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Research paper

Obesity, but not polycystic ovary syndrome, affects circulating markers of low-grade inflammation in young women without major cardiovascular risk factors

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ABSTRACT

OBJECTIVE: The aim of this study was to evaluate the influence of polycystic ovary syndrome (PCOS) and obesity on circulating markers of low-grade inflammation—tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6) and high sensitive C-reactive protein (hsCRP)—in young women without major cardiovascular (CV) risk factors (diabetes, dyslipidemia and arterial hypertension). DESIGN: Twenty-five young women with PCOS and 23 eumenorrheic women without major CV risk factors and matched for body mass index (BMI) were studied. They were subdivided according to BMI and PCOS status and comparisons were made between the PCOS and Control groups, regardless of BMI, and between the Obese and Lean groups, regardless of the presence of PCOS. RESULTS: Levels of TNF-α, IL-6 and hsCRP were similar between the PCOS group and the Control group (2.1 vs 1.9 pg/ml, *p*=0.397, 3.8 vs 5.7 pg/ml, *p*=0.805 and 0.9 vs 0.5 ng/ml, *p*=0.361, respectively). Levels of TNF-α were similar between the obese group and the lean group (2.1 vs 1.9 pg/ml, *p*=0.444). Levels of IL-6 and hsCRP were higher in the obese group than in the lean group (8.7 vs 2.0, *p* <0.001 and 1.4 vs 0.2 ng/ml, *p* <0.001, respectively). CONCLUSION: Obesity, but not polycystic ovary syndrome, affects circulating markers of low-grade inflammation in young women without major CV risk factors.

Key words: CV risk, Circulating markers of low-grade inflammation, Obesity, PCOS

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INTRODUCTION

Polycystic ovary syndrome (PCOS) is a heterogeneous disorder characterized by chronic anovulation, androgen excess and infertility, increased prevalence of obesity and increased risk for type 2 *diabetes mellitus* (DM) and cardiovascular (CV) disease.[1] There is evidence that PCOS is also a proinflammatory disorder, characterized by the presence of chronic low-

grade inflammation,[2,3] as variants in genes encoding several inflammatory cytokines and their receptors associated with insulin resistance (IR), obesity and/or DM have also been found to be associated with the syndrome.[4-8]

Cytokines are soluble molecules that are involved in intercellular communication, these produced by a wide variety of cells in the body including adipocytes,[9] and being involved in several biological processes, including atherosclerosis.[10,11] There are several subfamilies of cytokines, among them interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-α).

Interleukin 6 can predict CV disease and, experimentally, contributes to the development of early atherosclerotic lesions.[12-14] Another effect of IL-6 is the induction of hepatic C-reactive protein (CRP) production, which is known to be an independent *major* risk marker of CV complications,[14] even in individuals without known CV disease.[15] Women with the highest baseline high sensitive-CRP (hs-CRP) levels had a five times greater risk of suffering a vascular event and seven times the risk of myocardial infarction or stroke than did control subjects in a prospective Women's Health Study.[16]

Tumor necrosis factor-alpha is an important mediator of insulin resistance (IR)[17] and is related to components of metabolic syndrome,[18] such as impaired glucose tolerance (IGT) and type 2 DM,[19] higher blood pressure[20-22] and dyslipidemia.[23]

In recent years, several studies have been published concerning cytokines in PCOS patients, albeit with contradictory results. An important bias is the diagnostic criterion employed in the diagnosis of the syndrome. However, a question that remains unresolved is whether circulating cytokines are useful markers of the proinflammatory state and if this state could be due to PCOS itself or to the concomitant presence of obesity and/or CV risk factors, as obesity is a proinflammatory state *per se*[24] and CV risk factors could themselves trigger an inflammatory response.

Bearing all the above in mind, the aim of this study was to ascertain whether CRP is a better marker than TNF-α and IL-6 as inflammatory markers and if the low-grade inflammation described in women with PCOS could be attributed to the syndrome itself or to the concomitant presence of obesity and/or CV risk factors. With this objective, we adopted diagnostic criteria to restrict the number of PCOS phenotypes and employed a study design associated with a statistical methodology capable of determining the influence of PCOS and obesity, separately, on circulating levels of TNF-α, IL-6 and CRP in women with minor CV risk factors. As far we know, there is no study in the literature with this primary endpoint and involving the exclusion of CV risk factors.

PATIENTS AND METHODS

The procedures used were in accordance with the guidelines of the Helsinki Declaration on human experimentation. The study protocol was approved by the Ethical Committee of the Hospital das Clínicas da Universidade de São Paulo, and written informed consent was obtained from all women before beginning the study.

Twenty-five young Caucasian women with established PCOS (aged 19.0 to 33.0 yr) and 23 eumenorrheic women without evidence of hyperandrogenism (aged 18.0 to 35.0 yr) matched for body mass index (BMI) were studied. The data regarding these patients were already published.[25] All women were non-smokers, had a normal physical activity level, had no inflammatory or other diseases and had not taken chronic medications (hormones in general, hormonal contraceptives, glucocorticoids, nonsteroidal anti-inflammatory drugs, and drugs with an influence on body weight, glucose metabolism, lipid profile and on the CV system) for at least six months prior to the study.

The subjects were divided into two groups according to BMI: lean (18-25 kg/m^2) and obese (30-40 kg/m^2). Exclusion criteria for all subjects were age <18 or >35 years, pregnancy, arterial hypertension [systolic blood pressure (SBP) ≥140mmHg and/or diastolic blood pressure (DBP) ≥90mmHg], impaired fasting glucose, IGT on the oral glucose tolerance test (OGTT) or DM[26,27] and severe abnormalities of lipid profile (LDL-cholesterol >160 mg/dl or triglycerides >250 mg/dl).

Polycystic ovary syndrome diagnosis was based on the presence of menstrual dysfunction, hyperandro-

genism, hyperandrogenemia and polycystic ovaries morphology, after ruling out related disorders through appropriate tests. The presence of polycystic ovaries was established by the presence of 12 or more follicles in each ovary measuring 2-9 mm in diameter, and/or increased ovarian volume (>10 ml).[28]

Hirsutism was defined by a Ferriman and Gallwey score of ≥8.[29] Eumenorrhea was defined by the presence of menstrual cycles of 25-34 days for at least 12 months. Menstrual disturbance was defined by a cycle length ≥35 days and 8 or fewer menstrual cycles in the last year.

Body mass index was calculated by the formula: weight (kg)/height (m^2). The minimum waist measurements between the pelvic brim and the costal margin were used to determine waist circumference (WC).[30]

Blood pressure (BP) was measured twice in a mercury sphygmomanometer with cuffs that were adequate for the circumference of the patient's arm. The patients were in the supine position after a 20-minute rest period. The blood samples were collected at 08:00 AM after an overnight fast, up to the 7th day of the menstrual cycle for the control group, and was aleatory for the women with PCOS. Serum progesterone was determined to confirm the absence of ovulation (<1.0 ng/ml). For the OGTT, a 75 gr glucose load was given and a blood sample was collected before to determine glucose, insulin, total (TC), LDL (LDL-C) and HDL-cholesterol (HDL-C), triglycerides (TG), steroid hormone-binding globulin (SHBG), total testosterone (TT), progesterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), TNF-a, IL-6, high-sensitive C-reactive protein (hs-CRP) and then, at 30, 60, 90 and 120 minutes after, to measure glucose and insulin levels.

Standard methodologies for lipids, lipoproteins and glucose determination and for LDL-C calculation were used.[31]

Tumor necrosis factor-alpha and IL-6 were determined by Multiplex-Based immunoassays-HSCYTO-60SK (Millipore Corp., Missouri 63304, USA). The intra- and interassay coefficients of variation for these two cytokines were 3.1% and 5.8%, respectively. The lower limit detection for TNF-α and IL-6 were 0.05 pg/ml and 0.1 pg/ml, respectively. High-sensitive C-reactive protein was measured by ELISA-KAPDB4360 (Diasource ImmunoAssays S.A., Louvain-la-Neuve, Belgium). The intra- and interassay coefficients of variation for hs-CRP were <15.2% and <9.9%, respectively, and the lower limit detection was 0.08 ng/ml.

For hormone assays, blood samples were processed by centrifuging and serum was stored at -20° C until assayed. Progesterone and total testosterone were measured by a fluoroimmunometric assay (Wallac, Finland). Insulin, SHBG, LH and FSH were measured by an immunofluorometric assay (Wallac, Finland). All the assays were performed in duplicate and the intra-assay and interassay coefficients of variation did not exceed 10% and 15%, respectively.

Insulin resistance was estimated by HOMA-IR (insulin in μU/mL x glucose in mg/dL/22.5×18) and the area under the curve for insulin ($AUC_{insulin}$) was calculated using the trapezoidal rule.

Statistical analysis was performed using the software InStat (GraphPad Software Inc., San Diego, CA, USA). Data were presented as median and minimum-maximum values. Comparisons between the PCOS and Control groups, regardless of BMI, and between the Obese and Lean groups, regardless of PCOS, were performed using the Mann-Whitney *U* test followed by Bonferroni adjustment, with *p* values adjusted according to the number of comparisons; $p < 0.005$ was considered significant (*p* corrected). Spearman's correlation coefficient analyses were performed and *p*-value <0.05 was considered statistically significant.

RESULTS

Four groups were initially formed (Obese-PCOS, Lean-PCOS, Obese-Control and Lean-Control). The clinical and chemical characteristics of these groups have been previously described.[25] There was no difference between the medians of TNF-α among the four groups (p=0.296), while the medians of IL-6 levels were significantly higher in the Obese-Control group when compared with the Lean-Control group (p=0.009) and with the Lean-PCOS group (p=0.001). For hs-CRP, the median was higher in the Obese-Control and Obese-PCOS groups than in the Lean-Control group and in the Lean-PCOS group (Obese-Control vs Lean-Control, p=0.006 and

Obese-Control vs Lean-PCOS, p=0.004 and Obese-PCOS vs Lean-Control, p <0.001 and Obese-PCOS vs Lean-PCOS, p=0.001).

The data regarding the comparisons between the PCOS and Control groups, regardless of BMI, and between the Obese and Lean groups, irrespective of the presence of PCOS, are depicted in Table 1. For the comparison of the PCOS and Control groups, significant differences were seen only for HOMA-IR (p <0.001) and AUCi (p <0.001), these being higher in the PCOS group than in the Control group. Regarding the comparison between the Obese and Lean groups, significant differences were seen for BMI (p <0.001), HOMA-IR (p <0.001), AUCi (p=0.001), IL-6 (p <0.001) and hs-CRP (p <0.001), these being higher in the Obese than in the Lean group.

In the Obese group, Spearman's correlation coefficient showed a positive and a negative statistically significant correlation between hs-CRP and SBP (r=0.53; p=0.012) and between hs-CRP and HDL-C (r= -0.63; p=0.001), respectively. In the PCOS group, hs-CRP was positively and statistically significant correlated with BMI (r=0.62; p=0.001).

DISCUSSION

Although there is evidence of a state of a chronic low-grade inflammation in PCOS, studies of cytokines profiles in women with this syndrome have yielded controversial results.[32-37] We attribute these differences to several factors, such as the diagnostic criterion for the syndrome, the presence of CV risk factors and the presence of obesity itself. In this study we sought to select such a group of women that would avoid such bias and, through the employment of a select statistical method, to separate the confounding effect of PCOS and obesity.

It should be noted here that the early diagnostic criteria recognized only one phenotype of PCOS.[38] The Rotterdam[28] and the Androgen Excess and PCOS society diagnostic criteria for PCOS[39] identified several phenotypes of the syndrome each exhibiting a distinct phenotype, hormone profile, insulin resistance and metabolic disturbances.[40] We have used strict diagnostic criteria in order to restrict the number of phenotypes and our women with PCOS fulfilled all relevant criteria.

We did not observe significant differences in IL-6, TNF-α and hs-CRP circulating levels in our women with PCOS when compared to normal women, just as Ciaraldi et al did not observe significant differences in circulating cytokines, including TNF-α.[41] In our subjects, we attributed these results and the divergent results in other studies of the literature to a more homogeneous phenotype, but also to the exclusion of *major* CV risk factors (glucose metabolism disturbances, arterial hypertension and severe abnormalities of lipid profile), since CV risk factors by themselves could trigger an inflammatory response. The only CV risk factor that was not excluded was a low HDL-C, as this is so frequent in PCOS women that it would be impossible to have an adequate sample size.[42] In any case, there is no evidence that a low HDL-C could trigger an inflammatory response.

Table 1. Comparisons between PCOS group and control group and between obese group and lean group for insulin resistance parameters, tumor necrosis factor-α, interleukin-6 and high sensitive C-reactive protein

	PCOS group (n=25)	Control group (n=23)	p	Obese group (n=26)	Lean group (n=22)	p
HOMA-IR	2.2 (0.7 - 7.5)	1.2 (0.5 - 3.6)	<0.001	2.6 (0.7 - 7.5)	0.9 (0.5 - 3.7)	<0.001
AUCi (μIU/ml/min.10^{-2})	258.5 (32.6 - 1,927.1)	39.4 (8.6 - 404.6)	<0.001	261.1 (13.6 - 1,927.1)	44.2 (8.6 - 402.6)	0.001
TNF-α (pg/ml)	2.1 (0.8 - 7.3)	1.9 (0.6 - 3.8)	0.397	2.1 (0.8 - 7.3)	1.9 (0.6 - 3.8)	0.444
IL-6 (pg/ml)	3.8 (0.4 - 62.0)	5.7 (0.9 - 38.8)	0.805	8.7 (1.3 - 62.0)	2.0 (0.4 - 14.9)	<0.001
hs-CRP (ng/ml)	0.9 (0.1 - 5.7)	0.5 (0.1 - 4.6)	0.361	1.4 (0.2 - 5.7)	0.2 (0.1 - 1.0)	<0.001

Data are expressed as median (min-max); Mann-Whitney U test followed by Bonferroni adjustment was used to perform comparisons between PCOS and control groups, regardless of BMI, and between obese and lean groups, regardless of PCOS; p <0.005 was considered significant (p corrected); HOMA-IR: homeostatic model assessment of insulin resistance; AUCi: area under the curve of insulin during the oral glucose tolerance test; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6; hs-CRP: high-sensitive C-reactive protein.

The presence of classical CV risk factors, such as chronic hyperglycemia, can influence the inflammatory response *in vitro* and lead to misinterpretation of CV risk markers.[43] Moreover, it is established that the effect of TNF-α, through activation of the soluble receptors TNFR1 and sTNFR2, is related to components of the metabolic syndrome,[18] such as IGT and type 2 DM(19), higher blood pressure[20] and dyslipidemia,[23] which are common in women with PCOS, even in those with normal BMI.[42,44]

As for the inflammatory molecules, data on the relationship between PCOS and carotid intima-media wall thickness (cIMT), a marker of preclinical atherosclerosis, are not conclusive. In a previous study,[25] in this same cohort of women, we have demonstrated that by adopting stricter diagnostic criteria and excluding *major* CV risk factors[25,45] no differences were evident in vascular parameters related to early atherosclerosis in women with PCOS. This was also shown by Kahal et al[46] by studying obese women with PCOS diagnosed by strict diagnostic criteria and matched not only for BMI but also for abdominal obesity, blood pressure, lipid profile and smoking history. Although the main objective of this study was to examine cIMT and platelet function, our results also did not reveal any difference between normal and PCOS women with regard to hsCRP. As expected, we have found that the circulating levels of IL-6 and hs-CRP in obese women were higher compared to the lean women, regardless of their PCOS status, suggesting that obesity was the only major determinant of the circulating inflammatory markers.

It is well known that adipose tissue releases large amounts of IL-6 *in vivo* and this cytokine appears able to mediate several weight regulating processes. It is estimated that 15 to 30% of total circulating concentrations of IL-6 originate in adipose tissue in healthy subjects in the absence of acute inflammation, due to the fact that IL-6 adipose tissue production and systemic concentrations increase with adiposity.[13] As CRP synthesis is induced by IL-6, it was no surprise that hs-CRP concentration was also elevated in obese women when compared with lean women, regardless of PCOS status, and that hs-CRP was positively correlated with BMI in PCOS women. Similarly to IL-6, CRP is related to body fat mass and this association was seen to be somewhat stronger in women and in non-diabetic subjects.[13] It was also not surprising to observe in the obese women positive and negative correlations between hs-CRP and SBP and between hs-CRP and HDL-C, respectively. There is evidence that hs-CRP correlates with SBP, HDL-C and other components of the metabolic syndrome.[47]

On the other hand, TNF-α were not different between PCOS and control women, as related to the presence of obesity, and between obese and lean women, as related to PCOS status. These results are in accordance with other reports which did not display differences of TNF-α in PCOS women when compared with controls despite the presence of more IR in PCOS women.[34] This could be explained by the fact that the effect of TNF-α is a paracrine one, as adipose tissue did not release large amounts of this cytokine *in vivo*.[48]

The presence of overweight (BMI between 25 and 30 kg/m^2) is associated with the increase of some CV risk factors, which has been demonstrated in patients with PCOS.[41,49] For this reason, we considered it highly appropriate to compare obese subjects with women with normal BMI (BMI between 18-25 kg/m^2) and not with overweight (BMI between 25-30 kg/m^2).

CONCLUSION

The circulating levels of IL-6, TNF-α and hs-CRP were similar between women with and without PCOS, but the levels of IL-6 and hs-CRP were higher in obese women when compared with lean women, regardless of the presence of PCOS. We attributed these findings to a better homogenization of our sample of PCOS women due to stricter criteria adopted for the diagnosis of the syndrome and to the exclusion of CV risk factors, as the latter can trigger by themselves an inflammatory response. We suggest that these factors must be considered in designing future studies.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The

authors alone are responsible for the content and writing of this article.

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