EUROPEAN JOURNAL OF INFLAMMATION Vol. 11, no. 1, 179-191 (2013)

POLYCYSTIC OVARY SYNDROME AND HEPATIC STEATOSIS: COULD LOW-GRADE CHRONIC INFLAMMATION BE MEDIATED BY THE SPLEEN?

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Received July 3, 2012 – Accepted January 23, 2013

Polycystic Ovary Syndrome (PCOS) is characterized by an extreme variety of phenotypes and controversial metabolic implications. Hepatic Steatosis (HS) and low-grade chronic inflammation (LGCI) might be common findings in PCOS. We conducted a cross-sectional study to evaluate the LGCI and HS in young women with PCOS according to their Body Mass index (BMI), Insulin Resistance (IR), and PCOS phenotypes. Sixty young premenopausal PCOS women and 20 age-matched controls participated. Primary outcome measures were the presence/severity of HS; LGCI index evaluated as spleen longitudinal diameter (SLD) by UltraSound, C-Reactive Protein (CRP) and Interleukin (IL)-6 levels; BMI and the Homeostasis Model Assessment (HoMA) of IR. The second outcome measures were testosterone, Sex Hormone-Binding Globulin (SHBG) levels, and Free Androgen Index (FAI). The presence of HS and LGCI was not significantly different between NW and O/O patients, while there were significant differences particularly when the PCOS-women were grouped according to IR or to PCOS phenotypes. At multiple regression adjusted for BMI, HoMA-IR and the spleen size were the major determinants of the severity of HS (β= 0.36, p=0.007, and β= 0.28, p=0.034, respectively). At multiple regression SLD represented the unique predictor of FAI (β=0.32; p=0.018). In young women with PCOS, HS was detected independently from obesity and was well predicted not only by IR but also by spleen size, with variable expression of the liver-spleen axis across the different PCOS subtypes. A possible role of the spleen in determining LGCI also in women with PCOS is emphasized.

Considerable debate has surrounded the association between Polycystic Ovary Syndrome (PCOS) and non-alcoholic fatty liver disease (1), or generally speaking Hepatic Steatosis (HS), one of the most common causes of chronic liver disease in western countries (2). It has been reported that the prevalence of both entities rises proportionally to the degree of Insulin Resistance (IR) and the mass of adipose tissue (2, 3). Consequently, the majority of the studies had emphasized the relationship between PCOS and HS prevalently in the context of obesity (1, 4, 5). However, a number of confounding variables might account for the lack of HS evidence in NW PCOS women, such as age, ethnicity, the diagnosis

Key words: PCOS phenotypes, hepatic steatosis, low-grade chronic inflammation, spleen size

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1721-727X (2013)

DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

tool [Ultrasound (US)], single or combined laboratory liver tests), the prevalence of obesity in the cohorts, and more recently, of hyperandrogenism (6-9).

PCOS is characterized by an extreme phenotypic variety (10) and there are ongoing controversies concerning the metabolic implications of different PCOS subtypes. Thus, the relative role of obesity in the association between HS and PCOS still remains unravelled and the relative contribution of the different PCOS subtypes to HS in PCOS has not been considered previously.

A state of Low-Grade Chronic Inflammation (LGCI) has been involved in HS pathogenesis throughout the autocrine/paracrine/endocrine production of adipokines (11, 12). Although to date no specific single circulating marker has been found to definitely reflect inflammation at molecular levels, a key role in the LGCI has been depicted for Interleukin (IL)-6, a pro-inflammatory cytokine produced either by adipose tissue or by spleen macrophages that subsequently induce an increase in the production of C-Reactive Protein (CRP) by the liver (12). An increased volume of the spleen, an organ known to be implicated in both mechanisms of inflammation/immune disorders, has been recently proposed as a reliable and stable index of LGCI, strictly associated with severity of HS in obesity (13), in the so-called liver-spleen axis (14).

Apart from its clinical significance, the LGCI process has been emphasized as the main physiopathological mechanism in PCOS (15, 16). Recently, the role of hyperandrogenism *per se*, one of the main diagnostic features varying across the different PCOS phenotypic subgroups, has been extensively examined as grassroots of LGCI in PCOS (16, 18).

Finally, recent evidence points to an increased prevalence of both organ and non-organ specific autoimmunity in PCOS (19).

The aim of our study was to evaluate the role of the spleen in determining LGCI and the weight of this organ in the relationship between PCOS and HS in a group of young women with PCOS according to body weight, IR, and PCOS phenotypes.

MATERIALS AND METHODS

Study design

This is a cross-sectional study carried out in accordance with the guidelines of the Helsinki Declaration on human experimentation, after approval by the institutional review board of the University of Naples, Italy (#231/05, February 20, 2006). Primary outcome measures were the US quantification of HS, spleen longitudinal diameter (SLD), CRP and IL-6 circulating levels in addition to Body Mass Index (BMI) and the Homeostasis Model Assessment of IR (HoMA-IR). The second outcome measures were circulating levels of testosterone, Sex Hormone-Binding Globulin (SHBG), and Free Androgen Index (FAI), as one of the recommended methods to assess the biochemical androgen excess.

Subjects

Seventy-three women with PCOS diagnosis, from November 1st, 2009 to October 31st, 2011, were consecutively selected to enter this study at the outpatient clinic of our University Hospital. The diagnosis of PCOS was based on the Rott-PCOS criteria (20). In particular, patients with classical PCOS phenotype presented with the complete phenotype or with biochemical/clinical hyperandrogenism + oligo/anovulation without US-PCO, while patients with non-classical phenotype presented with biochemical/clinical hyperandrogenism + US-PCO or with oligo/anovulation + US-PCO. Patients were enrolled according to the following criteria: premenopausal status, age range (15-40 yr) for diagnoses of PCOS; anovulatory oligo-amenorrhea; Caucasian ethnicity.

Controls

Twenty healthy women, among clerks, paramedical and medical personnel of our Department, matched for age with the patients, from the same geographical area, with regular menstrual cycles (defined as 26-32 days in length), and no clinical/biochemical androgen excess or polycystic ovaries on US were recruited and agreed to participate in this study and were used as controls in order to set range of hyperandrogenism and of the LGCI parameters. All participants gave their informed consent before enrolment. All subjects included were neither on hypocaloric diet nor were taking weight loss drugs at least three weeks prior to admission.

Exclusion criteria

Patients were excluded according to the following criteria: smoking or alcohol consumption, pregnancy, hypothyroidism, hyperprolactinemia, Cushing's syndrome, non-classical congenital adrenal hyperplasia; previous (within the last 6 months) use of oral contraceptives, insulin sensitizing agents, glucocorticoids, anti-androgens, ovulation induction agents, anti-obesity drugs, presence of any acute viral, bacterial or fungal infection, chronic liver diseases of various nature, arthritis, bronchial asthma and chronic inflammatory bowel and cancer.

The final population included 60 individuals with median age of 25.5 yrs (range 16-38; 25-75 percentile 20.5-32.0) and median BMI of 25.2 kg/m^2 (range 18.2-46.6; 25-75 percentile 23.4-32.7).

Methods

The ovulatory state and the ultrasonographic feature of polycystic ovary (US-PCO) were investigated as reported elsewhere (21). Hirsutism was assessed using the Ferriman-Gallwey (FG)-score (22). The degree of normal weight, overweight, or obesity was established on the basis of BMI cut-off points of 18-24.9 (normal weight subjects: NW), 25->40 kg/m^2, respectively (overweight/obese subjects: O/O). Waist circumference was measured at the umbilicus level with a cut-off set at £88 cm (23).

Assays

All biochemical analyses including fasting plasma glucose (FPG), total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, transaminases, and uric acid were performed with a Roche Modular Analytics System in the Central Biochemistry Laboratory of our Institution. Aspartate aminotransferase (AST) and alanine aminotranferase (ALT) normal vales were < 35 U/L. Fasting Plasma Insulin (FPI), SHBG, and testosterone levels (Immulite, Diagnostic Products Co, Los Angeles, CA, USA) were measured by solid-phase chemiluminescent enzyme immunoassays. The intra-assay coefficients (CV) of variations were less than 5.5% for insulin and SHBG assays, and 10% for total T assay. FAI was calculated according to the formula=Testosterone (nmol/l)/SHBG (nmol/l) × 100. Among women with PCOS a cut off value of 8.1 for FAI was used to identify less *vs* more severe biochemical hyperandrogenism (sensitivity 100 and specificity 100). The HoMA-IR index was calculated according to the formula [FPI (μU/ml) × FPG (mmol/l)/22.5] (24). As a stringent measure of IR, a value of HoMA-IR > 2.0 was set (25). CRP was determined with a nephelomteric assay with CardioPhase from Siemens Healthcare Diagnostics (Marburg, Germany). IL-6 was measured by an enzyme-linked immunosorbent assay kit (Biosurce, Camarillo, California, USA). Its sensitivity was < 2 pg/ml, and the range 7.8–500 pg/ml. The intra-assay CV for low concentration was < 5%.

UltraSound analyses

Sonographic measurements were performed by the same operator, blinded to patients' data, using a *Vivid* system (General Electric Healthcare Company, Milan, Italy). The classification of "bright liver" or HS severity was based on the following scale of hyper-echogenity: 0 = absent, 1 = light, 2 = moderate, 3 = severe, pointing out the difference between the densities of the liver and the right kidney (26).

LGCI evaluation

LGCI was assessed taking into consideration two out of three elevated parameters suggestive of inflammation, i.e., CRP and IL-6 levels and spleen size measurements. For CRP and IL-6 the upper limits of the normal values were calculated as mean values plus 2 SD of controls. Spleen Longitudinal Diameter (SLD), the best single measurement well related to spleen size, was measured as previously reported and a cut off for LSD was set at 103 mm (13).

Statistical analysis

The minimal required sample size per group with a power of 99% [1-0.1 (step type II error)] was calculated taking into account the differences in spleen size between women with PCOS and controls and was set to 18 subjects. Results are expressed as mean ± SD, 95% CI or as median plus range, 25-75 percentile according to variables' distribution. Differences between the two groups were analysed by unpaired *t*-test or Mann-Whitney U-test. Frequencies were analyzed by c^2. After testing for equality of variance the Kruskal-Wallis test or ANOVA were used for comparison between controls and the two PCOS phenotypes found in this study, when appropriate. The pairwise comparisons were analyzed by the Conover-Inman or the Bonferroni tests. Correlations were evaluated by rho of Spearman. The presence of HS and LGCI was predicted by logistic regression (stepwise method) using HoMA-IR and FAI, respectively, as independent variables. Multiple linear regression analysis (enter method) was used to calculate the predictive value of LGCI parameters, expressed as Beta (β), on hyperandrogenism or HS severity, both of which were adjusted for BMI. To avoid multicollinearity, variables with a tolerance of 0.2 were excluded. Values ≤5% were considered statistically significant. The concordance correlation coefficient (ρc), which measures precision and accuracy adopted to evaluate the degree of intra-operator variation for HS detection and spleen measurements at US was 0.91. Data were stored and analysed using MedCalc® package.

RESULTS

The demographic, clinical and biochemical characteristics of the 60 PCOS-women and 20 controls are expressed in Table I. All the PCOS-women enrolled in this study presented clinical/biochemical hyperandrogenism and 51 (85%) also had chronic oligo-anovulation.

Table I. *Comparisons of demographic, clinical and biochemical characteristics and inflammatory markers in our study population of 60 PCOS-women and 20 controls.*

	PCOS women 60 subjects	Controls 20 subjects	*p* values
Age (yrs)	25.5(16.0-38.0)	25.0(21.0-34.0)	0.857
BMI (kg/m^2)	25.2(18.2-46.6)	22.6(18.7-24.6)	<0.001
Waist circumference (cm)	85.0(67.0-118.0)	75.0(65.0-79.0)	<0.001
Systolic blood pressure (mmHg)	120.0(110.0145.0)	120.0(110.0-125.0)	0.014
Diastolic blood pressure (mm Hg)	80.0(60.0-100.0)	78.0(60.0-89.0)	0.014
Plasma glucose (mg/dl)	89.9±8.5	79.2±4.7	<0.001
Insulin (μUI/ml)	12.0(3.5-41.7)	7.0(5.0-10.0)	<0.001
HoMA-IR	2.6(0.7-11.1)	1.43(0.94-1.93)	<0.001
N. patients with HoMA-IR >2	42 (70%)	0	<0.001
Cholesterol (mg/ml)	172.1±29.9	139.6±22.5	<0.001
HDL-cholesterol (mg/dl)	43.1±10.2	69.3±9.0	<0.001
LDL-cholesterol (mg/dl)	99.8±26.2	53.8±22.5	<0.001
Triglycerides (mg/dl)	158.5(52.0-230.0)	78.0(63.0-100.0)	<0.001
Testosterone (nmol/l)	3.8(1.8-5.48)	0.6(0.5-0.8)	<0.001
SHBG (nmol/l)	40.0(29.0-54.0)	70.0(65.0-75.0)	<0.001
FAI	9.1(3.4-16.9)	0.8(0.4-1.3)	<0.001
FG score	11.0(8.0-18.0)	5.0(4.0-8.0)	<0.001
AST (U/L)	33.2±8.4	24.1±6.2	<0.001
ALT (U/L)	37.25±9.0	23.50±9.3	<0.001
γGT (mg/ml)	40.0(17.0-144.0)	29.0(7.0-40.0)	<0.001
N. patients with HS	36 (58%)	0	<0.001
CRP (ng/ml)	1.4(0.0-8.0)	0.2(0.1-2.0)	<0.001
IL-6 (pg/ml)	10.2(2.0-79.4)	5.7(3.5-8.3)	<0.001
Spleen size (mm)	113.7±17.4	91.5±8.1	<0.001
N. patients with LGCI	46 (75%)	0	<0.001

Values are expressed as mean±SD or median plus range. As a stringent measure of IR, a value of HoMA-IR >2.0 was set (24). HS and spleen size were determined by Ultrasound. The LGCI was assessed taking into consideration two out of three elevated parameters (CRP and IL-6 levels, and spleen size). A cut-off for LSD was set at 103 mm (13), while for CRP and IL-6 the cut-off criteria (mean values plus 2 SD) were found in a cohort of 30 normal weight age-matched controls. PCOS: Polycystic Ovary Syndrome; BMI: Body Mass Index; HoMA-IR: Homeostasis Model Assessment of Insulin Resistance; SHBG: Sex Hormone-Binding Globulin; FAI: Free Androgen Index; FG score: Ferriman-Gallwey score; AST: aspartate aminotransferase; ALT: alanine aminotranferase; gGT: glutamyltransferase; HS: Hepatic Steatosis; CRP: C-Reactive Protein; IL-6: Interelukin-6; SLD: Spleen Longitudinal Diameter; LGCI: Low-grade Chronic Inflammation.

Subgroup analyses

NW vs O/O PCOS-women: Among O/O women there were 7 (23%) obese women. Although, as expected, the two groups presented significant differences in anthropometric and metabolic characteristics and biochemical hyperandrogenism, the presence of HS and LGCI was similarly evidenced (Table II). However, in NW PCOS-women both AST and ALT levels were higher than in controls (p=0.003 and p<0.001, respectively). All the O/O women with LGCI showed contextually IR and HS. In the NW subgroup the presence of LGCI

Table II. *Comparisons of demographic, clinical and biochemical characteristics and inflammatory markers in our study population of women with PCOS according to Body Mass Index (BMI).*

	PCOS women (60 subjects)		
	Normal weight (30)	**Overweight/Obese (30)**	***p* values**
Age (yrs)	25.0(16.0-38.0)	28.5(16.0-38.0)	0.495
BMI (kg/m^2)	23.4(18.2-24.9)	32.7(25.5-46.6)	<0.001
Waist circumference (cm)	75.0(67.0-82.0)	97.5(88.0-118.0)	<0.001
Systolic blood pressure (mm Hg)	120.0(110.0-128.0)	126.6(110.0-145.0)	<0.001
Diastolic blood pressure (mm Hg)	80.0(60.0-80.0)	90.0(75.0-100.0)	<0.001
Plasma glucose (mg/dl)	88.0±6.0	91.9±10.2	0.076
Insulin (μUI/ml)	11.0(3.5-17.0)	13.0(10.0-41.7)	<0.001
HoMA-IR	1.9(o.7-3.6)	3.3(2.0-11.1)	<0.001
N. patients with HoMA-IR >2	14 (47%)	28 (93%)	[a]<0.001
Cholesterol (mg/ml)	164.0±29.3	180.2±28.6	0.034
HDL-cholesterol (mg/dl)	50.2±8.7	36.1±5.8	<0.001
LDL-cholesterol (mg/dl)	91.9±27.7	107.6±22.3	0.019
Triglycerides (mg/dl)	105.0(52.0-170.0)	178.5(155.0-230.0)	<0.001
Testosterone (nmol/l)	3.6(1.8-4.4)	4.1(2.6-5.4)	0.004
SHBG (nmol/l)	42.5(36.0-54.0)	35.5(29.0-48.0)	<0.001
FAI	8.6(3.4-12.2)	11.3(6.0-16.9)	0.010
N. patients with FAI > 9.1	21 (70%)	23 (73%)	[b]0.001
FG score	10.0(8.0-12.0)	12.0(9.0-18.0)	<0.001
AST (U/L)	30.8±7.8 (40%)	35.5±8.4 (47%)	0.027
ALT (U/L)	34.2±8.6 (47%)	40.3±8.3 (80%)	0.007
γGT (mg/ml)	35.5(17.0-48.0)	70.5(39.0-144.0)	<0.001
N. patients with HS	15 (50%)	21 (70%)	[c]0.069
CRP (ng/ml)	1.4(0.0-2.5)	1.6(0.1-8.0)	0.068
IL-6 (pg/ml)	10.2(4.7-33.8)	17.0(2.0-79.4)	0.953
Spleen size (mm)	108.9±6.6	118.4±22.9	0.033
N. patients with LGCI	23 (77%)	23 (77%)	1.000

[a] χ^2=13.413; [b] χ^2=11.267; [c] χ^2=2.469

Values are expressed as mean±SD or median plus range. A cut-off for FAI was set at 9.1 (>median value) to identify less vs more severe biochemical hyperandrogenism). PCOS: Polycystic Ovary Syndrome; HoMA-IR: Homeostasis Model Assessment of Insulin Resistance; SHBG: Sex Hormone-Binding Globulin; FAI: Free Androgen Index; FG score: Ferriman-Gallwey score; AST: aspartate aminotransferase; ALT: alanine aminotranferase; gGT: glutamyltransferase; HS: Hepatic Steatosis; CRP: C-Reactive Protein; IL-6: Interelukin-6; SLD: Spleen Longitudinal Diameter; LGCI: Low-grade Chronic Inflammation.

was independent from HS or IR, while the presence of HS was always associated with IR.

IR vs non-IR PCOS-women: Again, as expected, the two groups presented significant differences in both anthropometric and metabolic characteristics and biochemical hyperandrogenism, but in this subgroup the significant differences in the presence of HS or with CRP and spleen size as markers of LGCI were highlighted (Table III).

PCOS phenotypes resulting from application of the Rott-Criteria: The classical phenotypes in comparison with the only non-classic subtype

Table III. *Comparisons of demographic, clinical and biochemical characteristics and inflammatory markers in our study population of women with PCOS according to Insulin Resistance (IR).*

	PCOS women		
	IR- (18 subjects)	IR+ (42 subjects)	*p* values
Age (yrs)	25.0(16.0-34.0)	28.0(16.0-38.0)	0.487
BMI (kg/m^2)	22.7(19.0-38.1)	30.0(18.2-46.6)	<0.001
Waist circumference (cm)	73.5(67.0-93.0)	92.5(67.0-118.0)	<0.001
Systolic blood pressure (mmHg)	120.0(110.0-130.0)	125.0(110.0-145.0)	0.077
Diastolic blood pressure (mmHg)	80.0(60.0-95.0)	81.0(70.0-100.0)	0.005
Plasma glucose (mg/dl)	85.5±4.1	91.8±9.2	0.007
Insulin (μUI/ml)	9.9(3.5-12.0)	13.0(10.0-41.7)	<0.001
Cholesterol (mg/ml)	159.8±24.4	177.4±30.7	0.036
HDL-cholesterol (mg/dl)	48.6±10.7	40.8±9.1	0.005
LDL-cholesterol (mg/dl)	89.3±21.8	104.3±26.9	0.042
Triglycerides (mg/dl)	98.5(52.0-188.0)	170.0(88.0-230.0)	<0.001
Testosterone (nmol/l)	3.5(1.8-4.4)	3.7(1.8-5.4)	0.006
SHBG (nmol/l)	43.0(36.0-54.0)	39.0(29.0-53.0)	0.004
FAI	8.3(3.4-12.2)	9.4(3.4-16.9)	0.004
N. patients with FAI > 9.1	5 (28%)	28 (67%)	[a]0.010
FG score	10.0(8.0-12.0)	12.0(8.0-18.0)	<0.001
AST (U/L)	28.0±7.9	35.4±7.6	0.001
ALT (U/L)	33.6±7.6	38.8±9.2	0.039
γGT (mg/ml)	35.0(17.0-100.0)	45.5(22.0-144.0)	<0.001
N. patients with HS	6 (33%)	30 (71%)	[b]0.019
CRP (ng/ml)	1.3(0.0-2.4)	1.6(0.1-8.0)	0.048
IL-6 (pg/ml)	9.0(2.7-31.0)	19.5(2.0-79.4)	0.160
Spleen size (mm)	105.4±12.5	117.2±18.1	0.016
N. patients with LGCI	13 (72%)	33 (79%)	[c]0.842

[a] χ^2=7.699; [b] χ^2=5.539; [c] χ^2=0.040

Values are expressed as mean±SD or median plus range. As a stringent measure of IR, a value of HoMA-IR >2.0 was set (24). PCOS: Polycystic Ovary Syndrome; HoMA-IR: Homeostasis Model Assessment of Insulin Resistance; SHBG: Sex Hormone-Binding Globulin; FAI: Free Androgen Index; FG score: Ferriman-Gallwey score; AST: aspartate aminotransferase; ALT: alanine aminotranferase; gGT: glutamyltransferase; HS: Hepatic Steatosis; CRP: C-Reactive Protein; IL-6: Interelukin-6; SLD: Spleen Longitudinal Diameter; LGCI: Low-grade Chronic Inflammation.

found and controls had higher BMI, and presented central obesity, more severe IR and biochemical hyperandrogenism, while the presence of HS and LGCI were much more evident (Table IV). Interestingly, the women with non-classic PCOS subtype presented an intermediate prevalence of HS and of LGCI in that it was different from controls, but it was lower than in classical PCOS subtypes.

Degree of hyperandrogenism: Using the cut off of 8.1 for FAI, the PCOS-women with more severe hyperandrogenism in comparison with those with less had higher BMI and presented central obesity, more severe IR, higher prevalence of HS, higher CRP levels, and larger spleen, although the prevalence of LGCI was not statistically different (Table V).

In Fig. 1 the comparisons of the main study

Table IV. *Comparisons of the main study variables in our sample population of women with PCOS according to PCOS subtypes.*

	Classic PCOS 46 subjects	Non classic PCOS 14 subjects	Controls 20 subjects	*p values*
Age (yrs)	27.0(16.0-38.0)	24.5(16.0-37.0)	25.0(21.0-34.0)	0.526
BMI (kg/m^2)	29.0(18.2-46.6)$^{*;**}$	23.4(19.0-29.0)	22.6(18.7-24.6)	<0.001
Waist circumference (cm)	91.5(67.0-118.0)$^{*;**}$	75.0(69.0-93.0)	75.0(65.0-79.0)	<0.001
HoMA-IR	2.8(1.6-11.1)$^{*;**}$	1.8(0.7-2.9)*	1.43(0.94-1.93)	<0.001
N. patients with HoMA-IR >2	38 (83%)	4 (29%)	0	^{a}<0.001
N. patients with HS	32 (70%)	4(29%)	0	^{b}0.015
FAI	9.5(3.4-16.9)$^{*;**}$	8.1(3.4-9.4)*	0.8(0.4-1.3)	<0.001
CRP (ng/ml)	2.0(0.0-8.0)$^{*;**}$	1.0(0.2-2.1)*	0.2(0.1-2.0)	<0.001
IL-6 (pg/ml)	19.5(2.0-79.4)*	7.0(4.6-21.6)	5.7(3.5-8.3)	0.002
Spleen size (mm)	117.5±16.8$^{*;**}$	100.9±13.0*	91.5±8.1	<0.001
N. patients with LGCI	39 (85%)	7 (50%)	0	^{c}0.020

** vs Controls; ** vs Non classic PCOS; $^{a}\chi^2=12.462$; $^{b}\chi^2=5.905$; $^{c}\chi^2=5.445$*

Values are expressed as mean±SD or median plus range. The Kruskal-Wallis test or ANOVA were used, when appropriate, and the pairwise comparisons were analyzed by the Conover-Inman or the Bonferroni tests. Differences between the prevalence of HoMA-IR, HS and LCGI between PCOS subtypes were analyzed by χ^2 test. PCOS: Polycystic Ovary Syndrome; BMI: Body Mass Index; HoMA-IR: Homeostasis Model Assessment of Insulin Resistance; HS: Hepatic Steatosis; FAI: Free Androgen Index; CRP: C-Reactive Protein; IL-6: Interelukin-6; SLD: Spleen Longitudinal Diameter; LGCI: Low-grade Chronic Inflammation.

variables across the different subgroup analyses were summarized.

Correlations

As none of the control subjects presented HS, the correlation analyses between HS severity and the other study variables, the logistic regression and the multiple regression analysis were calculated among the PCOS population only. As expected, in PCOS-women there was a significant correlation between HS severity and BMI (rho=0.302, p= 0.019). Similarly, HS severity well correlated to HoMA-IR (rho=0.420, p= 0.001), FAI (rho=0.364, p= 0.004), CRP (rho=0.408, p= 0.001), IL-6 (rho=0.307, p= 0.017), and, mainly with spleen size (rho=0.574, p=<0.001). Besides HS severity, the correlations between the other main study variables performed on the whole sample population (60 women with PCOS and 20 controls) was reported in Table VI. All the three markers of LGCI were associated with biochemical hyperandrogenism, with the strongest correlation between SLD and FAI.

At the logistic regression (stepwise method) in all PCOS women, the presence of HS was well predicted by HoMA-IR (OR 2.1; 95% CI: 1.1-3.9; p=0.0018). At multiple regression (enter method) adjusted for BMI among HoMA-IR and LGCI parameters, the major determinants of the severity of HS were HoMA-IR and the spleen size (β= 0.36, p=0.007, and β= 0.28, p=0.034, respectively).

At the logistic regression (stepwise method) FAI predicted the presence of LGCI, also when adjusted for BMI (OR 1.35; 95% CI: 1.1-1.65). At multiple regression, among the LGCI markers, SLD represented the unique predictor of FAI in our population (β=0.32; p=0.018).

DISCUSSION

The main findings of this study were firstly the presence of a liver-spleen axis in our sample population of PCOS-women and secondly the

Table V. *Comparisons of the main study variables in our sample population of women with PCOS according to biochemical hyperandrogenism (HA).*

	HA- (27 subjects)	HA+ (33 subjects)	*p* values
Age (yrs)	24.0(16.0-38.0)	30.0(16.0-38.0)	0.091
BMI (kg/m^2)	24.2(19.0-44.9)	32.0(18.2-46.6)	0.007
Waist circumference (cm)	79.5(67.0-118.0)	96.0(67.0-118.0)	0.005
HoMA-IR	2.0(0.7-5.5)	3.1(1.6-11.1)	0.001
N. patients with HoMA-IR >2	14 (52%)	28 (85%)	[a]0.010
N. patients with HS	11 (41%)	24 (73%)	[b]0.018
FAI	8.0(3.4-9)	10.9(8.8-16.9)	<0.001
CRP (ng/ml)	1.3(0.0-3.0)	2.1(0.5-8.0)	0.018
IL-6 (pg/ml)	9. 3(2.1-32.3)	22.0(2.0-79.4)	0.053
Spleen size (mm)	106.1±13.0	120.0±18.3	0.002
N. patients with LGCI	19 (70%)	27 (82%)	[c]0.365

[a] $\chi^2=7.699$; [b] $\chi^2=6.251$; [c] $\chi^2=1.088$

Values are expressed as mean±SD or median plus range. A cut-off for FAI was set at 9.1 (>median value) to identify less vs more severe biochemical hyperandrogenism). PCOS: Polycystic Ovary Syndrome; BMI: Body Mass Index; HoMA-IR: Homeostasis Model Assessment of Insulin Resistance; HS: Hepatic Steatosis; FAI: Free Androgen Index; CRP: C-Reactive Protein; IL-6: Interelukin-6; SLD: Spleen Longitudinal Diameter; LGCI: Low-grade Chronic Inflammation.

strongest correlations of the spleen enlargement among the other markers of LGCI with HS, IR and hyperandrogenism. In particular, we found that although HS and LGCI were similarly evidenced in both NW and O/O women with PCOS, these variables were much more different when the subjects were grouped according to IR or to PCOS phenotypes. As expected, PCOS-women with IR were characterized by a different profile of LGCI in comparison with the non-IR subjects, while the women with non-classic PCOS subtype presented an intermediate prevalence of HS and of LGCI, in that it was different from controls, but it was lower than in classical PCOS subtypes. The association between HS and spleen size was consistent with our previous data in O/O patients with HS (27), but to the best of our knowledge, this finding is novel in PCOS women. Similarly, the presence of the liver-spleen axis in PCOS and the different LGCI expression across the PCOS subtypes have not been reported previously. Therefore, these findings represent a further contribution to the knowledge on the possible, subsequent, metabolic implications of HS and LGCI in PCOS.

Due to their common association with IR, PCOS and HS are now considered, respectively, the ovarian (28) and hepatic manifestation of metabolic syndrome (MS) (29). However, the prevalence of HS among women with PCOS is variable (1). Using US to identify HS, up to 41% of young women with PCOS had evidence of liver disease *vs* 19% of the control group (7). Using a surrogate combined index of HS comprehending BMI, waist circumference, triglycerides, and gGT, this prevalence reached 89% among PCOS women with MS (30) *vs* 11.3% in PCOS women without MS. In contrast with common belief that obesity plays a determinant role in the association between HS and PCOS (4, 5, 9), but in line with Gambarin-Gelwan et al. (6), we found that the presence of HS in NW and O/O PCOS women was similar, although in NW PCOS-women transaminases were higher than in controls. Thus, the presence of different factors independent from obesity involved in the association between HS and PCOS is strongly suggested, the particular endocrine metabolic and inflammatory milieu in NW likely being responsible for the increased prevalence of hypertansaminasemia in these subjects, as also previously reported (5).

LGCI is considered a potential mechanism

Table VI. *Correlations between metabolic, inflammatory parameters and biochemical hyperandrogenism in our study population of 60 women with PCOS and 20 age-matched controls.*

	HoMA-IR	CRP	IL-6	SLD	T	SHBG	FAI
Waist circumference	0.614 *<0.001*	0.511 *<0.001*	0.526 *<0.001*	0.529 *<0.001*	0.581 *<0.001*	-0.538 *<0.001*	0.627 *<0.001*
HoMA-IR	/	0.650 *<0.001*	0.557 *<0.001*	0.683 *<0.001*	0.717 *<0.001*	-0.696 *<0.001*	0.719 *<0.001*
CRP	0.650 *<0.001*	/	0.658 *<0.001*	0.687 *<0.001*	0.693 *<0.001*	-0.666 *<0.001*	0.688 *<0.001*
IL-6	0.557 *<0.001*	0.658 *<0.001*	/	0.711 *<0.001*	0.537 *<0.001*	-0.582 *<0.001*	*0.548* *<0.001*
SLD	0.683 *<0.001*	0.687 *<0.001*	0.711 *<0.001*	/	0.723 *<0.001*	-0.699 *<0.001*	0.707 *<0.001*
Testosterone	0.717 *<0.001*	0.693 *<0.001*	0.537 *<0.001*	0.723 *<0.001*	/	-0.884 *<0.001*	0.935 *<0.001*
SHBG	-0.696 *<0.001*	-0.666 *<0.001*	-0.582 *<0.001*	-0.699 *<0.001*	-0.884 *<0.001*	/	-0.916 *<0.001*
FAI	0.719 *<0.001*	0.688 *<0.001*	*0.548* *<0.001*	0.707 *<0.001*	0.935 *<0.001*	-0.916 *<0.001*	/

Correlations are expressed as Spearman rho coefficients and corresponding p values. PCOS: Polycystic Ovary Syndrome; HoMA-IR: Homeostasis Model Assessment of Insulin Resistance; CRP: C-Reactive Protein; IL-6: Interelukin-6; SLD: Spleen Longitudinal Diameter; SHBG: Sex Hormone-Binding Globulin; FAI: Free Androgen Index.

whereby obesity determines IR (11), with a key role of IL-6 in the ectopic storage of lipid within other non-adipose tissues, such as liver, and in lipolysis in white adipose tissue (31). The involvement of the inflammatory cytokines have been generally related to the degree of obesity (1, 4, 5). Nevertheless, it is well known that many different classic inflammatory mediators, such as CRP or IL-6, could stimulate both ovarian and adrenal steroidogenesis (16-18). On the other hand, the androgen excess might induce *per se* or amplify the inflammatory state by increasing the amount of dysfunctional adipocytes, with subsequent major release of inflammatory cytokines and/or free fatty acids.

We previously reported that the increase in white blood cells, a marker of LGCI and cardiovascular risk, was well predicted by IR independently from obesity in a population of O/O PCOS-women (32); however, in that series we did not evidence any association between this marker of LGCI and androgens. In the present study, we found that three different markers of LGCI, mainly spleen enlargement, were correlated with hyperandrogenism, independently from obesity. In line with our data, Yang at al. (33) reported a similar association of IL-18 with testosterone levels independently from obesity. The presence of LGCI in NW and/or in non-IR subject PCOS-women independently from HS or IR was not reported previously. This finding might suggest that hyperandrogenism should represent one of the main factors involved in the early pathogenesis of LGCI and HS in PCOS independently from obesity.

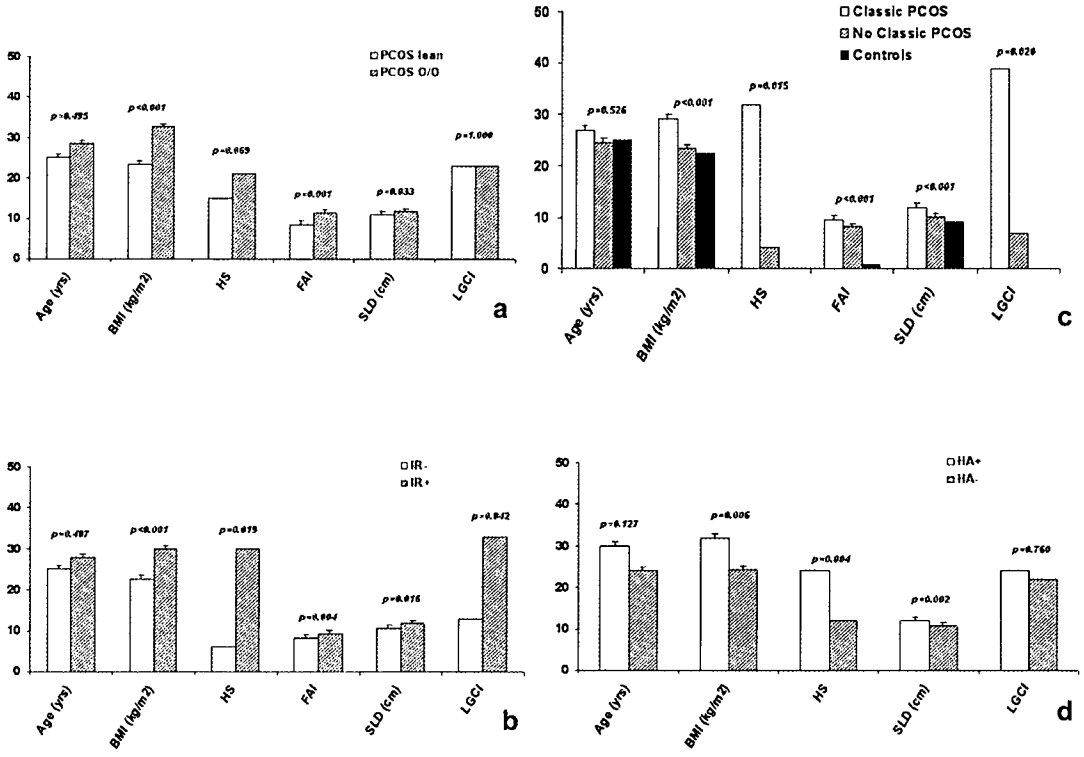

Fig. 1. *Comparisons of the main study variables in our sample population of women with PCOS according to Body Mass Index (BMI) (**a**), Insulin Resistance (IR) (**b**), PCOS phenotypes (**c**), and biochemical hyperandrogenism (HA) (**d**). The Kruskal-Wallis test or ANOVA were used for comparison between controls and the two PCOS phenotypes found in this study, when appropriate. The pairwise comparisons were analyzed by the Conover-Inman or the Bonferroni tests (c). Single p values are reported in Table 4. O/O: overweight/obese subjects; HS: number of subjects with Hepatic Steatosis; FAI: Free Androgen Index; SLD: Spleen Longitudinal Diameter; LGCI: number of subjects with Low-grade Chronic Inflammation.*

Nevertheless, the greater significant difference in HS and LGCI demonstrated when our study sample was grouped according to PCOS phenotypes compared to the other subgroups, allows us to hypothesize that the association of LGCI in PCOS is more likely due to a cluster of factors than to hyperandrogenism *per se*.

Taking into account the recently reported increased prevalence of both organ and non-organ specific autoimmunity in PCOS (19) and the well-established link between spleen and immune/autoimmune disorders (35), a possible pathway involving liver-spleen axis, hyperandrogenism, LGCI, immunity, and IR in the relationship between PCOS and HS is shown in Fig. 2.

There are some limitations to our research. Firstly, the type of study did not allow us to draw conclusions on the direction of the association. However, previous reports well supported our data on the independent associations between HS and PCOS and between inflammation and hyperandrogenism in PCOS. Secondly, the lack of computed tomography or magnetic resonance imaging, as well as of liver biopsy might have highlighted the presence of mild HS in NW women with PCOS and LGCI, who did not show HS at US. Thirdly, it could have been intriguing to investigate the serological aspect (auto-antibodies) or lymphocellular population in this syndrome to figure out whether the spleen enlargement could underlie an immune/autoimmune

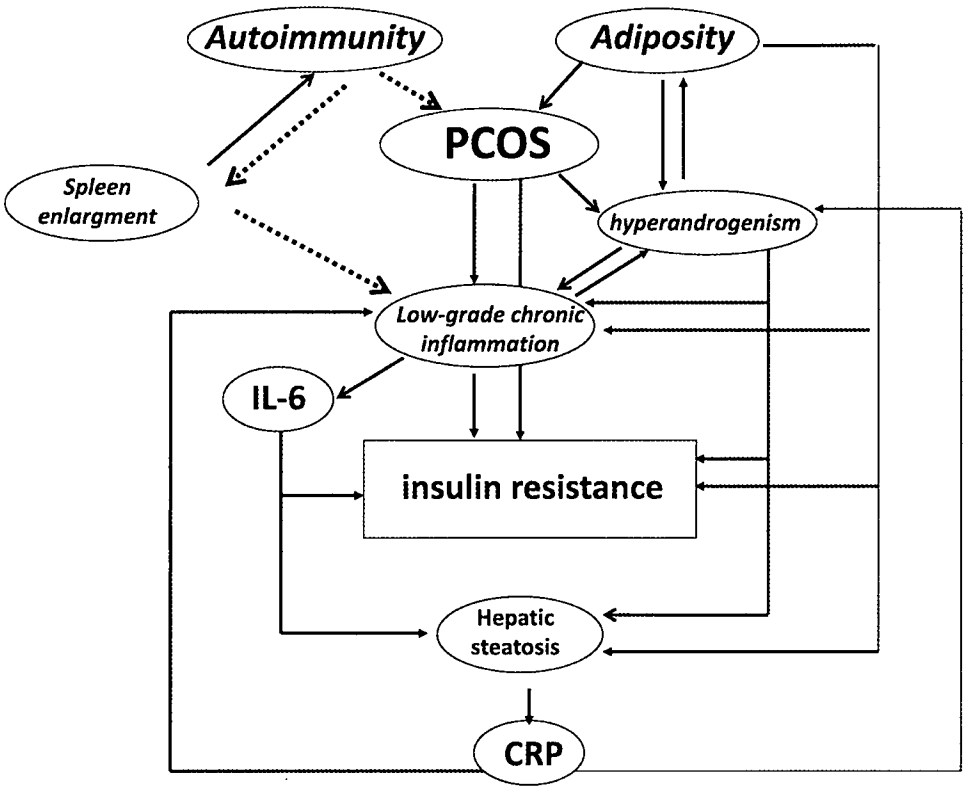

Fig. 2. *Hypothesized mechanism involving liver-spleen axis, hyperandrogenism, low-grade inflammation, and IR in the relationship between PCOS and HS. In PCOS, due to the favourable endogenous environment, i.e., androgen excess and IR (16, 18), the presence of LGCI might contribute to HS pathogenesis independently from obesity (17). Spleen enlargement, a marker of the underlying immune/autoimmune derangement in this syndrome (19), has been recently proposed as a reliable and a stable index of LGCI, strictly associated with severity of HS in obesity (13), in the so-called liver-spleen axis (14). Obesity and the androgen excess might induce per se or amplificate the inflammatory state by increasing the amount of dysfunctional adipocytes, with subsequent major release of inflammatory cytokines (15-17), such as IL-6 and CRP. PCOS: Polycystic Ovary Syndrome; HS: Hepatic Steatosis; IR: Insulin Resistance; LGCI: Low-grade Chronic Inflammation; CRP: C-Reactive Protein; IL-6: Interelukin-6.*

derangement in PCOS. Finally, the sample size including only young females of Caucasian ethnicity partially limited the strength of present findings that cannot be actually extended to populations with different nutrition state or environmental exposure to infectious diseases. However, our study group was homogeneously young and well characterized by a strict range of age and the findings of this study were significantly strengthened because of the presence of an age-matched control group which should have minimized potential bias.

In conclusion, although a causal role of the spleen in the relationship between PCOS and HS could be only speculated, we evidenced the presence of the liver-spleen axis in a group of young women with PCOS, with the variable expression of LGCI across the different PCOS subtypes. Further studies are warrant to elucidate the bidirectional relationships between PCOS, PCOS phenotypes and immunity.

ACKNOWLEDGEMENTS

This study was supported in part by the Ministry of University Research of Italy, PRIN, with the number 2007N4C5TY_005 and by Ricerca finalizzata - art.12 bis Decreto Legislativo 229/99.

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