J Assist Reprod Genet (2017) 34:1691–1698
DOI 10.1007/s10815-017-1030-3

REPRODUCTIVE PHYSIOLOGY AND DISEASE

Haptoglobin levels, but not Hp1-Hp2 polymorphism, are associated with polycystic ovary syndrome

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Received: 4 July 2017 / Accepted: 22 August 2017 / Published online: 13 September 2017

Abstract
Purpose Proteomic studies suggest an association between haptoglobin (Hp) and polycystic ovary syndrome (PCOS). Hp is a classic inflammatory marker and binds to the intravascular hemoglobin, avoiding the oxidative damages that can be caused by free hemoglobin. Inflammation and oxidative stress are important in the pathogenesis of the PCOS, one of the most frequent metabolic diseases in women.
Methods To validate these proteomic studies, we developed a controlled cross-sectional study that aimed to evaluate the Hp levels and allelic and genotypic frequencies of Hp1-Hp2 polymorphism in Brazilian women with PCOS. We also investigated the correlation between Hp levels and several important parameters in PCOS as follows: body mass index (BMI), waist circumference (WC), fasting glucose, post-prandial glucose, homeostatic model assessment (HOMA), lipid accumulation product (LAP), C-reactive protein (CRP), and metabolization test of tetrazolium salts (MTTs—serum antioxidant capacity).
Results Plasma Hp levels were higher in the PCOS group than in controls [8.20 (4.04) g/L; 7.98 (3.31) g/L; $p = 0.018$]. No significant difference was observed in the frequency of Hp1-Hp2 genotypes under additive, recessive, or dominant model of inheritance between the PCOS and the control groups. Plasma Hp levels did not differ according to the genotype. However, plasma Hp showed a negative correlation with MTT ($r = -0.383$; $p = 0.028$), as well as a positive correlation with CRP ($r = 0.361$; $p = 0.014$) in the PCOS group.
Conclusion Hp1-Hp2 polymorphism is not associated with PCOS but plasma Hp could be a potential biomarker for PCOS and its complications.

Keywords Polycystic ovary syndrome · Haptoglobin · Inflammation · Oxidative stress

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Introduction

Polycystic ovary syndrome (PCOS) is one of the most frequent metabolic diseases in women, which presents higher risks of insulin resistance (IR) and type 2 diabetes mellitus (DM2), dyslipidemia, metabolic syndrome, cardiovascular complications [1–3], infertility [4, 5], thrombosis [6], cancer [7], and obstetric complications [8].

Low-grade chronic inflammation is considered the link between PCOS and numerous metabolic disorders [9] and probably contributes to an increased risk of cardiovascular complications in PCOS patients [9, 10]. The visceral obesity commonly observed in this group also contributes to the increase of plasma levels of inflammatory mediators [9, 11]. Besides the chronic low-grade inflammation [11–13], PCOS women also present an imbalance of the oxidative profile, resulting in increased oxidative stress [14, 15].

Proteomic studies have suggested that haptoglobin (Hp)—an acute phase glycoprotein—may be associated to PCOS [16, 17]. The main function of Hp is to bind to the intravascular

hemoglobin (Hb) released during hemolysis, avoiding the oxidative damages that can be caused by free Hb, besides the iron loss [18]. The Hp-Hb soluble complex is recognized by CD163 receptor found on the surface of phagocytic cells [19], such as monocytes, macrophages, and Kupffer cells [20, 21]. The Hp-Hb-CD163 complex induces the production of several anti-inflammatory and antioxidative mediators [22].

Serum Hp levels increase considerably in inflammatory processes [19]. Hp is synthesized primarily in the liver, whose production is influenced by cytokines, such as TNF-α [23, 24], interleukin-6 [24, 25], and interleukin-1 [24], through the bind of these cytokines in regulatory sites of the Hp promoter gene [26]. Beyond the hepatic Hp production, *Hp* gene expression is observed in other tissues, including the lung, spleen, kidney, and heart [27, 28]. Moreover, since this protein is also expressed in adipose tissue [27–29], the presence of obesity may further increase serum Hp levels [30].

Hp is composed by two types of peptide chains, alpha and beta [31, 32], linked by disulfide bonds [33]. Both strands are encoded by the same gene, located in 16q22.2 [31, 34] as a pre-protein, which subsequently undergoes post-translational modifications, and a proteolytic process cleaves the two subunits [35]. A common polymorphism is present in this gene, which is characterized by two alleles. The Hp1 allele has five exons and is conserved among species. Hp2 allele is human-specific and presents seven exons that likely arose from a duplication (non-homologous crossing over) involving exons 3 and 4 of the Hp1 allele [21].

The final structure of the Hp protein is genotype-dependent [34]. There are three possible genotypes for *Hp* gene: Hp1-1, Hp2-1, and Hp2-2 [21, 31, 32, 34]. The Hp beta chain (40 kDa) is identical for both Hp alleles [32]. Hp from individuals with the Hp1-1 genotype presents an alpha-beta dimer with 86 kDa, whereas those from the Hp2-1 genotype have compositions ranging from 86 to 300 kDa. The Hp of individuals with the Hp2-2 genotype also has several possible structures, ranging from 170 to 900 kDa. The Hp1 allele presents a single exon that encodes the multimerization domain of the protein, while Hp2 has two copies of that same exon, which explains the different Hp structures [34].

Hp1-Hp1protein products have greater ability to bind Hb and the CD163 receptor, presenting greater anti-inflammatory and antioxidant properties [36]. Contrarily, Hp2 leads to a functional impairment in Hb clearance [19] and lower antioxidant activity [34].

In an attempt to validate proteomic studies that suggest the association between Hp and PCOS [16, 17], our study aimed to evaluate the Hp levels and allelic and genotypic frequencies of Hp1-Hp2 polymorphism in Brazilian women with PCOS. Our data can help to clarify a possible role of Hp and its use as a systemic marker for PCOS.

Material and methods

Experimental design

This is a cross-sectional study, which involved 86 women with PCOS (14–42 years old) and 86 women without this syndrome—controls (20–48 years old).

The group with PCOS was selected in the Hyperandrogenism Clinic of *Hospital das Clínicas*, Federal University of Minas Gerais, between 2011 and 2013, according to the European Society of Human Reproduction/Embryology and the American Society for Reproductive Medicine criteria (ESHRE/ASRM) [37], which considers the presence of at least two of three criteria: (1) menstrual dysfunction and anovulation, (2) clinical or laboratory evidence of hyperandrogenism, and (3) micropolycystic ovaries evidenced by ultrasonography, defined by the presence of 12 or more follicles in the ovary each, measuring 2 to 9 mm in diameter and/or increased ovarian volume (> 10 mL). In the same period, the women of the control group were recruited among students and employees of the same university. The control group showed no signs of hyperandrogenism or reported menstrual irregularity.

We considered the following as exclusion criteria: autoimmune, adrenal, kidney, liver, thyroid, thromboembolic or renal diseases, pregnancy, diabetes mellitus, sickle-cell anemia, cancer, hyperprolactinemia, hypogonadism, inflammatory/infectious process, orthopedic implant, C-reactive protein (CRP) levels > 10 mg/dL, and use of medications such as anti-inflammatory drugs (steroidal and non-steroidal), anabolic steroids, isotretinoin, cyclosporine, antiretroviral, insulin, and hormonal contraceptives, currently or recently (past 3 months).

Venous blood samples were obtained using tubes with EDTA, sodium citrate, and tubes without anticoagulant (Vacuette®), after 12 h of fasting. The samples were centrifuged at 1500×*g* at 4 °C for 20 min to obtain serum or plasma samples, which were stored at − 80 °C until the analyses. An aliquot of whole EDTA blood was also stored at − 20 °C for later genomic DNA extraction.

Clinical and laboratory parameters

Insulin and testosterone levels were measured using Abbott Architect®. Serum glucose and CRP were measured in plasma citrate using Vitros kits (Johnson and Johnson®). All procedures were conducted according to the manufacturer's instructions.

Hirsutism was assessed only in the PCOS group, according to the modified Ferriman–Gallwey scale [38], by a single observer, in order to avoid inter-examiner variation. Hyperandrogenism was considered for women that presented testosterone levels

> 77 ng/dL (biochemical) and/or Ferriman–Gallwey value ≥ 8 (clinical) [38].

Body mass index (BMI) was calculated by dividing the body mass (weight, kg) by the square of the body height (m^2), and waist circumference (WC) was measured midway between the lowest ribs and the iliac crest, as recommended by the World Health Organization and International Diabetes Federation [39].

Triglyceride levels were determined by using Vitros kits (Johnson and Johnson®) according to manufacturer's instructions. The lipid accumulation product (LAP) index was calculated by using the formula [(waist circumference − 58) × (triglycerides)] [40, 41]. The homeostatic model assessment (HOMA) for IR was calculated using the formula [insulin (mU/L) × glucose (mM/L)]/22.5 [42].

The serum antioxidant capacity was determined by the metabolization test of tetrazolium salts assay (MTT) [43], according to Duarte et al. [44] and Medina et al. [45]. It is based on the ability of plasma antioxidant factors to reduce the compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a colorimetric compound formed by the formazan crystals [43]. The results were expressed in arbitrary units.

Plasma Hp quantification was performed with R&D Systems® ELISA kit, using blood samples collected in citrate-coated tubes. The assay was applied according to the manufacturer's instructions, after validation with citrate samples.

Genotyping

Genomic DNA was obtained using Biopur Mini Spin Kit (BiometrixBiotecnologia®). The Hp1-Hp2 polymorphism genotyping was performed according to Koch et al. [46]. Hp1 and Hp2 alleles diverge in a DNA segment of approximately 1700 bp, duplicated in Hp2. Two PCR reactions were used, each containing a different pair of primers: first reaction, primer A (5′-GAGGGGAGCTTGCCTTTCCATTG-3′) and primer B (5′-GAGATTTTTGAGCCCTGGCTGGT-3′); second reaction, primer C (5′-CCTGCCTCGTATTAACTGCACCAT-3′) and primer D (5′-CCGAGTGCTCCACATAGCCATGT-3′).

PCR conditions were as follows: initial denaturing at 95 °C for 2 min, followed by 35 cycles of 95 °C for 1 min, 69 °C for 2 min (1 min for reaction 2), 69 °C for 2 min (1 min for reaction 2), and final extension of 7 min at 72 °C. PCR products were visualized in 1% agarose gel electrophoresis stained with ethidium bromide solution (10 mg/mL; Sigma-Aldrich, MO, USA).

Two PCR products were visualized in the first reaction, as a 1757-bp product (Hp1 allele) and a 3481-bp product (Hp2 allele). In the second reaction, a 349-bp PCR product was generated only in the presence of Hp2, which is useful in order to confirm the results. About 60% of the samples were re-genotyped using the two reactions, and the results were confirmed.

Statistical analysis

Statistical analyses were performed with the Statistical Package of the Social Sciences (SPSS) version 17.0. The charts in this article were generated in Microsoft Office Excel 2016. We considered significant all results that presented p value < 0.05.

To evaluate the normality of each parameter, we used the Shapiro–Wilk test. Variables with normal distribution were presented as mean and standard deviation and compared with Student's t test, whereas variables with non-normal distribution were presented as median and interquartile range (75th–25th percentiles) and compared with the Mann–Whitney test (two groups) or Kruskal–Wallis (three groups).

Correlation analyses between Hp levels and laboratorial parameters in the PCOS group were performed with Spearman's correlation test. Regarding the strength of the correlation, we considered it weak ($0 < r \leq 0.35$), moderate ($0.36 \geq r \leq 0.67$), or strong ($r \geq 0.68$), as proposed by Taylor [47].

Hardy–Weinberg equilibrium (HWE) was evaluated using exact tests by GENEPOP (http://genepop.curtin.edu.au/genepop_op1.html) [48] and OEGE (http://www.oege.org/software/hardy-weinberg.html) [49]. Differences in genotypic and allelic frequencies between the groups were investigated using the chi-square (χ^2) test.

Results

Table 1 summarizes the clinical and laboratorial characteristics of the women included in the study. The two groups did not differ regarding the age, fasting glucose, and CRP levels. However, the PCOS group presented significantly increased values of BMI, WC, insulin, HOMA, LAP, testosterone, and MTT when compared to controls.

Plasma Hp levels were higher in the PCOS group [8.20 (4.04) g/L] than in controls [7.98 (3.31) g/L] ($p = 0.018$) (Fig. 1). Hp also showed moderate and negative correlation with MTT ($r = -0.383$, $p = 0.028$; Fig. 2a), as well as moderate and positive correlation with CRP ($r = 0.361$, $p = 0.014$; Fig. 2b) in PCOS group. No correlation between Hp levels and BMI, WC, LAP, age, testosterone, fasting glucose, HOMA-IR, and insulin levels was observed ($p > 0.05$), if all individuals study were evaluated together or only patients in the PCOS group.

The polymorphism Hp1-Hp2 was in Hardy–Weinberg equilibrium ($p = 0.082$ for PCOS; $p = 0.369$ for controls) for either group. No significant difference was observed regarding the genotypic frequency between the PCOS and

Table 1 Clinical and laboratorial characterization of PCOS and control groups

Parameters	PCOS	Control	*p*
Age (years)	31.15 ± 4.92	29.0 ± 7.04	0.058
BMI (kg/m^2)	30.17 ± 5.44	23.27 ± 4.23	< 0.001*
WC (cm)	98.00 (17.0)	71.50 (16.0)	< 0.001*
FG (mmol/L)	87.10 ± 7.26	84.75 ± 10.42	0.230
Insulin (uUI/mL)	15.90 (17.3)	8.75 (5.2)	< 0.001*
HOMA-IR	3.54 (4.8)	1.68 (1.6)	< 0.001*
LAP	52.95 (63.4)	15.49 (19.1)	< 0.001*
Testosterone (ng/dL)	57.00 (43.4)	29.95 (18.5)	< 0.001*
Ferriman–Gallwey	11.00 (10.0)	–	–
CRP (mg/dL)	5.00 (8.6)	3.00 (1.0)	0.403
MTT[a]	0.34 (0.14)	0.24 (0.12)	< 0.001*

Student's *t* test—for normal distribution, mean ± standard deviation; Mann-Whitney test—for non-normal distribution, median (interquartile range)

PCOS polycystic ovary syndrome, *BMI* body mass index, *WC* waist circumference, *FG* fasting glucose, *HOMA* homeostatic model assessment, *LAP* lipid accumulation product, *CRP* C-reactive protein, *MTT* metabolization test of tetrazolium salts, – not evaluated for control group

*p<0.05 was considered statistically significant

[a]Measured in arbitrary units of absorbance

control groups, according to additive, recessive, or dominant model ($p > 0.05$) (Table 2). In both groups, the genotype Hp2-Hp1 was the most frequent [PCOS (60.5%); control (53.5%)]. The most frequent allele was Hp2 [PCOS (52.3%); control (60.5%)]; however, no significant difference in allelic frequency was observed between the groups ($p = 0.128$).

In order to evaluate if the Hp1-Hp2 polymorphism could change the circulating levels of the protein, plasma Hp levels were compared between the three genotypes. Plasma Hp levels did not differ according to the genotype in PCOS, nor in the control group (all $p > 0.05$). When compared the same genotype between the groups, no difference was also observed (all $p > 0.05$) (Table 3).

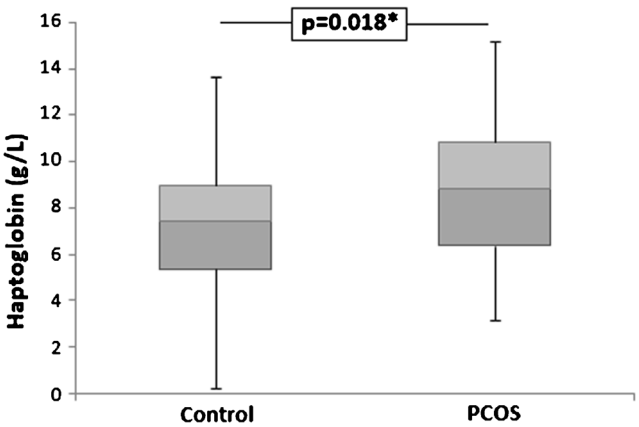

Fig. 1 Plasma haptoglobin levels in PCOS and control groups. PCOS polycystic ovary syndrome. Mann–Whitney test; *$p < 0.05$ was considered statistically significant

Discussion

In this study, we evaluated the association of plasma Hp levels, Hp1-Hp2 polymorphism, and biochemical variables in PCOS women. This is the first study that observed increased plasma Hp levels in patients with PCOS compared to controls, thereby validating the preliminary findings of proteomic studies. However, we did not find evidence that the Hp1-Hp2 polymorphism is related to the presence of the syndrome or to Hp levels in these groups.

The clinical and laboratorial characteristics of the patients reinforced common findings in patients with PCOS, as hyperandrogenism [50], increased abdominal fat [51], and IR—with consequent compensatory hyperinsulinemia [50]— as well as greater risk of metabolic syndrome, evaluated by LAP index [52]. The higher levels of MTT observed in the PCOS group could be associated to a compensatory effect, since this assay measures total plasmatic antioxidant capacity [43], and PCOS is commonly associated to a pro-oxidative profile [14, 15].

A low-grade chronic inflammation is characteristic of PCOS [9, 11–13]. Accordingly, in the present study, higher CRP levels were observed in PCOS women, but the difference between the groups was not significant, which could be explained by the low-grade inflammation in this syndrome that is not enough to significantly raise CRP values. In our study, CRP levels were minimally elevated, contrasting to conditions of acute, high-grade inflammation, such as tissue injury or infection, which typically cause CRP increase [53]. It is noteworthy that women who presented CRP levels > 10 mg/dL, associated with these conditions, were excluded from our study.

The PCOS group exhibited higher Hp levels than controls. This finding supports previous proteomic studies suggesting that Hp may be a biomarker of PCOS [16, 17]. In addition, a small study in the UK ($n = 30$ per group) using Western blotting to quantify Hp protein subunits found that total Hp and Hp beta chain protein abundance were elevated in women with PCOS compared with controls [54].

Additionally, we observed a positive correlation between Hp and CRP levels. This result corroborates the hypothesis that increased Hp levels are associated with inflammation in PCOS group. In fact, pro-inflammatory cytokines are important signal for *Hp* gene expression in white adipose tissue [55]. Moreover, the negative correlation with MTT indicates that higher Hp levels are observed with the increase of the pro-oxidative profile. Taken together, our findings suggest that

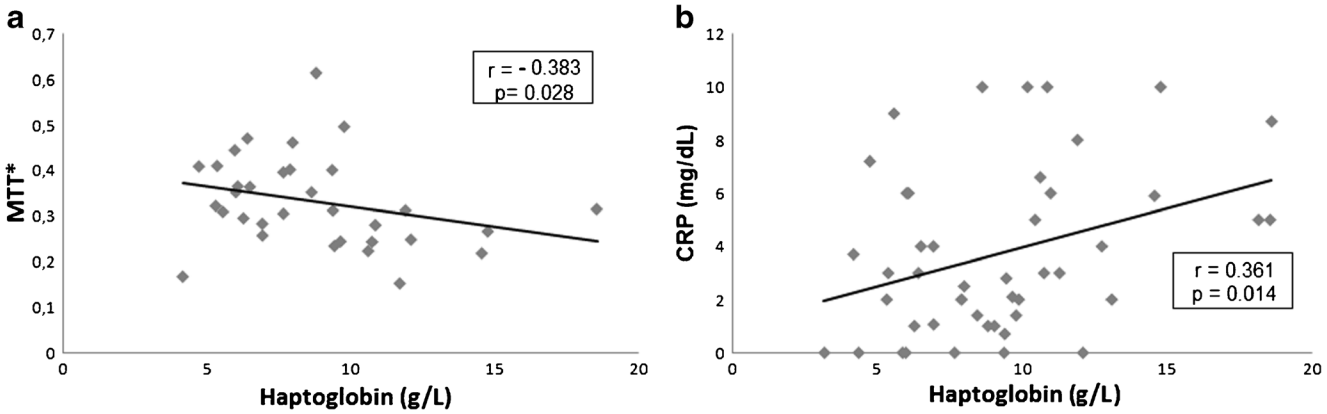

Fig. 2 Correlation coefficients between haptoglobin levels, MTT (**a**) and CRP levels (**b**) in PCOS group. PCOS polycystic ovary syndrome, CRP C-reactive protein, MTT* metabolization test of tetrazolium, expressed in arbitrary units. Spearman's correlation test; *$p < 0.05$ was considered statistically significant

higher Hp level in PCOS is a condition associated with inflammation and an increase in oxidative stress status.

Even though Hp levels increase with insulin resistance [56], neither Hp levels nor the Hp1-Hp2 polymorphism were related to glucose intolerance, since no correlation between these parameters and fasting glucose, HOMA-IR, and insulin levels was observed. Consequently, Hp does not appear to be involved in the insulin resistance of PCOS women.

Adiposity is an independent factor associated with Hp levels in humans [29]. It is known that Hp is produced by adipocytes, and that *Hp* gene is upregulated in white adipose tissue of the obese mice [22, 55]. The higher BMI and WC observed in PCOS could explain elevated Hp levels in this group. However, we did not observe any correlation between Hp levels and these parameters, suggesting that higher Hp concentration is independent from obesity status in this group investigated.

The genotypic and allelic frequencies of Hp1-Hp2 polymorphism were similar between the two groups. Langlois and Delanghe [21] showed that polymorphisms in alpha-chain influences circulating Hp concentrations to a lesser degree compared with its effect on the functionality of the protein. Consequently, our results suggest that higher Hp concentration in PCOS is independent of Hp1-Hp2 polymorphism, and that the protein is functionally analogous to that found in women without the syndrome.

Although no significant difference was observed, the results suggest a gene dosage effect of Hp1-Hp2 polymorphism on Hp levels in the control group. Decreased concentration was observed with the Hp2 allele, which expresses the protein

Table 2 Genotypic and allelic frequencies of *Hp* gene in PCOS and control groups

Genotype	PCOS (n = 86)	Control (n = 86)	OR	95% CI	p
Additive model					
Hp1-Hp1	15 (17.4%)	11 (12.8%)	0.480	0.162–1.408	0.136
Hp2-Hp1	52 (60.5%)	46 (53.5%)	0.580	0.270–1.237	0.126
Hp2-Hp2	19 (22.1%)	29 (33.7%)	Reference		
Recessive model					
Hp2-Hp2	19 (22.1%)	29 (33.7%)	1.794	0.911–3.533	0.091
Hp1-Hp1 + Hp2-Hp1	67 (77.9%)	57 (66.3%)			
Dominant model					
Hp2-Hp2 + Hp2-Hp1	71 (82.6%)	75 (87.2%)	1.440	0.620–3.347	0.396
Hp1-Hp1	15 (17.4%)	11 (12.8%)			
Allele					
Hp1	82 (47.7%)	68 (39.5%)	1.393	0.88–2127	0.128
Hp2	90 (52.3%)	104 (60.5%)			

Chi-square (χ^2) test with residual analysis

PCOS Polycystic ovary syndrome

*$p < 0.05$ was considered statistically significant

Table 3 Haptoglobin plasma levels (g/L) according to Hp genotypes

Genotype	PCOS	Control	
Hp1-Hp1	8.61 (2.70)	8.43 (7.68)	$p = 1.000$
Hp2-Hp1	9.23 (4.24)	7.49 (3.48)	$p = 0.790$
Hp2-Hp2	7.34 (7.82)	6.31 (4.73)	$p = 0.403$
	$p = 0.492$	$p = 0.349$	

Mann–Whitney and Kruskal–Wallis test

PCOS polycystic ovary syndrome

*$p < 0.05$ was considered statistically significant

with weakest antioxidant activities and reduced ability to bind free Hb. Interestingly, this profile was not observed in PCOS group, probably because other factors strongest influence on serum levels of Hp when compared to polymorphism effect.

Contrary to our results, Alvarez-Blasco et al. [36] reported an association between PCOS and the Hp2 allele [PCOS 62%; control 52%; $p = 0.023$]. In addition, this same study did not observe different Hp concentrations between patients with PCOS and non-hyperandrogenic controls, neither correlation of Hp levels and genotypes. It is noteworthy that Alvarez-Blasco et al. [36] used the same methodology for genotyping by Koch et al. [46], but their study involved Spanish women, while our study included Brazilian individuals. It is possible that the allelic and genotypic frequencies for the *Hp* gene substantially differ between these populations, since the genetic characteristic of the Brazilian population is resulted of European, African, and Amerindian miscegenation.

The main limitation of our study is the need of a relatively large effect size of PCOS to reach statistical significance in the available number of subjects, but the study was sufficiently powered to demonstrate inter-group differences in plasma Hp levels. Still, further studies involving more subjects should be conducted to confirm our results and search for possible correlations between factors, which were not observable under the current sample size.

Conclusion

We observed increased plasma Hp concentration in PCOS women compared to a control group. No association of Hp1-Hp2 polymorphism frequency was associated with PCOS or Hp levels, which suggests that the protein functionality is not changed in this group. Nevertheless, Hp levels are related to inflammatory status, as well as a pro-oxidative profile in PCOS group, indicating that Hp could be a potential biomarker for PCOS complications.

Acknowledgements Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) for the financial support. FMR and KBG are grateful to CNPq Research Fellowship. Simone Martins Gonçalves, Dalva Maria de Resende, for technical support. Special thanks to the patients involved in this study.

Compliance with ethical standards This study was approved by the ethics committees of Federal University of Minas Gerais (Minas Gerais, Brazil)—CAAE 0379.0.203.000-11. All procedures performed were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All the women who participated in this study signed a free and informed consent form.

Conflict of interest The authors declare that they have no conflict of interest.

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