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ORIGINAL ARTICLE

Increased serum advanced glycation end-products is a distinct finding in lean women with polycystic ovary syndrome (PCOS)

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Summary

Background Nonenzymatic advanced glycation and oxidation end-products, advanced glycation end-products (AGEs), impart a potent impact on vessels and other tissues in diabetic state and in euglycaemic conditions with increased oxidative stress.

Insulin resistant (IR) polycystic ovary syndrome (PCOS) women, have elevated serum AGEs, increased receptor (RAGE) expression, and increased deposition with differential localization in the polycystic ovarian tissue (theca and granulosa) compared to normal.

Objective To determine whether the raised AGE levels in non-insulin resistant women with PCOS is a distinct finding compared with those presenting the isolated components of the syndrome and among PCOS subphenotypes. Noninsulin resistant women were selected in order to show that serum AGEs are elevated in PCOS independently of the presence of IR.

Design Clinical trial.

Patients One hundred and ninety-three age- and BMI-matched young lean noninsulin resistant women were studied. Among them, 100 women were diagnosed with PCOS according to Rotterdam criteria, and divided to subphenotypes (hyperandrogenaemia with or without PCO morphology and with or without anovulation). Sixty-eight women with the isolated components of the PCOS phenotype were also studied along with 25 healthy women.

Measurements Serum AGE levels, metabolic, hormonal profiles and intravaginal ultrasound were determined in all subjects.

Results The studied population did not differ in BMI, fasting insulin concentration, waist : hip and glucose : insulin ratios. PCOS women exhibited statistically higher AGEs levels (7·96 ± 1·87 U/ml, $P < 0·001$) compared with those with isolated hyperandrogenaemia (5·61 ± 0·61 U/ml), anovulation (5·53 ± 1·06 U/ml), US-PCO morphology (5·26 ± 0·25 U/ml) and controls (5·86 ± 0·89 U/ml).

Conclusions In PCOS, serum AGEs are distinctly elevated compared with women having the isolated characteristics of the syndrome. No difference was observed between PCOS subphenotypes. As chronic inflammation and increased oxidant stress have been incriminated in the pathophysiology of PCOS, the role of AGEs as inflammatory and oxidant mediators, may be linked with the metabolic and reproductive abnormalities of the syndrome.

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Introduction

Polycystic ovary syndrome (PCOS) is a common reproductive and metabolic disorder, which affects 6–7% of premenopausal women.[1,2] The aetiology of the syndrome is unknown and it is diagnosed by the presence of hyperandrogenism, chronic anovulation and/or PCO morphology, with the exclusion of the adrenal, ovary and pituitary disorders.[3,4] PCOS is also characterized by metabolic aberrations, such as impaired glucose tolerance (IGT), diabetes mellitus (DM2)[5–7] dyslipidaemia[8] and increased several cardiovascular risk markers, both functional and morphological.[9–12]

Advanced glycation end-products (AGEs), which are products of nonenzymatic glycation and oxidation (glycoxidation) of proteins and lipids, have been proposed to be among the main mediators of molecular damage in the vascular bed and other tissue structures in diverse biological settings, such as diabetes, inflammation, renal failure and ageing.[13–16]

AGEs are a heterogenous group of compounds, generated endogenously via multiple routes *in vivo*, by nonenzymatic glycation in hyperglycaemic conditions and also in euglycaemic states spontaneously with amino peptides, lipids and nucleic acids. AGEs are also absorbed from exogenously derived sources such as tobacco[17] and certain foods, particularly those that are overcooked.[18]

The term AGEs, while literally referring to nonreactive glycoxidation products such as εN-carboxymethyllysine (CML, the most representative and well-studied AGE) or pentosidine, also includes many reactive intermediates or AGE-precursors. The most common

methods used for detection are HPLC, ELISA and immunohistochemistry and only one monoclonal antibody which recognizes specifically CML called 6D12 has become commercially available.[19]

Oxidative stress and reactive oxygen species (ROS) have been shown to catalyse the chemical modification of proteins by Maillard reactions (nonenzymatic glycation reactions) *in vivo*.[20] In addition to their immediate effects on protein structure and function, AGEs in turn, induce oxidative stress, leading to inflammation and propagation of tissue damage. Thus, formation of AGEs and resultant oxidative stress, which accelerate Maillard reactions, can initiate an autocatalytic cycle of deleterious reactions in tissues.[20,21] If oxidation accompanies glycation, then the products formed are also known as glycoxidation products.

In vitro studies have shown that AGEs are also implicated in the pathogenesis of insulin resistance (IR).[22]

Although the initial chemistry behind their formation has been known for years, the intracellular signalling of AGEs and ultimately, the mechanisms of tissue damage linked with the broad spectrum of diseases, remain unknown. However, several of their deleterious effects appeared to be mediated via a multiligand receptor, RAGE, activating NF-κB and initiating the generation of a cascade of proinflammatory cytokines (VCAM-1, IL-1α, IL-6, TNF-α), coagulatory (PAI-1, decreased platelets survival, glycation of anti thrombin III[16] and vasoconstrictive molecules (ET-1).[23,24]

The study of AGEs represents one of the most promising areas of research today, involving synthesis, absorption as well as the clearance of these atherogenic molecules and more importantly, their accumulation on tissues (including ovarian tissue), independently of hyperglycaemia. In AGEs accumulation, the receptor responsible for their clearance (macrophage scavenger receptor-1, MSR-1), activated via PI-3K, plays a fundamental role.[25,26]

In particular, the presence of IR in normoglycaemic women with PCOS, as we have suggested in previous work, could have altered AGEs clearance via decreased activity of PI-3K which has been demonstrated in *in vivo* studies in PCOS.[26,27]

The present study was undertaken in order to investigate whether increased AGE levels is a specific finding of noninsulin resistant women with PCOS, compared with women presenting with the isolated diagnostic features of the syndrome, like hyperandrogenaemia, anovulation or polycystic ovaries.

Subjects and methods

Patients and study protocol

A total of 193 Greek Caucasian women were recruited, between 2004 and 2006, at the Outpatient Departments of Reproductive Endocrinology and Endocrinology of the two participant University Hospitals in Thessaloniki and in Athens, respectively. All these women visited the outpatient's Departments because of hirsutism and/or menstrual abnormalities and/or subfertility. The ethics boards approved the study protocol and informed consents were obtained from all participants.

The diagnosis of PCOS was established on the basis of ESHRE/ASRM Consensus diagnostic criteria in 100 women. Hyperandrogenaemia was assessed as total testosterone levels above the 95th percentile of the levels detected in the group of normal menstruating women. PCOS subjects were subdivided into four well-characterized subgroups (1 A–4 A) as follows:

Group 1 A: Biochemical hyperandrogenaemia, chronic anovulation and PCO morphology

Group 2 A: Biochemical hyperandrogenaemia with chronic anovulation and normal ovaries on ultrasound

Group 3 A: Biochemical hyperandrogenaemia with PCO on ultrasound and confirmed regular ovulation

Group 4 A: Chronic anovulation and PCO

Three groups of age- and BMI-matched women (68 women total) with the isolated signs of hyperandrogenaemia (GROUP : HYPER), PCO morphology (GROUP : PCO) and anovulation (GROUP : ANOV) also participated in the study with the following description:

Group HYPER: Hyperandrogenaemia, normal ovaries on ultrasound and confirmed regular ovulation

Group PCO: PCO on ultrasounds and confirmed regular ovulation. No hyperandrogenaemia

Group ANOV: Chronic anovulation, normal ovaries on ultrasound and no hyperandrogenaemia

A final group of 25 women, well-selected, volunteered to participate in the study as control group, from infertile couples due to male infertility, with normal menses (27–32 days), normal androgens levels, nonhirsute (assessed by F–G scale), and with proven ovulatory cycles (progesterone confirmed in two consecutive cycles). These women did not have a history of hypertension or diabetes mellitus or family history in a first-degree relative of diabetes mellitus. All control women were studied during the follicular phase (days 5–8) of a spontaneous menstrual cycle. Serum progesterone levels were determined on days 21–24 of their menstrual cycle. All participants were carefully selected to be nonsmokers.

Two doctors in each centre performed physical examination in each woman. The enrolled population was in good health and not suffering from chronic or acute diseases. Chronic anovulation was assessed by < 8 cycles per year and serum progesterone levels below 3 ng/ml. Studies were performed in the follicular phase in ovulatory subjects confirmed with serum progesterone level < 3 ng/ml. Ovarian morphology and ovarian size, in three dimensions, were determined and registered in films in all subjects (in each case by the same operator at each centre and all sonographic records were reviewed and scored by a third sonographer for the statistical analysis assessment) according to Rotterdam criteria by transvaginal ultrasound.

A Synacthen test was performed by tetracosactide (Synacthène 0·25 mg/ml; Novartis Pharma S.A.S. Rueil-Malmaison, France), to each woman with a basal 17α-OH-progesterone (17OHP) plasma level greater than 1·5 ng/ml to exclude congenital adrenal hyperplasia. All women had normal serum PRL levels and normal thyroid function.

Oral contraceptives or other drugs, known to affect sex hormone or carbohydrate metabolism, if had been administrated, were discontinued for at least 3 months before study.

All women underwent an OGTT, data not shown. Women with IGT were not included. All participants in the study had fasting blood obtained for the measurements of LH, FSH, total testosterone (TT), dehydroepiandrosterone sulphate (DHEAS), androstenedione (Δ4), sex hormone-binding globulin (SHBG), insulin (INS), glucose (GLU). Blood samples were collected from all patients and healthy

controls between 08·00 and 13·00 hours, after an overnight fast. The samples were centrifuged immediately, and serum was stored at –80 °C until assayed.

Parameters studied

Weight, height, waist and hip circumferences were measured. Body weight was measured using analogue scales in light clothing; height was measured bare-foot using a stadiometer. Body mass index (BMI, kg/m^2) was calculated by dividing weight by height squared (kilograms per square meter) to assess obesity. Waist circumference was obtained as the smallest circumference at the level of the umbilicus. Hip circumference was obtained as the widest circumference at the level of the buttocks. Waist : hip ratio (WHR) was calculated to assess fat distribution.

Ultrasound assessment

Transvaginal ultrasound scans of the ovaries were performed by experienced sonographers in all the subjects who participated in the study (PCOS women and controls). The presence of polycystic ovaries was diagnosed by the presence of 12 or more follicles in each ovary measuring 2–9 mm in diameter, and/or increased ovarian volume ($> 10\ cm^3$). Ovarian volume was calculated by the formula:

$$V = (\pi/6) \times (D_{\text{length}} \times D_{\text{width}} \times D_{\text{thickness}})$$

Where, D: dimension.

Adding the volume of each ovary and dividing by 2 calculated the mean ovarian volume for each participant.

Only one ovary fitting this definition was sufficient to define PCO. Subjects with a developing follicle (defined as largest follicle with mean diameter > 10 mm) or an ovarian cyst were excluded from the study.

Chemicals and reagents

Alkaline phosphatase-conjugated goat antimouse immunoglobulin G (IgG) and p-nitrophenyl phosphate (pNPP) tablets were purchased from Sigma Chemical (St Louis, MO). Superblock' blocking buffer was obtained from Pierce (Rockford, IL) and normal goat serum from Gibco-BRL (Gaithersburg, MD). Mouse antihuman AGE monoclonal antibody (6D12) and AGE-modified bovine serum albumin (AGE–BSA) were obtained from Research Diagnostics Inc. (Concord, MA).

Competitive AGE enzyme-linked immunosorbent assay (ELISA)

The competitive AGE–ELISA procedure was performed as described previously in a PCOS population by Diamanti-Kandarakis *et al.* (2005).[28]

Assays

Serum samples were collected from each participant for determination of baseline levels of biochemical and hormonal parameters and stored at –80 °C prior to analysis. Just prior to assay by competitive AGE-ELISA, sera were thawed and diluted 1 : 5 with dilution buffer [phosphate-buffered saline (PBS), 0·02% Tween-20 and 1 mM sodium azide (NaN_3)]. AGE measurements were performed at the Chemwell analyser (Awareness, FL).

Plasma glucose concentrations were measured with the glucose oxidase technique using an auto analyser (Roche/Hitachi 902; Roche Diagnostics GmBH, Manheim, Germany). LH, FSH and PRL, Δ4 levels were measured with RIA method, while SHBG levels were measured with IRMA method, using commercial kits (FSH: Radioisotopic Kit, Nichols Institute Diagnostics, San Juan Capistrano, CA; LH: Radioisotopic Kit, Nichols Institute Diagnostics; PRL: Radioisotopic Kit, Nichols Institute Diagnostics; TT: Radioisotopic Kit, Diagnostic Systems Laboratories, Webster, TX; Δ4: Radioisotopic Kit, Diagnostic Systems Laboratories; DHEAS: Radioisotopic Kit, Diagnostic Systems Laboratories; SHBG: Immunoradiometric Assay (IRMA) Kit, Diagnostic Systems Laboratories). Serum insulin levels were measured with an enzyme immunoassay (ELISA Kit, Mercodia AB, Uppsala, Sweden). The intra-assay coefficients of variation (CV) were 1·5% for FSH, 0·7% for LH, 2·7% for PRL, 1·3% for TT, 5·9 for Δ4, 9·4% for DHEAS, 5·8% for SHBG and 3·8% for insulin. The average interassay CV were 3·2% for FSH, 1·7% for LH, 3·4% for PRL, 2·2% for TT, 9·2% for Δ4, 12·1% for DHEAS, 7·8% for SHBG, 4·4% for insulin.

Hyperandrogenimic index

Hyperandrogenaemia was estimated by the free androgen index (FAI), defined as: FAI (%) = [total testosterone (nmol/l)/SHBG (nmol/l)] × 100.

IR indices

IR was estimated by the quantitative insulin sensitivity check index (QUICKI), defined as: QUICKI = 1/[log (fasting insulin) + log (fasting glucose)].

Statistical analysis

The statistical analysis was performed mainly using the statistical package SPSS for Windows (version 15·0), and Stata (version 8·0).

The properties of the Normal distributed characteristics of interest are reported as mean value ± SE, apart from non-Normal characteristics which are reported as median ± IQR (interquartile range). Furthermore, to determine if there are differences among the means of these characteristics we have used both parametric and nonparametric tests. Parametric tests have been used only for normally distributed variables, while appropriate nonparametric tests were used in the case of non-Normal variables.

The assumption of Normality has been assessed by applying both the nonparametric Kolmogorov–Smirnov test as well as the test of Shapiro-Wilks. The first step in our analysis was to check the assumption of normality for the continuous variables under study (health measurements). From the analysis, it was revealed that only the variables age, height, weight, BMI (anthropometric characteristics) are Normally distributed. On the contrary all the other continuous

Table 1. Anthropometric characteristics of all subjects under study

Characteristics	PCOS					Controls	Hyper	PCO	ANOV	*P*-value
	1 A	2 A	3 A	4 A	Total					
Number of subjects	25	25	25	25	100	25	25	22	21	
Age (year)	24·20 ± 0·94	25·20 ± 0·80	25·76 ± 1·10	24·80 ± 1·20	24·99 ± 0·52	27·02 ± 0·99	24·16 ± 1·24	27·90 ± 1·03	27·45 ± 2·02	0·152
Height (m)	1·653 ± 0·01	1·652 ± 0·01	1·636 ± 0·01	1·658 ± 0·01	1·649 ± 0·01	1·646 ± 0·01	1·67 ± 0·01	1·659 ± 0·01	1·70 ± 0·02	0·219
Weight (kg)	60·76 ± 1·22	60·53 ± 1·28	59·09 ± 1·05	60·94 ± 1·29	60·33 ± 0·60	60·67 ± 1·29	62·20 ± 1·29	60·11 ± 1·13	61·28 ± 2·86	0·863
BMI (kg/m^2)	22·21 ± 0·38	22·17 ± 0·36	22·07 ± 0·35	22·14 ± 0·34	22·15 ± 0·18	22·36 ± 0·30	22·41 ± 0·36	21·84 ± 0·35	21·18 ± 0·66	0·668
Waist : hip	0·76 (0·11)	0·74 (0·07)	0·72 (0·05)	0·75 (0·05)	0·74 (0·08)	0·72 (0·05)	0·76 (0·09)	0·74 (0·07)	0·76 (0·09)	0·495

Data are given means ± SE (except for the variable waist : hip ratio which is expressed as medians and interquartile range). *P*-value refers to the ANOVA procedure for testing the hypothesis that several means are equal (except the *P*-value for the variable waist : hip ratio which refers to the Kruskal–Wallis test).

variables under study do not follow a normal distribution (tests were performed using a 5% significance level). The list of non-Normal characteristics contains WHR, hormonal profile variables (FSH, LH, PRL, testosterone, Δ4, DHEAS, SHBG, FAI) and metabolic variables (AGEs, insulin, glucose, glucose : insulin ratio, QUICKI).

In case of Normality, the statistical test used for determining the equality of group means was the ANOVA test (supplemented with the appropriate, for each case, *post hoc* procedure). On the contrary, in case of non-Normal variables the statistical test used for determining the equality of means (in all groups) was the Kruskal–Wallis nonparametric test (supplemented again with the appropriate, for each case, *post hoc* procedure). In order to explore graphically the difference we have constructed the corresponding means plots. A means plot shows the means of the 8 groups as well as the corresponding confidence interval around each mean.

Also, correlations between variables were evaluated both by Pearson's coefficient (in case of Normal variables) and Spearman's coefficient (in case of non-Normal variables). *P*-values < 0·05 have been considered statistically significant.

Results

In Table 1, the mean values as well as the SE of the mean for all Normal distributed anthropometric characteristics are given. In case, of the non-Normal variable WHR the median as well as the interquartile range are given. Also, in Table 1, the *P*-value of the ANOVA procedure for age, height, weight and BMI is given. Additionally, the *P*-value of the Kruskal–Wallis test for the non-Normal variable WHR is given. As all the *P*-values are greater than 0·05, we can not reject the null hypothesis that there are no statistically significant differences between the means of all the anthropometric characteristics among the eight distinct groups.

In Table 2 the median as well as the interquartile range for all non-Normal distributed hormonal profile characteristics is given. Also, in Table 2, the *P*-value of the nonparametric test Kruskal–Wallis test is given, which is used to test the hypothesis that several medians are equal. As we can see the medians of the variables FSH, LH and PRL are not statistically significant. As all the *P*-values for the rest of the variables are < 0·01, we can reject the null hypothesis that there are no statistically significant differences between the medians for these variables among the eight distinct groups.

Furthermore, the appropriate nonparametric *post hoc* analysis gave the following results. Concerning the testosterone values, as was revealed from the analysis pairs (1 A, 4 A) (1 A, Controls) (1 A, PCO) (1 A, ANOV) (2 A, 4 A) (2 A, Controls) (2 A, PCO) (2 A, ANOV) (3 A, 4 A) (3 A, Controls) (3 A, PCO) (3 A, ANOV) (4 A, Hyper) (5 A, Hyper) (Hyper, PCO) (Hyper, ANOV) showed statistically significant differences at the 99% confidence level ($P < 0{\cdot}01$). All the other groups appeared to form a homogeneous group. Concerning the D4 values, as was revealed from the analysis pairs (1 A, 4 A) (1 A, Controls) (1 A, PCO) (1 A, ANOV) (2 A, Controls) (2 A, PCO) (3 A, Controls) (3 A, PCO) (3 A, ANOV) (4 A, Controls) (Controls, Hyper) (Hyper, PCO) showed statistically significant differences at the 99% confidence level ($P < 0{\cdot}01$). All the other groups appeared to form a homogeneous group. Concerning the FAI values, as was revealed from the analysis pairs (1 A, 4 A) (1 A, Controls) (1 A, PCO) (1 A, ANOV) (2 A, 4 A) (2 A, Controls) (2 A, PCO) (2 A, ANOV) (3 A, 4 A) (3 A, Controls) (3 A, PCO) (3 A, ANOV) (4 A, Controls) (Controls, Hyper) (Hyper, PCO) (Hyper, ANOV) showed statistically significant differences at the 99% confidence level ($P < 0{\cdot}01$). All the other groups appeared to form a homogeneous group. Concerning the SHBG values, as was revealed from the analysis pairs (1 A, Controls) (2 A, Controls) (2 A, PCO) (3 A, Controls) (3 A, PCO) (Controls, Hyper), showed statistically significant differences at the 99% confidence level ($P < 0{\cdot}01$). All the other groups appeared to form a homogeneous group. Concerning the DHES values, as was revealed from the analysis pairs (2 A, Controls) (3 A, Controls) (3 A, ANOV) showed statistically significant differences at the 99% confidence level ($P < 0{\cdot}01$). All the other groups appeared to form a homogeneous group.

In Table 3, the median as well as the interquartile range for all non-Normal distributed hormonal profile characteristics is given. Also, in Table 3, the *P*-value of the nonparametric test Kruskal–Wallis test is given, which is used to test the hypothesis that several medians are equal. As we can see the medians of the variables QUICKI, glucose : insulin ratio, glucose and insulin are not statistically significant. Glucose levels were not significantly different between groups ($P = 0{\cdot}937$) as well as insulin levels ($P = 0{\cdot}655$, Table 3).

Table 2. Hormonal profile of all subjects under study

	PCOS									
Hormonal parameters	1 A	2 A	3 A	4 A	Total	Controls	Hyper	PCO	ANOV	*P*-value
FSH (mIU/ml)	6·72 (1·66)	6·72 (3·28)	6·02 (2·56)	5·95 (2·11)	6·12 (2·18)	6·22 (2·09)	6·29 (2·09)	6·10 (3·03)	6·20 (1·55)	0·871
LH (mIU/ml)	10·25 (11·56)	6·92 (4·49)	6·23 (5·26)	6·32 (4·85)	6·62 (5·55)	5·90 (3·66)	5·64 (4·72)	7·23 (3·17)	4·10 (5·16)	0·122
PRL (ng/ml)	15·00 (18·54)	13·00 (8·68)	15·05 (9·36)	11·00 (10·20)	13·85 (10·33)	14·40 (12·63)	14·87 (15·91)	14·05 (9·76)	10·97 (15·20)	0·495
Testosterone (ng/dl)	82·00 (25·70)	70·00 (16·50)	74·40 (23·14)	49·95 (13·43)	71·85 (26·00)	45·00 (17·35)	67·95 (16·15)	44·50 (13·83)	40·00 (14·60)	0·000
Δ4 (ng/ml)	3·01 (1·86)	2·53 (0·97)	2·91 (1·06)	2·25 (0·88)	2·55 (1·33)	1·70 (0·90)	2·55 (0·90)	1·79 (0·57)	1·70 (0·61)	0·000
DHEAS (mg/ml)	2750·50 (2100·00)	3525·58 (1922·18)	3405·00 (2090·92)	1863·45 (946·16)	2496·00 (2070·00)	1861·54 (1610)	3138·96 (1569·50)	1853·50 (1079·25)	1714·00 (1130·00)	0·000
SHBG (nmol/l)	48·00 (32·00)	39·20 (23·16)	40·90 (39·43)	53·47 (35·99)	45·12 (30·00)	65·18 (38·32)	53·36 (34·89)	59·00 (45·03)	53·00 (32·60)	0·000
FAI	6·01 (4·75)	5·89 (3·64)	6·80 (4·45)	3·24 (1·91)	5·51 (5·30)	2·43 (1·37)	5·36 (3·42)	2·36 (1·08)	2·68 (1·91)	0·000

Data are given as medians (interquartile range). *P*-value refers to the Kruskal–Wallis test for testing the hypothesis that several medians are equal.

Table 3. Metabolic parameters of all subjects under study

	PCOS									
Metabolic parameters	1 A	2 A	3 A	4 A	Total	Controls	Hyper	PCO	ANOV	*P*-value
Ages (U/ml)	8·19 (2·01)	8·50 (2·51)	7·57 (3·00)	6·30 (2·10)	7·90 (2·89)	6·32 (1·56)	5·57 (0·50)	5·17 (0·36)	5·26 (0·12)	0·000
Insulin (pmol/l)	7·82 (6·53)	7·40 (4·84)	6·23 (4·47)	6·00 (5·39)	6·37 (4·90)	6·28 (3·19)	6·84 (4·40)	6·33 (3·88)	6·10 (3·26)	0·655
Glucose (nmol/l)	94·00 (15·75)	98·50 (21·50)	101·00 (20·00)	103·50 (20·00)	91·00 (11·00)	98·00 (16·50)	92·50 (11·25)	104·00 (22·00)	99·00 (30·00)	0·937
Glucose : insulin	12·91 (12·05)	14·42 (10·83)	15·54 (12·01)	15·03 (13·13)	14·50 (9·00)	19·11 (10·95)	12·25 (7·41)	15·11 (8·50)	17·08 (7·58)	0·320
QUICKI	0·35 (0·50)	0·35 (0·03)	0·36 (0·04)	0·36 (0·04)	0·35 (0·04)	0·36 (0·03)	0·36 (0·04)	0·36 (0·05)	0·36 (0·03)	0·857

Data are given as medians (interquartile range). *P*-value refers to the Kruskal–Wallis test for testing the hypothesis that several medians are equal.

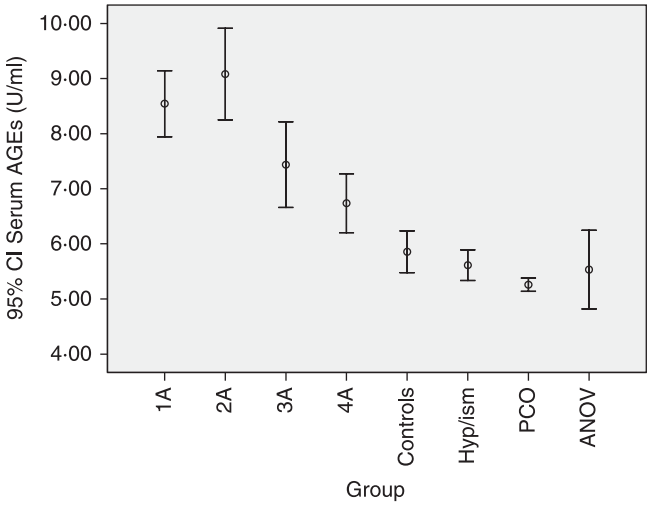

Fig. 1 Serum AGE levels of women with established PCOS, isolated components of PCOS (single hyperandrogenaemia, anovulation, PCO), and control subjects.

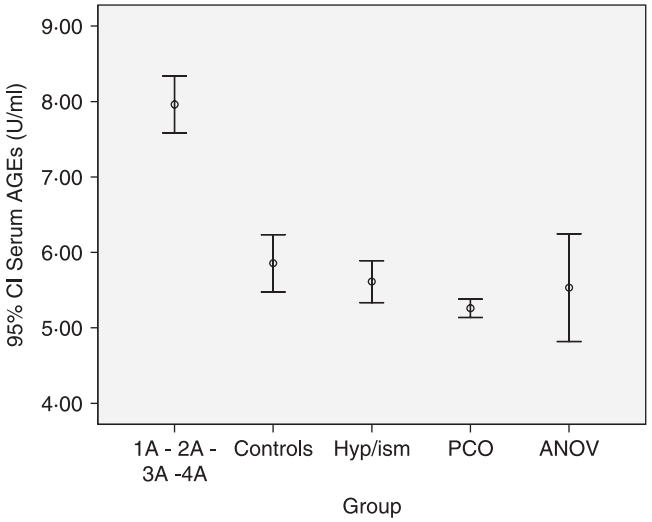

Fig. 2 Serum AGE levels of PCOS subgroups, women with the isolated components of PCOS and controls.

Accordingly, the same holds also for glucose : insulin ratio (P = 0·320) as well as for QUICKI (P = 0·857). As the P-value for the AGEs is < 0·01, we can reject the null hypothesis that there is no statistically significant difference between the median among the eight distinct groups.

Concerning the AGEs values, a multiple comparison procedure, to determine which means are significantly different from which others, was applied (Figs 1 and 2). The method that was used to discriminate among the means is an appropriate nonparametric procedure available from the statistical package stata 8·0. With this method, there is a α% risk of calling each pair of means significantly different when the actual difference equals to zero. As was revealed from the analysis pairs (1 A, Controls) (1 A, HYPER) (1 A, PCO) (1 A, ANOV) (2 A, 4 A) (2 A, Controls) (2 A, HYPER) (2 A, PCO) (2 A, ANOV) (3 A, Controls) (3 A, HYPER) (3 A, PCO) (3 A, ANOV) (4 A, PCO), showed statistically significant differences at the 99% confidence level (Figs 1 and 2 $P < 0·01$). All the other groups appeared to form a homogeneous group.

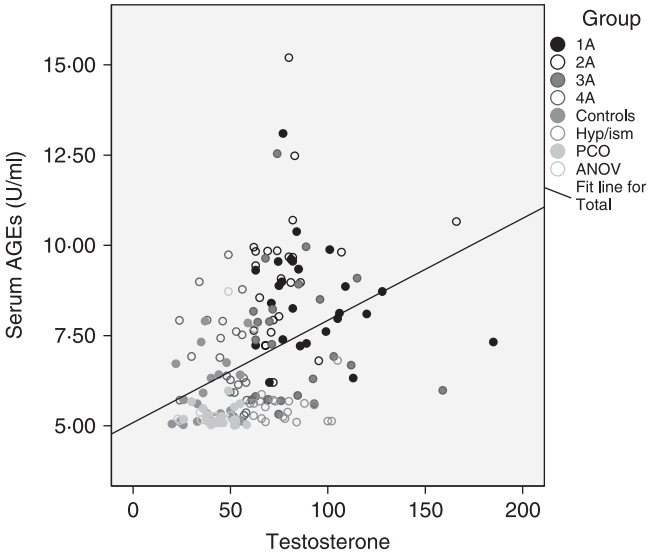

Fig. 3 Correlation of serum AGE levels and testosterone in total PCOS population.

Correlations

A positive correlation was identified between AGEs and testosterone levels using both the Pearson's correlation coefficient as well as Spearman's correlation coefficient ($r_P = 0·395$ with $P < 0·001$ and $r^S = 0·489$ with $P < 0·001$, Fig. 3), Δ4 ($r_P = 0·375$ with $P < 0·001$ and $r^S = 0·473$ with $P < 0·001$), DHEAS ($r_P = 0·238$ with $P = 0·002$ and $r^S = 0·252$ with $P = 0·001$) and FAI ($r_P = 0·248$ with $P = 0·001$ and and $r^S = 0·445$ with $P < 0·001$) while they were negative correlated with the variable SHBG ($r_P = –0·220$ with $P = 0·004$ and $r^S = –0·241$ with $P = 0·004$). Testosterone levels were positively correlated with insulin ($r_P = 0·207$ with $P = 0·005$ and $r^S = 0·167$ with $P = 0·026$) and negatively correlated with glucose : insulin ($r_P = –0·208$ with $P = 0·005$ and $r^S = –0·211$ with $P = 0·005$). A multiple regression analysis confirms to us the fact that only testosterone, FAI and Δ4 are significantly correlated with AGEs.

Discussion

The present study demonstrates, for the first time, that elevated AGEs and specifically CML levels are a distinct finding in lean young, noninsulin resistant women, with PCOS compared with women presenting the isolated components of the syndrome and controls. No difference was found in serum AGE levels among PCOS sub phenotypes.

IR assessed by mathematical indices, was not detected in the total as well as in the women with isolated features of the syndrome and in each one of the phenotypes of PCOS, compared with the control group, suggesting that serum AGEs are elevated in PCOS independently of the presence of IR. However it should be taken into

consideration that the calculated mathematical indices have been shown not to be adequately sensitive to detect IR in lean PCOS women, compared to the gold standard technique of the hyperinsulinaemic euglycaemic clamp.[29]

It is further confirmed in the present study that AGEs are strongly and positively correlated with androgen levels in women with the syndrome, an association that is was previously found by our group in a small sample of patients. However hyperandrogenaemia per se could not account for this finding, since in women with isolated hyperandrogenaemia, AGEs levels did not differ from the other non-PCOS groups with normal androgen levels, suggesting that the syndrome and not the isolated components were responsible for the elevation of these harmful molecules.

The fact that the serum AGEs levels did not differ in different PCOS sub phenotypes, according to current diagnostic criteria, suggests that a comparable inflammatory or pre-atherogenic load is present among these phenotypes. However Carmina *et al.*[30] found that PCOS sub phenotypes which differ in the degree of obesity and IR, between the compared sub phenotypes, presented different levels of cardiovascular risk factors. The difference in the degree of obesity could have account for this observation.

In the present study, it is noticeable that BMI and age-matched women with the syndrome when compared with their counterparts with the isolated features of the syndrome demonstrated elevated AGEs. This suggests that women diagnosed with PCOS distinctly carry increased low grade chronic inflammation[31] and pre-atherogenic load indicated in this case by elevated levels of serum AGEs.

Another study by Barber *et al.*[32] showed that normoandrogenaemic, oligomenorrhoeic women with PCOS, were metabolically similar to controls with significantly fewer metabolic features than PCOS women who were also hyperandrogenaemic. This observation is in accordance with our results (Table 3).

Recently it was shown that serum elevated AGE levels were associated with endothelial dysfunction in nondiabetic individuals.[33] Therefore the increased AGE levels could be a contributing factor in endothelial dysfunction associated with PCOS.

The differential immunohistochemical localization of AGE, RAGE and NF-κB in the ovarian tissue from PCOS compared to controls is suggestive of AGEs involvement in the reproductive abnormalities characterizing the syndrome.[34] Moreover it was found that AGEs and RAGE were excessively localized immunohistochemically in the ovaries of normal animals, fed exclusively with high AGEs diet compared to controls and low-AGE-fed animals.[35] Therefore, this evidence suggests that AGEs, endogenous or exogenous, may be involved in the ovarian dysfunction, a feature of the syndrome.

However the mechanisms of elevated AGEs in this common and heterogeneous syndrome remain at present speculative. Serum AGEs are increased in diabetes mellitus, other IR states, obesity, advanced age and during oxidative stress.[36–39] Interestingly the oxidative stress has been implicated as one of the potential mechanisms of increased AGEs, in normoglycaemic individuals.[33]

Several studies provide evidence for increased oxidative stress in lean normoglycaemic women with PCOS[40,41] and therefore this could account for the elevated levels of serum AGEs in these women.

Furthermore, it has been suggested that in women with PCOS oxidative stress induces hyperandrogenism[41] and ROS generation is directly related to androgen levels. On these grounds, the role of AGEs could also be considered, to participate directly or indirectly, in the pathophysiology of the syndrome, as oxidative stress has been shown to be involved in the development of IR and hyperandrogenism in PCOS.

Additionally, other factors [central obesity, age, serum glucose, insulin levels and IR,[41] which have been shown to be related to oxidative status of women with PCOS are not present and therefore could not be considered as contributors to the elevated levels of AGEs in the women of this study.

In summary, the present data demonstrate for the first time that AGEs are distinctly elevated, in women with PCOS and not in women with the isolated components of the syndrome. The pathological significance of these harmful molecules (AGEs), in metabolic and cardiovascular abnormalities, as well as in the ovarian dysfunction in PCOS, clearly requires further investigation.

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