Polycystic ovary syndrome influences the level of serum amyloid A and activity of phospholipid transfer protein in HDL$_2$ and HDL$_3$

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STUDY QUESTION: Is polycystic ovary syndrome (PCOS) associated with altered levels of pro-inflammatory high-density lipoproteins (HDL) and activity of HDL-associated enzymes?

SUMMARY ANSWER: In PCOS, HDL contained increased levels of the inflammatory marker serum amyloid A (SAA) and altered functioning of HDL-associated phospholipid transfer protein (PLTP), with these changes being independent of BMI, body fat and insulin resistance (IR).

WHAT IS KNOWN ALREADY: PCOS is associated with adipocyte-derived inflammation, which potentially increases the risk of cardiovascular disease and diabetes. SAA is an inflammatory marker that is released from hypertrophic adipocytes and interacts with HDL, reducing their anti-atherogenic properties. No studies have previously investigated if SAA-associated HDL influences the HDL-associated enzymes namely, PLTP and cholesterol ester transfer protein (CETP) in women with PCOS.

PARTICIPANTS/MATERIALS, SETTINGS, METHODS: Obese women with PCOS were matched with controls for BMI and percentage body fat ($n = 100$ group; cohort-1); a subset of these women ($n = 64$ group; cohort-2) were further matched for IR. HDL in blood samples was subfractionated into HDL$_2$ and HDL$_3$ by rapid ultracentrifugation. SAA was measured in serum, HDL$_2$ and HDL$_3$ by an enzyme-linked immunosorbent assay and the activities of PLTP and CETP were measured in HDL$_2$ and HDL$_3$ by fluorimetric assays.

MAIN RESULTS AND THE ROLE OF CHANCE: In the PCOS women from cohort-1, SAA was increased in serum, HDL$_2$ and HDL$_3$ ($P = 0.038, 0.008$ and $0.001$ versus control, respectively), as was the activity of PLTP in HDL$_2$ and HDL$_3$ ($P = 0.006$ and $0.009$ versus controls, respectively). In the PCOS women from cohort-2, SAA was increased in serum, HDL$_2$ and HDL$_3$, although only significantly in HDL$_3$ ($P = 0.083, 0.120$ and $0.034$ versus controls, respectively), as was the activity of PLTP in HDL$_2$ and HDL$_3$, although this was only significant in HDL$_2$ ($P = 0.045$ and $0.070$ versus controls, respectively).

LIMITATIONS, REASONS FOR CAUTION: First, insulin sensitivity was not determined by the euglycaemic-hyperinsulinaemic clamp. Secondly, the method used to estimate body fat was not able to discriminate between visceral and peripheral fat. Thirdly, larger study groups would be required to confirm if PCOS independently contributed to SAA-related HDL and functional changes to this lipoprotein, independent of BMI, percentage body fat and IR.

WIDER IMPLICATIONS OF THE FINDINGS: This is the first study to highlight the usefulness of HDL-associated SAA as a marker to identify increased inflammation in women with PCOS. This study also identified that the functioning of HDL was altered in women with PCOS. These findings illustrate a mechanism through which cardiovascular disease may increase in PCOS.

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Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder affecting women of reproductive age, with a prevalence of 5–7% (Diamanti-Kandarakis et al., 1999; Michelmore et al., 1999; Asuncion et al., 2000; Sirmans and Pate, 2013). Women with PCOS are insulin resistant (IR) (Pasquali and Gambineri, 2013) and at increased risk of type-2 diabetes (Book and Dunaif, 1999).

Approximately 40–70% of women with PCOS are obese, typically in a central distribution (Borruel et al., 2013), and this contributes to IR, hyperandrogenism, dyslipidaemia, inflammation and risk of type-2 diabetes (Book and Dunaif, 1999; Sirmans and Pate, 2013). Centrally distributed adipose tissue is associated with increased fatty acid release (Horejsi et al., 2004), which in turn increases IR and alters the assembly of lipoproteins to an atherogenic phenotype (Holte et al., 1994; Macut et al., 2008). Centrally distributed adipose tissue also plays a key role in endocrine and metabolic control, secreting a range of adipokines into the circulation, many of which increase inflammation (Yang et al., 2006).

PCOS, however, has not been unequivocally associated with inflammation independent of the effects of obesity or IR (Mannerås-Holm et al., 2011; Tan et al., 2011; Scicchitano et al., 2012). One inflammatory marker that might be more specific to PCOS-related inflammation is serum amyloid A (SAA). This inflammatory molecule, which is released chronically from hypertrophic adipocytes (Jernås et al., 2006), associates with high-density lipoprotein (HDL), particularly HDL₃, rendering this lipoprotein dysfunctional (Artl et al., 2000; Ancsin and Kisilevsky, 2001). This association reduces the anti-atherogenic properties of HDL, including impairment of its reverse cholesterol transport (RCT) capabilities and alterations in the activities of several HDL-associated enzymes involved in HDL remodelling (Navab et al., 1997; Jonas, 2000; Van Lenten et al., 2001; Tailleux et al., 2002). These enzymes include phospholipid transfer protein (PLTP) and cholesteryl ester transfer protein (CETP) (Tall, 2008; Masson et al., 2009). To date, neither of these enzymes has been examined in serum or isolated HDL in women with PCOS.

Only three studies to date have investigated the relationship between PCOS and serum SAA. In the first, it was reported that serum SAA did not differ between 31 mildly overweight PCOS and 31 weight-matched controls (Mannerås-Holm et al., 2011). In the second study, it was reported that serum SAA was increased in PCOS women, matched for BMI and waist:hip ratio (WHR) (Tan et al., 2011). In the third study, carried out by our group in a different and smaller PCOS cohort to that described here, we found that serum SAA was increased in the PCOS women, when matched with control women for BMI and IR and that this increase was independent of changes to other inflammatory markers, including high-sensitive C-reactive protein (hsCRP), myeloperoxidase and neopterin (Blair et al., 2013). None of these studies examined the relationship between SAA and HDL, or if the functioning of any of the HDL-associated enzymes were altered by the presence of SAA and PCOS.

The current study is the first to examine if PCOS was independently associated with inflammatory changes within HDL, specifically HDL₃ and HDL₄, and to relate such changes to the activities of PLTP and CETP within these subfractions. The population numbers utilized in this current study were much larger than those previously reported (Mannerås-Holm et al., 2011; Tan et al., 2011; Blair et al., 2013), thus increasing the statistical power to detect PCOS-related changes. In the first of our analysis, women with PCOS were compared with a BMI-matched cohort. In the second analysis, a subgroup of the PCOS women were further matched with control women for IR, with the purpose of determining whether PCOS was associated with HDL function, independent of IR.

Materials and Methods

Study population

The study population was a sub-set of a cohort previously reported in the publications of O’Connor et al. (2010) and Phelan et al. (2013). In the current analysis, 100 of these women with PCOS were matched for BMI and percentage body fat with 100 of the healthy control women (these two groups are described as cohort-1). This population was selected based on the availability of matched-serum sample from the primary analysis. A subset of this cohort (64 healthy control women and 64 PCOS women) was then further matched in a case–controlled manner for age and IR (described as cohort-2). IR was primarily estimated using the Avignon index (SM), which was calculated using glucose and insulin levels measured fasting and post-glucose load, and which has been previously demonstrated to closely approximate to results obtained using clamp techniques in women with PCOS (Ciampelli et al., 2005).

Women with PCOS were recruited from Endocrinology Outpatient Clinics (Adelaide and Meath Hospital, Tallaght, Dublin). Control women were recruited by local advertisement. PCOS was defined according to the US National Institutes of Health criteria as chronic oligomenorrhoea (fewer than nine menstrual cycles per year) and clinical and/or biochemical evidence of hyperandrogenism, in the absence of other disorders causing the same phenotype (Zawadzki and Dunaif, 1992). Clinical criteria included hirsutism with a Ferriman-Galwey score >9, and acne or male pattern alopecia. Biochemical criteria included total testosterone, androstenedione or dehydroepiandrosterone sulphate (DHEAS) greater than the laboratory reference range. All control women were eumenorrhoeic with testosterone levels within the normal range and were studied in the follicular phase of the menstrual cycle. Women were excluded if they were <18 or >40 years old, non-Caucasian, pregnant or lactating, had a BMI <18 or >50 kg/m², had a recent illness or any chronic illness likely to influence the results or were taking any medications likely to influence the results, including hormonal contraception, anti-hypertensives, lipid-lowering therapy, antiplatelet agents, anti-inflammatory agents or non-prescription agents.

Ethical approval

All study women gave their written signed consent to the study, which was approved by the Research Ethics Committee of the Adelaide and Meath Hospital and St. James’ Hospital (Dublin, Ireland). This trial was registered at clinicaltrials.gov as NCT001195168.
Laboratory methods and anthropometric measurements

Blood samples were taken following a 12-h fast; in addition, all women underwent a standard 75 g oral glucose tolerance test. DHEAS and sex hormone-binding globulin (SHBG) were measured in serum by chemiluminescence immunoassays (Siemens, Munich, Germany) [coefficients of variation (CV) <5%]. Total testosterone was measured in serum by an electrochemiluminescence immunoassay (Cobas Roche Diagnostics, West Sussex, UK) on the Roche E Module Analyser. Androstenedione was measured by a radio-immune assay (Siemens, Munich, Germany) (CV < 5%). Fasting insulin was measured by electrochemiluminescence immunoassay (Cobas Roche Diagnostics, West Sussex, UK) on the Roche E Module Analyser. Glucose was measured by an enzymatic hexokinase method (Cobas Roche Diagnostics, West Sussex, UK) on a Roche P Module Analyser. Glucose and insulin levels in the fasting state and post-glucose load were used to calculate homeostasis model assessment of insulin resistance (HOMA-IR) and the Avignon index of insulin sensitivity (SiM). Total and HDL cholesterol were measured by enzymatic colorimetric assay (Cobas Roche Diagnostics, West Sussex, UK). Low-density lipoprotein (LDL)-cholesterol was calculated by the Friedewald equation. hsCRP was measured by an enzyme-linked immunosorbent assay (ELISA) using a commercial available kit (BioCheck, Inc., Foster City, USA).

Percentage body fat was estimated using the Body Stat 1500 system (Body stat Ltd, Isle of Man, UK). Height was measured by Harpenden stadiometer and weights were measured in a hospital gown. Waist and hip circumference were measured with a non-distensible flexible tape measure to estimate the WHR. The above analyses were carried out at AMNCH, Dublin.

Isolation of HDL<sub>2</sub> and HDL<sub>3</sub>

Fasting peripheral venous blood samples were collected into K<sub>2</sub>EDTA (potassium ethylenediaminetetraacetic acid) tubes. The samples were immediately centrifuged at 1100 g for 10 min at 4°C and the separated plasma was removed and stored as 2 ml aliquots at −80°C.

HDL<sub>2</sub> and HDL<sub>3</sub> subfractions were isolated from plasma by rapid ultracentrifugation (McPherson et al., 2007). Protein concentration in HDL<sub>2</sub> and HDL<sub>3</sub> was determined by the Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, UK), which was based on the Bradford method and was used to standardize SAA, PLTP and CETP in each of the HDL subfractions (McPherson et al., 2007).

SAA concentration and the activities of PLTP and CETP

SAA levels in serum, HDL<sub>2</sub> and HDL<sub>3</sub> were measured by an ELISA. The ELISA kit was supplied by Invitrogen (HUMAN SAA, KHA001) and the assay was performed on a Grifols Triturus (Italy) automated ELISA system, as per the manufacturer’s instructions. PLTP and CETP activity in HDL<sub>2</sub> and HDL<sub>3</sub> using fluorimetric assays (Roar Biomedical, NY, USA), as per the manufacturer’s instructions.

Statistical analysis

Results were analysed using the Statistical Package for the Social Sciences PASW Statistics version 20 for Windows. Variables were assessed for normality and logarithmically transformed where required. Between-group comparisons were analysed by an independent Student’s t-test. Correlations between variables were assessed by Pearson’s two-tailed bivariate analysis. Results are presented as mean (SEM), mean (SD) or geometric mean (interquartile range) when normally distributed after log transformation.

Significance was set as P < 0.05 for all analyses.

Results

Baseline characteristics

Baseline characteristics for cohorts-1 and -2 are shown in Table I. In cohort-1, women with PCOS were significantly younger (P < 0.001) and, as expected, WHR and HOMA-IR were higher compared with control women (P < 0.001).

The matching process in cohort-2 resulted in no significant differences in BMI, percentage body fat, age and both estimates of IR (SiM and HOMA-IR) between groups. WHR was higher in women with PCOS compared with control women (P < 0.05).

In the PCOS women in cohorts-1 and -2 and compared with their respective control women, testosterone, androstenedione and DHEAS were greater, while SHBG was lower (P < 0.05 for all comparisons). Tri-glycerides, total cholesterol, HDL and LDL did not differ between the groups in either cohort-1 or cohort-2.

Compared with their respective control groups, hsCRP was higher in the women with PCOS in cohort-1 (P < 0.05), but not in cohort-2.

Protein concentration in HDL<sub>2</sub> and HDL<sub>3</sub>

The concentration of protein was similar in HDL<sub>2</sub> and HDL<sub>3</sub> between the control and PCOS groups in both cohort-1 and cohort-2 (results not shown).

SAA concentration in serum, HDL<sub>2</sub> and HDL<sub>3</sub> (Fig. 1)

**Cohort-1**

Compared with the control women, SAA was greater in serum, HDL<sub>2</sub> and HDL<sub>3</sub> in the PCOS women (P = 0.038, 0.008 and 0.001, respectively).

**Cohort-2**

Compared with the control women, SAA was greater in HDL<sub>3</sub> (P = 0.034) in the PCOS women, and although serum and HDL<sub>2</sub>-SAA were numerically higher in the PCOS women, these differences were not significant (P = 0.083 and P = 0.120, respectively).

PLTP activity in HDL<sub>2</sub> and HDL<sub>3</sub> (Table II)

**Cohort-1**

Compared with the control women, the activity of PLTP was greater in HDL<sub>2</sub> and HDL<sub>3</sub> in the PCOS women (P < 0.05 for both comparisons).

**Cohort-2**

Compared with the control women, the activity of PLTP was greater in HDL<sub>2</sub> (P < 0.05) and HDL<sub>3</sub> in the PCOS women, although the latter did not reach significance in HDL<sub>3</sub> (P = 0.070).

CETP activity in HDL<sub>2</sub> and HDL<sub>3</sub> (Table II)

**Cohort-1**

Although the activity of CETP was numerically greater in the PCOS women in HDL<sub>2</sub> and HDL<sub>3</sub>, these differences were not significant compared with controls (P > 0.05 for both comparisons).
were not significant compared with controls ($P$ = 0.410, respectively). In the control women, serum HDL cholesterol and SAA did not correlate with CETP in HDL 2, but not in HDL 3 ($r = -0.049, P = 0.723$, respectively). Similarly, serum HDL cholesterol negatively correlated with the activity of CETP in HDL 2, but not in HDL 3 ($r = -0.466, P < 0.001; r = 0.152, P = 0.250$).

### Correlations between serum HDL cholesterol, SAA and the activities of PLTP and CETP

**Serum HDL cholesterol and SAA**

In the PCOS women, serum HDL cholesterol did not correlate with SAA in serum, HDL 2 or HDL 3 ($r = 0.104, P = 0.410; r = 0.221, P = 0.085; P = 0.117, P = 0.385$, respectively). In the control women, serum HDL cholesterol did not correlate with SAA in serum, HDL 2 or HDL 3 ($r = -0.041, P = 0.745; r = -0.164, P = 0.215; r = -0.113, P = 0.410$, respectively).

**Serum HDL cholesterol and HDL-associated enzymes**

In the PCOS women, serum HDL cholesterol negatively correlated with the activities of PLTP in HDL 2 and HDL 3 ($r = -0.521, P < 0.001; r = -0.396, P = 0.003$, respectively) and with the activity of CETP in HDL 2 and HDL 3, although this narrowly missed being significant in HDL 3 ($r = -0.476, P < 0.001; r = -0.250, P = 0.056$, respectively). In the control women, serum HDL cholesterol negatively correlated with the activities of PLTP in HDL 2, but not in HDL 3 ($r = -0.427, P < 0.001; r = -0.049, P = 0.723$, respectively). Similarly, serum HDL cholesterol negatively correlated with the activity of CETP in HDL 2, but not in HDL 3 ($r = -0.466, P < 0.001; r = 0.152, P = 0.250$).

### Discussion

This is the first study in women with PCOS to examine serum and HDL-associated SAA and the activities of several HDL-associated enzymes with the activity of CETP in HDL 2 and HDL 3, although this narrowly missed being significant in HDL 3 ($r = -0.476, P < 0.001; r = -0.250, P = 0.056$, respectively). In the control women, serum HDL cholesterol negatively correlated with the activities of PLTP in HDL 2, but not in HDL 3 ($r = -0.427, P < 0.001; r = -0.049, P = 0.723$, respectively). Similarly, serum HDL cholesterol negatively correlated with the activity of CETP in HDL 2, but not in HDL 3 ($r = -0.466, P < 0.001; r = 0.152, P = 0.250$).
enzymes. The principal findings were that serum and HDL-associated SAA were increased in PCOS women versus control women, as was the activity of HDL-associated PLTP. Serum markers of inflammation are elevated in PCOS, which was first noted by González et al. (1999), who reported increased serum tumour necrosis factor (TNF)-α independent of BMI. It is not clear to what extent this is an effect of IR, and whether there is also an effect of PCOS status per se or hyperandrogenism. We have recently reported an increased white cell count (WCC), hsCRP, and the cytokines TNF-α and interleukin (IL)-6 in women with PCOS, compared with control women of similar BMI (Phelan et al., 2013). However, in that study only WCC remained elevated in PCOS women following further adjustment for IR (Phelan et al., 2013). The current study adds to our understanding of the inflammatory process in PCOS by demonstrating increased SAA in serum and HDL₂ and HDL₃ in women with PCOS, independent of BMI and percentage body fat. Similarly to WCC, but unlike hsCRP, TNF-α and IL-6, the increased SAA in HDL₃ (and non-significantly in serum and HDL₂) persisted following further matching of PCOS and normal women for IR.

The importance of SAA extends beyond being a marker of inflammation, as it is known to influence HDL function, reducing its anti-atherogenic potential (Artl et al., 2000) in part through influencing its role in RCT (McGillicuddy et al., 2009). This property was not confirmed by de Beer et al. (2010) in a mouse model; the authors, however, acknowledged that findings could differ in human subjects. Typically, an inverse relationship is thought to exist between HDL cholesterol and cardiovascular disease (CVD) risk, with low HDL cholesterol identifying patients at elevated CVD risk. However, recent randomized trials designed to test the ‘HDL hypothesis’ have failed to show benefit of an inverse relationship between raised HDL cholesterol and decreased CVD risk (Toth et al., 2013). This illustrates that a simple measure of serum HDL cholesterol cannot fully identify the anti-atherogenic potential of HDL and that functional/compositional aspects of HDL must also be considered. Correlation analysis in the current study demonstrated that serum HDL cholesterol was not predictive of the inflammatory condition of HDL, as no significant correlations were observed between serum HDL cholesterol and HDL-SAA in either PCOS or control women. Although there are few data regarding the influence of androgens on HDL metabolism, one study illustrated that PCOS was associated with smaller HDL particles (Sidhwani et al., 2011), a property that reduces their involvement in RCT (El Harchaoui et al., 2009). The current study extends these findings demonstrating that in women with PCOS, the HDL subfractions, HDL₂ and HDL₃, were associated with increased SAA levels, suggesting that HDL may possess proatherogenic properties in such women. In support of this concept, only in the control group was an inverse and potentially protective association observed between SHBG and SAA in serum, HDL₂ and HDL₃.

The increased HDL-associated SAA potentially contributed to the observed increases in the activity of PLTP in women with PCOS. PLTP is necessary for the maturation of HDL (Huusokonen et al., 2001; Masson et al., 2009) and increased activity in serum has been associated with inflammation (Cheung et al., 2006), decreased HDL (Pussinen et al., 1998), obesity (Dullaart et al., 1994; Kaser et al., 2004) and coronary artery disease (Schlitt et al., 2005). Conversely, low serum PLTP activity has also been associated with peripheral arterial disease (Schgoer et al., 2008). However, the current study clearly identified that the activity of PLTP was increased in HDL₂ and HDL₃ from the PCOS women in cohort-1 and remained increased in HDL₂ (with a trend towards an increase in HDL₃, P = 0.070) following further matching the women for IR (cohort-2). The contradictory findings previously reported in the literature (Dullaart et al., 1994; Pussinen et al., 1998; Kaser et al., 2004; Schlitt et al., 2005; Cheung et al., 2006; Schgoer et al., 2008) possibly result from measuring PLTP activity in serum, which is subject to daily variation. We suggest that because of the approximate 4-day half-life of HDL in the circulation (Scanu and Hughes, 1962), measured activity of PLTP in HDL particles might be more reflective of its true long-term activity. We also observed that the activities of both PLTP and CETP negatively correlated with serum HDL cholesterol and SHBG in the

**Figure 1** Serum amyloid A (SAA) concentration in serum and high-density lipoprotein (HDL)₂ and HDL₃ for the controls and women with PCOS.
PCOS group, which was mirrored to a lesser extent in the control group. These findings suggest that increased HDL cholesterol and SHBG might be associated with anti-atherogenic changes to the activity of these enzymes, although this concept would require further investigation.

Overall, therefore, this study provides evidence that HDL particles are abnormal in women with PCOS, which was not completely explained by IR. The observed changes are likely to have a detrimental effect on HDL, which is a key lipoprotein.

Some limitations should be considered. First, insulin sensitivity was not determined using the euglycaemic-hyperinsulinaemic clamp method. In order to obtain an adequate number of matched pairs, it was necessary to estimate insulin sensitivity in more than 200 women and it was not feasible to carry out clamp studies in this sample size. The groups, however, were very closely matched for IR estimated by two different methods, HOMA and SiM, the latter of which takes into account post-challenge glucose and insulin levels, as well as those measured in the fasting state. Furthermore, SiM has been shown to be the marker of insulin sensitivity that most closely correlates with clamp derived data in PCOS (Ciampelli et al., 2005).

Secondly, we acknowledge that the method used to estimate percentage body fat was unable to discriminate between visceral and peripheral fat, which may be particularly relevant as it is visceral fat that is more closely associated with SAA release (Yang et al., 2014). Thirdly, the study may have been underpowered to detect small changes in some variables. This was more likely to be the case in the cohort of women further matched for IR (cohort-2), which had 30% fewer women than in cohort-1, meaning that between-group differences would be expected to be smaller and would reduce study power. As an example, a retrospective power calculation indicated that 109 women per group (a similar number to that of cohort-1) would have been sufficient to detect a statistical difference between the groups in serum, HDL2 and HDL3 SAA, when matched for BMI, percentage body fat and IR (cohort-2).

In summary, we have identified that PCOS was associated with an increase in serum levels of SAA, making it a potentially useful marker of inflammation in such a cohort. Additionally, PCOS was associated
with increased HDL-associated SAA and altered PLTP activity, both of which would indicate HDL particles with altered anti-atherogenic function.

**Authors’ roles**

J.M. devised this research, supervised S.G. and J.M. and also prepared the manuscript, construction of tables and figures and its submission. S.G. and J.M. undertook the laboratory analysis and assisted in the preparation of the manuscript. N.P., A.M. and A.O. were responsible for recruitment and sample collection. I.S.Y. co-supervised S.G. J.G. supervised J.M. undertook the laboratory analysis and assisted in the preparation of J.M. devised this research, supervised S.G. and J.M. and also prepared the manuscript.

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**Conflict of interest**

None declared.

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