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Assessing C-reactive protein/albumin ratio as a new predictor of Polycystic Ovary Syndrome: a case-control study

Short title: CRP/Albumin as a predictor of PCOS

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Abstract

Objective: Polycystic ovary syndrome (PCOS) is an endocrine disorder affecting approximately one in seven women who experience androgen excess, menstrual cycle irregularities, frequent anovulation, and a tendency for central obesity and insulin resistance. Chronic subclinical inflammation is now recognized as being common in the context of PCOS, which led to the postulation that PCOS may fundamentally be an inflammatory process. This study aimed to: 1) evaluate serum CRP/albumin ratio as a predictor of PCOS; 2) compare the relationship between CRP/albumin and PCOS to classical predictors of the syndrome.

Design: Case-control study.

Setting: Adult obstetrics/gynaecology, endocrinology and outpatient clinics; university hospital in Bahrain.

Participants: 200 premenopausal women with a diagnosis of PCOS, and 119 ethnically-matched eumenorrheic premenopausal women.

Main Outcome Measures: CRP/albumin ratio, anthropometric measures, insulin resistance, androgen excess.

Results: Independent of body mass index (BMI), receiver operating characteristic (ROC) curve for CRP/albumin ratio as predictor of PCOS was 0.865 (95% CI: 0.824–0.905), which was more sensitive than CRP alone. Binary regression analysis showed that CRP/albumin ratio outperformed classical markers, free androgen index and insulin resistance, in predicting PCOS for every BMI category.

Conclusion: CRP/albumin ratio, a marker for inflammation related to metabolic dysfunction, better predicts PCOS than either androgen excess or insulin resistance. Inflammation is known to be influenced by adiposity, but relative to controls, women with PCOS have higher

levels of CRP/albumin irrespective of BMI. These findings support the view that inflammation plays a central role in the pathophysiology of PCOS.

Article Summary

Strengths and limitations of this study

- This analysis addressed previous limitations of studies, namely small sample sizes, heterogeneous populations, and confounding factors (such as BMI), that have attempted to show PCOS is an inflammatory process
- The relationship between inflammation and PCOS was assessed using CRP/albumin ratio, which may be a better marker for inflammation in the context of metabolic dysfunction
- Limitation: study used waist circumference as a substitute for visceral adiposity; gold standard is computed tomography (CT) or magnetic resonance imaging (MRI)

Introduction

Polycystic ovary syndrome (PCOS) is the most common reproductive disorder, affecting 5 to 15 % of premenopausal women worldwide [1, 2]. The prevalence of PCOS is increasing [1], which may partly be attributed to improved diagnosis, as well as an increase in environmental factors that may predispose to the development of this complex metabolic condition. PCOS is characterized by androgen excess, menstrual irregularities, ovulatory disturbances, and is often associated with central obesity and insulin resistance [3-5]. As such, women with PCOS are at an increased risk for a number of health issues, including infertility, cardiovascular disease and diabetes [3, 6, 7].

Possibly related to the constellation of endocrine and metabolic dysfunction they experience, women with PCOS are also found to have greater chronic subclinical inflammation [8-11], which is often clinically assessed by measuring serum levels of C-reactive protein (CRP). CRP is a liver-derived acute phase protein produced in response to IL-6 secreted from activated cells such as macrophages and adipocytes [12, 13]. A meta-analysis of 31 studies concluded that systemic CRP levels are 96% higher in women with PCOS compared to control women [14]. Collectively, these findings have given rise to the speculation that inflammation may play a pivotal role in the pathophysiology of PCOS [8-10].

Elevated serum CRP levels are linked to several health risk factors experienced by women with PCOS, particularly insulin resistance and heightened risk of type 2 diabetes [7, 15, 16]. Chronic inflammation also contributes to endothelial dysfunction, exacerbating the development of atherosclerotic plaques, triggering the onset of cardiovascular disease (CVD) [17]. As such inflammation has been associated with both CVD and coronary artery disease in women with PCOS [10].

In contrast to CRP, albumin is a negative acute phase response protein produced by the liver. Serum levels of albumin are reduced in individuals experiencing chronic inflammation [18]. In addition to its role as a binding molecule for sex steroids [19], albumin also provides the majority of the total antioxidant capacity of normal plasma [18]. The ratio of serum CRP levels over serum albumin (CRP/albumin) was found to be strongly associated with more severe metabolic dysfunction in premenopausal women with induced alterations to their ovarian hormone status [20]. CRP/albumin ratio was also found to be significantly higher in premenopausal women with PCOS relative to controls, and adversely predicted their bone quality [21]. Given the ability of CRP/albumin to simultaneously capture chronic inflammation and metabolic dysfunction in premenopausal women, we hypothesized that CRP/albumin ratio would, in itself, serve as a strong predictor of PCOS in a cohort of similarly aged women. This case-control study investigated CRP/albumin ratio along with classical markers, androgen excess and insulin resistance, in their ability to predict PCOS in 319 premenopausal Bahraini Arab women.

Methods

Study subjects

Women with PCOS (n = 200) were recruited from adult obstetrics/gynecology, endocrinology and outpatient clinics in Manama, Bahrain. Women without PCOS (n = 119) were ethnically-matched, eumenorrheic university employees and students, and healthy volunteers representative of the Bahraini population. Women serving as controls were examined in the follicular phase of their menstrual cycle, and had their testosterone levels were within range. A diagnosis of PCOS was based on the 2003 Rotterdam Criteria, which requires two of the three following criteria to be met: ultrasound evidence of polycystic ovarian morphology, anovulation, and hyperandrogenism [22]. Exclusion criteria included

hyperprolactinemia, non-classical adrenal hyperplasia, androgen-producing tumors, 21-hydroxylase deficiency, Cushing's syndrome, and active thyroid disease. Additional exclusion criteria included extremes of body mass index (BMI; <18 kg/m^2 or >45 kg/m^2), recent/present illness, and treatment affecting carbohydrate metabolism or hormonal levels, for three months or longer before inclusion the study. Women using anti-hypertensive, oral contraceptive, anti-inflammatory, and lipid-lowering drugs were also excluded. Demographic information, along with detailed personal and family history of diabetes, hypertension, infertility, hypercholesterolemia, and ischemic heart disease were obtained from all participants. This study was conducted in accord with the Helsinki II Declaration guidelines, and all participants gave written informed consent to participate. Study approval was obtained from the Bahraini Ministry of Health and Arabian Gulf University Research and Ethics Committees (IRB number: 35-PI-01/15) and the Clinical Research Ethics Board of the University of British Columbia (H16-02101).

Biochemical analysis

Peripheral venous fasting blood samples were obtained between 7:00 and 9:00 am following an overnight (> 12 h) fast during the early follicular phase of the menstrual cycle (days 2 ± 5) for control women, or women with PCOS who did not present with menstrual irregularities, or any day for women with PCOS with menstrual irregularities. Serum samples were analyzed for sex hormone binding globulin (SHBG) by sandwich ELISA (R&D Systems, Minneapolis, MN); assay sensitivity was 0.01 nmol/ml, and inter-assay and intra-assay precision (CV %) were 5.3% and 4.3%, respectively.

Serum luteinizing hormone (LH), follicular stimulating hormone (FSH), thyroid stimulating hormone (TSH), testosterone, glucose (ADVIA Centaur, Bayer Vital, Fernwald, Germany), and insulin (IMMULITE 2000, DPC Biermann, Bad Nauheim, Germany), were measured by

automated chemiluminescence immunoassays. Free (FT) and bioactive (BT) testosterone and free androgen index (FAI) were determined using Free & Bioavailable Testosterone Calculator (www.issam.ch/freetesto.htm). Concentrations of serum albumin were analyzed by photospectrometry with albumin bromocresol purple assay on a COBAS c701 Chemistry Analyzer (Roche Diagnostics, Dubai, UAE). Insulin resistance (IR) was estimated by the homeostasis model assessment (HOMA-IR), defined as fasting serum insulin (IU/mL) × fasting plasma glucose (mmol/L)/22.5.

Measurement of plasma high sensitivity CRP levels was done by latex-enhanced nephelometry on a BN II Nephelometer (Dade Behring, Milan, Italy). Samples were assayed in duplicate in each analytical run; the lower limit of detection was 0.15 mg/L, and the assay range was 0.175–11.0 mg/L (initial dilution). Serial serum dilutions were made in measuring high CRP (>30 mg/L) levels. Percentile CRP values were estimated for comparison purposes.

Statistical analysis

The *core outcome set* of variables included assessment of CRP/albumin as a predictor of PCOS while controlling for relevant factors, such as BMI and age, and to subsequently compare the strength of the relationship between CRP/albumin and PCOS with classical predictors of the syndrome, androgen excess and insulin resistance. Baseline characteristics were compared using the Mann-Whitney U test and the independent samples t-test for the continuous variables, and the $\chi 2$ test for categorical variables. Numerical variables are presented as mean ± standard deviation (SD). Because distribution of CRP/albumin levels was skewed to the right, correlations between CRP/albumin ratio and other continuous variables were assessed using Spearman's rho. Univariate general linear models were applied to test independent associations between CRP/albumin ratio and other independent variables.

The optimal cut-off level for the CRP/albumin ratio was determined by a receiver operating characteristic (ROC) curve analysis, and the areas under the curve (AUC) were measured and compared to assess the power of a model to identify patients who experienced metabolic disturbances. Cut-off values showing the greatest accuracy were determined using a sensitivity/specificity versus criterion value plot. Quartiles of CRP and CRP/albumin ratio were calculated separately in PCOS and control groups; and since 25% quartile of CRP value in patient group was very close to standard value of normal level of CRP, we decided to use 25 quartile values for CRP and CRP/albumin ratio in PCOS group as cut-off values for the two predictors respectively.

Using the calculated cut-off values, regression analysis was performed to determine how well each variable predicted PCOS. To fully explore the role of the CRP/albumin ratio as a biomarker in the prediction of metabolic disturbances, CRP and CRP/albumin ratio were additionally assessed as binary variables. Subjects were categorized into two groups based on the cut-off values and their means (SD) for metabolic markers. Insulin resistance (HOMA-IR), free testosterone, total adiponectin, BMI and TSH were compared between subjects with normal values, and those who had higher than normal values. P-values <0.05 were considered as statistically significant. All statistical analyses were performed using the IBM SPSS statistics software program version 22 (IBM, Armonk, NY).

Results

Study subjects

The sociodemographic, anthropometric, clinical, and biochemical characteristics of the 200 women with PCOS and 119 controls women are summarized in **Table 1**. Relative to controls, women with PCOS had fewer pregnancies and live births (p<0.001), were more likely to

have insulin resistance ($p<0.001$), and differed in education attainment with those with PCOS, having a higher number of high school and post-secondary graduates ($p=0.012$).

Metabolic characteristics

The proportion of women with a BMI greater than 30 kg/m^2 were higher in the PCOS cohort than controls ($p<0.001$), but the waist-to-hip ratio was not significantly different (**Table 1**). Serum levels of adiponectin, an adipocyte-associated protein that tends to be inversely linked to visceral adiposity [23], was markedly lower in women with PCOS compared to controls. Fasting plasma glucose, cholesterol, HDL, LDL and triglyceride levels did not differ significantly between women with and without PCOS. However, indices of insulin resistance and insulin sensitivity (HOMA-IR and QUICKI) indicated that women with PCOS had greater impaired regulation of insulin compared to controls (**Table 1**).

Table 1: Demographic, clinical and hormonal characteristics of study population: women with polycystic ovary syndrome (PCOS) and controls.

Data are presented as means (standard deviation).

	All (N=319)	**PCOS** (N=200)	**Controls** (N=119)	Mean difference	95 % CI of Mean Difference	
					lower	upper
Age (yrs)	27.9 (6.4)	28.4 (5.9)	27.2 (7.2)	1.24	-0.22	2.71
BMI (kg/m^2)	28 (5.9)	29 (6.3)	26.5 (5)	2.53	1.19	3.87
Waist/hip ratio	0.94 (0.09)	0.94 (0.09)	0.93 (0.09)	0.0067	-0.017	0.031
Menarche (yrs)	12.5 (1.4)	12.5 (1.5)	12.4 (1.2)	0.12	-0.21	0.45
HOMA.IR	3.2 (0.18)	3.8 (0.26)	2.1 (0.2)	1.67	0.94	2.4
QUICKI	0.6 (0.006)	0.57 (0.008)	0.65 (.01)	-0.078	-0.10	-0.05
Total adiponectin (ng/L)	33.8 (1.4)	28.4 (1.3)	44.6 (2.9)	-16.25	-21	-10

Albumin (g/L)	37.3 (0.46)	32.7 (0.46)	45 (0.35)	-12.22	-13.52	-10.93
CRP (mg/L)	11.1 (1.1)	15.5 (1.6)	3.6 (0.85)	11.89	7.61	16.17
CRP/Alb ratio	0.36 (0.04)	0.53 (0.06)	0.08 (0.02)	0.45	0.29	0.61
SHBG (nmol/L)	60.1 (1.5)	52.2 (1.4)	72 (2.8)	-19.72	-26.5	-13.93
Free testosterone index	0.025 (0.001)	0.029 (0.001)	0.017 (0.001)	0.011	0.007	0.015

BMI: Body Mass Index, HOMA.IR: Homeostatic Model Assessment for Insulin Resistance, QUICKI: Quantitative insulin sensitivity check index, CRP: C-reactive protein, SHBG: Sex hormone binding hormone.

Reproductive hormone characteristics

There were no statistically significant differences in plasma levels of estradiol, progesterone, total testosterone, prolactin, FSH, LH and DHES between women with and without PCOS. Free testosterone was higher and SHBG lower in women with PCOS (**Table 1**).

C-reactive protein (CRP)/albumin ratio as a predictor of polycystic ovary syndrome (PCOS) stratified by body mass index (BMI)

Women with PCOS had markedly higher levels of CRP and lower levels of serum albumin relative to controls ($p<0.001$; **Table 1**). ROC curve analysis showed that the CRP/albumin ratio had greater discriminatory power to differentiate between women with PCOS and controls (AUC: 0.865, 95% CI: 0.824-0.905) compared to CRP alone (AUC: 0.820, 95% CI: 0.773-0.867); **Figure 1**. This greater efficacy of CRP/albumin ratio to discriminate between cases and controls was also evident when taking into account the presence of insulin resistance at every measure of sensitivity; for a sensitivity level of 75%, CRP/albumin ratio had a specificity of 85% compared to 69% for CRP alone.

Spearmen correlation analysis between CRP/albumin ratios and clinical and biochemical markers was subsequently performed. Variables found to be univariately linked to CRP/albumin values were included in a general linear model testing the relationship amongst PCOS diagnosis, BMI, and CRP/albumin levels. The model revealed that for any given BMI value, women with PCOS have markedly elevated CRP/albumin levels ($p<0.001$, **Figure 2**).

Classical predictors of PCOS, free androgen index and insulin resistance, were compared to CRP/albumin ratio as predictors of PCOS in a binary regression analysis stratified by three BMI categories: <25 kg/m^2 [normal], 25 – 29.9 kg/m^2 [overweight], >30 kg/m^2 [obese]); **Table 2**. A CRP/albumin ratio of ≥0.097 outperformed both insulin resistance and free androgen index in predicting PCOS for every BMI category (**Table 2**).

Table 2: Summary of binary Regression Analysis for Variables Predicting PCOS with the Odds Ratio of each risk factor adjusted for other variables in the model

	Odds Ratio (95% Confidence Interval)		
	BMI < 25 (normal)	BMI 25-29.9 (overweight)	BMI ≥ 30 (obese)
CRP/Albumin Ratio[1]			
< 0.097	1	1	1
≥0.097	11.21 (3.28-39.75)	19.32 (5.07-72.17)	34.5 (7.75-153.52)
Insulin Resistance[2]			
No	1	1	1
Borderline	3.34 (0.645-17.33)	5.58 (0.907-34.41)	3.13 (0.53-18.48)
Yes	9.21 (1.63-51.93)	8.81 (1.75-44.31)	17.94 (1.81-177.61)
Free Androgen Index[3]			
<3.95	1	1	1

≥3.95	2.28 (.536-9.75)	0.86 (.22-3.35)	3.79 (0.59-24.42)

[1]Cut-off values were derived from the sensitivity and specificity analysis of the receiver operating characteristic curve

[2]Categories of insulin resistance based on normal values used for HOMA-IR

[3]Cut-off values from normal laboratory reference ranges for free androgen index

Discussion

This study demonstrated that CRP/albumin ratio is a better predictor of PCOS than both free androgens and insulin resistance in 319 ethnically-matched premenopausal women, and this relationship was independent of BMI. Despite being the most common reproductive disorder to affect women, the etiology of PCOS has remained elusive to date. In the absence of a definitive cure, treatment has focused on symptom management, and a goal to prevent the progression of serious health conditions, such as type 2 diabetes and CVD, for which women with PCOS are at heightened risk. Chronic low-grade inflammation has emerged as a common underlying state in women with PCOS, and a likely direct contributor to insulin resistance and heart disease risk. This has raised the question whether PCOS is fundamentally an inflammatory condition [8, 10, 24].

Small sample sizes, heterogeneous populations, and an inability to correct for confounding factors, such as BMI and use of oral contraceptives, both of which influence inflammatory markers such as CRP [21], has in part hampered efforts in assigning inflammation as truly a defining feature of PCOS. This current analysis has overcome some of the main issues in assessing the independent relationship between PCOS and chronic low-grade inflammation. This was accomplished by accounting for many of the confounding variables and by using a more refined marker for inflammation, the CRP/albumin ratio, which may have greater

specificity and sensitivity for inflammation associated with metabolic dysfunction. The CRP/albumin ratio was first found to be useful in assessing cardiometabolic and inflammatory status following ovariectomy surgery [20]. It was subsequently used to show the influence of chronic subclinical inflammation on bone quality in women with PCOS [21]. Although CRP is used to predict cardiovascular risk and is associated with metabolic disorders associated with obesity and insulin resistance [25-28], it has been criticized for being too general and non-specific a marker for inflammation [29].

When compared to CRP alone, we found that the CRP/albumin ratio had an improved ROC curve for predicting PCOS. Serum albumin, which is commonly measured to assess liver function and malnutrition, is not widely considered as an analyte of interest for PCOS. However, this study showed for the first time that albumin is markedly reduced in women with PCOS relative to controls. This may, at least in part, be due to albumin being a negative acute phase protein [18]. It is also possible that there is increased oxidation and glycation of albumin in women with PCOS - which can impact the structure, function and metabolism of the protein [18]. As one of the most abundant serum proteins, among albumin's many roles is the transport of hormones [19]. Thus, reduced albumin levels can potentially contribute to higher free androgens in women with PCOS and exacerbation of disease phenotype.

This analysis was limited by a lack of a more sensitive measure of visceral adiposity; the gold standard being imaging with computed tomography (CT) or magnetic resonance imaging (MRI) [30]. Furthermore, the case-control design limited the ability to assess how CRP/albumin performs in predicting health outcomes in women with PCOS. Prospective studies are now needed to determine the use of CRP/albumin in predicting the progression of disorders linked to chronic inflammation and metabolic dysfunction that women with PCOS are at increased risk. These include not only cardiovascular disease and diabetes, but also depression [31-34]. Importantly, CRP/albumin ratio may be particularly useful in assessing the effectiveness of

new interventions targeting inflammation in women with PCOS as a novel approach to managing the condition and its long-term health consequences.

Conclusion: CRP/albumin ratio, a marker for inflammation related to metabolic dysfunction, is a better predictor of PCOS than either androgen excess or insulin resistance. Inflammation is known to be influenced by adiposity, but relative to controls, women with PCOS have higher levels of CRP/albumin ratio irrespective of BMI. This supports the view that inflammation may play a central role in the pathophysiology of PCOS.

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Competing Interests Statement

SK is Director of Scientific Innovation at Qu Biologics Inc., a clinical-stage biotechnology company. All other authors have no conflict of interest to declare.

Author Contributions

SK designed the study, interpreted the data and wrote the first draft of the manuscript. AG performed the statistical analysis and helped interpret the analysis. SS and WA collected all the data, performed the biochemical analysis and managed the clinical operations of the study. AJ assisted with literature review and manuscript preparation. All authors reviewed and approved the final manuscript.

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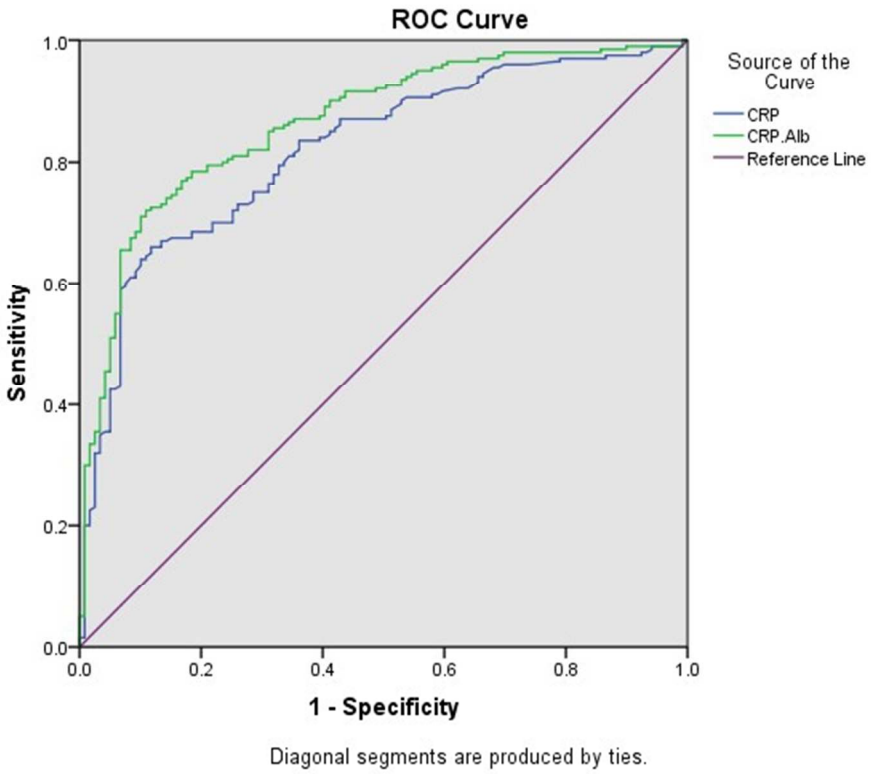
Figure Legends

Figure 1. Receiver Operating Characteristic (ROC) curve plotting the true positive rate against the false positive rate for CRP/Albumin (green line) and CRP (blue line) in differentiating women with and without PCOS. The area under the curve (AUC) for CRP/Albumin: 0.865, 95% CI: 0.824-0.905; for CRP: 0.820, 95% CI: 0.773-0.867.

Figure 2. Scatter plot analysis showing the age-adjusted CRP/albumin values by body mass index (BMI) in women with PCOS and controls.



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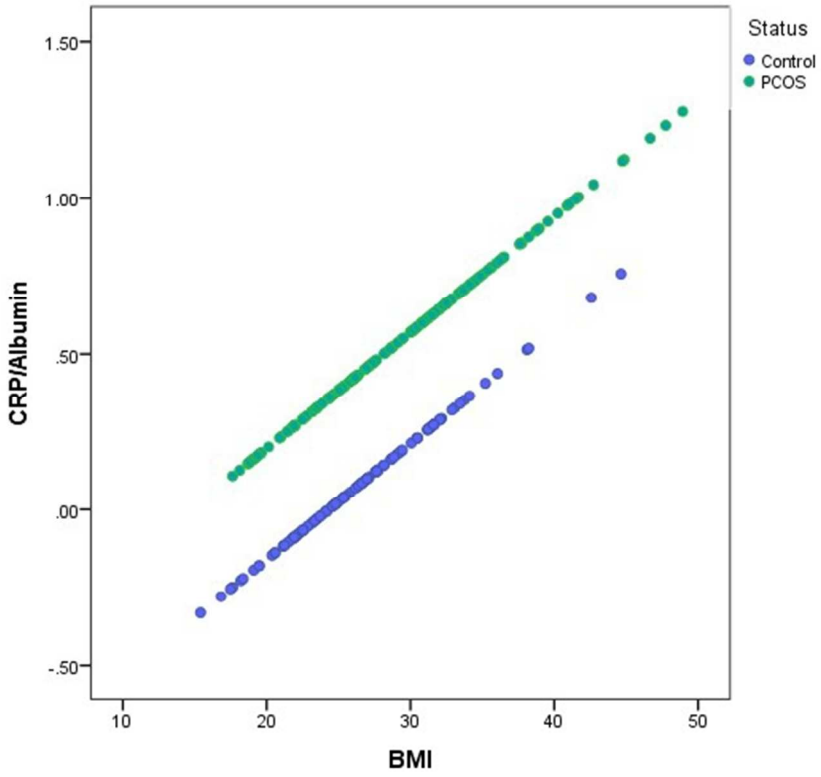

Figure 2. Scatter plot analysis showing the age-adjusted CRP/albumin values by body mass index (BMI) in women with PCOS and controls.

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Assessing C-reactive protein/albumin ratio as a new predictor of Polycystic Ovary Syndrome: a case-control study of women from Bahraini medical clinics

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Assessing C-reactive protein/albumin ratio as a new predictor of Polycystic Ovary Syndrome: a case-control study of women from Bahraini medical clinics

Short title: CRP/Albumin as a predictor of PCOS

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Abstract

Objective: Polycystic ovary syndrome (PCOS) is an endocrine disorder affecting approximately one in seven women who experience androgen excess, menstrual cycle irregularities, frequent anovulation, and a tendency for central obesity and insulin resistance. Chronic subclinical inflammation is now recognized as being common in the context of PCOS, which led to the postulation that PCOS may fundamentally be an inflammatory process. This study aimed to: 1) evaluate serum CRP/albumin ratio as a predictor of PCOS; 2) compare the relationship between CRP/albumin and PCOS to variables classically associated with the syndrome.

Design: Case-control study.

Setting: Adult obstetrics/gynaecology, endocrinology and outpatient clinics; university hospital in Bahrain.

Participants: 200 premenopausal women with a diagnosis of PCOS, and 119 ethnically-matched eumenorrheic premenopausal women.

Main Outcome Measures: CRP/albumin ratio, anthropometric measures, insulin resistance, androgen excess.

Results: Independent of body mass index (BMI), receiver operating characteristic (ROC) curve for CRP/albumin ratio as predictor of PCOS was 0.865 (95% CI: 0.824–0.905), which was more sensitive than CRP alone. Binary regression analysis showed that CRP/albumin ratio outperformed classical correlates, free androgen index and insulin resistance, in predicting PCOS for every BMI category.

Conclusion: CRP/albumin ratio, a marker for inflammation related to metabolic dysfunction, better predicts PCOS than either androgen excess or insulin resistance. Inflammation is known to be influenced by adiposity, but relative to controls, women with PCOS have higher

levels of CRP/albumin irrespective of BMI. These findings support the view that inflammation plays a central role in the pathophysiology of PCOS.

Article Summary

Strengths and limitations of this study

- This analysis addressed previous limitations of studies, namely small sample sizes, heterogeneous populations, and confounding factors (such as BMI), that have attempted to show PCOS is an inflammatory process
- The relationship between inflammation and PCOS was assessed using CRP/albumin ratio, which may be a better marker for inflammation in the context of metabolic dysfunction
- Limitation: study used waist circumference as a substitute for visceral adiposity; gold standard is computed tomography (CT) or magnetic resonance imaging (MRI)

Introduction

Polycystic ovary syndrome (PCOS) is the most common reproductive disorder, affecting 5 to 15 % of premenopausal women worldwide, [1, 2] and the prevalence of PCOS appears to be increasing. [1] This rise may partly be attributed to improved diagnosis as well as to an increase in environmental factors that predispose to the development of this complex metabolic condition. PCOS is characterized by androgen excess, menstrual irregularities, ovulatory disturbances, and is often associated with central obesity and insulin resistance. [3-5] As such, women with PCOS are at an increased risk for a number of health issues, including infertility, cardiovascular disease and diabetes. [3, 6, 7]

Possibly related to the constellation of endocrine and metabolic dysfunction they experience, women with PCOS are also found to have greater chronic subclinical inflammation, [8-11] which is often clinically assessed by measuring serum levels of C-reactive protein (CRP). CRP is a liver-derived acute phase protein produced in response to IL-6 secreted from activated cells such as macrophages and adipocytes. [12, 13] A meta-analysis of 31 studies concluded that systemic CRP levels are 96% higher in women with PCOS compared to control women. [14] Collectively, these findings have given rise to the speculation that inflammation may play a pivotal role in the pathophysiology of PCOS. [8-10]

Elevated serum CRP levels are linked to several health risk factors experienced by women with PCOS, particularly insulin resistance and heightened risk of type 2 diabetes. [7, 15, 16] Chronic inflammation also contributes to endothelial dysfunction, exacerbating the development of atherosclerotic plaques, triggering the onset of cardiovascular disease (CVD). [17] As such inflammation has been associated with both CVD and coronary artery disease in women with PCOS. [10]

In contrast to CRP, albumin is a negative acute phase response protein produced by the liver. Serum levels of albumin are reduced in individuals experiencing chronic inflammation. [18] In addition to its role as a binding molecule for sex steroids, [19] albumin also provides the majority of the total antioxidant capacity of normal plasma. [18] The ratio of serum CRP levels over serum albumin (CRP/albumin) was found to be strongly associated with more severe metabolic dysfunction in premenopausal women with induced alterations to their ovarian hormone status. [20] CRP/albumin ratio was also found to be significantly higher in premenopausal women with PCOS relative to controls, and adversely predicted their bone quality. [21] Given the ability of CRP/albumin to simultaneously capture chronic inflammation and metabolic dysfunction in premenopausal women, we hypothesized that CRP/albumin ratio would, in itself, serve as a strong predictor of PCOS in a cohort of similarly aged women. This case-control study investigated CRP/albumin ratio along with classical markers, androgen excess and insulin resistance, in their ability to predict PCOS in 319 premenopausal Bahraini Arab women.

Methods

Patient and public involvement

The development of the research question and the study's setting was influenced by the wish many women in Bahrain have to bear children. Infertility is a consequence of PCOS, [22] and the combination of both can impact women's health-related quality of life. [23] Patients were not involved in the design of the study or the recruitment. Study participants will not be re-contacted by the study investigators; however, the results of this study will be disseminated to the Bahraini community through press releases of the open access publication.

Study subjects

Women with PCOS (n = 200) were recruited from adult obstetrics/gynecology, endocrinology and outpatient clinics in Manama, Bahrain. Women without PCOS (n = 119) were ethnically-matched, eumenorrheic university employees and students, and healthy volunteers representative of the Bahraini population. The sample size was based on the ability to detect differences between cases and controls with 10% precision (two-tailed t-test with a=0.05) and 80% power, taking into account the estimated prevalence of PCOS in Bahrain of 7.5-8.0% (unpublished data; Bahraini Ministry of Health) and Z-value for 95% CI (confidence interval). Women serving as controls were examined in the follicular phase of their menstrual cycle and had their testosterone levels were within range. A diagnosis of PCOS was based on the 2003 Rotterdam Criteria, which requires two of the three following criteria to be met: ultrasound evidence of polycystic ovarian morphology, anovulation, and hyperandrogenism. [24] Exclusion criteria included hyperprolactinemia, non-classical adrenal hyperplasia, androgen-producing tumors, 21-hydroxylase deficiency, Cushing's syndrome, and active thyroid disease. Additional exclusion criteria included extremes of body mass index (BMI; <18 kg/m^2 or >45 kg/m^2), recent/present illness, and treatment affecting carbohydrate metabolism or hormonal levels, for three months or longer before inclusion the study. Women using anti-hypertensive, oral contraceptive, anti-inflammatory, and lipid-lowering drugs were also excluded. Demographic information, along with detailed personal and family history of diabetes, hypertension, infertility, hypercholesterolemia, and ischemic heart disease were obtained from all participants. This study was conducted in accord with the Helsinki II Declaration guidelines, and all participants gave written informed consent to participate. Study approval was obtained from the Bahraini Ministry of Health and Arabian Gulf University Research and Ethics Committees (IRB number: 35-PI-01/15) and the Clinical Research Ethics Board of the University of British Columbia (H16-02101).

Biochemical analysis

Peripheral venous fasting blood samples were obtained between 7:00 and 9:00 am following an overnight (> 12 h) fast during the early follicular phase of the menstrual cycle (days 2 ± 5) for control women, or women with PCOS who did not present with menstrual irregularities, or any day for women with PCOS with menstrual irregularities. Serum samples were analyzed for sex hormone binding globulin (SHBG) by sandwich ELISA (R&D Systems, Minneapolis, MN); assay sensitivity was 0.01 nmol/ml, and inter-assay and intra-assay precision (CV %) were 5.3% and 4.3%, respectively.

Serum luteinizing hormone (LH), follicular stimulating hormone (FSH), thyroid stimulating hormone (TSH), testosterone, glucose (ADVIA Centaur, Bayer Vital, Fernwald, Germany), and insulin (IMMULITE 2000, DPC Biermann, Bad Nauheim, Germany), were measured by automated chemiluminescence immunoassays. Free (FT) and bioactive (BT) testosterone and free androgen index (FAI) were determined using Free & Bioavailable Testosterone Calculator (www.issam.ch/freetesto.htm). Concentrations of serum albumin were analyzed by photospectrometry with albumin bromocresol purple assay on a COBAS c701 Chemistry Analyzer (Roche Diagnostics, Dubai, UAE). Insulin resistance (IR) was estimated by the homeostasis model assessment (HOMA-IR), defined as fasting serum insulin (IU/mL) × fasting plasma glucose (mmol/L)/22.5. HOMA-IR values were characterized as Normal (insulin-sensitive) if <2.40; Borderline if between 2.40-3.50, and High (insulin-resistant) if > 3.50.

Measurement of plasma high sensitivity CRP levels was done by latex-enhanced nephelometry on a BN II Nephelometer (Dade Behring, Milan, Italy). Samples were assayed in duplicate in each analytical run; the lower limit of detection was 0.15 mg/L, and the assay

range was 0.175–11.0 mg/L (initial dilution). Serial serum dilutions were made in measuring high CRP (>30 mg/L) levels. Percentile CRP values were estimated for comparison purposes.

Statistical analysis

The *core outcome set* of variables included assessment of CRP/albumin as a predictor of PCOS while controlling for relevant factors, such as BMI and age, and to subsequently compare the strength of the relationship between CRP/albumin and PCOS with variables known to strongly link to the syndrome, namely androgen excess and insulin resistance. The Shapiro-Wilk test was used to evaluate the distribution of the variables, and many variables for the PCOS cohort were flagged as being non-normally distributed. However, upon testing for skewness, we found the values for asymmetry and kurtosis fell between -2 to +2 for all. Thus, we used parametric tests, as suggested, [25] given the sample sizes were >30. We reassessed the validity of our analysis by running the Mann–Whitney U test in addition to student t test for variables we had detected as being non-normal and confirmed that the results were similar. Baseline characteristics were compared using the Mann-Whitney U test and the independent samples t-test for the continuous variables, and the $\chi 2$ test for categorical variables. Numerical variables are presented as mean ± standard deviation (SD). Because distribution of CRP/albumin levels was skewed to the right, correlations between CRP/albumin ratio and other continuous variables were assessed using Spearman's rho. Univariate general linear models were applied to test independent associations between CRP/albumin ratio and other independent variables.

The optimal cut-off level for the CRP/albumin ratio was determined by a receiver operating characteristic (ROC) curve analysis, and the areas under the curve (AUC) were measured and compared to assess the power of a model to identify patients who experienced metabolic disturbances. Cut-off values showing the greatest accuracy were determined using a

sensitivity/specificity versus criterion value plot. Quartiles (i.e. 0-25%, 26-50%, 51-75%, and 76-100%) of CRP and CRP/albumin ratio were calculated separately in PCOS and control groups. Because the 25% quartile of CRP value in the PCOS cohort was similar to the standard normal values of CRP, we used the 25% quartile of CRP and CRP/albumin ratio as the cut-off point for both predictors..

Using the calculated cut-off values, regression analysis was performed to determine how well each variable predicted PCOS. To fully explore the role of the CRP/albumin ratio as a biomarker in the prediction of metabolic disturbances, CRP and CRP/albumin ratio were additionally assessed as binary variables. Subjects were categorized into two groups based on the cut-off values and their means (SD) for metabolic markers. Insulin resistance (HOMA-IR), free testosterone, total adiponectin, BMI and TSH were compared between subjects with normal values, and those who had higher than normal values. P-values <0.05 were considered as statistically significant. All statistical analyses were performed using the IBM SPSS statistics software program version 22 (IBM, Armonk, NY).

Results

Study subjects

The sociodemographic, anthropometric, clinical, and biochemical characteristics of the 200 women with PCOS and 119 controls women are summarized in **Table 1**. Relative to controls, women with PCOS had fewer pregnancies and live births (p<0.001), were more likely to have insulin resistance (p<0.001), and differed in education attainment with those with PCOS, having a higher number of high school and post-secondary graduates (p=0.012).

Metabolic characteristics

The proportion of women with a BMI greater than 30 kg/m^2 were higher in the PCOS cohort than controls ($p<0.001$), but the waist-to-hip ratio was not significantly different (**Table 1**). Serum levels of adiponectin, an adipocyte-associated protein that tends to be inversely linked to visceral adiposity, [26] was markedly lower in women with PCOS compared to controls. Fasting plasma glucose, cholesterol, HDL, LDL and triglyceride levels did not differ significantly between women with and without PCOS. However, indices of insulin resistance and insulin sensitivity (HOMA-IR and QUICKI) indicated that women with PCOS had greater impaired regulation of insulin compared to controls (**Table 1**).

Table 1: Demographic, clinical and hormonal characteristics of study population: women with polycystic ovary syndrome (PCOS) and controls.

Data are presented as means (standard deviation).

	All (N=319)	**PCOS** (N=200)	**Controls** (N=119)	Mean difference	95 % CI of Mean Difference	
					lower	upper
Age (yrs)	27.9 (6.4)	28.4 (5.9)	27.2 (7.2)	1.24	-0.22	2.71
BMI (kg/m^2)	28 (5.9)	29 (6.3)	26.5 (5)	2.53	1.19	3.87
Waist/hip ratio	0.94 (0.09)	0.94 (0.09)	0.93 (0.09)	0.0067	-0.017	0.031
Menarche (yrs)	12.5 (1.4)	12.5 (1.5)	12.4 (1.2)	0.12	-0.21	0.45
HOMA.IR	3.2 (0.18)	3.8 (0.26)	2.1 (0.2)	1.67	0.94	2.4
QUICKI	0.6 (0.006)	0.57 (0.008)	0.65 (.01)	-0.078	-0.10	-0.05
Total adiponectin (ng/L)	33.8 (1.4)	28.4 (1.3)	44.6 (2.9)	-16.25	-21	-10
Albumin (g/L)	37.3 (0.46)	32.7 (0.46)	45 (0.35)	-12.22	-13.52	-10.93
CRP (mg/L)	11.1 (1.1)	15.5 (1.6)	3.6 (0.85)	11.89	7.61	16.17
CRP/Albumin ratio	0.36 (0.04)	0.53 (0.06)	0.08 (0.02)	0.45	0.29	0.61

SHBG (nmol/L)	60.1 (1.5)	52.2 (1.4)	72 (2.8)	-19.72	-26.5	-13.93
Free testosterone index	0.025 (0.001)	0.029 (0.001)	0.017 (0.001)	0.011	0.007	0.015

BMI: Body Mass Index, HOMA.IR: Homeostatic Model Assessment for Insulin Resistance, QUICKI: Quantitative insulin sensitivity check index, CRP: C-reactive protein, SHBG: Sex hormone binding hormone.

Reproductive hormone characteristics

There were no statistically significant differences in plasma levels of estradiol, progesterone, total testosterone, prolactin, FSH, LH and DHES between women with and without PCOS. Free testosterone was higher and SHBG lower in women with PCOS (**Table 1**).

C-reactive protein (CRP)/albumin ratio as a predictor of polycystic ovary syndrome (PCOS) stratified by body mass index (BMI)

Women with PCOS had markedly higher levels of CRP and lower levels of serum albumin relative to controls ($p<0.001$; **Table 1**). ROC curve analysis showed that the CRP/albumin ratio had greater discriminatory power to differentiate between women with PCOS and controls (AUC: 0.865, 95% CI: 0.824-0.905) compared to CRP alone (AUC: 0.820, 95% CI: 0.773-0.867); **Figure 1**. This greater efficacy of CRP/albumin ratio to discriminate between cases and controls was also evident when taking into account the presence of insulin resistance at every measure of sensitivity; for a sensitivity level of 75%, CRP/albumin ratio had a specificity of 85% compared to 69% for CRP alone.

Spearmen correlation analysis between CRP/albumin ratios and clinical and biochemical markers was subsequently performed. Variables found to be univariately linked to CRP/albumin values were included in a general linear model testing the relationship amongst

PCOS diagnosis, BMI, and CRP/albumin levels. The model revealed that for any given BMI value, women with PCOS have markedly elevated CRP/albumin levels ($p<0.001$, **Figure 2**).

Variables that are known to strongly associate with PCOS, namely free androgen index and insulin resistance, were compared to CRP/albumin ratio as predictors of PCOS in a binary regression analysis stratified by three BMI categories: <25 kg/m^2 [normal], 25 – 30 kg/m^2 [overweight], >30 kg/m^2 [obese]); **Table 2**. A CRP/albumin ratio of ≥0.097 outperformed both insulin resistance and free androgen index in predicting PCOS for every BMI category (**Table 2**).

Table 2: Summary of binary Regression Analysis for Variables Predicting PCOS with the Odds Ratio of each risk factor adjusted for other variables in the model

	Odds Ratio (95% Confidence Interval)		
	BMI < 25 (normal)	BMI 25-30 (overweight)	BMI ≥ 30 (obese)
CRP/Albumin Ratio[1]			
< 0.097	1	1	1
≥0.097	11.21 (3.28-39.75)	19.32 (5.07-72.17)	34.5 (7.75-153.52)
Insulin Resistance[2]			
No	1	1	1
Borderline	3.34 (0.645-17.33)	5.58 (0.907-34.41)	3.13 (0.53-18.48)
Yes	9.21 (1.63-51.93)	8.81 (1.75-44.31)	17.94 (1.81-177.61)
Free Androgen Index[3]			
<3.95	1	1	1
≥3.95	2.28 (.536-9.75)	0.86 (.22-3.35)	3.79 (0.59-24.42)

[1]Cut-off values were derived from the sensitivity and specificity analysis of the receiver operating characteristic curve

[2]Categories of insulin resistance based on normal values used for HOMA-IR

[3]Cut-off values from normal laboratory reference ranges for free androgen index

Discussion

This study demonstrated that CRP/albumin ratio is a better predictor of PCOS than both free androgens and insulin resistance in 319 ethnically-matched premenopausal women, and this relationship was independent of BMI. Despite being the most common reproductive disorder to affect women, the etiology of PCOS has remained elusive to date. In the absence of a definitive cure, treatment has focused on symptom management, and a goal to prevent the progression of serious health conditions, such as type 2 diabetes and CVD, for which women with PCOS are at heightened risk. Chronic low-grade inflammation has emerged as a common underlying state in women with PCOS, and a likely direct contributor to insulin resistance and heart disease risk. This has raised the question whether PCOS is fundamentally an inflammatory condition. [8, 10, 27]

Small sample sizes, heterogeneous populations, and an inability to correct for confounding factors, such as BMI and use of oral contraceptives, both of which influence inflammatory markers such as CRP, [21] has in part hampered efforts in assigning inflammation as truly a defining feature of PCOS. This current analysis has overcome some of the main issues in assessing the independent relationship between PCOS and chronic low-grade inflammation. This was accomplished by accounting for many of the confounding variables and by using a more refined marker for inflammation, the CRP/albumin ratio, which may have greater specificity and sensitivity for inflammation associated with metabolic dysfunction. The CRP/albumin ratio was first found to be useful in assessing cardiometabolic and

inflammatory status following ovariectomy surgery. [20] It was subsequently used to show the influence of chronic subclinical inflammation on bone quality in women with PCOS. [21] Although CRP is used to predict cardiovascular risk and is associated with metabolic disorders associated with obesity and insulin resistance, [28-31] it has been criticized for being too general and non-specific a marker for inflammation. [32]

When compared to CRP alone, we found that the CRP/albumin ratio had an improved ROC curve for predicting PCOS. Serum albumin, which is commonly measured to assess liver function and malnutrition, is not widely considered as an analyte of interest for PCOS. However, this study showed for the first time that albumin is markedly reduced in women with PCOS relative to controls. This may, at least in part, be due to albumin being a negative acute phase protein. [18] It is also possible that there is increased oxidation and glycation of albumin in women with PCOS - which can impact the structure, function and metabolism of the protein. [18] As one of the most abundant serum proteins, among albumin's many roles is the transport of hormones. [19] Thus, reduced albumin levels can potentially contribute to higher free androgens in women with PCOS and exacerbation of disease phenotype.

This analysis was limited by a lack of a more sensitive measure of visceral adiposity; the gold standard being imaging with computed tomography (CT) or magnetic resonance imaging (MRI). [33] Furthermore, the case-control design limited the ability to assess how CRP/albumin performs in predicting health outcomes in women with PCOS. Prospective studies are now needed to determine the use of CRP/albumin in predicting the progression of disorders linked to chronic inflammation and metabolic dysfunction that women with PCOS are at increased risk. These include not only cardiovascular disease and diabetes, but also depression. [34-37] Importantly, CRP/albumin ratio may be particularly useful in assessing the effectiveness of new interventions targeting inflammation in women with PCOS as a novel approach to managing the condition and its long-term health consequences.

Conclusion: CRP/albumin ratio, a marker for inflammation related to metabolic dysfunction, is a better predictor of PCOS than either androgen excess or insulin resistance. Inflammation is known to be influenced by adiposity, but relative to controls, women with PCOS have higher levels of CRP/albumin ratio irrespective of BMI. This supports the view that inflammation may play a central role in the pathophysiology of PCOS.

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Data Sharing Statement

Due to subject confidentiality, the complete data cannot be made publicly available. However, researchers who would like controlled access to the data are welcome to contact Dr. Wassim Y. Almawi at: wassim.almawi@outlook.com.

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Competing Interests Statement

SK is Director of Scientific Innovation at Qu Biologics Inc., a clinical-stage biotechnology company. All other authors have no conflict of interest to declare.

Author Contributions

SK designed the study, interpreted the data and wrote the first draft of the manuscript. AG performed the statistical analysis and helped interpret the analysis. SS and WA collected all the data, performed the biochemical analysis and managed the clinical operations of the study. AJ assisted with literature review and manuscript preparation. All authors reviewed and approved the final manuscript.

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Figure Legends

Figure 1. Receiver Operating Characteristic (ROC) curve plotting the true positive rate against the false positive rate for CRP/Albumin (green line) and CRP (blue line) in differentiating women with and without PCOS. The area under the curve (AUC) for CRP/Albumin: 0.865, 95% CI: 0.824-0.905; for CRP: 0.820, 95% CI: 0.773-0.867.

Figure 2. Linear regression analysis of adjusted CRP/albumin values by body mass index (BMI) in women with PCOS and controls.

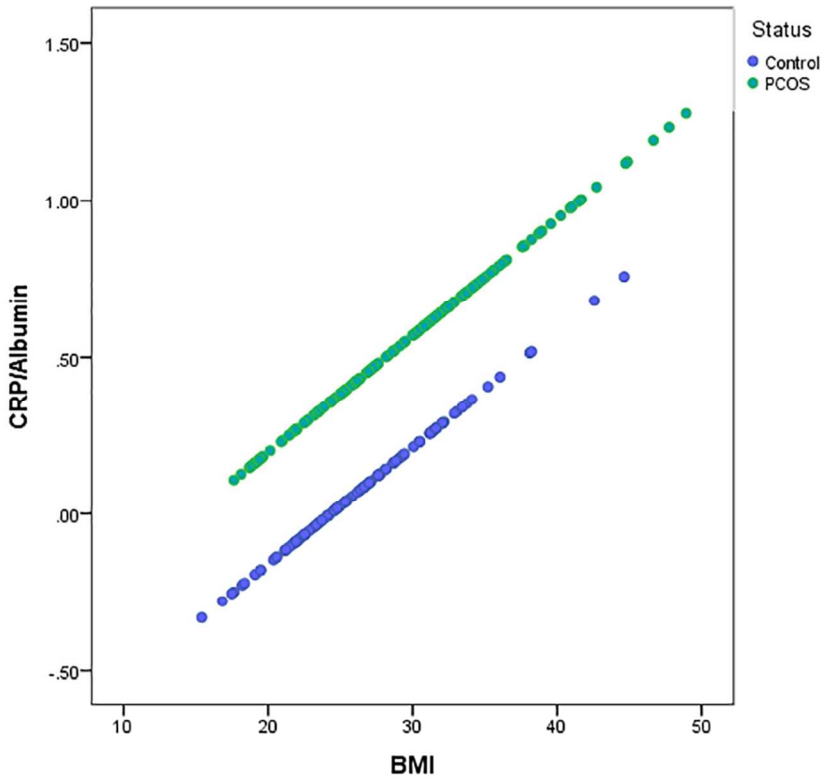

Figure 2. Linear regression analysis of adjusted CRP/albumin values by body mass index (BMI) in women with PCOS and controls. A univariate generalized linear model was computed investigating the relationship between CRP/albumin and BMI, adjusting for the variables found to associate with CRP/albumin (insulin, free testosterone, progesterone and adiponectin) plus age stratified by PCOS diagnosis.

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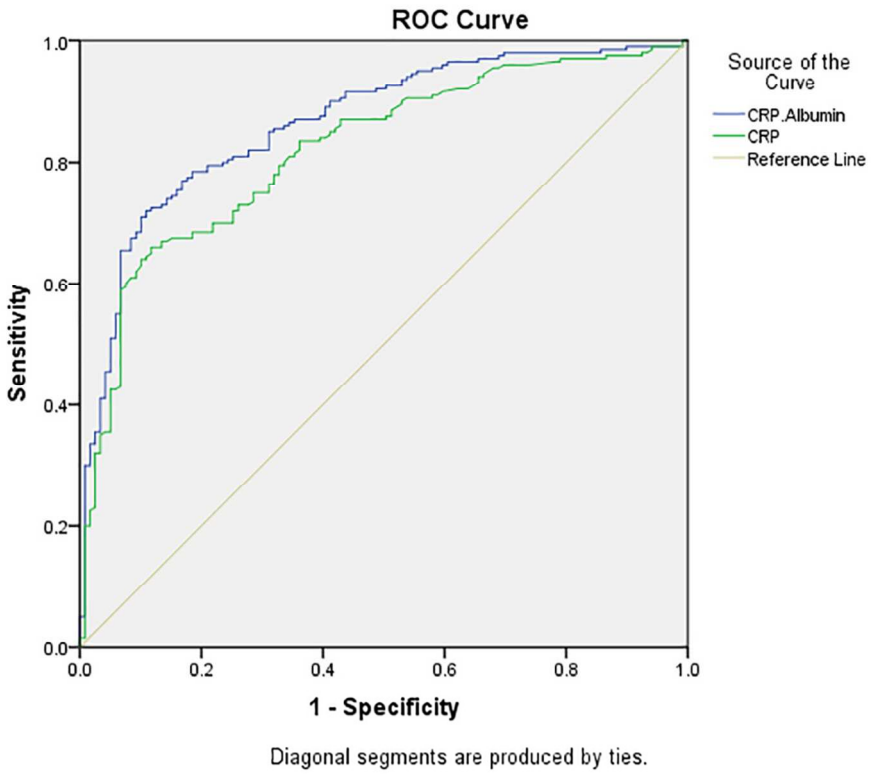

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STROBE Statement

Checklist of items that should be included in reports of observational studies

Section/Topic	Item No	Recommendation	Reported on Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2,3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4-5
Objectives	3	State specific objectives, including any prespecified hypotheses	5
Methods			
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5-6
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	5-6
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	5-7
Data sources/measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	6,7
Bias	9	Describe any efforts to address potential sources of bias	5,6
Study size	10	Explain how the study size was arrived at	5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	7,8
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	7,8
		(*b*) Describe any methods used to examine subgroups and interactions	-
		(*c*) Explain how missing data were addressed	-
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	5
		(*e*) Describe any sensitivity analyses	-

Section/Topic	Item No	Recommendation	Reported on Page No
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	9
		(b) Give reasons for non-participation at each stage	-
		(c) Consider use of a flow diagram	-
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	Table 1
		(b) Indicate number of participants with missing data for each variable of interest	Table 1
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	9, Table 1
		Cross-sectional study—Report numbers of outcome events or summary measures	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	9-11
		(*b*) Report category boundaries when continuous variables were categorized	Table 2, 11
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	-
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	Figures 1-2, Table 2
Discussion			
Key results	18	Summarise key results with reference to study objectives	12-13
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	13-14
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	14
Generalisability	21	Discuss the generalisability (external validity) of the study results	13, 14
Other Information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	14

**Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.*

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ Open

Assessing C-reactive protein/albumin ratio as a new biomarker for Polycystic Ovary Syndrome: a case-control study of women from Bahraini medical clinics

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Keywords:	polycystic ovary syndrome, inflammation, C-reactive protein, albumin, pathophysiology

Assessing C-reactive protein/albumin ratio as a new biomarker for Polycystic Ovary Syndrome: a case-control study of women from Bahraini medical clinics

Short title: CRP/Albumin as a biomarker of PCOS

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Keywords: polycystic ovary syndrome, inflammation, pathophysiology, C-reactive protein, albumin

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Abstract

Objective: Polycystic ovary syndrome (PCOS) is an endocrine disorder affecting approximately one in seven women who experience androgen excess, menstrual cycle irregularities, frequent anovulation, and a tendency for central obesity and insulin resistance. Chronic subclinical inflammation is now recognized as being common in the context of PCOS, which led to the postulation that PCOS may fundamentally be an inflammatory process. This study aimed to: 1) evaluate serum CRP/albumin ratio as a potential predictive biomarker for PCOS; 2) compare the relationship between CRP/albumin and PCOS to variables classically associated with the syndrome.

Design: Case-control study.

Setting: Adult obstetrics/gynaecology, endocrinology and outpatient clinics; university hospital in Bahrain.

Participants: 200 premenopausal women with a diagnosis of PCOS, and 119 ethnically-matched eumenorrheic premenopausal women.

Main Outcome Measures: CRP/albumin ratio, anthropometric measures, insulin resistance, androgen excess.

Results: Independent of body mass index (BMI), receiver operating characteristic (ROC) curve for CRP/albumin ratio as a selective biomarker for PCOS was 0.865 (95% CI: 0.824–0.905), which was more sensitive than CRP alone. Binary regression analysis showed that CRP/albumin ratio outperformed classical correlates, free androgen index and insulin resistance, in predicting PCOS for every BMI category.

Conclusion: CRP/albumin ratio, a marker for inflammation related to metabolic dysfunction, was found to have a stronger association with PCOS than either androgen excess or insulin resistance. Inflammation is known to be influenced by adiposity, but relative to controls,

women with PCOS have higher levels of CRP/albumin irrespective of BMI. These findings support the view that inflammation plays a central role in the pathophysiology of PCOS.

Article Summary

Strengths and limitations of this study

- This analysis addressed previous limitations of studies, namely small sample sizes, heterogeneous populations, and confounding factors (such as BMI), that have attempted to show PCOS is an inflammatory process
- The relationship between inflammation and PCOS was assessed using CRP/albumin ratio, which may be a better marker for inflammation in the context of metabolic dysfunction
- Limitation: study used waist circumference as a substitute for visceral adiposity; gold standard is computed tomography (CT) or magnetic resonance imaging (MRI)

Introduction

Polycystic ovary syndrome (PCOS) is the most common reproductive disorder, affecting 5 to 15 % of premenopausal women worldwide, [1, 2] and the prevalence of PCOS appears to be increasing. [1] This rise may partly be attributed to improved diagnosis as well as to an increase in environmental factors that predispose to the development of this complex metabolic condition. PCOS is characterized by androgen excess, menstrual irregularities, ovulatory disturbances, and is often associated with central obesity and insulin resistance. [3-5] As such, women with PCOS are at an increased risk for a number of health issues, including infertility, cardiovascular disease and diabetes. [3, 6, 7]

Possibly related to the constellation of endocrine and metabolic dysfunction they experience, women with PCOS are also found to have greater chronic subclinical inflammation, [8-11] which is often clinically assessed by measuring serum levels of C-reactive protein (CRP). CRP is a liver-derived acute phase protein produced in response to IL-6 secreted from activated cells such as macrophages and adipocytes. [12, 13] A meta-analysis of 31 studies concluded that systemic CRP levels are 96% higher in women with PCOS compared to control women. [14] Collectively, these findings have given rise to the speculation that inflammation may play a pivotal role in the pathophysiology of PCOS. [8-10]

Elevated serum CRP levels are linked to several health risk factors experienced by women with PCOS, particularly insulin resistance and heightened risk of type 2 diabetes. [7, 15, 16] Chronic inflammation also contributes to endothelial dysfunction, exacerbating the development of atherosclerotic plaques, triggering the onset of cardiovascular disease (CVD). [17] As such inflammation has been associated with both CVD and coronary artery disease in women with PCOS. [10]

In contrast to CRP, albumin is a negative acute phase response protein produced by the liver. Serum levels of albumin are reduced in individuals experiencing chronic inflammation. [18] In addition to its role as a binding molecule for sex steroids, [19] albumin also provides the majority of the total antioxidant capacity of normal plasma. [18] The ratio of serum CRP levels over serum albumin (CRP/albumin) was found to be strongly associated with more severe metabolic dysfunction in premenopausal women with induced alterations to their ovarian hormone status. [20] CRP/albumin ratio was also found to be significantly higher in premenopausal women with PCOS relative to controls, and adversely predicted their bone quality. [21] Given the ability of CRP/albumin to simultaneously capture chronic inflammation and metabolic dysfunction in premenopausal women, we hypothesized that CRP/albumin ratio may serve as a strong predictor of PCOS in a cohort of similarly aged women. This case-control study investigated CRP/albumin ratio along with classical markers, androgen excess and insulin resistance, in their association with PCOS in 319 premenopausal Bahraini Arab women.

Methods

Patient and public involvement

The development of the research question and the study's setting was influenced by the wish many women in Bahrain have to bear children. Infertility is a consequence of PCOS, [22] and the combination of both can impact women's health-related quality of life. [23] Patients were not involved in the design of the study or the recruitment. Study participants will not be re-contacted by the study investigators; however, the results of this study will be disseminated to the Bahraini community through press releases of the open access publication.

Study subjects

Women with PCOS (n = 200) were recruited from adult obstetrics/gynecology, endocrinology and outpatient clinics in Manama, Bahrain. Women without PCOS (n = 119) were ethnically-matched, eumenorrheic university employees and students, and healthy volunteers representative of the Bahraini population. The sample size was based on the ability to detect differences between cases and controls with 10% precision (two-tailed t-test with a=0.05) and 80% power, taking into account the estimated prevalence of PCOS in Bahrain of 7.5-8.0% (Bahraini Ministry of Health; www.moh.gov.bh/Content/Files/Publications/statistics/HS2015) and Z-value for 95% CI (confidence interval). Women serving as controls were examined in the follicular phase of their menstrual cycle and had their testosterone levels were within range. A diagnosis of PCOS was based on the 2003 Rotterdam Criteria, which requires two of the three following criteria to be met: ultrasound evidence of polycystic ovarian morphology, anovulation, and hyperandrogenism. [24] Exclusion criteria included hyperprolactinemia, non-classical adrenal hyperplasia, androgen-producing tumors, 21-hydroxylase deficiency, Cushing's syndrome, and active thyroid disease (overt, central and subclinical hypothyroidism and hyperthyroidism). Additional exclusion criteria included extremes of body mass index (BMI; <18 kg/m^2 or >45 kg/m^2), recent/present illness, and treatment affecting carbohydrate metabolism or hormonal levels, for three months or longer before inclusion the study. Women using anti-hypertensive, oral contraceptive, anti-inflammatory, and lipid-lowering drugs were also excluded. Demographic information, along with detailed personal and family history of diabetes, hypertension, infertility, hypercholesterolemia, and ischemic heart disease were obtained from all participants. This study was conducted in accord with the Helsinki II Declaration guidelines, and all participants gave written informed consent to participate. Study approval was obtained from the Bahraini Ministry of Health and Arabian Gulf

University Research and Ethics Committees (IRB number: 35-PI-01/15) and the Clinical Research Ethics Board of the University of British Columbia (H16-02101).

Biochemical analysis

Peripheral venous fasting blood samples were obtained between 7:00 and 9:00 am following an overnight (> 12 h) fast during the early follicular phase of the menstrual cycle (days 2 ± 5) for control women, or women with PCOS who did not present with menstrual irregularities, or any day for women with PCOS with menstrual irregularities. Serum samples were analyzed for sex hormone binding globulin (SHBG) by sandwich ELISA (R&D Systems, Minneapolis, MN); assay sensitivity was 0.01 nmol/ml, and inter-assay and intra-assay precision (CV %) were 5.3% and 4.3%, respectively. Samples were tested in duplicates for adiponectin levels (Cat. No. DRP300) by sandwich enzyme-linked immunosorbent assay (R&D Systems); assay sensitivity was 0.891 ng/ml, and inter-assay and intra-assay precision (CV%) were 6.5% and 3.5%, respectively.

Serum luteinizing hormone (LH), follicular stimulating hormone (FSH), thyroid stimulating hormone (TSH), testosterone, glucose (ADVIA Centaur, Bayer Vital, Fernwald, Germany), and insulin (IMMULITE 2000, DPC Biermann, Bad Nauheim, Germany), were measured by automated chemiluminescence immunoassays. Free (FT) and bioactive (BT) testosterone and free androgen index (FAI) were determined using Free & Bioavailable Testosterone Calculator (www.issam.ch/freetesto.htm). Progesterone and estradiol serum levels were quantitated by radioimmunoassay, with comparable CV% (< 5%), while DHEA-S levels were measured by solid-phase competitive immunoassay (Immulite; Siemens); inter- and intra-assay CV were 9.4 and 7.0%, respectively.

Concentrations of serum albumin were analyzed by photospectrometry with albumin bromocresol purple assay on a COBAS c701 Chemistry Analyzer (Roche Diagnostics, Dubai, UAE). Insulin resistance (IR) was estimated by the homeostasis model assessment (HOMA-IR), defined as fasting serum insulin (IU/mL) × fasting plasma glucose (mmol/L)/22.5. HOMA-IR values were characterized as Normal (insulin-sensitive) if <2.40; Borderline if between 2.40-3.50, and High (insulin-resistant) if > 3.50.

Measurement of plasma high sensitivity CRP levels was done by latex-enhanced nephelometry on a BN II Nephelometer (Dade Behring, Milan, Italy). Samples were assayed in duplicate in each analytical run; the lower limit of detection was 0.15 mg/L, and the assay range was 0.175–11.0 mg/L (initial dilution). Serial serum dilutions were made in measuring high CRP (>30 mg/L) levels. Percentile CRP values were estimated for comparison purposes.

Statistical analysis

The *core outcome set* of variables included assessment of CRP/albumin as a predictor of PCOS while controlling for relevant factors, such as BMI and age, and to subsequently compare the strength of the relationship between CRP/albumin and PCOS with variables known to strongly link to the syndrome, namely androgen excess and insulin resistance. The Shapiro-Wilk test was used to evaluate the distribution of the variables, and many variables for the PCOS cohort were flagged as being non-normally distributed. However, upon testing for skewness, we found the values for asymmetry and kurtosis fell between -2 to +2 for all. Thus, we used parametric tests, as suggested, [25] given the sample sizes were >30. We reassessed the validity of our analysis by running the Mann–Whitney U test in addition to student t test for variables we had detected as being non-normal and confirmed that the

results were similar. Baseline characteristics were compared using the Mann-Whitney U test and the independent samples t-test for the continuous variables, and the $\chi 2$ test for categorical variables. Numerical variables are presented as mean ± standard deviation (SD). Because distribution of CRP/albumin levels was skewed to the right, correlations between CRP/albumin ratio and other continuous variables were assessed using Spearman's rho. Univariate general linear models were applied to test independent associations between CRP/albumin ratio and other independent variables.

The optimal cut-off level for the CRP/albumin ratio was determined by a receiver operating characteristic (ROC) curve analysis, and the areas under the curve (AUC) were measured and compared to assess the power of a model to identify patients who experienced metabolic disturbances. Cut-off values showing the greatest accuracy were determined using a sensitivity/specificity versus criterion value plot. Quartiles (i.e. 0-25%, 26-50%, 51-75%, and 76-100%) of CRP and CRP/albumin ratio were calculated separately in PCOS and control groups. Because the 25% quartile of CRP value in the PCOS cohort was similar to the standard normal values of CRP, we used the 25% quartile of CRP and CRP/albumin ratio as the cut-off point for both predictors.

Using the calculated cut-off values, regression analysis was performed to determine how well each variable predicted PCOS. To fully explore the role of the CRP/albumin ratio as a biomarker in the prediction of metabolic disturbances, CRP and CRP/albumin ratio were additionally assessed as binary variables. Subjects were categorized into two groups based on the cut-off values and their means (SD) for metabolic markers. Insulin resistance (HOMA-IR), free testosterone, total adiponectin, BMI and TSH were compared between subjects with normal values, and those who had higher than normal values. P-values <0.05 were considered as statistically significant. All statistical analyses were performed using the IBM SPSS statistics software program version 22 (IBM, Armonk, NY).

Results

Study subjects

The sociodemographic, anthropometric, clinical, and biochemical characteristics of the 200 women with PCOS and 119 controls women are summarized in **Table 1**. Relative to controls, women with PCOS had fewer pregnancies and live births ($p<0.001$), were more likely to have insulin resistance ($p<0.001$), and differed in education attainment with those with PCOS, having a higher number of high school and post-secondary graduates ($p=0.012$).

Metabolic characteristics

The proportion of women with a BMI greater than 30 kg/m^2 were higher in the PCOS cohort than controls ($p<0.001$), but the waist-to-hip ratio was not significantly different (**Table 1**). Serum levels of adiponectin, an adipocyte-associated protein that tends to be inversely linked to visceral adiposity, [26] was markedly lower in women with PCOS compared to controls. Fasting plasma glucose, cholesterol, HDL, LDL and triglyceride levels did not differ significantly between women with and without PCOS. However, indices of insulin resistance and insulin sensitivity (HOMA-IR and QUICKI) indicated that women with PCOS had greater impaired regulation of insulin compared to controls (**Table 1**).

Table 1: Demographic, clinical and hormonal characteristics of study population: women with polycystic ovary syndrome (PCOS) and controls.

Data are presented as means (standard deviation).

	All (N=319)	**PCOS** (N=200)	**Controls** (N=119)	**Mean difference**	**95 % CI of Mean Difference**	**p values**

					lower	upper	
Age (yrs)	27.9 (6.4)	28.4 (5.9)	27.2 (7.2)	1.24	-0.22	2.71	0.09
BMI (kg/m^2)	28 (5.9)	29 (6.3)	26.5 (5)	2.53	1.19	3.87	0.000
Waist/hip ratio	0.94 (0.09)	0.94 (0.09)	0.93 (0.09)	0.0067	-0.017	0.031	0.58
Menarche (yrs)	12.5 (1.4)	12.5 (1.5)	12.4 (1.2)	0.12	-0.21	0.45	0.47
HOMA.IR	3.2 (0.18)	3.8 (0.26)	2.1 (0.2)	1.67	0.94	2.4	0.000
QUICKI	0.6 (0.006)	0.57 (0.008)	0.65 (.01)	-0.078	-0.10	-0.05	0.000
Total adiponectin (ng/L)	33.8 (1.4)	28.4 (1.3)	44.6 (2.9)	-16.25	-21	-10	0.000
Albumin (g/L)	37.3 (0.46)	32.7 (0.46)	45 (0.35)	-12.22	-13.52	-10.93	0.000
CRP (mg/L)	11.1 (1.1)	15.5 (1.6)	3.6 (0.85)	11.89	7.61	16.17	0.000
CRP/Albumin ratio	0.36 (0.04)	0.53 (0.06)	0.08 (0.02)	0.45	0.29	0.61	0.000
SHBG (nmol/L)	60.1 (1.5)	52.2 (1.4)	72 (2.8)	-19.72	-26.5	-13.93	0.000
DHEAS (nmol/L)	6.2 (0.28)	6.2 (0.29)	6.3 (0.95)	-0.18	-0.174	1.36	0.81
Total testosterone (nmol/L)	1.7 (0.06)	1.8 (0.07)	1.5 (0.1)	0.23	-0.02	0.49	0.08
Bioavailable testosterone (nmol/L)	0.49 (0.02)	0.52 (0.03)	0.42 (0.03)	0.098	0.015	0.18	0.02
Free Androgen Index	3.4 (0.16	4 (0.22)	2.4 (0.16)	1.57	0.93	2.2	0.000
Free testosterone	0.025 (0.001)	0.029 (0.001)	0.017 (0.001)	0.011	0.007	0.015	0.000

index							

BMI: Body Mass Index, HOMA.IR: Homeostatic Model Assessment for Insulin Resistance, QUICKI: Quantitative insulin sensitivity check index, CRP: C-reactive protein, SHBG: Sex hormone binding hormone.

Reproductive hormone characteristics

There were no statistically significant differences in plasma levels of estradiol, progesterone, total testosterone, prolactin, FSH, LH and DHES between women with and without PCOS. Free testosterone was higher and SHBG lower in women with PCOS (**Table 1**).

C-reactive protein (CRP)/albumin ratio as a predictor of polycystic ovary syndrome (PCOS) stratified by body mass index (BMI)

Women with PCOS had markedly higher levels of CRP and lower levels of serum albumin relative to controls ($p<0.001$; **Table 1**). ROC curve analysis showed that the CRP/albumin ratio had greater discriminatory power to differentiate between women with PCOS and controls (AUC: 0.865, 95% CI: 0.824-0.905) compared to CRP alone (AUC: 0.820, 95% CI: 0.773-0.867); **Figure 1**. This greater efficacy of CRP/albumin ratio to discriminate between cases and controls was also evident when taking into account the presence of insulin resistance at every measure of sensitivity; for a sensitivity level of 75%, CRP/albumin ratio had a specificity of 85% compared to 69% for CRP alone.

Spearmen correlation analysis between CRP/albumin ratios and clinical and biochemical markers was subsequently performed. Variables found to be univariately linked to CRP/albumin values were included in a general linear model testing the relationship amongst PCOS diagnosis, BMI, and CRP/albumin levels. The model revealed that for any given BMI value, women with PCOS have markedly elevated CRP/albumin levels ($p<0.001$, **Figure 2**).

Variables that are known to strongly associate with PCOS, namely free androgen index and insulin resistance, were compared to CRP/albumin ratio as predictors of PCOS in a binary regression analysis stratified by three BMI categories: <25 kg/m^2 [normal], 25 – 30 kg/m^2 [overweight], >30 kg/m^2 [obese]); **Table 2**. A CRP/albumin ratio of ≥0.097 outperformed both insulin resistance and free androgen index in predicting PCOS for every BMI category (**Table 2**).

Table 2: Summary of binary Regression Analysis for Variables Predicting PCOS with the Odds Ratio of each risk factor adjusted for other variables in the model

	Odds Ratio (95% Confidence Interval)		
	BMI < 25 (normal)	BMI 25-30 (overweight)	BMI ≥ 30 (obese)
CRP/Albumin Ratio[1]			
< 0.097	1	1	1
≥0.097	11.21 (3.28-39.75)	19.32 (5.07-72.17)	34.5 (7.75-153.52)
Insulin Resistance[2]			
No	1	1	1
Borderline	3.34 (0.645-17.33)	5.58 (0.907-34.41)	3.13 (0.53-18.48)
Yes	9.21 (1.63-51.93)	8.81 (1.75-44.31)	17.94 (1.81-177.61)
Free Androgen Index[3]			
<3.95	1	1	1
≥3.95	2.28 (.536-9.75)	0.86 (.22-3.35)	3.79 (0.59-24.42)

[1]Cut-off values were derived from the sensitivity and specificity analysis of the receiver operating characteristic curve

[2]Categories of insulin resistance based on normal values used for HOMA-IR

[3]Cut-off values from normal laboratory reference ranges for free androgen index

Discussion

This study demonstrated that CRP/albumin ratio is a stronger correlate of PCOS than both free androgens and insulin resistance in 319 ethnically-matched premenopausal women, and this relationship was independent of BMI. Despite being the most common reproductive disorder to affect women, the etiology of PCOS has remained elusive to date. In the absence of a definitive cure, treatment has focused on symptom management, and a goal to prevent the progression of serious health conditions, such as type 2 diabetes and CVD, for which women with PCOS are at heightened risk. Chronic low-grade inflammation has emerged as a common underlying state in women with PCOS, and a likely direct contributor to insulin resistance and heart disease risk. This has raised the question whether PCOS is fundamentally an inflammatory condition. [8, 10, 27]

Small sample sizes, heterogeneous populations, and an inability to correct for confounding factors, such as BMI and use of oral contraceptives, both of which influence inflammatory markers such as CRP, [21] has in part hampered efforts in assigning inflammation as truly a defining feature of PCOS. This current analysis has overcome some of the main issues in assessing the independent relationship between PCOS and chronic low-grade inflammation. This was accomplished by accounting for many of the confounding variables and by using a more refined marker for inflammation, the CRP/albumin ratio, which may have greater specificity and sensitivity for inflammation associated with metabolic dysfunction. The CRP/albumin ratio was first found to be useful in assessing cardiometabolic and inflammatory status following ovariectomy surgery. [20] It was subsequently used to show the influence of chronic subclinical inflammation on bone quality in women with PCOS. [21] Although CRP is used to predict cardiovascular risk and is associated with metabolic

disorders associated with obesity and insulin resistance, [28-31] it has been criticized for being too general and non-specific a marker for inflammation. [32]

When compared to CRP alone, we found that the CRP/albumin ratio has an improved ROC curve for predicting PCOS. Serum albumin, which is commonly measured to assess liver function and malnutrition, is not widely considered as an analyte of interest for PCOS. However, this study showed for the first time that albumin is markedly reduced in women with PCOS relative to controls. This may, at least in part, be due to albumin being a negative acute phase protein. [18] It is also possible that there is increased oxidation and glycation of albumin in women with PCOS, which can impact the structure, function and metabolism of the protein. [18] Albumin is one of the most abundant serum proteins, and among its many roles is the transport of hormones. [19] Thus, reduced albumin levels can potentially contribute to higher free androgens in women with PCOS and exacerbation of disease phenotype.

This analysis was limited by a lack of a more sensitive measure of visceral adiposity; the gold standard is imaging with computed tomography (CT) or magnetic resonance imaging (MRI). [33] Furthermore, the case-control design limited the ability to assess how CRP/albumin performs in predicting health outcomes in women with PCOS. Prospective studies are now needed to determine the use of CRP/albumin in predicting the progression of disorders linked to chronic inflammation and metabolic dysfunction that women with PCOS are at increased risk. These include not only cardiovascular disease and diabetes, but also depression. [34-37] Importantly, CRP/albumin ratio may be particularly useful in assessing the effectiveness of new interventions targeting inflammation in women with PCOS as a novel approach to managing the condition and its long-term health consequences.

Conclusion: CRP/albumin ratio, a marker for inflammation related to metabolic dysfunction, was found to have a stronger association with PCOS than either androgen excess or insulin resistance. Inflammation is known to be influenced by adiposity, but relative to controls, women with PCOS have higher levels of CRP/albumin ratio irrespective of BMI. This supports the view that inflammation may play a central role in the pathophysiology of PCOS.

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Data Sharing Statement

Due to subject confidentiality, the complete data cannot be made publicly available. However, researchers who would like controlled access to the data are welcome to contact Dr. Wassim Y. Almawi at: wassim.almawi@outlook.com.

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Competing Interests Statement

SK is Director of Scientific Innovation at Qu Biologics Inc., a clinical-stage biotechnology company. All other authors have no conflict of interest to declare.

Author Contributions

SK designed the study, interpreted the data and wrote the first draft of the manuscript. AG performed the statistical analysis and helped interpret the analysis. SS and WA collected all the data, performed the biochemical analysis and managed the clinical operations of the study. AJ assisted with literature review and manuscript preparation. All authors reviewed and approved the final manuscript.

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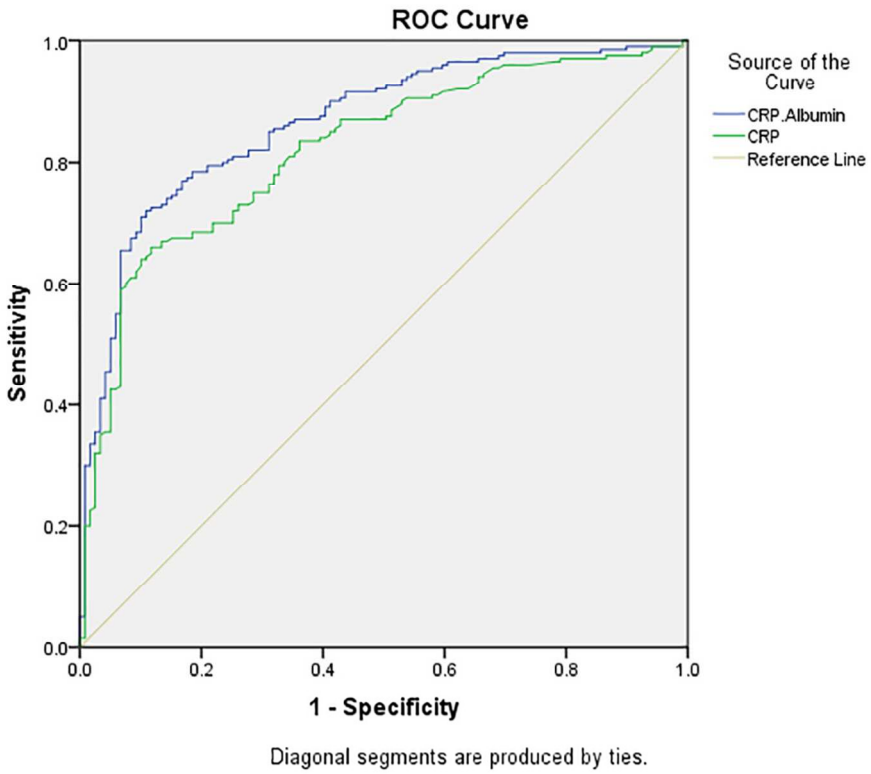
Figure Legends

Figure 1. Receiver Operating Characteristic (ROC) curve plotting the true positive rate against the false positive rate for CRP/Albumin (green line) and CRP (blue line) in differentiating women with and without PCOS. The area under the curve (AUC) for CRP/Albumin: 0.865, 95% CI: 0.824-0.905; for CRP: 0.820, 95% CI: 0.773-0.867.

Figure 2. Linear regression analysis of adjusted CRP/albumin values by body mass index (BMI) in women with PCOS and controls. A univariate generalized linear model was computed investigating the relationship between CRP/albumin and BMI, adjusting for the variables found to associate with CRP/albumin (insulin, free testosterone, progesterone and adiponectin) plus age stratified by PCOS diagnosis.



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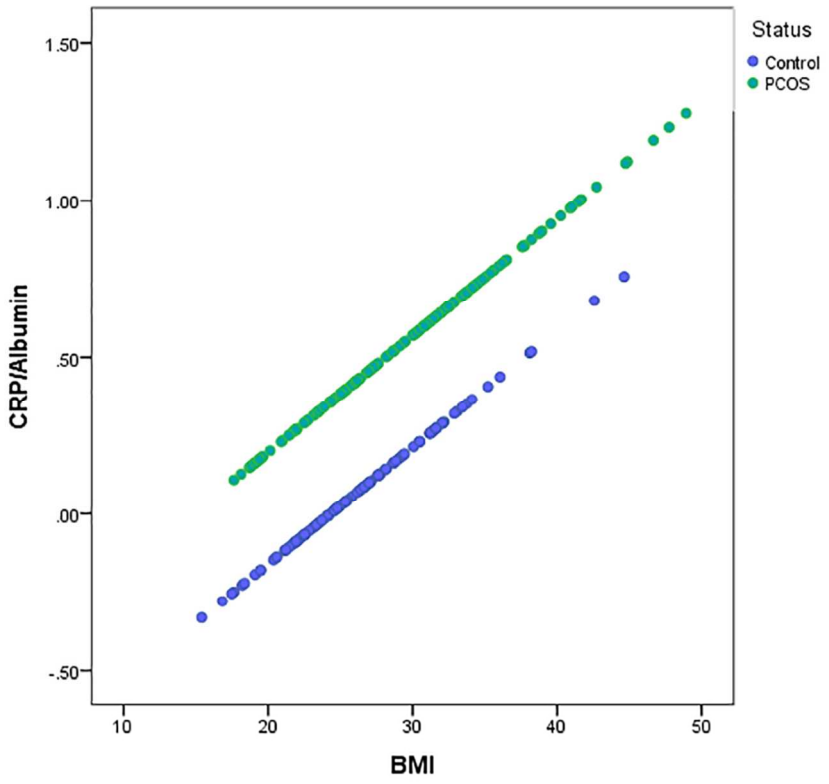

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STROBE Statement

Checklist of items that should be included in reports of observational studies

Section/Topic	Item No	Recommendation	Reported on Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2,3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4-5
Objectives	3	State specific objectives, including any prespecified hypotheses	5
Methods			
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5-6
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	5-6
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	5-7
Data sources/measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	6,7
Bias	9	Describe any efforts to address potential sources of bias	5,6
Study size	10	Explain how the study size was arrived at	5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	7,8
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	7,8
		(*b*) Describe any methods used to examine subgroups and interactions	-
		(*c*) Explain how missing data were addressed	-
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	5
		(*e*) Describe any sensitivity analyses	-

Section/Topic	Item No	Recommendation	Reported on Page No
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	9
		(b) Give reasons for non-participation at each stage	-
		(c) Consider use of a flow diagram	-
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	Table 1
		(b) Indicate number of participants with missing data for each variable of interest	Table 1
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	9, Table 1
		Cross-sectional study—Report numbers of outcome events or summary measures	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	9-11
		(*b*) Report category boundaries when continuous variables were categorized	Table 2, 11
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	-
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	Figures 1-2, Table 2
Discussion			
Key results	18	Summarise key results with reference to study objectives	12-13
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	13-14
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	14
Generalisability	21	Discuss the generalisability (external validity) of the study results	13, 14
Other Information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	14

**Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.*

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.