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ORIGINAL ARTICLE

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Activation profiles of monocyte-macrophages and HDL function in healthy women in relation to menstrual cycle and in polycystic ovary syndrome patients

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Abstract

Purpose Hormonal status and menopause affect human macrophage function and cardiometabolic risk. In polycystic ovary syndrome (PCOS) patients the cardiometabolic risk increases through mechanisms that are largely unknown. We tested the hypotheses that macrophage activation is influenced by menstrual cycle and that ovarian dysfunction in PCOS patients is associated with altered macrophage inflammatory responses and cholesterol efflux capacity of serum HDL.

Methods Blood samples were obtained in the follicular and luteal phases from cycling women ($n = 10$) and on a single visit from PCOS patients with ovarian dysfunction ($n = 11$). Monocyte-derived macrophage activation and monocyte subsets were characterized ex vivo using flow cytometry. The capacity of HDL to promote cell cholesterol efflux through the main efflux pathways, namely aqueous diffusion, ATP-binding cassette A1 and G1, was also evaluated.

Results Hormone and metabolic profiles differed as expected in relation to menstrual cycle and ovulatory dysfunction. Overall, macrophage responses to activating stimuli in PCOS patients were blunted compared with cycling women. Macrophages in the follicular phase were endowed with enhanced responsiveness to LPS/interferon-γ compared with the luteal phase and PCOS. These changes were not related to baseline differences in monocytes. HDL cholesterol efflux capacity through multiple pathways was significantly impaired in PCOS patients compared to healthy women, at least in part independent from lower HDL-cholesterol levels.

Conclusions Regular menstrual cycles entailed fluctuations in macrophage activation. Such dynamic pattern was attenuated in PCOS. Along with impaired HDL function, this may contribute to the increased cardiometabolic risk associated with PCOS.

Keywords Macrophages · Macrophage activation · Cholesterol efflux · Polycystic ovary syndrome · Menstrual cycle · Monocytes

Introduction

During fertile age, women are protected from cardiovascular disease (CVD) by their estrogen-replete status. By contrast, the CVD risk profile worsens after menopause, yet the causative mechanisms are incompletely understood [1].

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Disorders of estrogen secretion, primary ovarian insufficiency, and polycystic ovary syndrome (PCOS) are associated with subclinical atherosclerosis and increased CVD risk [2, 3]. In particular, PCOS affects 5–10% of women of reproductive age, and increases the risk for metabolic disease and CVD during reproductive age as well as after menopause. Hormonal contraceptives are first-line treatment, but this option might be inadequate to reverse CVD risk [4, 5].

The monocyte-macrophage system is a key player in vascular inflammation and atherosclerosis [6, 7]. Atherosclerotic lesion progression correlates with the dominance of classical/pro-inflammatory (M1) over alternative/anti-inflammatory (M2) macrophage phenotypes [8]. We recently demonstrated that menopausal estrogen deprivation is associated with impaired alternative activation of human macrophages [9], and the contribution of estrogen-regulated pathways to macrophage activation is actively investigated [9, 10]. Although immune cell variation during menstrual cycle has been reported in the female reproductive tract and in circulating cells [11–14], whether the menstrual cycle affects blood-derived macrophage phenotype and function is at present unclear.

Beyond inflammatory activation of monocyte/macrophages, intracellular cholesterol content regulation is pivotal for atherogenesis. Macrophage cholesterol content is dynamically governed by endogenous synthesis, uptake from extracellular donors, and efflux. These pathways are modulated by the functional expression of membrane receptors and transporters including ATP binding cassette (ABC)A1 and G1 [15]. However, macrophage cholesterol content is highly influenced by the capacity of serum high density lipoprotein (HDL) to interact with ABCA1 and ABCG1 thereby promoting cholesterol efflux. HDL dysfunction facilitates foam cell formation, occurs in several conditions including inflammatory diseases [16], and is considered an independent marker of CVD risk. Serum cholesterol efflux capacity (CEC) is a metric of HDL activity in promoting cholesterol efflux from macrophages, thus opposing to their pro-inflammatory activation and transformation into foam cells. HDL function, measured as CEC, is a stronger inverse predictor of CVD than HDL-cholesterol levels [17, 18]. Estrogen not only modulates inflammatory response and immune cell function [10, 19, 20], but also affects human macrophage cholesterol handling [21, 22]. While suggesting reduced CEC along with a more atherogenic lipoprotein profile in women with PCOS compared with controls [3, 23], previous reports do not provide data on specific cholesterol efflux pathways, macrophage function or menstrual cycle status.

We herein hypothesized that macrophage function would be modulated during regular menstrual cycle-related fluctuations as well as by PCOS-associated hormone disorders. Thus, we investigated the activation profile of blood-derived macrophages through analysis of individual and combined phenotypic markers as well as assessment of M1/M2 macrophage ratio in all groups. We also investigated whether specific CEC pathways would be affected by the menstrual cycle as well as by ovulatory dysfunction in PCOS.

Materials and methods

Study design/subjects

Experiments were performed using blood samples taken from $n = 10$ healthy women aged 22–32 years at two different time points during the menstrual cycle and from $n = 11$ women aged 18–36 years with PCOS diagnosis [24] and amenorrhea since at least 3 months. Written informed consent was obtained from each participant included in the study. The protocol was in accordance with the principles set out in the 1975 Declaration of Helsinki and was approved by Padua University Hospital's ethics committee. Subjects were deemed eligible, pending meeting of inclusion/exclusion criteria (Supplemental Table 1). Descriptions and comparisons of participating women are shown in Table 1 and Supplemental Fig. 1. Healthy women had regular menstrual cycles and no signs of clinical hyperandrogenism (e.g. hirsutism or acne), and were not taking medications. In these cycling women, blood was taken during the follicular (F, day 6–9) and the luteal (L, day 20–24) phases. Serum samples were obtained from a subset of $n = 8$ healthy donors at F and L, and from all 11 PCOS patients on a single visit for CEC assays as described below. On a subsequent visit, blood was taken from a further subset of $n = 4$ healthy donors at both F and L to assess the subset distribution and intracellular cholesterol level of circulating monocytes.

Cell culture

For generation of human monocyte-derived macrophages, peripheral blood mononuclear cells were isolated from freshly heparinized blood essentially as described [25, 26] with minor modifications. Briefly, monocytes were isolated first by Ficoll-Paque (GE Healthcare) density gradient centrifugation followed by a second, high-density hyperosmotic Percoll gradient (GE Healthcare), and seeded at a density of 5×10^4 cells/cm^2 in RPMI 1640 + 10% fetal

Table 1 Clinical characteristics of study subjects

Variable	Healthy ($n = 10$)	PCOS ($n = 11$)	p-value
Age, years	25.9 ± 3.0	28.2 ± 6.1	0.343
Body mass index, kg/m^2	20.8 ± 1.9	30.2 ± 9.2	0.012
Waist circumference, cm	74.8 ± 7.4	101.4 ± 19.1	0.002
Systolic blood pressure, mm Hg	113.1 ± 9.6	131.0 ± 18.6	0.026
Diastolic blood pressure, mm Hg	78.1 ± 4.6	83.1 ± 9.1	0.179
Fasting plasma glucose, mg/dL	83.0 ± 7.5	90.3 ± 11.1	0.126
Fasting insulin, mU/L	9.4 ± 2.9	17.8 ± 16.6	0.176
HOMA-IR	1.9 ± 0.5	4.0 ± 3.7	0.129
Total cholesterol, mg/dL	172.4 ± 24.8	188.8 ± 35.5	0.278
HDL cholesterol, mg/dL	72.8 ± 17.8	51.7 ± 11.3	0.005
LDL cholesterol, mg/dL	98.9 ± 25.6	127.8 ± 33.7	0.029
Triglycerides, mg/dL	57.4 ± 18.2	74.0 ± 24.1	0.120
Homocysteine, μM	8.8 ± 2.5	11.9 ± 1.2	0.018
C-reactive protein, mg/L	0.36 ± 0.06	1.75 ± 0.39	0.002
Estrone, ng/L	22.3 ± 9.3	28.6 ± 15.8	0.332
17β-estradiol, pM	202.1 ± 150.6	189.0 ± 64.2	0.797
17OH-progesterone, nM	3.2 ± 1.2	3.9 ± 1.8	0.431
Progesterone, nM	1.8 ± 1.5	1.0 ± 0.9	0.171
Total testosterone, nM	1.0 ± 0.6	2.2 ± 0.9	0.002
Androstenedione, nM	8.4 ± 4.1	11.9 ± 3.9	0.075
DHEAS, μM	5.8 ± 2.8	4.6 ± 2.0	0.319
SHBG, nM	70.1 ± 14.2	40.3 ± 18.0	0.010
LH, U/L	7.0 ± 1.5	12.5 ± 7.2	0.051
FSH, U/L	7.0 ± 1.6	5.3 ± 1.4	0.027
LH/FSH ratio	1.0 ± 0.4	2.2 ± 0.8	<0.001

Data are expressed as mean ± SD. Hormone levels for healthy women are shown for the follicular phase

bovine serum in the presence of 20 nM macrophage colony stimulating factor (CSF-1). Cells were cultured over 7 days and the medium was changed every 3 days, adding fresh CSF-1.

Protocols of macrophage activation

After replacing the culture medium, unpolarized M0 macrophages were activated toward the M1 or M2 phenotypes using LPS + interferon (IFN)-γ and interleukin (IL)-4+IL-13, respectively, as described previously [25, 26]. The M1 phenotype was defined as % of $CD80^+/CD68^+$ cells, whereas the M2 phenotype was defined as % of $CD206^+/CD163^+$ cells.

Flow cytometry

At the end of stimulations, cells were washed and harvested essentially as described [25, 26]. Briefly, resting and activated macrophages were labeled with fluorochrome-tagged monoclonal antibodies against the surface markers CD68 FITC and CD80-PE to typify the M1 phenotype, and against CD206-FITC and CD163-PE to characterize the M2 phenotype, respectively [27]. All antibodies were from BD Biosciences. Analyses were performed on a FacsCanto II flow cytometer (BD Biosciences), and data analyzed using the FacsDiva software. Isotype-matched controls were used as a reference. The macrophage activation profile for individual study subjects in each group, expressed as Δ(M1-M2 ratio), was calculated as $[(\%CD80^+CD68^+_{LPS+IFN\gamma} - \%CD80^+CD68^+_{resting})/\%CD80^+CD68^+_{resting}] - [(\%CD206^+CD163^+_{IL\text{-}4+IL\text{-}13} - \%CD206^+CD163^+_{resting})/\%CD206^+CD163^+_{resting}]$.

HDL cholesterol efflux capacity

HDL CEC occurring through the main pathways was evaluated by a standardized radioisotopic technique as previously described [28]. We evaluated total (t)-CEC and its major contributors, aqueous diffusion (AD) and ABCA1-mediated efflux, in the J774 mouse macrophage cell model. In particular, resting cells were used to assess AD, while cells incubated with a cAMP analogue inducing ABCA1 expression were used to measure HDL tCEC [29]. The specific ABCA1-mediated contribution was calculated as the difference between tCEC and AD CEC [28]. Serum HDL CEC mediated by ABCG1 was evaluated using Chinese hamster ovary (CHO) cells transfected or not with the human ABCG1 gene. The specific ABCG1 contribution was calculated as the difference between CEC in ABCG1-transfected and in nontransfected cells [30].

J774 macrophages were grown in 10% FCS-containing DMEM (Lonza, Verviers, Belgium) in the presence of antibiotics (penicillin-streptomycin, Thermo Fisher Scientific, Carlsbad, CA, USA), and labeled with 2 μCi/mL [1,2-^{3}H] cholesterol (Perkin Elmer, Waltham, MA, USA) for 24 h in the presence of an acyl-CoA:cholesterol acyltransferase inhibitor (Sandoz 58035, 2 μg/ml; Sigma-Aldrich, Milano, Italy). Cells were then incubated in the presence or absence of a cAMP analogue (cpt-cAMP, 0.3 mM, Sigma-Aldrich) in 0.2% BSA-containing medium for 18 h, and subsequently exposed to the HDL fraction of sera from healthy and PCOS donors (2%, v/v) for 4 h. The HDL fraction was isolated from serum by precipitating the apoB-containing lipoproteins with polyethylene glycol [30].

HDL CEC was expressed as a percentage of radiolabeled cholesterol released into the medium over the total radioactivity incorporated by cells. Lipid-free human apoA-I and the HDL fraction from a serum pool of normolipidemic subjects were used as controls.

CHO cells were cultured in Ham's F-12 medium (Lonza) supplemented with 10% FCS and antibiotics (zeocin and geneticin, Waltham, MA, US). After labeling with 1 µCi/mL [1,2-^{3}H] cholesterol, CHO cells were equilibrated in 0.2% BSA-containing medium for 90 min. Cells were subsequently exposed to the HDL fraction of sera (1% v/v) from control and PCOS donors for 6 h. HDL CEC was expressed as a percentage of radiolabeled cholesterol released into the medium over the total radioactivity incorporated by cells. The intra-assay coefficient of variation for HDL CEC assays was <10%.

Monocyte cell cholesterol assay

Monocytes from a subgroup of $n = 4$ healthy donors in F and L phases were analyzed for intracellular cholesterol content and subset distribution. Monocytes were isolated by Ficoll-Percoll gradient centrifugation as described above. For cholesterol assay, the cell pellet was resuspended in 1% sodium cholate (Sigma-Aldrich) supplemented with 10 U/mL DNase (Sigma-Aldrich) at 2×10^6 cells/mL. Cell cholesterol was then measured by a fluorimetric technique [31] using the Amplex Red Cholesterol Assay Kit (Molecular Probes, Eugene, OR, USA) following the manufacturer's instructions. An aliquot of cell lysates was used to measure cell protein using the bicinchoninic acid assay (Thermo Fisher Scientific). Cholesterol content was expressed as µg cholesterol per mg protein.

Monocyte subtype analysis

Identification and quantification of monocyte subtypes was performed on fresh blood samples from $n = 4$ healthy donors in F and L phases, within 3 h after collection, using polychromatic flow cytometry as described [32]. Cells were stained with PE-conjugated anti-CD14 and FITC-conjugated anti-CD16 monoclonal antibodies (Beckman-Coulter).

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software). Data are expressed as mean values ± SEM. Nonnormal variables were log-transformed before analysis. Changes in the variables of interest between the F and L phases were performed using paired Student's *t*-test. Comparisons between two independent groups were performed using unpaired Student *t*-test. The Bonferroni correction was used to adjust for multiple comparisons when there were more than two groups. Linear correlations were checked using the Pearson's *r* coefficient. For HDL CEC analyses, we used one-way ANOVA to compare groups, with significance expressed as F value and the probability of the null hypothesis for the whole model as Prob > F, and general linear model to adjust for HDL-C levels (SAS Statistical package v. 9.4, SAS Institute Inc.). A p value ≤ 0.05 was considered to be statistically significant.

Results

Subjects' demographic

The lipid and hormone profiles were determined in healthy donors ($n = 10$) in both the F and L phases and in PCOS patients ($n = 11$). As expected, cycling subjects in L had higher levels of estrone, LH, 17β-estradiol and LH/FSH ratio, and lower levels of FSH compared with F (Supplemental Fig. 1). Preliminary data indicated that the remaining biochemical values did not significantly differ between F and L phases of healthy women (data not shown). Compared with healthy donors in the F phase, as expected, PCOS donors had a worse CVD risk profile showing higher values of body weight and waist circumference, systolic blood pressure, LDL-C, homocysteine, total testosterone, C-reactive protein and LH/FSH ratio. Women with PCOS had also lower FSH and SHBG concentrations (Table 1).

Macrophage activation profile

Analysis of individual phenotypic markers of healthy women in the F phase showed that the M1 marker CD80 was significantly upregulated (Supplemental Fig. 2A), whereas CD68 was unchanged following LPS+IFN-γ (M1 stimuli) activation (Supplemental Fig. 2B). In line with our previous study [9], the M2 markers CD206 (Supplemental Fig. 2C) and CD163 (Supplemental Fig. 2D) tended to be downregulated following LPS+IFN-γ but were unchanged after IL-4+IL-13 activation. A comparable response profile was observed in macrophages isolated from the same subjects during the L phase. The response to M1 stimuli of macrophages from PCOS patients was comparable to that of cycling women (Supplemental Figs 2A, B), with a significant increase in $CD80^{+}$ cells. In contrast, the fraction of macrophages expressing CD206 (Supplemental Fig. 2C) and CD163 (Supplemental Fig. 2D) following activation with LPS+IFN-γ or IL-4+IL-13 remained fairly stable compared to resting.

Analysis of combinatorial antigenic phenotypes provides a more robust picture of macrophage activation with respect to single phenotypic markers. Thus, a significant increase in

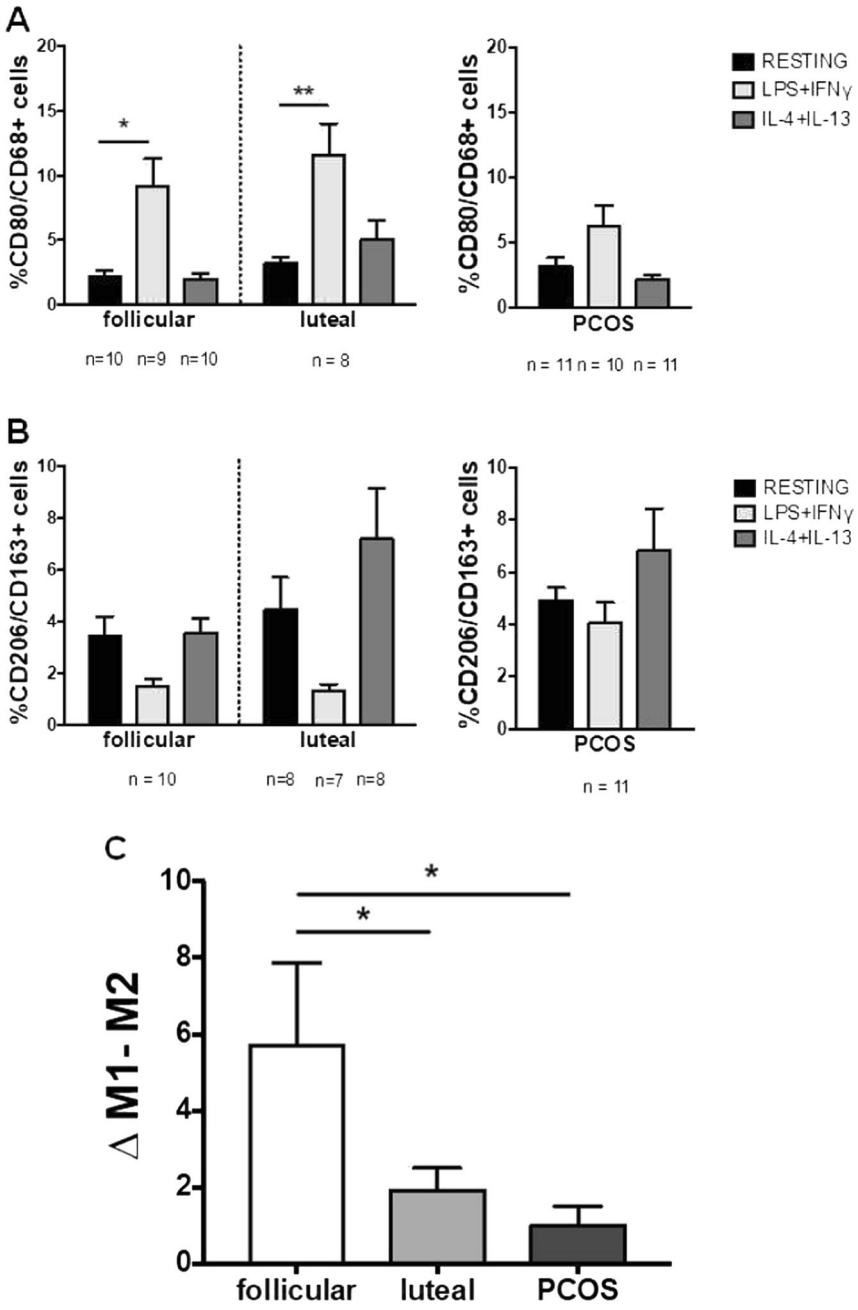

Fig. 1 Phenotypes of resting and activated blood-derived macrophages. Monocytes from healthy women in the follicular and luteal phases and from PCOS patients were cultured for 7 days in RPMI1640 + 10% FBS in the presence of 20 nM CSF-1 and differentiated into macrophages. After washing, macrophages were activated to M0 (resting), M1 (LPS + IFN-γ), and M2 (IL-4 + IL-13) for 24 h. Cells were then phenotyped using conjugated anti-human CD68, CD80, CD163 and CD206 specific antibodies. Subpopulations of $CD80^+/CD68^+$ (M1; **a**) and $CD206^+/CD163^+$ (M2; **b**) macrophages are shown. Results are shown as mean ± SEM. $*p < 0.05$, $**p < 0.01$. **c** Delta of $CD80^+/CD68^+$ and $CD206^+/CD163^+$ subsets following activation with LPS + IFN-γ (M1) or IL-4 + IL-13 (M2) for 24 h, respectively, vs. resting macrophages (Δ M1-M2) in the three donor groups. $*p < 0.05$

the classically activated ($CD80^+/CD68^+$) macrophage subfraction in response to M1-inducing stimuli was observed in healthy women in either phase (Fig. 1a). However, this phenotypic induction was lost in PCOS macrophages (Fig. 1a). The M2 stimuli did not change the fraction of $CD80^+/CD68^+$ macrophages. In healthy women, the M2 phenotype ($CD206^+/CD163^+$) again tended to be attenuated in response to LPS+IFN-γ compared with resting, whereas no major changes were induced by IL-4+IL-13. In PCOS patients, the fraction of $CD206^+/CD163^+$ macrophages was not affected by LPS+IFN-γ or IL-4+IL-13 (Fig. 1b). Of note, the macrophage activation profile of individual subjects, expressed as Δ(M1–M2) ratio, was twice as high in macrophages in the F phase as in those in the L phase or in PCOS patients, suggesting that macrophages in the F phase are endowed with enhanced responsiveness to inflammatory stimuli (Fig. 1c).

To rule out the possibility that the observed changes in macrophage activation were due to baseline differences in blood monocytes, we compared the distribution of circulating monocytes in a subset of cycling women ($n = 4$) in the F and L phases. No significant changes in the different

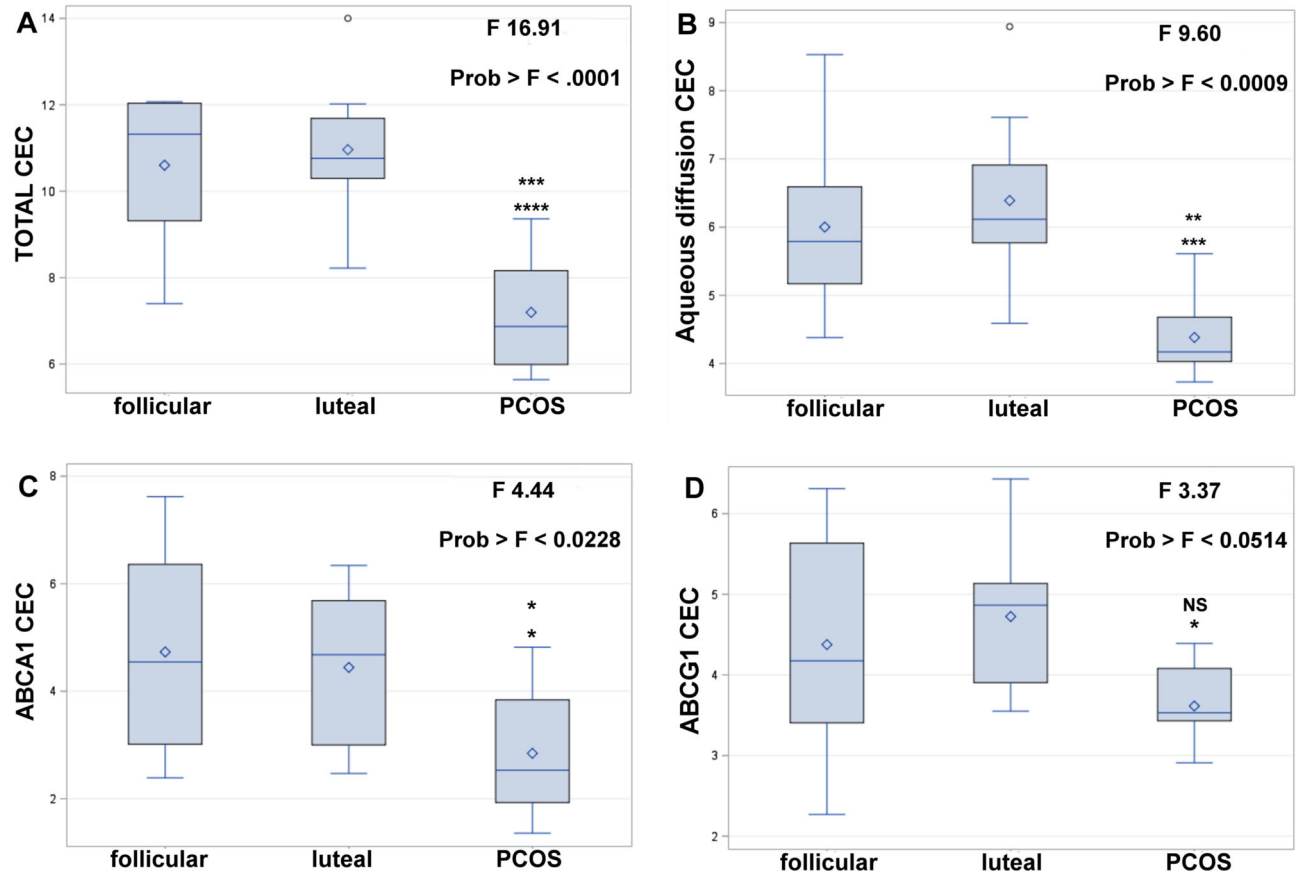

Fig. 2 Serum HDL cholesterol efflux capacity (CEC) in cycling and PCOS donors. Total (**a**), aqueous diffusion (**b**) and ABCA1-mediated CEC (**c**) were measured in cycling women ($n = 8$) in the follicular and luteal phases and in PCOS patients ($n = 11$) using a standardized J774 mouse macrophage model. Panel **d** shows ABCG1-mediated CEC measured using a standardized CHO cell-based model. CEC is expressed as percentage of released cholesterol over that incorporated by cells. One-way ANOVA was applied, reporting in the upper right corner the significance expressed as F value and the probability of the null hypothesis for the whole model as Prob > F. In the box plots, upper horizontal line indicates 75th percentile, lower horizontal line the 25th percentile; horizontal bar within box, median; upper horizontal bar outside box, 90th percentile; lower horizontal bar outside box, 10th percentile Asterisks denote significant differences between PCOS and follicular (above) or luteal phase (below); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$

monocyte subsets were observed in L compared with F phase (Supplemental Fig. 3). Similarly, the intracellular cholesterol content in monocytes was comparable between the two phases (Supplemental Fig. 4).

HDL cholesterol efflux capacity

CEC was measured using specific cell models in order to explore the capacity of circulating HDL to promote cell cholesterol efflux through the main known routes. In cAMP-stimulated J774 macrophages, cholesterol release to HDL mainly occurs through aqueous diffusion (AD-CEC) and the ABCA1-mediated pathway (ABCA1-CEC), while in unstimulated J774 it occurs only by AD-CEC. The menstrual cycle had no impact on serum CEC irrespective of the pathway tested (Fig. 2a–d). By contrast, total CEC (tCEC), i.e. AD-CEC plus ABCA1-CEC, was significantly lower in PCOS patients than in cycling women in both F and L phases ($p < 0.001$; Fig. 2a). Accordingly, PCOS diagnosis was associated with a significant drop in HDL AD-CEC compared to healthy women in both phases ($p = 0.003$; Fig. 2b). In PCOS the specific ABCA1-CEC was also significantly impaired with respect to control women in F ($p = 0.012$) and L phase ($p = 0.031$, Fig. 2c). HDL ABCG1-mediated CEC (ABCG1-CEC) was significantly impaired in PCOS patients as compared only to control women in L phase ($p = 0.020$, Fig. 2d). When adjusted for serum HDL cholesterol levels, the difference between PCOS and F or L was maintained for tCEC and AD-CEC, while it was lost for ABCA1- and ABCG1-CEC (Table 2). Similar results were obtained when sera from PCOS donors were compared to those from cycling women in both phases pooled together.

Table 2 Statistical analysis of HDL-CEC data before and after adjustment for HDL-C levels

CEC pathway	PCOS vs. follicular		PCOS vs. luteal		PCOS vs. luteal + follicular	
	p-value	*p*-value after correction for HDL-C	*p*-value	*p*-value after correction for HDL-C	*p*-value	*p*-value after correction for HDL-C
Total	**<0.001**	**0.002**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
ABCA1	**0.012**	0.141	**0.031**	0.312	**0.006**	0.153
Aqueous diffusion	**0.003**	**0.002**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
ABCG1	0.100	0.299	**0.020**	0.114	**0.019**	0.142

p-values were obtained by one-way ANOVA with post-hoc analyses and general linear model to adjust for HDL-C. *p*-values expressing significant differences are shown in bold

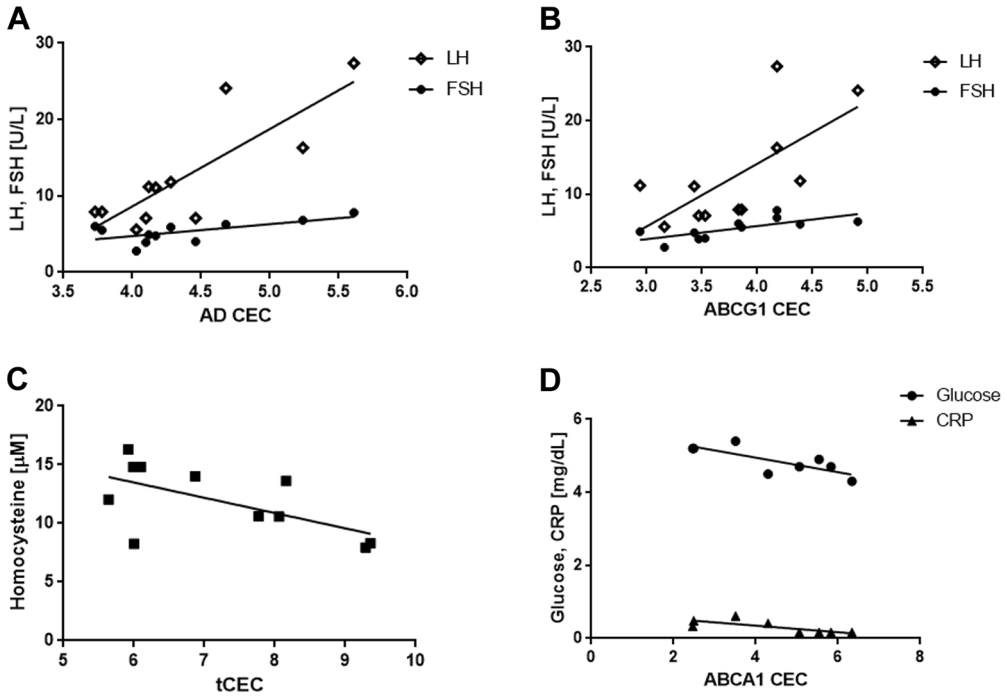

Fig. 3 Correlations of HDL CEC with sex hormones and inflammation. Panels **a** and **b** show the direct correlation between HDL aqueous diffusion (AD)-CEC and ABCG1-CEC with FSH and LH in PCOS patients ($n = 11$). Panel **c** shows the inverse correlation between total (t)CEC and serum homocysteine levels in PCOS patients. Panel **d** shows the direct correlation of ABCA1-CEC with serum glucose and CRP in healthy women in the luteal phase ($n = 8$)

Correlations of HDL CEC with sex hormones and inflammation markers

On a separate analysis of the three groups, we found a direct correlation between AD-CEC and ABCG1-CEC with FSH (AD-CEC $r^2 = 0.48$, $p = 0.034$; ABCG1-CEC $r^2 = 0.51$, $p = 0.013$; Fig. 3a) and LH levels (AD-CEC $r^2 = 0.68$, $p = 0.002$; ABCG1-CEC $r^2 = 0.46$, $p = 0.021$; Fig. 3b) in PCOS patients. In contrast, no correlation with sex hormones was found in cycling women in either phase. Again, HDL tCEC inversely correlated with serum homocysteine levels in PCOS patients only ($R^2 = 0.37$, $p = 0.047$; Fig. 3c). Finally, we found a direct correlation between ABCA1-CEC and glycemia ($R^2 = 0.61$, $p = 0.022$) and serum CRP levels ($R^2 = 0.58$, $p = 0.028$) in healthy women in the L phase (Fig. 3d). No significant correlations were found between CEC and BMI, waist circumference, blood pressure, HOMA index or insulin in study subjects (data not shown).

Discussion

The impact of female hormone fluctuations and/or disorders on CVD risk is unquestioned, but the precise mechanisms involved are not fully clarified. Both pro-inflammatory activation and cholesterol metabolism disorders in the monocyte-derived macrophage system contribute to the atherosclerotic process [7]. We are reporting herein an extensive

characterization of the monocyte-macrophage system in relation to menstrual cycle and PCOS-associated ovarian dysfunction. We also approached the question of HDL function, which is strongly involved in macrophage cholesterol homeostasis through its capacity to trigger cell cholesterol efflux. The main output from the present study is that (1) the activation profile of blood-derived macrophages was modulated by the menstrual cycle; (2) CEC was impaired in PCOS patients, and (3) macrophage responsiveness to activating stimuli was blunted in PCOS. These findings may reflect the static nature of PCOS as opposed to dynamic changes in the menstrual cycle of healthy women.

We showed for the first time that macrophages in the F phase are endowed with enhanced responsiveness to inflammatory stimuli as compared with the L phase and PCOS. This observation might be explained by the particular hormonal pattern typical of the F phase, characterized by low levels of estrone, 17β-estradiol, and progesterone. The functional implications of this finding as well as mechanistic interpretations thereof deserve further investigation. However, it can be argued that such a dynamic fluctuation of blood-derived macrophage function as related to the menstrual cycle underlies a variety of implications for tissue homeostasis (e.g. endometrial turnover) that are likely impaired by the static nature of ovarian dysfunction linked to the PCOS condition. Accordingly, elevated levels of proinflammatory cytokines have been detected during menses [33]. This is also consistent with a previous study from our group demonstrating anti-inflammatory effects of 17β-estradiol in vitro and impaired alternative activation (M2) response ex vivo in macrophages from post-menopausal women, whose estrogen levels are lower compared with women of reproductive age [9].

A further novel aspect of this study is the extensive ex vivo characterization of the monocyte-macrophage system along with the assessment of specific impairment of HDL capacity to promote cell cholesterol efflux in PCOS as compared to healthy donors. Cholesterol efflux is the only mechanism by which macrophages can dispose of excess cholesterol. This may occur via aqueous diffusion or the membrane transporters ABCA1 and ABCG1. In all cases, the efficiency of this process strongly depends on the cholesterol acceptor capacity of circulating HDL, which comprises heterogeneous particles differing in size and composition. Menstrual cycle did not influence HDL function measured as CEC in healthy women. Conversely, HDL CEC impairment in PCOS was observed for all specific pathways, some of which untested in previous studies [3, 23], indicating an overall reduction of HDL activity in regulating cell cholesterol homeostasis. This observation may be of relevance to CVD risk assessment in PCOS, as CEC is an independent predictor of CVD [17, 18]. The mechanisms of impaired CEC appear to be related not only to reduced serum HDL concentrations but also to intrinsic HDL particle dysfunction in PCOS patients. In fact, tCEC and aqueous diffusion-mediated CEC values remained significantly lower in PCOS than in controls following adjustment for HDL-C levels. This suggests that dysfunction affects particularly large mature HDL, known to be the preferential cholesterol acceptors involved in these efflux pathways. Indeed, HDL qualitative particle modifications are known to be even more important than plasma levels for HDL function, and other authors have reported alterations in HDL size and composition in PCOS [34]. Moreover, depletion of estrogen fatty acyl derivatives might be involved in HDL dysfunction in PCOS patients [35].

We found that the menstrual cycle affected neither the distribution of circulating subsets and intracellular cholesterol levels of monocytes nor serum CEC in healthy women. These findings stand against a role for hormonal tuning of these parameters in resting monocytes. This also suggests that the above readouts are more relevant as long-term disease markers than short-term homeostatic factors in healthy women. One obvious limitation of this study is the lack of data with monocytes from PCOS patients, thus we are unable to support the hypothesis that monocyte subset distribution is also altered in PCOS, as it is the case for instance in CVD patients [32, 36].

A hallmark of PCOS is hormonal imbalance resulting from an abnormal ratio between LH and FSH [2] resulting in increased androgen production by theca cells and insufficient conversion of androgens to estradiol, leading in turn to failure in selecting a dominant follicle and chronic anovulation [37]. Hyperandrogenism is associated with upregulation of adipokines, increased recruitment of inflammatory monocytes and macrophage unbalance in the ovaries, suggesting that PCOS is associated with the dysregulation at the reproductive–immunological interface [38]. The LH/FSH ratio is diagnostic and assists in grading the severity of hormonal derangements. We report here for the first time that HDL CEC positively correlated with both LH and FSH serum levels in PCOS patients. This condition is characterized by abnormal pituitary hormone levels, which are stable and do not follow the typical cyclic fluctuations of normal menstrual cycle. Thus, PCOS may represent a favourable condition to unravel a relationship between pituitary hormones and HDL function. Interestingly, although PCOS patients had higher LH and FSH levels and lower CEC, the correlation between the two parameters was direct. Thus, other as yet unknown factors, such as inflammatory mediators and oxidative stress (see below), are conceivably involved in HDL dysfunction in PCOS, and pituitary hormones appear to be somewhat protective. However, such correlations do not imply causality, and underlying mechanisms as well as physiologic implications thereof need to be clarified. Yet, if confirmed in a larger cohort, this novel observation may provide additional mechanistic insights into the biology of the vessel wall. Of interest is also the inverse

correlation between homocysteine concentrations and tCEC in PCOS (Fig. 3c). Homocysteine is a biomarker of oxidant stress, and higher levels were found in PCOS donors compared to controls. HDL homocysteinylation may reduce HDL function. Fasting insulin and homocysteine levels are significantly higher in PCOS patients with an ABCA1 mutant genotype than those with heterozygote and wild-type genotypes [39], with potential implications for HDL function [40]. This mechanism, however, is unlikely to be specific for PCOS.

The main limitations of this study are the small size of groups, the significant difference in BMI between healthy and PCOS women as a potential confounding factor, the limited set of preliminary analyses on monocytes from healthy women and the lack of monocyte characterization in PCOS patients. In addition, patients having higher testosterone, weight and waist circumference values such as those enrolled in this study may not represent the whole PCOS population due to the variety of disease phenotypes. Even so, our pilot study generated novel findings in terms of macrophage activation, serum HDL CEC, and correlations to be confirmed in larger studies.

In conclusion, we showed that regular menstrual cycles were associated with fluctuations in blood-derived macrophage activation, but not in monocytes from healthy women. Macrophage responses to test stimuli in women with PCOS and ovarian dysfunction were attenuated when compared with those in healthy women having normal menstrual cycle. Along with the global impairment of HDL antiatherogenic function in PCOS, these findings provide novel features of the complex relationship between female hormonal status and cardiovascular health [41].

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Author Contributions G.P.F., C.B., and A.C. conceived the idea and planned the experiments. R.M., M.B., S.P., C.S., and G.P.F. conducted the clinical part of the study, S.T. and R.C performed cell culture and flow cytometry analyses, S.T. and G.P.F. carried out statistical analysis, M.P.A., N.R., and F.B performed CEC and intracellular cholesterol assays and refined statistical analyses, M.P. was responsible for laboratory medicine data. A.C. wrote the draft and all authors made substantial contributions to the final manuscript.

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Compliance with ethical standards

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

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of Padova University Hospital Ethics Committee and with the 1964 Helsinki declaration and its later amendments.

Informed consent Informed consent was obtained from all individual participants included in the study.

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