMI) 27·18 ± 7·71 kg/m^2] with the diagnosis of PCOS were studied. Their diagnosis was based on National Institute of Child Health and Human Development conference criteria: chronic anovulation and hyperandrogenaemia (eight or less menses per year, elevated serum levels of testosterone and clinical symptoms of hyperandrogenism), excluding nonclassical congenital adrenal hyperplasia, androgen-secreting neoplasm, hyperprolactinaemia and thyroid disease by appropriate tests. One woman with PCOS exhibited impaired glucose tolerance. Including this woman in the study group could have introduced a bias in the study. However, our results were similar, with and without inclusion of this woman. Thus, we decided to include her in the study group.

The control group consisted of 22 healthy women (mean age 28·12 ± 4·00 years; BMI 23·28 ± 5·73 kg/m^2) who had regular menstrual cycles, normal plasma androgen levels, no acne or hirsutism, hypertension and no first-degree relative with type 1 or 2 diabetes.

Study protocol

The evaluations were conducted within 10 days from the onset of menstrual flow in control women and at any time in the PCOS women who were chronically anovulatory. In the amenorrhoeic women, recent ovulation was excluded by progesterone measurement (< 5 nmol/l). Blood samples were collected at 08:00 h after an overnight fast to determine serum levels of total testosterone (T, nmol/l), SHBG (nmol/l), serum fasting insulin (pmol/l), serum fasting glucose (mmol/l) and fructosamine (F, mmol/l). The glucose-to-insulin ratio (GLU/INS) and the T-to-SHBG ratio × 100 (free androgen index, FAI, %) were also estimated.

Chemicals and reagents

Bovine serum albumin (BSA), D-glucose, alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG) and anti-goat IgG-fluorescein isothiocyanate (FITC) as well as *p*-nitrophenyl phosphate (pNPP) tablets were purchased from Sigma Chemicals (St Louis, MO, USA). Superblock™ blocking buffer was from Pierce (Rockford, IL, USA) and normal goat serum (NGS) from Gibco-BRL (Gaithesburg, MD, USA). Mouse anti-human AGE monoclonal antibody (mAb) (6D12) and goat anti-human RAGE mAb were obtained from Research Diagnostics Inc., USA.

Preparation of AGE-modified proteins

AGE-modified BSA (AGE-BSA) was prepared as described by Makita *et al.*[24] Briefly, pure BSA protein was incubated under sterile conditions with 0·5 M D-glucose in 0·2 M phosphate buffer (pH 7·4) at 37 °C for 8 weeks, and then low molecular weight reactants and glucose were removed by dialysis (10 kDa cut-off) against phosphate-buffered saline (PBS, pH 7·4). The degree of AGE modification of the protein was further determined by competitive AGE-ELISA as described below.

Assays

After a 10- to 12-h overnight fast, serum and plasma samples were obtained from the three groups and stored at –20 °C prior to analysis. Just before assay by competitive AGE enzyme-linked immunosorbent assay (ELISA), sera were thawed and diluted 1 : 5 with dilution buffer [PBS, 0·02% Tween-20 and 1 mM sodium azide (NaN_3)].

All measurements were performed at the Chemwell analyser (Awareness, USA), unless stated otherwise. Glucose levels were determined by the glucose oxidase technique, using the commercially available reagent from Linear Chemicals (Spain). Insulin levels were determined immunoenzymatically by using the ELISA kit from Biosource Europe SA. This kit includes a highly specific monoclonal anti-insulin antibody, therefore minimizing any possible cross-reactivity with proinsulin. Fructosamine levels were measured by using the specific reagents from Raichem (San Diego, USA).

Testosterone and SHBG levels were measured spectrophotometrically using the appropriate kits from Linear Chemicals and IBL Immuno-biological laboratories (Germany), respectively.

Oral glucose tolerance test (OGTT)

At 08:00 h, after a 10- to 12-h overnight fast, all the subjects received a 75-g OGTT. Blood samples were taken at 0, 30, 60, 90 and 120 min for the measurement of plasma glucose concentrations. The area under the curve for glucose (AUCGLU) in the OGTT was estimated.

Insulin resistance estimation

The estimate of insulin resistance was calculated by the quantitative insulin sensitivity check index (QUICKI) and the homeostasis model assessment (HOMA). QUICKI is defined as $1/(\log I_0 + \log G_0)$, where I_0 is the fasting insulin and G_0 is the fasting glucose. HOMA

Table 1. Anthropometric characteristics and metabolic and hormonal profile of PCOS patients and normal women

Variable	PCOS group ($N = 29$)	Control group ($N = 22$)	P
Age (years)	25·79 ± 4·98	28·12 ± 4·00	NS
BMI (kg/m^2)	27·18 ± 7·71	23·28 ± 5·73	NS
Waist/hip ratio	0·78 ± 0·01	0·74 ± 0·01	< 0·04
Glucose (mmol/l)	(5·69 ± 0·24)	(5·19 ± 0·12)	NS
Fructosamine (mmol/l)	2·07 ± 0·08	1·92 ± 0·10	NS
Insulin (pmol/l)	73·68 ± 5·69	39·79 ± 2·98	< 0·0001
Testosterone (nmol/l)	3·36 ± 0·22	1·19 ± 0·10	< 0·0001
SHBG (nmol/l)	42·28 ± 6·60	51·47 ± 8·37	NS
FAI (%)	387·83 ± 55·36	83·57 ± 11·17	< 0·0001
GLU/INS	11·63 ± 1·03	19·63 ± 2·39	< 0·002
AUCGLU	16 077·69 ± 436·63	12 272·50 ± 1596·12	< 0·0001
QUICKI	0·331 ± 0·004	0·367 ± 0·005	< 0·0001
HOMA	2·90 ± 0·26	1·41 ± 0·10	< 0·0001

Data are given as means ± SE, except BMI and waist/hip ratio, which are given in means ± SD. $P < 0·05$ statistically significant.
FAI, free androgen index; GLU/INS, glucose-to-insulin ratio; AUCGLU, area under the curve of the OGTT for glucose; QUICKI, quantitative insulin sensitivity check index; HOMA, homeostasis model assessment.

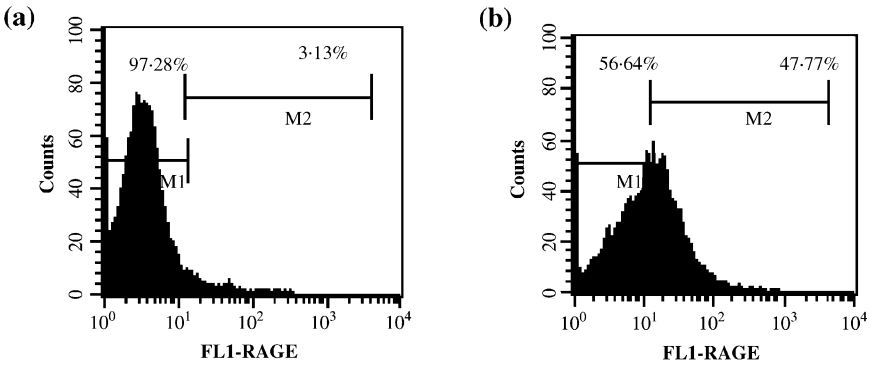

Fig. 1 Flow cytometric analysis of RAGE expression in monocytes of control (a) and PCOS (b) women. The M1 marker region shows the percentage of negatively stained cells and the M2 region shows the percentage of positively stained cells for RAGE.

was applied by using the formula HOMA = [fasting insulin (μU/ml) × fasting glucose (mmol/l)]/22·5.

Competitive AGE-ELISA

The competitive AGE-ELISA procedure was performed as described by Mitsuhashi *et al.*[25] In brief, 96-well ELISA plates were coated with 100 μl/well of 3 μg/ml AGE-BSA in coating buffer overnight at 4°C. Wells were washed three times with 150 μl washing buffer, and then blocked with 100 μl Superblock™ blocking buffer in PBS at room temperature for 1 h. After three rinses with washing buffer, 50 μl of competing antigen in dilution buffer was added, followed by 50 μl of anti-AGE mAb (1 : 1000) in dilution buffer containing 2% NGS.

Plates were incubated at room temperature for 2 h with gentle agitation on a horizontal rotary shaker. Wells were then rinsed three times with washing buffer. Secondary antibody in dilution buffer with 1% NGS was then added to each well and the plates were incubated at 37 °C for 1 h. After rinsing three times with washing buffer, 100 μl pNPP substrate was added to each well. Optical density (OD) at 405 nm was determined by a SPECTRA Fluor Plus TECAN reader after 30–60 min. Results were expressed as B/Bo, calculated as [experimental OD – background OD (i.e. no antibody)]/[total OD (i.e. no competitor) – background OD].

One AGE unit was defined as the mean percentage inhibition resulting from 1 : 5 diluted normal human serum (NHS) in the competitive AGE-ELISA format and the amount of AGE in NHS was set at 5 units/ml. The AGE-BSA was used as a competing antigen to generate an AGE standard curve (0·1–100 AGE units/ml) for each experiment. Sample AGE values were calculated from a linear regression of the standard curve.

Flow cytometric analysis of RAGE expression

Peripheral blood mononuclear cells were isolated from 50 ml of fresh heparinized blood using density gradient centrifugation on Ficoll-Paque (Pharmacia Biotech LKB, Vienna, Austria). After washing with sterile PBS, cells were counted and adjusted to 1×10^6 cells/ml. Labelling with goat anti-human RAGE mAb (50 μl/sample, diluted 1 : 400 in PBS) was performed for 1 h at room temperature. Isotype controls were used throughout the experiment. After washing, cells were incubated with 100 μl secondary anti-goat IgG-FITC for 1 h at 4 °C (anti-IgG-FITC was diluted 1 : 1000 in PBS). At the end of the incubation, cells were resuspended in 500 μl PBS and analysed.

Flow cytometry analysis was performed using a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) equipped with a single 488 nm-argon laser. RAGE-FITC labelling was analysed

Table 2. Levels of advanced glycation end-products (AGE) and their receptor (RAGE) expression

Variable	Control group ($N = 22$)	PCOS group ($N = 29$)	P
Serum AGE (U/ml)	5·11 ± 0·16	9·81 ± 0·16	< 0·0001
RAGE expression (% +ve)	7·97 ± 2·61	30·91 ± 10·11	< 0·02

Data are given as means ± SE, $P < 0·05$ statistically significant.

using excitation and emission settings of 488 nm and 535 nm (FL-1 channel). In all cases, a total of 10 000 events per sample were acquired and the forward and side scatters were used to gate out cellular fragments. All data were analysed with CellQuest software version 7·5·3 (Becton Dickinson).

Statistics

All results are reported as mean value ± SE, apart from BMI and age, which are reported as mean value ± SD, with P less than 0·05 being considered statistically significant. Statistical analysis was performed with Student's t-test between the control group and PCOS. Normal distribution of continuous variables was assessed by applying the nonparametric Kolmogorov–Smirnov test. All variables were normally distributed. Correlations between variables were evaluated by Pearson's coefficient. Partial correlation coefficient was used to describe the correlation between AGE proteins and testosterone adjusted by BMI, insulin levels and the area under the glucose curve. Multiple regression analysis was applied to estimate which of T, BMI, INS, SHBG or AUCGLU as independent variables best predict the value of AGE plasma levels as a dependent variable. Analysis was performed using the SPSS for Windows (version 10·0).

Results

There were no statistically significant differences in the mean age (25·79 ± 4·98 *vs.* 28·12 ± 4·00 years) and BMI (27·18 ± 7·71 *vs.* 23·28 ± 5·73 kg/m^2) between the PCOS and the controls group, although the women with PCOS were heavier and younger than the controls (Table 1).

PCOS women showed a significant rise at the AGE protein levels in serum (9·81 ± 0·16 U/ml) compared with controls (5·11 ± 0·16 U/ml, $P < 0·0001$, Table 2). Furthermore, analysis of monocytes from the two groups regarding expression of RAGE, studied by flow cytometry, showed significant changes between groups (Fig. 1). The percentage of positive cells for RAGE expression was significantly increased in PCOS (30·91 ± 10·11%) compared to normal subjects (7·97 ± 2·61%, $P < 0·02$, Table 2).

PCOS women had increased serum concentrations of testosterone (3·36 ± 0·22 nmol/l) compared with the controls (1·19 ± 0·10 nmol/l, $P < 0·0001$) and increased FAI ratios compared with controls (387·83 ± 55·36 *vs.* 83·57 ± 11·17%, $P < 0·0001$), although no difference was observed in SHBG in PCOS *vs.* controls (42·28 ± 6·60 *vs.* 51·47 ± 8·37 nmol/l, P = NS). Glucose levels were not significantly different between PCOS and controls (5·69 ± 0·24 *vs.* 5·19 ± 0·12 mmol/l, P = NS). Waist-to-hip ratio (WHR) differed between the groups (PCOS: 0·78 ± 0·01 *vs.* controls: 0·74 ± 0·01, $P < 0·04$). Insulin was increased in women with PCOS (73·68 ± 5·69 pmol/l) compared with the healthy individuals (39·79 ± 2·98 pmol/l, $P < 0·0001$)]. Accordingly, the GLU/INS ratio was lower in PCOS when compared with the control group (11·63 ± 1·03 *vs.* 19·63 ± 2·39, $P < 0·002$). Fructosamine levels showed no significant difference between the PCOS and controls (2·07 ± 0·08 *vs.* 1·92 ± 0·10 mmol/l, P = NS, Table 1).

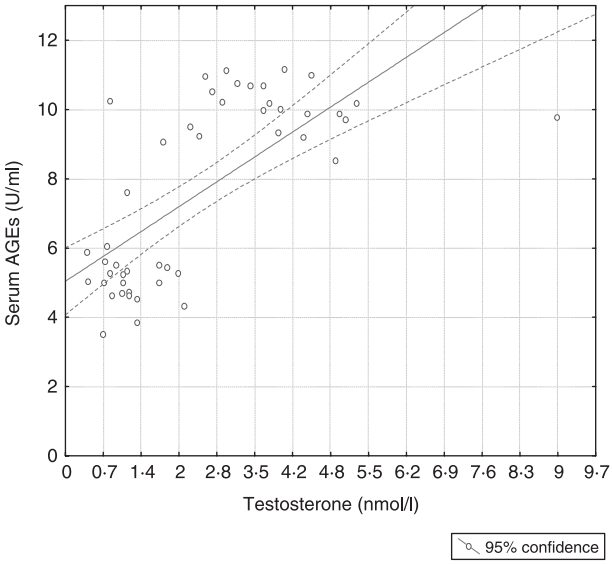

Fig. 2 Positive correlation of serum AGE levels and testosterone in the total population studied (PCOS and controls; $r = 0·65$, $P < 0·001$).

Women with PCOS had significantly higher AUCGLU and HOMA values than healthy women (16 077·69 ± 436·63 *vs.* 12 272·50 ± 1596·12, $P < 0·0001$ and 2·90 ± 0·26 *vs.* 1·41 ± 0·10, $P < 0·0001$, respectively) and QUICKI was significantly higher in the control group than in PCOS (0·367 ± 0·005 *vs.* 0·331 ± 0·004, $P < 0·0001$, Table 1).

A positive correlation was observed between AGE proteins and testosterone levels ($r = 0·73$, $P < 0·0001$, Fig. 2). The correlation between AGE proteins and testosterone levels remained high (partial correlation coefficient = 0·61, $P = 0·0001$) after controlling for BMI, insulin levels and AUCGLU. A positive correlation was also observed between AGE proteins and FAI ($r = 0·58$, $P < 0·0001$), WHR ($r = 0·31$, $P < 0·02$), insulin ($r = 0·46$, $P < 0·001$), HOMA ($r = 0·47$, $P < 0·0001$), AUCGLU ($r = 0·52$, $P < 0·002$) and RAGE ($r = 0·59$, $P < 0·01$). A negative correlation was observed between AGE proteins and GLU/INS ratio ($r = –0·35$, $P < 0·01$), and QUICKI ($r = –0·50$, $P < 0·01$).

In multiple regression analysis T was the only independent predictor of AGE levels ($P < 0·0001$, $b = 0·044$) between BMI, insulin, SHBG and AUCGLU (adjusted $R^2 = 0·59$, $F = 44·41$, $P < 0·0001$).

Discussion

There are two main findings in the present study: (1) women with PCOS exhibited higher levels of circulating AGE when compared with normoandrogenic women with regular cycles and similar BMI;

and (2) increased expression of RAGE was observed in monocytes of PCOS compared to controls.

AGE are toxic by-products of a nonenzymatic (Amadori product) reaction of proteins or lipoproteins.[26] AGE-modified proteins induce several cellular responses, such as mitogenic activity in macrophages and chemotactic activity in vascular smooth muscle cells.[27] Their presence and/or accumulation of AGE in arterial walls are thought to play a key role in the pathogenesis of diabetic micro- and macrovascular complications.

AGE usually exert their effects by interaction with specific cell membrane receptors on macrophages, monocytes and endothelial cells, termed collectively RAGE. Three types of AGE-receptors have been identified, including the macrophage scavenger receptor (MSR;[28]), the receptor for AGE (RAGE;[29]) and the receptor complex of OST-48, 80K-H and galectin-3 (previously called p60 and p90;[30]) *In vivo* studies with MSR knockout mice have shown that the receptor plays a major role in endocytotic uptake of AGE proteins by macrophages. Specifically, liver endothelial cells, which express a large amount of MSR, were responsible for elimination of 60–65% of intravenously injected AGE proteins from plasma.[31,32]

AGE stimulate the expression of RAGE in a homologous up-regulation phenomenon, originally observed in DM patients, and seen also in this PCOS group with insulin resistance. AGE-binding might therefore be enhanced in PCOS and diabetic patients in a teleological model to compensate for the elevated levels of circulating AGE proteins. It is of interest to note that the PCOS group is placed in between normal and type 2 diabetic women regarding AGE levels as well as RAGE expression, in a small group of diabetic women studied by our group (data not shown).

Previous *in vitro* studies have shown an interconnection between the insulin receptor signalling (IRS) pathway and the AGE elimination system. Specifically, insulin up-regulates receptor-mediated endocytotic uptake of AGE proteins through the IRS/PI-3K pathway, in which IRS following insulin receptor tyrosine autophosphorylation plays an important role. It seems that PI-3K stimulates MSR-mediated endocytotic uptake of AGE proteins, one of the main pathways of AGE elimination.[20] Thus, the presence of effective coupling between insulin-stimulated PI-3K and activation of receptor-mediated endocytotic uptake of ligands is possibly a requirement for efficient elimination of AGE.[33] In women with PCOS, earlier studies have shown a defective insulin intracellular signalling at the level of PI-3K (i.e. down-regulation of the PI-3K pathway) in skeletal muscle under physiological circumstances.[23] Therefore, among known insulin action intrinsic defects, the PI-3K pathway could be responsible for the defective elimination of AGE proteins in PCOS women and their accumulation in tissues. In support of the insulin-mediated AGE elimination, cells transfected with a mutant inactive human insulin receptor did not show insulin-enhanced endocytotic uptake of AGE proteins.[20]

In this study, the insulin resistance indices QUICKI and HOMA showed a positive correlation to serum AGE levels. Considering that these young women with PCOS had normal fasting glucose, normal OGTT, except one case with impaired glucose tolerance (which did not alter the results), and normal fructosamine levels, overt hyperglycaemia cannot be implicated as a causal or major contributing factor to the elevated serum AGE levels.

Interestingly, AGE levels were positively correlated with testosterone concentrations in PCOS. This positive correlation remained high even when the two groups were adjusted for BMI, insulin and the AUCGLU during the OGTT. A possible interaction between testosterone and AGE in hyperandrogenaemic women cannot be excluded.

Previous studies in PCOS women have also shown a positive correlation of testosterone with the other atherogenic factors such as endothelin-1,[34] adhesion molecules (unpublished data), plasminogen activator inhibitor 1 (PAI-1)[35] and von Willebrand factor.[36] Therefore, multiple interlinked factors in PCOS associated with insulin resistance may all contribute to the process of vascular pathology in atherogenesis.

AGE proteins found to be elevated in this group of PCOS women could possibly belong to the group of potentially atherogenic factors, as they have been found in atherosclerotic lesions of human coronary arteries and aorta in patients with atherosclerosis.[17,37]

In this study, we have demonstrated that young normoglycaemic women with PCOS have increased levels of AGE compared to a group of ovulatory normoandrogenic women. As the insulin resistance indices as well as AUCGLU differed between the PCOS and the control group, it is likely that insulin resistance is involved at some level in the pathway of AGE synthesis and clearance in the PCOS group. Interestingly, the correlation between AGE proteins and testosterone levels remained high controlling for BMI, insulin levels and AUCGLU, implying an interaction between AGE proteins and hyperandrogenaemia. Multiple regression analysis further confirmed this interaction as testosterone remained the only predictor factor for the elevated AGE proteins.

The implications of these findings to the metabolic and reproductive aspects of the PCOS require further investigation. Additionally, the effectiveness of therapeutic modalities such as diet, insulin sensitizers, lipase inhibitors or antiandrogens to reduce AGE levels in patients with PCOS should also be examined.

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References

1 Knochenhauer, E.S., Key, T.J., Kahsar-Miller, M., Waggoner, W., Boots, L.R. & Azziz, R. (1998) Prevalence of the polycystic ovarian syndrome in unselected Black and White women of the Southeastern United States: a prospective study. *Journal of Clinical Endocrinology and Metabolism*, **83**, 3078–3082.

2 Diamanti-Kandarakis, E., Kouli, C., Bergiele, A., Filandra, F., Tsianateli, T., Spina, G., Zapanti, E. & Bartzis, M. (1999) A survey of the PCOS in the Greek island of Lesbos: hormonal and metabolic profile. *Journal of Clinical Endocrinology and Metabolism*, **84**, 4006–4011.

3 Diamanti-Kandarakis, E. & Dunaif, A. (1996) New perspectives in PCOS. *Trends in Endocrinology and Metabolism*, **7**, 267–271.

4 Legro, R.S., Kunselman, A.R., Dodson, W.C. & Dunaif, A. (1999) Prevalence and predictors of risk for type 2 diabetes mellitus and

impaired glucose tolerance in polycystic ovary syndrome: a prospective, controlled study in 254 affected women. *Journal of Clinical Endocrinology and Metabolism*, **84**, 165–169.

5 Ehrmann, D.A., Bames, R.B., Rosenfild, R.L., Cavagham, M.K. & Imperial, J. (1999) Prevalence of impaired glucose tolerance and diabetes in women with polycystic ovary syndrome. *Diabetes Care*, **22**, 141–146.

6 Dunaif, A., Segal, K.R., Futterweit, W. & Dobrjansky, A. (1989) Profound peripheral insulin resistance, independent of obesity, in polycystic ovary syndrome. *Diabetes*, **38**, 1165–1174.

7 Dunaif, A. (1997) Insulin resistance and the polycystic ovary syndrome: mechanism and implications for pathogenesis. *Endocrinology Reviews*, **18**, 774–800.

8 Conway, G.S., Agrawal, R., Betteridge, D.J. & Jacobs, H.S. (1992) Risk factors for coronary artery disease in lean and obese women with the polycystic ovary syndrome. *Clinical Endocrinology*, **37**, 119–125.

9 Dahlgren, E., Janson, P.O., Johansson, S., Lapidus, L. & Oden, A. (1992) Polycystic ovary syndrome and risk for myocardial infarction. Evaluated from a risk factor model based on a prospective population study of women. *Acta Obstetrica et Gynecologica Scandinavica*, **71**, 599–604.

10 Talbott, E., Guzick, D., Clerici, A., Berga, S., Detre, K., Weimer, K. & Kuller, L. (1995) Coronary heart disease risk factors in women with polycystic ovary syndrome. *Arteriosclerosis, Thrombosis and Vascular Biology*, **15**, 821–826.

11 Guzick, D.S. (1996) Cardiovascular risk in women with polycystic ovarian syndrome. *Seminars in Reproductive Endocrinology*, **14**, 45–49.

12 Birdsall, M.A., Farquhar, C.M. & White, H.D. (1997) Association between polycystic ovaries and extent of coronary artery disease in women having cardiac catheterization. *Annals of Internal Medicine*, **126**, 32–35.

13 Reaven, G.M. & Chen, Y.D. (1996) Insulin resistance, its consequences, and coronary heart disease. Must we choose one culprit? *Circulation*, **93**, 1780–1783.

14 McCance, D.R., Dyer, D.G., Dunn, J.A., Bailie, K.E., Thorpe, S.R., Baynes, J.W. & Lyons, T.J. (1993) Maillard reaction products and their relation to complications in insulin-dependent diabetes mellitus. *Journal of Clinical Investigation*, **91**, 2470–2478.

15 Vlassara, H., Bucala, R. & Striker, L. (1994) Pathogenic effects of advanced glycosylation: biochemical, biologic, and clinical implications for diabetes and aging. *Laboratory Investigations*, **70**, 138–151.

16 Makino, H., Shikata, K., Hironaka, K., Kushiro, M., Yamasaki, Y., Sugimoto, H., Ota, Z., Araki, N. & Horiuchi, S. (1995) Ultrastructure of nonenzymatically glycated mesangial matrix in diabetic nephropathy. *Kidney International*, **48**, 517–526.

17 Kume, S., Takeya, M., Mori, T., Araki, N., Suzuki, H., Horiuchi, S., Kodama, T., Miyauchi, Y. & Takahashi, K. (1995) Immunohistochemical and ultrastructural detection of advanced glycation end products in atherosclerotic lesions of human aorta with a novel specific monoclonal antibody. *American Journal of Pathology*, **147**, 654–667.

18 Vitek, M.P., Bhattacharya, K., Glendening, J.M., Stopa, E., Vlassara, H., Bucala, R., Manogue, K. & Cerami, A. (1994) Advanced glycation end products contribute to amyloidosis in Alzheimer disease. *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 4766–4770.

19 Yan, S.D., Yan, S.F., Chen, X., Fu, J., Chen, M., Kuppusamy, P., Smith, M.A., Perry, G., Godman, G.C. & Nawroth, P. (1995) Non-enzymatically glycated tau in Alzheimer's disease induces neuronal oxidant stress resulting in cytokine gene expression and release of amyloid beta-peptide. *Nature Medicine*, **1**, 693–699.

20 Sano, H., Higashi, T., Matsumoto, K., Melkko, J., Jinnouchi, Y., Ikeda, K., Ebina, Y., Makino, H., Smedsrod, B. & Horiuchi, S. (1998) Insulin enhances macrophage scavenger receptor-mediated endocytic uptake of advanced glycation end products. *Journal of Biological Chemistry*, **273**, 8630–8637.

21 Dunaif, A., Segal, K.R., Shelley, D.R., Green, G., Dobrjansky, A. & Licholai, T. (1992) Evidence for distinctive and intrinsic defects in insulin action in polycystic ovary syndrome. *Diabetes*, **41**, 1257–1266.

22 Dunaif, A., Xia, J., Book, C.B., Schenker, E. & Tang, Z. (1995) Excessive insulin receptor serine phosphorylation in cultured fibroblasts and in skeletal muscle. A potential mechanism for insulin resistance in the polycystic ovary syndrome. *Journal of Clinical Investigation*, **96**, 801–810.

23 Dunaif, A., Wu, X., Lee, A. & Diamanti-Kandarakis, E. (2001) Defects in insulin receptor signaling *in vivo* in the polycystic ovary syndrome (PCOS). *American Journal of Physiological Endocrinology and Metabolism*, **281**, E392–E399.

24 Makita, Z., Vlassara, H., Cerami, A. & Bucala, R. (1992) Immunochemical detection of advanced glycosylation end products *in vivo*. *Journal of Biological Chemistry*, **267**, 5133–5138.

25 Mitsuhashi, T., Vlassara, H., Founds, H.W. & Li, Y.M. (1997) Standardizing the immunological measurement of advanced glycation endproducts using normal human serum. *Journal of Immunological Methods*, **207**, 79–88.

26 Yui, S., Sasaki, T., Araki, N., Horiuchi, S. & Yamazaki, M. (1994) Induction of macrophage growth by advanced glycation end products of the Maillard reaction. *Journal of Immunology*, **152**, 1943–1949.

27 Higashi, T., Sano, H., Saishoji, T., Ikeda, K., Jinnouchi, Y., Kanzaki, T., Morisaki, N., Rauvala, H., Shichiri, M. & Horiuchi, S. (1997) The receptor for advanced glycation end products mediates the chemotaxis of rabbit smooth muscle cells. *Diabetes*, **46**, 463–472.

28 Kodama, T., Freeman, M., Rohrer, L., Zabrecky, J., Matsudaira, P. & Krieger, M. (1990) Type I macrophage scavenger receptor contains alpha-helical and collagen-like coiled coils. *Nature*, **343**, 531–535.

29 Neeper, M., Schmidt, A.M., Brett, J., Yan, S.D., Wang, F., Pan, Y.C., Elliston, K., Stern, D. & Shaw, A. (1992) Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. *Journal of Biological Chemistry*, **267**, 14998–15004.

30 Li, Y.M., Mitsuhashi, T., Wojciechowicz, D., Shimizu, N., Li, J., Stitt, A., He, C., Banerjee, D. & Vlassara, H. (1996) Molecular identity and cellular distribution of advanced glycation endproduct receptors: relationship of p60 to OST-48 and p90 to 80K-H membrane proteins. *Proceedings of the National Academy of Sciences of the United States of America*, **93**, 11047–11052.

31 Araki, N., Higashi, T., Mori, T., Shibayama, R., Kawabe, Y., Kodama, T., Takahashi, K., Shichiri, M. & Horiuchi, S. (1995) Macrophage scavenger receptor mediates the endocytic uptake and degradation of advanced glycation end products of the Maillard reaction. *European Journal of Biochemistry*, **230**, 408–415.

32 Smedsrod, B., Melkko, J., Araki, N., Sano, H. & Horiuchi, S. (1997) Advanced glycation end products are eliminated by scavenger-receptor-mediated endocytosis in hepatic sinusoidal Kupffer and endothelial cells. *Biochemical Journal*, **322**, 567–573.

33 Vlassara, H., Brownlee, M. & Cerami, A. (1985) High-affinity-receptor-mediated uptake and degradation of glucose-modified proteins: a potential mechanism for the removal of senescent macromolecules. *Proceedings of the National Academy of Sciences of the United States of America*, **82**, 5588–5592.

34 Diamanti-Kandarakis, E., Spina, G., Kouli, C. & Migdalis, I. (2001) Increased endothelin-1 levels in women with polycystic ovary syndrome and the beneficial effect of metformin therapy. *Journal of Clinical Endocrinology and Metabolism*, **86**, 4666–4673.

35 Diamanti-Kandarakis, E., Palioniko, G., Alexandraki, K., Bergiele, A., Koutsouba, T. & Bartzis, M. (2004) The prevalence of 4G5G polymorphism of plasminogen activator inhibitor (PAI-1) gene in polycystic ovarian syndrome and its association with plasma PAI-1 levels. *European Journal of Endocrinology*, **150**, 795–800.

36 McCrohon, J.A., Jessup, W., Handelsman, D.J. & Celermajer, D.S. (1999) Androgen exposure increases human monocyte adhesion to vascular endothelium and endothelial cell expression of vascular cell adhesion molecule-1. *Circulation*, **99**, 2317–2322.

37 Nakamura, Y., Horii, Y., Nishino, T., Shiiki, H., Sakaguchi, Y., Kagoshima, T., Dohi, K., Makita, Z., Vlassara, H. & Bucala, R. (1993) Immunohistochemical localization of advanced glycosylation end products in coronary atheroma and cardiac tissue in diabetes mellitus. *American Journal of Pathology*, **143**, 1649–1656.