# $CD4^+CD28^{null}$ T lymphocyte frequency, a new marker of cardiovascular risk: relationship with polycystic ovary syndrome phenotypes

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**Objective:** To study the frequency of $CD4^+CD28^{null}$ T cells, which are aggressive T lymphocytes associated with recurrent coronary instability and type 2 diabetes mellitus, in different polycystic ovary syndrome (PCOS) phenotypes and in age- and body mass index–matched healthy women.
**Design:** Retrospective cohort observational study.
**Setting:** Unit of human reproductive pathophysiology, university hospital.
**Patient(s):** A total of 167 PCOS patients and 102 control subjects.
**Intervention(s):** None.
**Main Outcome Measure(s):** $CD4^+CD28^{null}$ T cell frequency, high-sensitive C-reactive protein levels, and other glucose-metabolic parameters.
**Result(s):** $CD4^+CD28^{null}$ frequency was significantly higher in all PCOS groups than in control subjects. $CD4^+CD28^{null}$ frequency was significantly higher in nonhyperandrogenic phenotype (5.7%, range 3.2–7.1) than in phenotypes with hyperandrogenism (H) + oligoamenorrhea (O) + polycystic ovary (PCO) (3.5%, range 1–5.8), H + O (3%, range 1.8–4.7), and H + PCO (2.63%, range 1.2–4.1). The relative risk of non-H phenotype for PCOS women in the highest quartile for $CD4^+CD28^{null}$ frequency compared with PCOS women with the lowest quartile was 3.2 (95% confidence interval 1.9–5.8).
**Conclusion(s):** Cardiovascular risk evaluation should be performed in all PCOS phenotypes. In particular, we demonstrated that the non-H phenotype has potentially increased cardiovascular risk in terms of $CD4^+CD28^{null}$ frequency. (Fertil Steril® 2012;98:1609–15. ©2012 by American Society for Reproductive Medicine.)
**Key Words:** Polycystic ovary syndrome, $CD4^+CD28^{null}$, phenotypes, cardiovascular risk, Rotterdam criteria

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Polycystic ovary syndrome (PCOS) is the most common female endocrinopathy in reproductive age (1, 2). This syndrome is a heterogeneous condition characterized by several symptoms and clinical signs related to reproductive, cardiometabolic, and psychologic disorders (3, 4).

The diagnostic criteria have undergone several changes in recent years. In 1990, the National Institutes of Health (NIH) criteria assessed that PCOS diagnosis required the presence of both biochemical or clinical hyperandrogenism (H) and oligo- or anovulation (O), regardless of the presence of polycystic ovary (PCO) on ultrasound (5). According to this definition, the estimated

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prevalence of the condition was 4%–8% (1, 6–8). In 2003, at the PCOS Consensus Workshop Group meeting held in Rotterdam, the American Society for Reproductive Medicine and the European Society for Reproductive Medicine revised this definition and defined PCOS by the presence of two of the following three features (excluding other etiologies): biochemical or clinical H and O, and PCO on ultrasound (9). Thus, with the Rotterdam Consensus, PCOS prevalence has increased to 12%–18%, also highlighting the clinical heterogeneity of PCOS (4, 10). Moreover, a consequence of that workshop was the generation of two additional phenotypes of PCOS, which were not included in the NIH definition of PCOS: H and PCO without O, and O and PCO without H.

Interestingly, PCOS is often associated with pathologic conditions, such as insulin resistance (IR), type 2 diabetes (DM2), and obesity, and it has potentially increased risk for cardiovascular disease (CVD) (11). Of note, risk factors for CVD, including dyslipidemia, hypertension, oxidative stress, and inflammation, are associated with PCOS (12). We previously demonstrated that young PCOS women exhibit an expansion of an unusual T-cell population that mediate vascular damage, identified by $CD4^+CD28^{null}$ T lymphocytes, and we hypothesized that $CD4^+CD28^{null}$ cells might be involved in the increased long-term cardiovascular risk observed in PCOS women (13).

$CD4^+CD28^{null}$ represent an aggressive T-lymphocyte subset that exerts proinflammatory, proatherogenic, and plaque-destabilizing functions by the production of high levels of interferon (IFN) $\gamma$, tumor necrosis factor $\alpha$, interleukin-2, and cytolytic enzymes (14). Clonality and longevity of $CD4^+CD28^{null}$ T cells, and their well known resistance to apoptosis-inducing signals, suggest that they might be only partially susceptible to immunoregulation (15). These T lymphocyte are rarely found in healthy individuals, are slightly expanded in the elderly, probably because of aging of the immune system, and have been implicated in the pathogenesis of various inflammatory disorders (16, 17). In these conditions, their presence is significantly associated not only with specific disease manifestations indicating their pathogenetic importance, but also with preclinical atherosclerotic changes, including arterial endothelial dysfunction and carotid artery wall thickening (18). A correlation has also been shown between atherosclerosis and the degree of expansion of $CD4^+CD28^{null}$ cells in subjects with chronic kidney disease (19). $CD4^+CD28^{null}$ cell frequency seems to correlate with the extent of coronary artery disease (20). Moreover, patients with DM2 show an expansion of $CD4^+CD28^{null}$ cells in absence of clinical evidence of CVD; $CD4^+CD28^{null}$ expansion is strongly associated with a first cardiovascular event in this population (21). Finally, a raised frequency of these cells has been found in peripheral blood of acute coronary syndrome (ACS) patients and has been associated with recurrent coronary instability (22–24).

Based on these observations, the aim of the present study was to investigate the frequency of $CD4^+CD28^{null}$ T lymphocytes in the different phenotypes of PCOS patients and in healthy age and body mass index (BMI)–matched women. The role of modulating factors, such as IR, H, chronic anovulation, dyslipidemia, or adiposity was also evaluated. Moreover, high-sensitivity C-reactive protein (hs-CRP) levels and other glucose-metabolic parameters were evaluated.

## MATERIALS AND METHODS

### Subjects

This study was approved by the Institutional Review Board of the Policlinico Gemelli, Rome, Italy. It was a retrospective cohort observational study enrolling 167 PCOS patients, aged 18–35 years and with BMI $\leq 25$ kg/m$^2$, and 102 healthy age- and BMI-matched women. PCOS women were recruited at the Day Hospital of Physiopathology of Human Reproduction from January 2006 to September 2011, where they were referred due to problems of hyperandrogenism, acne, or irregular menstruation. Healthy control women were recruited at the Outpatient Clinic for Human Papillomavirus Screening, with negative viral screening results, from January 2006 to August 2011. The control patients were chosen according to normal androgen levels, regular menstrual pattern, absence of hirsutism and acne, and normal ovarian morphology at ultrasound examination.

All PCOS patients were diagnosed and divided into four phenotypes according to the Rotterdam criteria (9): 82 patients with complete phenotype with H + O + PCO (A phenotype); 24 patients with H + O (B phenotype); 30 patients with H + PCO (C phenotype); and 31 patients with O + PCO (D phenotype).

The exclusion criteria were chronic or acute inflammatory disease, neoplasm, autoimmune disease, treatment with clomiphene citrate, oral contraceptives, antiandrogens, drugs to control their appetite or insulin-sensitizing drugs (metformin, pioglitazone and rosiglitazone) during the 6 months before our evaluation, DM2, major surgery in the past 3 months, or other hormonal dysfunction (hypothalamic, pituitary, thyroidal, or adrenal causes).

The following data were obtained from our database: age, BMI, waist-hip ratio (WHR), hirsutism, acne, O, IR, PCO, blood pressure, and cardiovascular risk factors. The cardiovascular risk factors were family history of early coronary artery disease (first-degree relative with a history of myocardial infarction at <60 years of age), smoking and hypertension (systolic blood pressure >140 mm Hg and/or diastolic blood pressure >85 mm Hg or treated hypertension). Hirsutism was evaluated with Ferriman-Gallwey (FG) map scoring system (hirsutism was diagnosed if FG >8) (25), and clinical acne was defined by a history of persistent acne (presence of acne on most days for $\geq 3$ years), recent acne treatment, and presence of >10 inflammatory acne lesions (26). Oligoamenorrhea was defined by <8 spontaneous menstrual cycles per year for $\geq 3$ years before the first examination. IR was determined by calculation of the homeostasis model assessment (HOMA) score as fasting plasma glucose (mg/dL) × fasting plasma insulin ($\mu$U/mL)/405.

For the diagnosis of PCO morphology, all women underwent transvaginal ultrasonography during the early follicular phase. Ovarian volume was calculated by following formula: $V = (\pi/6) \times D_{length} \times D_{width} \times D_{thickness}$, where D denotes the

dimension. The presence of PCO was diagnosed by the presence of ≥12 follicles measuring 2–9 mm in diameter in each ovary and/or increased ovarian volume (>10 $cm^3$ ) (9).

At day 3 of a spontaneous or induced (with medroxyprogesterone acetate, 10 mg/day for 7 days) menstrual cycle, both PCOS patients and control subjects were tested for plasma levels of testosterone (T), sex hormone–binding globulin (SHBG), androstenedione (A), 17-hydroxyprogesterone (17-OHP), dehydroepiandrosterone sulphate (DHEAS), triglycerides, total cholesterol, and high- and low-density lipoprotein cholesterol (HDL and LDL), and complete blood counts were assayed. A second venous blood sample was obtained to analyze T-cell subset frequencies and hs-CRP levels. The free androgen index (FAI) was calculated by the following formula: T × 100/SHBG.

All patients also underwent an oral glucose tolerance test (OGTT; 75 g of glucose); glycemia and insulinemia were assayed basally and every 30 minutes for the 2 following hours. The OGTT data were analyzed as the insulinemic area under the curve calculated by the trapezoidal rule ($AUC_{i-2h}$) (27).

## Laboratory Assays

All hormones were measured in our laboratory. Levels of A (normal range 0.40–3 ng/mL), T (0.20–0.60 ng/mL), SHBG (25–100 nmol/L), DHEAS (800–3000 ng/mL), and 17-OHP (0.2–1.2 ng/mL) were measured in duplicate by radioimmunoassay methods with the use of a commercial kit (Radim). The intra- and interassay coefficients of variation for all these hormones were <7% and <12%, respectively. Insulin (basal 5.0–20.0 μUI/mL) was determined by an immunoradiometric assay (Diasorin), and the intra-assay and interassay coefficients of variation were 2.1%–2.6% and 2.9%–4.7%, respectively.

Plasma glucose (basal 65–110 mg/dL) was determined by the glucose oxidase method. Glucose plasma concentrations were determined by the glucose oxidase technique with a glucose analyzer (Beckam). Total cholesterol (130–200 mg/dL) and triglyceride (20–170 mg/dL) concentrations were determined by an enzymatic assay (Bristol). HDL (>45 mg/dL) concentrations were determined after precipitation of chylomicrons, very-low-density lipoprotein, and LDL (Boehringer, Mannheim, Germany). A magnesium chloride/phosphotungstic acid technique was used to precipitate LDL (<130 mg/dL), from the bottom fraction after ultracentrifugation.

Total and differential white blood cell counts and T-cell subset distributions were analyzed on fresh blood samples with a Bayer H-3 hematology analyzer using automated cytochemistry in flow. Heparinized (10 IU/mL) whole-blood samples were stained with fluorescein isothiocyanate–conjugated anti-CD4 (Becton Dickinson) and phycoerythrin-conjugated anti-CD28 (Pharmingen) monoclonal antibodies (mAb) and were analyzed by two-color flow cytometry on the Coulter Epics XL (Beckman Coulter). Nonspecific staining with isotype-matched control mAb was <1%; the intra-assay and interassay variabilities were <10%. The frequencies of total $CD4^+$ and $CD4^+CD28^{null}$ were determined with the use of WinMDI software (Joseph Trotter, Scripps Research Institute,). $CD4^+CD28^{null}$ frequency was expressed as the percentage of the entire population of $CD4^+$ T cells. Coded plasma and serum samples were stored at −80°C and analyzed for hs-CRP in a single batch at the end of the study. hs-CRP was measured using a high-sensitivity latex-enhanced immunonephelometric assay (Latex/BN II; Dade Behring). The working range of the assay was 0.175–1,100 mg/L, and the coefficient of variation was <5%. The median normal value for hs-CRP is 0.8 mg/L, with 90% of normal values <3 mg/L.

## Sample Size

A sample size of 82, 24, 30, and 31 PCOS patients and 102 control subjects was obtained from the five groups whose $CD4^+CD28^{null}$ frequencies were compared. The total sample of 267 subjects achieved 100% power to detect the differences observed within the groups with a .05 significance level.

Owing to the small sample size of our study and the multiple comparisons performed among different study groups, we cannot exclude that a type 1 B error might have been observed in our statistical analysis.

## Statistical Analysis

Data distribution was assessed by the Kolmogorov-Smirnov test. $CD4^+CD28^{null}$ frequency and hs-CRP levels had a skewed distribution. Continuous variables were expressed as mean ± SD or median and interquartile range as appropriate; dichotomous variables were expressed as percentages. Continuous variables among the four groups of subjects in the study population were compared with analysis of variance or Kruskal-Wallis test as appropriate; unpaired *t* test or Mann-Whitney *U* test were used, as appropriate, for pair-wise comparisons; categorical variables were compared with the use of chi-square test or Fisher exact test, as appropriate. Bonferroni (Ba) or Dunn correction for multiple comparisons was applied. Correlations between variables were performed with the Pearson or Spearman correlation test as appropriate. Analysis of trends was used to test for associations between increasing levels of $CD4^+CD28^{null}$ T lymphocytes and the risk of belonging to PCOS D phenotype, after the sample including all PCOS women was divided into quartiles. Adjusted risk estimates were obtained with use of logistic-regression models in addition to accounting for the variables used for matching (age and BMI). All analyses were performed with the use of SPSS v. 16.0 software.

# RESULTS

Among the 167 PCOS patients retrospectively screened, 82 women (49.1%) exhibited complete phenotype with H + O + PCO (A group), 24 (14.5%) had H + O (B group), 30 (17.9%) had H + PCO (C group), 31 (18.5%) had O + PCO (D group). No differences were observed among the four phenotypes and control group regarding age, BMI, or WHR (Table 1).

Clinical and biochemical characteristics (including anthropometric measurements and hormonal and metabolic

## TABLE 1

**Baseline characteristics of the study population according to polycystic ovary syndrome (PCOS) phenotypes and in control subjects.**

| | PCOS phenotype (n = 167) | | | | | |
|---|---|---|---|---|---|---|
| **Parameter** | **A (n = 82)** | **B (n = 24)** | **C (n = 30)** | **D (n = 31)** | **Control (n = 102)** | ***P* value (overall)** |
| Age (y), mean ± SD | 25 ± 4 | 26 ± 5 | 25 ± 5 | 25 ± 6 | 25 ± 4 | NS |
| BMI (kg/m$^2$), median (range) | 24 (21–24) | 22.1 (19–23) | 22 (21–24) | 22 (19–24) | 23 (21–24) | NS |
| WHR, median (range) | 0.75 (0.75–0.8) | 0.7 (0.7–0.8) | 0.74 (0.77–0.84) | 0.8 (0.7–0.88) | 0.75 (0.6–0.79) | NS |
| Smoking, n (%) | 8 (9.7) | 2 (8.3) | 3 (10) | 4 (12,9) | 11 (10.7) | NS |
| Hypertension, n (%) | 9 (10.9)[c] | 3 (12,5)[c] | 3 (10)[c] | 4 (12.9)[c] | 0 (0) | NS |
| Family history, n (%) | 33 (40)[b] | 8 (33)[b] | 11 (36.6)[b] | 12 (38.7)[b] | 0 (0) | NS |
| Total cholesterol (mg/dL), mean ± SD | 178.98 ± 32.59[c] | 187.5 ± 25.5[c] | 179.2 ± 35.8[c] | 192.6 ± 59[c] | 159 ± 18 | NS |
| LDL cholesterol (mg/dL), mean ± SD | 104.6 ± 28.1[d] | 107.2 ± 9.8[d] | 100.9 ± 26.6[d] | 121.9 ± 56[c] | 67 ± 7 | NS |
| HDL cholesterol (mg/dL), mean ± SD | 65.53 ± 13.57[d] | 56 ± 13.64[d] | 65.46 ± 14.13[d] | 55.74 ± 15.23[d] | 89.12 ± 19.1 | .001[3,6] |
| Triglycerides (mg/dL), mean ± SD | 88.9 ± 34.2 | 72.3 ± 33.4 | 63.9 ± 17.2 | 120 ± 36.69[a] | 71 ± 19 | .001[3,5,6] |
| Glycemia (mg/dL), mean ± SD | 82.8 ± 10.3[d] | 76 ± 4.9 | 74.3 ± 15.1 | 79.4 ± 5.8 | 71 ± 5 | NS |
| Insulin (μIU/mL), mean ± SD | 11.8 ± 8[a] | 9 ± 3.8[b] | 12 ± 10.5[a] | 9.3 ± 5.1[b] | 5.9 ± 1.3 | NS |
| Insulinemic area (μIU/mL × 120), mean ± SD | 9.277 ± 5.650[b] | 3.438 ± 2.362 | 10.190 ± 3.121[b] | 6.151 ± 3.981[d] | 4.024 ± 1.114 | .004[1,4] |
| HOMA-IR, median (range) | 1.85 (1.29–2.94)[c] | 1.68 (0.6–2.12)[c] | 1.6 (0.9–2.03)[c] | 1.46 (0.8–2)[d] | 1.1 (0.7–1.7) | NS |
| T (ng/mL), median (range) | 0.62 (0.3–0.8)[c] | 0.7 (0.4–1.2)[c] | 0.55 (0.3–0.6)[d] | 0.31 (0.29–0.55) | 0.35 (0.3–0.5) | .002[2,3,4,5] |
| FAI, mean ± SD | 8.4 ± 1.1[a] | 9.1 ± 1.2[a] | 4.1 ± 0.8[c] | 2.6 ± 0.7 | 1.4 ± 0.3 | .000[2,3,4,5] |
| SHBG (nmol/L), median (range) | 32.4 (19.7–58)[d] | 38.9 (11.2–56.5)[d] | 37.6 (20–54)[d] | 51 (24.4–71.2) | 56 (25.4–68.3) | NS |
| A (ng/mL), median (range) | 3.5 (2.7–4.3)[d] | 3.2 (2.2–4.3)[d] | 2.6 (2–3.6)[d] | 2 (1.83–2.68) | 1.7 (1.4–2.4) | .000[2,3,5] |
| DHEAS (ng/mL), median (range) | 3,025 (2,200–3,842)[d] | 3,485 (2,962–4,341)[d] | 2,645 (2,322–3,283)[d] | 2,164 (1,651–2,685) | 2,000 (1,478–2,895) | .000[3,4,5] |
| 17-OHP (ng/mL), median (range) | 0.9 (0.6–1.1)[c] | 0.75 (0.52–1.5)[c] | 0.8 (0.5–0.9)[d] | 0.6 (0.5–0.7) | 0.5 (0.3–0.8) | .041[3] |
| Ovarian volume (cm$^3$), median (range) | 11.17 (8.6–15.1)[c] | 7.7 (6.5–12.1) | 9.8 (6.4–14.8)[d] | 9.48 (7.6–12.8)[d] | 7.6 (4.6–8.9) | .020[1,4,5] |

*Note:* Family history = family history of early coronary artery disease; FAI = free androgen index; HOMA-IR = homeostasis model assessment of insulin resistance; WHR = waist-hip ratio.
Significant differences in the post hoc comparisons between PCOS phenotypes and control subjects: [a]$P<.001$; [b]$P<.005$; [c]$P<.01$; [d]$P<.05$. Significant differences ($P<.05$) in the post hoc comparisons among the four PCOS phenotypes: [1]between A and B phenotypes; [2]between A and C phenotypes; [3]between A and D phenotypes; [4]between B and C phenotypes; [5]between B and D phenotypes; [6]between C and D phenotypes.

*Moro. CD4$^+$CD28$^{null}$ T cells and PCOS phenotypes. Fertil Steril 2012.*

## TABLE 2

**$CD4^+CD28^{null}$ T-cell frequencies and high-sensitivity C-reactive protein (hs-CRP) levels among polycystic ovary syndrome (PCOS) phenotypes and in control subjects.**

| | PCOS phenotype (n = 167) | | | | | |
|---|---|---|---|---|---|---|
| **Parameter** | **A (n = 82)** | **B (n = 24)** | **C (n = 30)** | **D (n = 31)** | **Control (n = 102)** | ***P* value (overall)** |
| Lymphocyte count ($\times 10^9$/L) | 2.1 (1.7–3.9) | 2 (1.8–3.5) | 2.3 (1.5–3.8) | 2.1 (1.6–3.4) | 1.9 (1.1–3.4) | NS |
| Total $CD4^+$ frequency (%) | 44.1 (39–51) | 43 (38–52.3) | 41.9 (37.1–53.7) | 42.5 (37.6–52.4) | 41 (35–49) | NS |
| $CD4^+CD28^{null}$ frequency (%) | 3.5 (1–5.8)[b] | 3 (1.8–4.7)[b] | 2.63 (1.2–4.1)[d] | 5.7 (3.2–7.1)[a] | 0.3 (0.1–0.8) | .002[1,2,3] |
| hs-CRP (mg/L) | 1.5 (0.8–2.95) | 1.2 (0.8–2.02) | 1.3 (1.1–2.1) | 2 (0.9–2.45)[d] | 1 (0.8–2.1) | NS |

*Note:* Values are presented as median (range). Significant differences in the post hoc comparisons between PCOS phenotypes and control subjects: [a]$P<.001$; [b]$P<.005$; [c]$P<.01$; [d]$P<.05$. Significant differences ($P<.05$) in the post hoc comparisons among the four PCOS phenotypes: [1]between A and D phenotypes; [2]between B and D phenotypes; [3]between C and D phenotypes.

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profiles) of the 167 PCOS and 102 control subjects are presented in Table 1.

### Clinical and Biochemical Characteristics

In univariate analysis, all PCOS groups had higher total cholesterol and LDL and lower HDL levels than the control group (Table 1). Of note, the D phenotype showed significantly higher levels of triglycerides and lower HDL levels compared with A, B, and C phenotypes (Table 1).

Basal glycemic levels were higher only in A phenotype compared with control subjects, whereas basal insulin level was higher in all PCOS groups compared with control subjects. All PCOS phenotypes were more insulin resistant (HOMA-IR) than the control group, and only the A, C, and D groups showed higher $AUC_{i-2h}$ than control subjects (Table 1). B phenotype showed a reduced $AUC_{i-2h}$ compared with A and C groups, but no significant differences were found in HOMA-IR, basal glycemia, and insulin among the four groups (Table 1).

### Hormonal Profiles

Circulating androgens were higher in women with A, B, and C phenotypes than in the control group (Table 1). Total T and FAI were higher in A and B phenotypes compared with C or D. A was significantly higher in A and B phenotypes than in D. Significantly higher DHEAS levels were detected in the A and B phenotypes than in the D phenotype, and 17-OHP levels were higher in the A group than in the D population (Table 1).

### Ultrasound Evidence

As expected, ovarian volume was higher in A, C, and D groups compared with B. However, only the A phenotype showed a median ovarian volume $>10$ $cm^3$ (Table 1).

### $CD4^+CD28^{null}$ T-Cell Frequencies and hs-CRP Levels

$CD4^+CD28^{null}$ frequency was significantly higher in all PCOS groups than in control subjects (0.3%, range 0.1–0.8; Table 2). $CD4^+CD28^{null}$ frequency was significantly higher in D phenotype patients (5.7%, range 3.2–7.1) than in A (3.5%, range 1–5.8; Ba $P=.021$), B (3%, range 1.8–4.7; Ba $P=.016$), and C groups (2.63%, range 1.2–4.1; Ba $P=.003$). No other statistical differences in $CD4^+CD28^{null}$ frequencies were observed between A and B, A and C, or B and C patients (Table 2). Of note, no significant differences were found between groups regarding lymphocyte count or total $CD4^+$ T-cell frequency (Table 2). hs-CRP levels were higher in women with D phenotype compared with the control group, whereas no differences were found in hs-CRP levels among the four PCOS phenotypes (Table 2). Of note, $CD4^+CD28^{null}$ frequency did not correlate with hs-CRP levels ($P=.34$).

Table 3 shows the relative risks of belonging to D phenotype according to quartiles of $CD4^+CD28^{null}$ when considering all PCOS women. Measurements of higher $CD4^+CD28^{null}$ appeared to be predictive of D phenotype. Of note, the relative risk of belonging to D phenotype for PCOS women with the highest quartile for $CD4^+CD28^{null}$ frequency compared with PCOS women with the lowest quartile was 3.2 (95% confidence interval 1.9–5.8; $P<.001$).

## DISCUSSION

The present study aimed to evaluate a new cardiovascular risk marker, $CD4^+CD28^{null}$ T-lymphocyte frequency, in the four different PCOS phenotypes. Our attention was focused on this subset of T lymphocytes because of their proatherogenic and plaque-destabilizing properties. They release large amounts of proinflammatory cytokines, particularly IFN-γ (thus activating monocytes and macrophages) (28), and

## TABLE 3

**Relative risk (RR) and 95% confidence interval (CI) of D phenotype by $CD4^+CD28^{null}$ quartiles in all polycystic ovary syndrome phenotypes.**

| $CD4^+CD28^{null}$ quartile | RR | 95%CI | *P* value for trend |
|---|---|---|---|
| First quartile | 1.0 | 0.8–1.2 | .01 |
| Second quartile | 1.9[a] | 1.4–2.6 | |
| Third quartile | 2.7[b] | 2.0–4.6 | |
| Fourth quartile | 3.2[c] | 1.9–5.8 | |

[a] Second quartile vs. first quartile: $P=.04$.
[b] Third quartile vs. first quartile: $P=.01$.
[c] Fourth quartile vs. first quartile: $P<.001$.

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have direct cytolytic effects on endothelial cells, amplified by hs-CRP (29), and on vascular smooth muscle cells (30). By directly stimulating apoptosis of vascular smooth muscle or by coordinating and activating macrophages to kill these cells through the elevated production of IFN-γ, $CD4^+CD28^{null}$ T cells could weaken the fibrous cap and destabilize angiogenic vessels, precipitating atherosclerotic plaque rupture (14). In acute coronary syndrome (ACS), $CD4^+CD28^{null}$ T cells are increased in peripheral blood (22) and infiltrate unstable coronary plaques, where they undergo clonal expansion (23) probably triggered by specific antigens (31, 32). Moreover, the frequency of these T cells in peripheral blood is strongly associated with recurrent episodes of instability in patients with ACS. In particular, patients with recurrent instability showed about fourfold higher $CD4^+CD28^{null}$ T-cell frequency compared with patients with unstable angina as first-ever event (24). $CD4^+CD28^{null}$ T cells have also been demonstrated to be expanded in DM2 patients, where they are associated with poor glycemic control. They were also strongly linked to the first cardiovascular event in the same patients (21). In various inflammatory disorders, such as systemic lupus erythematosus and rheumatoid arthritis, $CD4^+CD28^{null}$ T-cell frequency is significantly associated with preclinical atherosclerotic changes (18). A correlation has also been shown between atherosclerosis and the degree of expansion of $CD4^+CD28^{null}$ cells in subjects with chronic kidney disease (19), and $CD4^+CD28^{null}$ frequency seems to correlate with the extent of coronary artery disease (20).

We recently demonstrated that young women with PCOS exhibit an expansion of this aggressive T-cell population compared with control subjects. Additionally, $CD4^+CD28^{null}$ frequency was not associated with hyperinsulinemia, hs-CRP levels, or androgen levels, but only with PCOS status (13). In the present study, we showed that $CD4^+CD28^{null}$ frequency was significantly higher in all PCOS phenotypes than in the control group. Moreover, we showed that $CD4^+CD28^{null}$ frequency is significantly higher in the nonhyperandrogenic PCOS (D) phenotype than in the other three PCOS phenotypes. Moreover, the detection of high $CD4^+CD28^{null}$ frequency even among nonobese PCOS patients may underline the need to screen all PCOS patients independently from weight and hyperandrogenic status.

The reason for expansion of this population in young women with PCOS is not known. PCOS is a state of inflammatory activation (12). It has been suggested that chronic inflammation and persistent infection can induce loss of CD28 from the cell surface (17). The circulating hs-CRP levels were significantly increased in the PCOS population, but we did not find a statistically significant association of this parameter with the $CD4^+CD28^{null}$ T-cell population.

Our results seem to be in contrast with some recent data supporting the hypothesis that the hyperandrogenic phenotypes are associated to the highest cardiovascular risk (33–39). Different data suggested that, rather than H, chronic anovulation could play a key role in the cardiometabolic risk in PCOS patients (40–42). In this scenerio, other authors did not find any differences regarding cardiometabolic characteristics among the various PCOS phenotypes (4, 43).

The present study presents some limitations. We retrospectively recruited subjects with PCOS based on the Rotterdam criteria. Therefore, our distribution of patients into the four different PCOS phenotypes may not be accurate, and a prospective study will be needed to confirm the distribution of women with PCOS in these phenotypes. Moreover, the majority of patients seen in our hospital are white (>90%), and the distribution of the PCOS phenotypes in other ethnic groups, especially those with higher rates of metabolic complications (African Americans and Hispanic Americans), needs to be evaluated. Moreover, we lacked additional markers of cardiovascular risk, such as endothelial cell markers, flow-mediated dilation, or carotid intima-media thickness. Indeed, our aim was only to assess the association between $CD4^+CD28^{null}$ T-lymphocyte frequency and the four different PCOS phenotypes.

In conclusion, our results show that all PCOS phenotypes have increased $CD4^+CD28^{null}$ frequency compared with control subjects. Moreover, we demonstrated that the non-H phenotype shows the highest levels of $CD4^+CD28^{null}$. Based on these results, we suggest evaluating the cardiovascular risk factors in the whole PCOS population and not only in the H phenotypes.

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