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Evaluation of immunoglobulins subclasses and free-light chains in non-obese patients with polycystic ovary syndrome and correlations with hormonal and metabolic parameters: preliminary data

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Abstract. – OBJECTIVE: Polycystic ovary syndrome (PCOS) is characterized by hyperandrogenism and hyperinsulinemia that contribute to create a state of chronic low-grade inflammation. We performed an observational case-control study to investigate inflammatory and immunological parameters, such as IgG subclasses and free light chains (FLCs) and hemolytic complement activity (CH50) in non-obese PCOS, evaluating their relations with metabolic and hormonal parameters.

PATIENTS AND METHODS: 36 subjects were studied: 16 PCOS patients (mean±SEM 27.13±1.82 age; BMI 24.1±0.9 kg/m²); 20 controls (aged 26.05±0.73; BMI 20.8 ± 0.4 kg/m²). The blood sample was collected for metabolic and hormonal parameters, IgG subclasses, k and λ FLCs, CH50. Hormones were measured by immunochemiluminometric assays; metabolic parameters by enzymatic assays; subclasses of IgG, FLCs, and CH50 were evaluated by the turbidimetric method.

RESULTS: PCOS patients showed *vs.* controls lower IgG1, IgG2, IgG3 (mean±SEM 3.76±0.29 g/l, 2.63±0.20, 0.62±0.06, 0.34±0.08 *vs.* 6.49±0.35, 4.28±0.25, 0.84±0.07, 0.33±0.04, respectively) and higher levels of FLCs (k 12.22±0.71 *vs.* 6.03±0.30, λ 10.10±0.79 *vs.* 8.04±0.48 g/l) and CH50 (48.64±2.65 *vs.* 36.51±1.38 U/ml); we found correlation between IgG2 and free-testosterone (r=0.72, *p*=0.005) and CH50 and vitamin D (r=0.54, p=0.04); an inverse correlation was found between IgG1 and, respectively, ACTH (r=-0.57, *p*=0.02) and cortisol (r=0.78, *p*=0.001) in PCOS.

CONCLUSIONS: In the complex scenario of low-grade inflammation in non-obese PCOS, we showed lower levels of main subclasses of IgG and higher CH50 levels, suggesting the involvement of other mechanisms other than the "classical" pathway of complement activation; FLCs could be attractive to monitor inflammation degree, disease activity and influence on hormonal status.

Key Words:
Immunoglobulins, Insulin-resistance, PCOS, Personalized medicine, Inflammation.

Introduction

Polycystic ovary syndrome (PCOS) is a multifactorial disease characterized by reproductive and metabolic impairments. PCOS patients present a higher prevalence of anovulation, hirsutism, and infertility, as well as metabolic, together with hyperandrogenism, syndrome and diabetes[1]. Insulin-resistance (IR), together with hyperandrogenism, represents the main players, with reciprocal influences in a vicious circle: insulin promotes androgen secretion in thecal cells[2], and hyperandrogenism has been related to adipose tissue dysfunction[3,4] that led to IR and hyperinsulinemia[5].

Both factors contribute to create in PCOS patients a state of chronic low-grade inflammation (LGI), which may have an important role in the physiopathology of the disease[6,7], which is predominantly evaluated in obese PCOS[8]. Visceral adipose tissue, indeed, produces high levels of cytokines and other inflammatory mediators,

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which influence glucose metabolism promoting IR[9], and androgen production[3,10]. Several inflammatory markers have been evaluated in PCOS[11], such as C-reactive protein (CRP), which is markedly increased versus control; TNF-a and IL-6 have been evaluated with controversial data; a recent meta-analysis has established that no significant difference between PCOS patients and control[9] was present.

Other markers of LGI have been investigated; we demonstrated high levels of free light chains of immunoglobulins in PCOS patients[12] and also in other conditions characterized by insulin resistance and cardiovascular risk[13,14].

The complement cascade comprises over 20 serum proteins, and it is part of the innate immune system. Complement acts in clearing pathogens through bacterial lysis. Complement total activity (CH50) is a screening assay of the activation of the classical complement pathway, the immunoglobulin mediated-one. It is sensitive to the reduction, absence and/or inactivity of any component of the pathway. CH50 tests the functional capability of serum complement components of the classical pathway to lyse sheep red blood cells pre-coated with rabbit anti-sheep red blood cell antibody (hemolysin). CH50 blood test is often ordered to evaluate complement component deficiency and evaluate complement activity in cases of immune complex diseases, such as glomerulonephritis and autoimmune diseases[15].

The subclasses of IgG may be useful to better understand a scenario of altered immune response. The distribution of the IgG subclasses depends on the type of antigen and duration of antigen exposure, a concept known as "subclass restriction". Thus, the elevations of a given IgG subclass could reflect the nature of the underlying driving antigen. In humans, IgG is the predominant antibody class (7-15 g/L) and the four IgG subclasses, termed IgG1, IgG2, IgG3, and IgG4, functionally distinct because of different heavy chain gene usage, differ in their ability to fix complement and bind Fc receptors. The antigen driven subclass switching is sequential in the order IgG3, IgG1, IgG2 and IgG4, and it is under T cell control[16].

To the best of our knowledge, no data are reported on CH50 and IgG subclasses behavior in PCOS. Therefore, we performed a case-control study in a group of non-obese young women with PCOS evaluating CH50 levels and IgG subclasses to further explore the pattern of inflammatory/immune markers in this condition.

Patients and Methods

Study Population

We enrolled a total amount of 36 subjects; the protocol study was approved by the Institutional Review Board of our Department.

The subjects were divided into two groups. Group 1: 16 patients with PCOS (mean ± SEM age 27.13±1.82; mean ± SEM BMI 24.1 ± 0.9 years kg/m^2), diagnosed according to the Rotterdam's criteria[17]. Group 2: 20 volunteers, with normal menstrual cycles and ultrasound ovarian pattern as control group (mean ± SEM age 26.35±0.73 years, mean ± SEM BMI 20.8 ± 0.4 kg/m^2).

All the subjects enrolled in the study gave their informed written consent, conducted in accordance with the declaration of Helsinki, as revised in 2013.

Exclusion criteria were BMI <18.5 or >30 kg/m^2, diabetes mellitus, renal, liver, and heart failure, chronic inflammatory and autoimmune diseases, malignancies.

Blood samples were collected in the morning, in the early follicular phase of the menstrual cycle, after overnight fasting, into pyrogen-free tubes with heparin as an anticoagulant; after centrifugation, separate plasma aliquots were stored at 80°C until assayed.

Hormonal and Metabolic Parameters

Basal determinations of the following metabolic parameters were performed: glucose, total cholesterol, high-density lipoproteins (HDL), low-density lipoproteins (LDL), triglycerides, and insulin. Homeostasis Model Assessment Index (HOMA-IR index) was calculated according to the formula: {[fasting insulin (U/mL)] [fasting glucose (mmol/L)]}/22.5. Moreover, basal determinations of the following hormones were assayed: free triiodothyronine (FT3), free thyroxine (FT4), thyroid-stimulating hormone (TSH), adrenocorticotropic hormone (ACTH), cortisol, insulin-like growth factor 1 (IGF-1), dehydroepiandrosterone-sulfate (DHEA-S), follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (T).

Free and bioavailable testosterone were calculated according to the Vermeulen formula[18]. Plasma concentrations of glucose, total cholesterol, HDL cholesterol, triglycerides were measured by using enzymatic assays and on Olympus AU2700 chemistry analyzer (Olympus America Inc., Center Valley, PA, USA). The intra- and inter-assay

coefficients of variation (CV) for total cholesterol and triglycerides were <1.5% and <2.5%, respectively. The intra- and inter-assay CV for HDL cholesterol were <2.5% and <3.0%, respectively. LDL cholesterol was calculated by Friedewald's equation: LDL = [total cholesterol] – [HDL] – [triglycerides]/5.

All serum concentrations of hormones were measured by immunochemiluminometric assays on a Roche Modular E170 analyzer (Roche Diagnostics, Indianapolis, IN, USA). The intra- and inter-assay CV were, respectively, <5.0% and <7.0%.

Immunoglobulins Subclasses, Free Light Chains, and CH50

The Optilite CH50 Reagent is intended for the quantitative *in vitro* determination of total classical complement activity (CH50) in human serum and EDTA plasma using the Binding Site Optilite Turbidimetric assays analyzer (The Binding Site, Birmingham, UK). The test consists of liposomes encapsulating glucose-6-phosphate dehydrogenase (G6PDH) used to mimic an invading microorganism. In addition to the sample, antibodies in the reagent combine with dinitrophenyl groups on the surface of the liposomes. The resultant complex activates complement in the sample, which lyses the liposome, releasing G6PDH to react with glucose-6-phosphate and NAD in the reagent. The change in absorbance can be measured and is proportional to the complement activity in the sample. The comparison to a calibration curve gives a value for the unknown patient sample.

The four IgG subclasses concentrations were measured by turbidimetry through the employment of Human IgG and IgG subclass liquid reagent kits (The Binding Site, Birmingham, UK) with Optilite instrument according to the manufacturer's recommendations. These kits are intended for quantifying human IgG and IgG subclasses. Concentrations are automatically calculated by reference to a standard curve stored within the instrument. Normal range for subclasses: 3.82-9.29 g/L for IgG1; 2.42-7.0 g/L for IgG2; 0.22-1.76 g/L for IgG3; 0.04-0.86 g/L for IgG4. Samples were tested according to the manufacturer's instructions, and serum dilutions, where necessary, were performed according to the manufacturer's recommendations.

FLCs were assessed using the Freelite™ Human Kappa and Lambda Free Kits (The Binding Site, Birmingham, UK) on an Optilite instrument (The Binding Site, Birmingham, UK; free κ normal range: 3.3-19.4 mg/L; free λ normal range: 5.7-26.3 mg/L). A ratio of κ/λ <0.26 or >1.65 is abnormal, according to the manufacturer's recommendations.

Statistical Analysis

Statistical evaluation was performed using Mann-Whitney U-test for unpaired data to evaluate differences between groups. Spearman correlation coefficient was used to investigate the association between Ig subclasses and hormonal and metabolic parameters. Statistical significance was assessed when $p<0.05$. All statistical analyses were performed using the software GraphPad Prism 8, San Diego, CA, USA.

Results

Metabolic and hormonal parameters in PCOS and control subjects are reported in Table I. As expected, PCOS group showed higher levels of free-testosterone and HOMA-IR.

Figure 1 shows median and interquartile ranges of IgG subclasses in the two groups. PCOS showed lower levels of IgG1, IgG2, and IgG3 subclasses and higher levels of k and λ FLCs compared with controls ($p<0.05$).

Figure 2 shows median and interquartile ranges of CH50 in the two groups: PCOS showed higher levels of CH50 compared with controls ($p<0.05$).

Regarding correlations, we found direct correlation between IgG2 and free T (r 0.72, $p=0.005$) and CH50 and vitamin D (r=0.54, p=0.04); moreover, an inverse significant correlation was found between IgG1 and, respectively, ACTH (r= -0.57, $p=0.02$) and cortisol (r= -0.78, $p=0.001$).

Discussion

Our data, while confirming increased levels of k and λ FLCs as previously reported, show reduced levels of immunoglobulins subclasses mainly involved in immune response (IgG1, IgG2, IgG3), in comparison to normal weight female controls, although remaining in the normal range. It is also known that female subjects have lower levels than males[19]. This is concordant with higher CH50 level, suggesting lower "classical" component of complement activation; this datum, coupled with elevated levels of inflammatory markers, suggests that other pathways could be

Table I. Mean ± SEM levels of metabolic and hormonal parameters.

	PCOS	Controls
HOMA-IR	1.78 ± 0.35*	0.96 ± 0.19
Total Cholesterol (mg/dl)	184.87 ± 10.84	166.71 ± 10.80
HDL (mg/dl)	62.53 ± 4.49	61.5 ± 5.47
LDL (mg/dl)	97.04 ± 4.45	101.33 ± 8.00
Triglycerides (mg/dl)	81.14 ± 20.45	55.83 ± 7.03
FT3 (pg/ml)	2.95 ± 0.14	2.97 ± 0.14
FT4 (pg/ml)	10.09 ± 0.33	11.29 ± 0.40
TSH (µUI/ml)	1.75 ± 0.23	1.47 ± 0.21
ACTH (pg/ml)	21.13 ± 2.28	18.61 ± 1.91
Cortisol (ng/ml)	121.22 ± 16.73	113.55 ± 15.62
LH (mU/ml)	5.11 ± 1.75	5.27 ± 0.96
FSH (mU/ml)	3.38 ± 0.53*	6.43 ± 0.73
Testosterone (ng/dl)	44 ± 4	40 ± 4
SHBG	86.09 ± 22.22	91.40 ± 17.94
Free Testosterone (ng/dl)	0.39 ± 0.01*	0.33 ± 0.01
Bio-available Testosterone (ng/ml)	10.3 ± 2.0	7.788 ± 2.2
DHEA-S (ng/mL)	2970.94 ± 354.74	2199.46 ± 265.47

*p<0.05.

involved in low-grade inflammation of PCOS, at least in non-obese patients.

Although autoimmune mechanisms have been supposed to contribute to the development of the syndrome, our data did not show significant differences in IgG4.

FLCs have a short serum half-life, and the large clinical range provides a sensitive marker for assessment of response to treatment. FLCs indicate response may vary between the different degrees of diseases[20]. Polyclonal FLCs level provides an indication of total immunoglobulin syn-

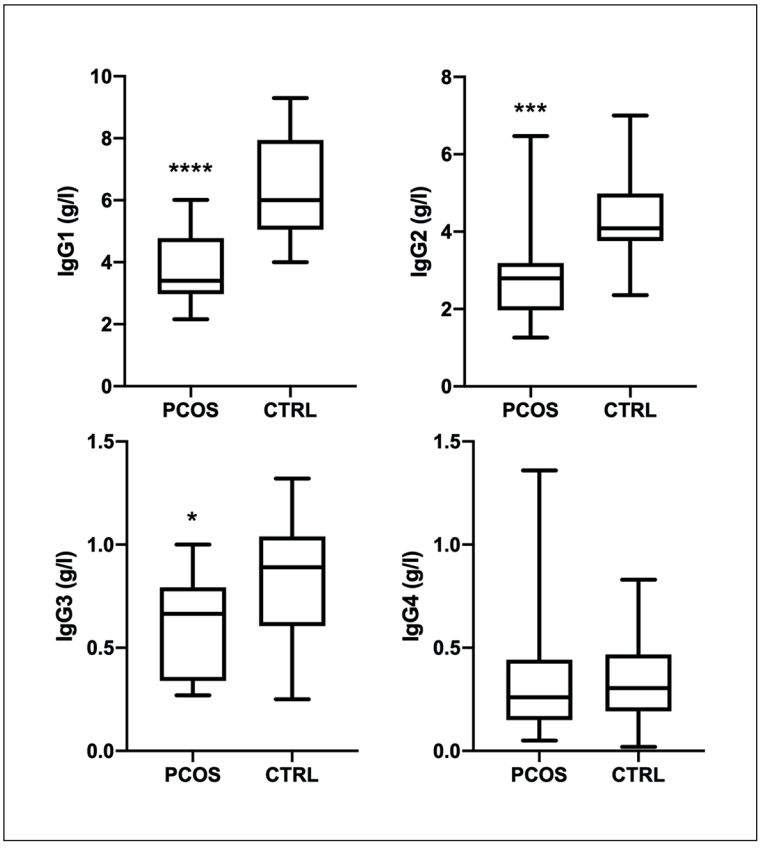

Figure 1. Median and interquartile ranges of immunoglobulin subclasses and FLCs between the two groups. *p=0.02; ***p=0.0002; ****p<0.0001.

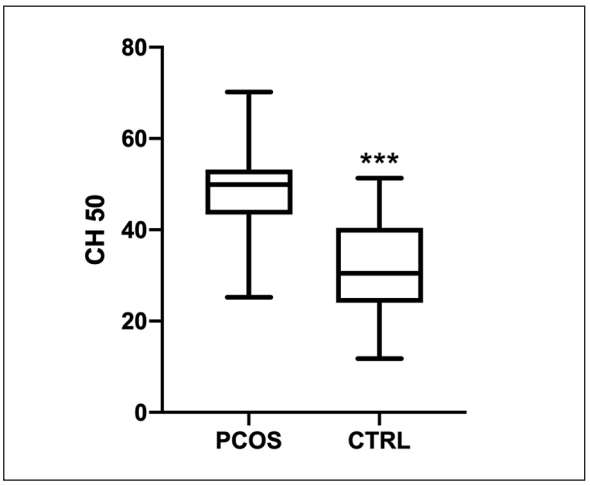

Figure 2. Median and interquartile ranges of CH50 between the two groups. ***p=0.0002.

thesis that may serve as a biomarker of immune stimulation and inflammation.

Prolonged inflammation in these disorders drives B-cell dysfunction and activation, resulting in FLC overproduction due to the immune system's ongoing efforts to search a new equilibrium. In a multifactorial disease, variable structure of both monoclonal and polyclonal FLCs could influence an individual FLCs antigen-binding capacity, pathogenic potential, and ability to interact with immune cells[21].

The scenario of chronic-low-grade inflammatory state has been extensively studied in PCOS, even if a clear and unequivocal picture still needs to be completed, especially in normal weight patients; indeed, despite the large part of literature concern obese PCOS, also in non-obese ones insulin-resistance is present and can be related to inflammation[22]. This pathophysiological mechanism has been related to both hyperandrogenism and long-term consequences of the syndrome[23,24]. In fact, on one side, *in vitro* studies suggest that proinflammatory stimuli can induce up-regulation of the steroidogenic enzymes for the production of androgens in ovarian theca cells[1], suggesting the possibility that a direct relationship between inflammation and hyperandrogenism in PCOS is underlined.

On the other side, among inflammation-related phenomena, high levels of leukocytes, endothelial dysfunction, and disorder of the proinflammatory cytokines have been described[25,26]. Direct correlations have been found between increased levels of inflammation markers (CRP, ferritin, leukocyte TNF-α, IL-6, IL-18) and the development of PCOS. Other contributors include elevated levels of plasminogen activator inhibitor (PAI1) and free fatty acids, causing phosphorylation of serine residues, and consequently, a rise in insulin resistance[27]. Other mechanisms recently included concern markers of iron overload: increased levels of ferritin and transferrin and a higher frequency of the HP2/HP2 genotype of the haptoglobin α chain have been observed, in turn, reduction of anti-inflammatory cytokines and antioxidant molecules[28].

Another study reported higher median serum level of IL-1α and IL-1β in the PCOS group versus the control group, while the median serum level of IL-17 was significantly lower in the pathological groups. The authors connected these cytokines release to the activation of sympathoadrenal axis, also well described in PCOS[29].

Among other inflammatory characteristics, low levels of vitamin B12 and increased ratio neopterin/lymphocytes ratio have been reported[30]. Finally, oxidative stress has been documented at intracellular levels also in non-obese PCOS[31].

On the other hand, dysregulation of cellular immune response has been reported.

Nasri et al[32] reported that the frequency of Th1 cells was increased compared to Th2 cells in infertile PCOS when considering Th1/Th2 ratio, with a bias toward Th17 dominancy in PCOS. The proportion of CD4+CD25+Foxp3+ regulatory T cells was significantly lower in PCOS patients than that of healthy fertile women (p=0.02). In summary, Th1 and Th17 bias and reduction of Treg and Th2 cells as regulators of immune responses might be involved in the pathogenesis of PCOS; this datum is in agreement with our report of low immunoglobulins and contributes to the hypothesis of an altered immune response to inflammatory status in PCOS patients. Moro et al[33] reported an augment expression of CD4+CD28null lymphocytes, an aggressive subset with proinflammatory and proatherogenic functions.

Other than blood, large amounts of immunocompetent cells, including T cells, B cells, macrophages, and dendritic cells, have been found in human preovulatory follicles; therefore, the attention was also addressed to such evaluation in PCOS. Li et al[34] showed a decrease of the percentage of follicular CD8+ T, CD69, and IFN-γ, while the level of PD-1 was increased in both CD4+ and CD8+T cells from infertile patients with PCOS (p<0.05).

Moreover, the expression of PD-1 on CD4+ or CD8+T cells was positively correlated with the estradiol (E2) levels in the serum and reversely correlated with the expression of IFN-γ in CD4+ or CD8+ T cells in infertile patients with PCOS. They therefore suggested an involvement of T cell dysfunction in the pathogenesis of PCOS: the higher expression of PD-1 in CD4+ T and CD8+ T cells in follicular fluid in PCOS patients with infertility probably cannot induce T cell activation or recruitment, which, in turn, leads to the failure of dominant follicle selection and development. Finally, it has been reported that increased expression of PD-1 and significantly decreased expression of IFN-γ were detected in CD4+ T and CD8+ T cells in infertile patients with PCOS ($p<0.05$). The authors speculated that the dysfunction of T cells, which may be an immunological feature, might participate in the immune pathogenesis in the ovary of PCOS patients with infertility. These results suggest that chronic inflammation may be one of the underlying mechanisms for the pathogenesis of PCOS. On the other hand, elevated C3 levels have been demonstrated[34].

We also observed a correlation of immune dyscrasia with T levels, in favor of the hypothesis of the relationships with hyperandrogenism, as above described; a role of the activation of ACTH-cortisol axis is suggested by the inverse correlation of IgG with the two hormones; finally, we showed that a significant correlation was present between CH50 and vitamin D; Vitamin D alterations are well described in PCOS[35], but the scenario of a protective role of vitamin D against classical complement activation could be open by our data.

Conclusions

In the complex scenario of low-grade inflammation in PCOS, we showed lower levels of main subclasses of IgG, with higher levels of CH50, in non-obese PCOS patients. This suggests that other mechanisms could be involved other than "classical" pathway of complement activation.

Specific structural features with ability to distinguish pathological and nonpathological FLCs have not been well described. FLCs have become an attractive novel target in precision medicine to monitor inflammation degree and disease activity.

Extended data on a larger group of patients are needed to confirm these preliminary observations.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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