$CD4^+CD28^{null}$ T lymphocytes are expanded in young women with polycystic ovary syndrome

Women affected by polycystic ovary syndrome (PCOS) have an increased risk of cardiovascular disease. We demonstrated that women with PCOS showed an expansion of $CD4^+CD28^{null}$ T cells, an aggressive population of T lymphocytes that has been recently associated with recurrent coronary instability and type 2 diabetes mellitus. This sheds new light on possible mechanisms responsible for the higher rate of cardiovascular disease among women with PCOS. (Fertil Steril® 2011;95:2651–4. ©2011 by American Society for Reproductive Medicine.)

Key Words: Hyperinsulinemia, inflammation, polycystic ovary syndrome, T lymphocytes

Young women have a lower risk of cardiac events, but this protection fades after menopause, leaving them vulnerable to developing cardiovascular disease (CVD) (1). Endocrine and gynecologic diseases may influence this paradigm. Indeed, polycystic ovary syndrome (PCOS), the most common female endocrinopathy in reproductive age, seems to be associated with an increased cardiovascular risk (2, 3). The syndrome is characterized by chronic anovulation and hyperandrogenism (4, 5). Other features of PCOS such as insulin resistance (IR), obesity, dyslipidemia, chronic low-grade inflammation, and prothrombotic modifications may contribute to the association with CVD (6–14). Moreover the high prevalence of IR in women with PCOS suggests common ground between this syndrome and type 2 diabetes (T2DM). Recent data have demonstrated that patients with T2DM show an expansion of an unusual T-cell population, identified by $CD4^+CD28^{null}$ T lymphocytes in absence of clinical evidence of CVD (15). $CD4^+CD28^{null}$ represent an aggressive T-lymphocyte subset that differs from conventional $CD4^+CD28^+$ helpers. $CD4^+CD28^{null}$ have proinflammatory functions by the production of high levels of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and interleukin-2 (IL-2) (16). In addition, $CD4^+CD28^{null}$ are cytotoxic (17) by releasing cytolytic enzymes (18). $CD4^+CD28^{null}$ have been found to be expanded in patients with unstable angina and have recently been associated to recurrent coronary instability (19–21).

Starting from these observations, we compared $CD4^+CD28^{null}$ frequencies in young women with PCOS but without overt CVD compared with healthy controls. Because high-sensitivity C-reactive protein (HS-CRP) levels are often increased in PCOS (8), we also assessed this biomarker to evaluate the relationship between chronic low-grade inflammation and $CD4^+CD28^{null}$.

The study population was made by 30 hyperinsulinemic and 30 normoinsulinemic women with PCOS (range: 18 to 37 years of age). All participants were volunteers who gave informed consent to participate to the study, which was approved by the institutional review board of our institute. Polycystic ovary syndrome was diagnosed in accordance with the Rotterdam Consensus Conference (22). The exclusion criteria were [1] chronic or acute inflammatory disease, [2] neoplasm, [3] autoimmune disease, [4] intake of drugs in the last 30 days, [5] history of CVD, [6] diabetes mellitus, [7] major surgery in the last 3 months, or [8] other hormonal dysfunction (hypothalamic, pituitary, thyroidal, or adrenal causes).

Twenty-three healthy, normally ovulating, age-matched women served as controls. Moreover, the controls were matched for body mass index (BMI) with the normoinsulinemic PCOS patients. All controls had ovaries of normal appearance on ultrasound examination, and had normal luteinizing (LH) and follicle-stimulating hormone (FSH) levels. None had elevated androgen levels.

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At day 3 of a spontaneous or induced (with medroxyprogesterone acetate, 10 mg/day for 7 days) menstrual cycle, all patients underwent a clinical examination, which included measurements of blood pressure, BMI, and waist-hip ratio (WHR) as well as transvaginal ultrasonography. An abnormal WHR was considered >0.85. Cardiovascular risk factors were carefully examined: family history of early coronary artery disease (first-degree relative with an history of myocardial infarction at <60 years), hypercholesterolemia (total cholesterol >200 mg/dL or treated hypercholesterolemia), smoking, and hypertension (systolic blood pressure >140 mm Hg and/or diastolic blood pressure ≥85 mm Hg or treated hypertension). Metabolic syndrome was diagnosed according to the Adult Treatment Panel III criteria (23). On the same day, testosterone (T), sex hormone–binding globulin (SHBG), free androgen index (FAI), androstenedione (A), 17-hydroxyprogesterone (17-OHP), dehydroepiandrosterone sulfate (DHEAS), triglycerides, total cholesterol, high- and low-density lipoprotein cholesterol (HDL and LDL), and complete blood count were assayed. A second venous blood sample was obtained to analyze T-cell subset frequencies and HS-CRP levels.

All women underwent an oral glucose tolerance test (OGTT; 75 g of glucose); glycemia and insulinemia were assayed basally and every 30 minutes for the two following hours. The OGTT data were analyzed as the area under the curve (AUC) (insulinemic area) calculated by the trapezoidal rule. Hyperinsulinemia was defined as an insulinemic area value ≥7000 μIU/mL (24). According to insulinemic area, PCOS was classified as normoinsulinemic or hyperinsulinemic.

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 (Bayer Diagnostic Division, Tarrytown, NY) using automated cytochemistry in flow. Heparinized (10 IU/mL) whole blood samples were stained with fluorescein isothiocyanate-conjugated anti-CD4 (Becton Dickinson, San Jose, CA) and phycoerythrin-conjugated anti-CD28 (Pharmingen, San Diego, CA) monoclonal antibodies (mAb), and were analyzed by two-color flow cytometry on the Coulter Epics XL (Beckman Coulter, Fullerton, CA). Nonspecific staining with isotype-matched control mAb was <1%; the intra-assay and interassay variability was <10%. The frequencies of total $CD4^+$ and $CD4^+CD28^{null}$ were determined using WinMDI software (Joseph Trotter, Scripps Research Institute, La Jolla, CA). $CD4^+CD28^{null}$ 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. The C-reactive protein was measured using a high-sensitivity latex-enhanced immunonephelometric assay (Latex/BN II; Dade Behring, Marburg, Germany). The working range of the assay was 0.175 to 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.

Data distribution were assessed by the Kolmogorov-Smirnov test. $CD4^+CD28^{null}$ frequency and HS-CRP levels had a skewed distribution. Continuous variables were expressed as mean and standard deviation (mean ± SD) or median and interquartile range, as appropriate; dichotomous variables were expressed as percentages. Continuous variables among the three groups of subjects in the study population were compared with analysis of variance (ANOVA) 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 using the chi-square test or Fisher's exact test, as appropriate. Bonferroni correction for multiple comparison was applied. Correlations between variables were performed using the Pearson or Spearman correlation test, as appropriate. Multivariable linear regression analysis was performed to assess independent predictors of $CD4^+CD28^{null}$ frequency. At this scope, in the model we included all variables showing a statistically significant association at univariate analysis ($P<.05$), including PCOS status, glycemia levels, HS-CRP levels, and FAI. $P<.05$ was considered statistically significant. All analyses were performed by use of SPSS, v. 16.0 software (SPSS, Inc., Florence, Italy).

Table 1 shows comparisons of age, physical findings, cardiovascular risk factors, and main laboratory data among the normoinsulinemic (n = 30; age: 25 ± 5 years) and hyperinsulinemic (n = 30; age: 25 ± 6) women with PCOS and the controls. $CD4^+CD28^{null}$ frequency was statistically significantly higher in normoinsulinemic (2.7%: 1.2% to 4.0%) and in hyperinsulinemic women with PCOS (2.5%: 1.2% to 5.0%) than in controls (0.2%: 0.1% to 0.9%; $P<.001$, respectively), whereas no statistically significant difference was found between normoinsulinemic and hyperinsulinemic women with PCOS ($P=1$). Conversely, HS-CRP levels were statistically significantly higher in hyperinsulinemic (2.5: 1.2–4.6 mg/L) than in normoinsulinemic (1.4: 0.6–1.8, $P=.003$) women with PCOS and in controls (1.1: 0.8–2.1 mg/L, $P<.001$). A statistically significant positive correlation was found between $CD4^+CD28^{null}$ frequency and glycemia ($r = 0.48$, $P<.001$), HS-CRP serum levels ($r = 0.21$, $P=.05$), and FAI ($r = 0.42$, $P<.001$). Multivariable linear regression analysis showed that PCOS status only independently predicted $CD4^+CD28^{null}$ frequency (B = 1.21, ES = 0.23, $P<.001$).

Our study has demonstrated, for the first time, that young women with PCOS exhibit an expansion of an aggressive subset of $CD4^+CD28^{null}$ T lymphocytes. Of note, neither hyperinsulinemia nor HS-CRP levels were associated with $CD4^+CD28^{null}$ frequency. Although a correlation was found between FAI and $CD4^+CD28^{null}$ frequency at univariate analysis, this androgenic index was not a predictive factor in the multivariable analysis. However, the small sample size of our study may have underpowered a possible role of androgens in $CD4^+CD28^{null}$ expansion.

Our attention was focused on this subset of T lymphocytes for several reasons. First, they exert a potent proinflammatory activity through the production of large amounts of INF−γ and TNF−α (16). Second, they are also efficient killer cells: they can lyse endothelial target cells and resist to proapoptotic signals (17, 25). Finally, a raised frequency of these cells has been found in peripheral blood of acute coronary syndrome patients (19–21), and seems to correlate with the extent of coronary artery disease (26). Taken together these observations suggest that expanded $CD4+CD28^{null}$ might be involved in the increased long-term cardiovascular risk observed in PCOS. Similar to our findings, patients with autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis have an increased risk of developing atherosclerosis (27, 28). $CD4^+CD28^{null}$ are a key component of the immune response that mediates vascular damage as observed both in autoimmune disorders and coronary artery disease (19, 29).

TABLE 1

Comparison of baseline features between women with polycystic ovary syndrome and controls.

Parameters	Normoinsulinemic PCOS (n = 30)	Hyperinsulinemic PCOS (n = 30)	Controls (n = 23)	*P* value
Age (y), mean ± SD	25 ± 5	25 ± 6	26 ± 6	NS
Smoking, n (%)	3 (10)	11 (38)	2 (8)	.07
Dyslipidemia, n (%)	5 (17)	10 (33)	2 (9)	.09
Family history, n (%)	1 (3)	16 (53)	0 (0)	<.001
Hypertension, n (%)	1 (3)	8 (27)	0 (0)	.003
Metabolic syndrome, n (%)	0 (0)	6 (20)	0 (0)	<.001
SBP (mm Hg), mean ± SD	112 ± 11	120 ± 12	117 ± 4	.016
DBP (mm Hg), mean ± SD	75 ± 8	81 ± 8	74 ± 5	.004
WHR >0.85, n (%)	7 (23)	16 (53)	0 (0)	<.001
BMI >30, n (%)	0 (0)	7 (24)	0 (0)	<.001
Total cholesterol (mg/dL), mean ± SD	176 ± 45	183 ± 27	164 ± 20	.14
LDL cholesterol (mg/dL), mean ± SD	101 ± 42	107 ± 24	62 ± 8	.10
HDL cholesterol (mg/dL), mean ± SD	62 ± 12	54 ± 17	88 ± 22	.04
Triglycerides (mg/dL), mean ± SD	61 ± 26	98 ± 44	71 ± 22	<.001
Glycemia (mg/dL), mean ± SD	82 ± 7	87 ± 6	73 ± 5.22	<.001
Insulin (μIU/mL), mean ± SD	6.2 ± 2.7	14.1 ± 9.1	5.7 ± 1.4	<.001
Insulinemic area (μIU/mL × 120), mean ± SD	4,537 ± 1,508	1,2911 ± 6,218	4,021 ± 1,223	<.001
Lymphocyte count ($\times 10^9$/L)	1.7 (1.1–3.9)	2.2 (1.7–3.0)	2.0 (1.2–3.3)	.92
Total $CD4^+$ T-cell frequency (%), median (range)	45 (39–51)	43 (37–50)	40 (33–48)	.48
$CD4^+CD28^{null}$ frequency (%), median (range)	2.7 (1.2–4)	2.5 (1.2–5)	0.2 (0.1–0.9)	<.001
HS-CRP (mg/L), median (range)	1.4 (0.6–1.8)	2.5 (1.2–4.6)	1.1 (0.8–2.1)	<.001
T (ng/mL), median (range)	0.56 (0.34–0.52)	0.60 (0.41–1.2)	0.46 (0.43–1.27)	.001
SHBG (nmol/L), median (range)	71.2 (69.2–73.4)	54.9 (35.3–83)	31.2 (28.9–54.8)	<.001
FAI, mean ± SD	4.7 ± 2.9	11.8 ± 8	1.2 (1–1.4)	<.001
A (ng/mL), median (range)	2.4 (1.2–1.5)	3.8 (2.1–3.5)	1.4 (2.8–4.6)	<.001
DHEAS (ng/mL), median (range)	2,559 (1,654–2,134)	3,081 (1,875–3,065)	2,000 (2,361–4156)	<.001
17-OHP (ng/mL), median (range)	0.70 (0.40–0.70)	0.95 (0.50–1.10)	0.50 (0.77–1.2)	.002

Note: Pair-wise comparisons between groups are reported in the text. 17-OHP = 17-hydroxyprogesterone; A = androstenedione; BMI = body mass index; DBP = diastolic blood pressure; DHEAS = dehydroepiandrosterone sulfate; FAI = free androgen index; HDL = high-density lipoprotein; HS-CRP = high-sensitivity C-reactive protein; LDL = low-density lipoprotein; PCOS = polycystic ovary syndrome; SBP = systolic blood pressure; SD = standard deviation; SHBG = sex hormone–binding globulin; T = testosterone; WHR = waist-hip ratio.

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In our study, hyperinsulinemic PCOS showed a clustering of adverse features (higher levels of HS-CRP, altered lipidic and glycemic profile, high blood pressure, and features of the metabolic syndrome) which may further increase the risk of coronary artery disease (30, 31). Higher levels of $CD4^+CD28^{null}$ in PCOS could be part of the complex pathogenic mechanism of this syndrome. Of course, further studies are needed to better understand the causes and mechanisms underlying the expansion of $CD4^+CD28^{null}$ in PCOS and its possible link with hyperandrogenism. Our study demonstrated the presence of higher levels of $CD4^+CD28^{null}$ in young women with PCOS. We also found higher levels of HS-CRP in women with PCOS, in particular in the subset who are insulin resistant. Taken together, our findings suggest that both activation of innate immunity as well as dysregulation of adaptive immunity play a pathogenetic role in this complex syndrome.

REFERENCES

1. Stramba-Badiale M, Fox KM, Priori SG, Collins P, Daly C, Graham I, et al. Cardiovascular diseases in women: a statement from the policy conference of the European Society of Cardiology. Eur Heart J 2006;27:994–1005.
2. Talbott E, Guzick D, Clerici A, Berga S, Detre K, Weimer K, et al. Coronary heart disease risk factors in women with polycystic ovary syndrome. Arterioscler Thromb Vasc Biol 1995; 15:821–6.
3. Cibula D, Cífková R, Fanta M, Poledne R, Zivny J, Skibová J. Increased risk of non-insulin dependent diabetes mellitus, arterial hypertension, and coronary artery disease in perimenopausal women with a history of the polycystic ovary syndrome. Hum Reprod 2000;15:785–9.
4. Franks S. Polycystic ovary syndrome. N Engl J Med 1995;333:853–61.
5. Scarpitta AM, Sinagra D. Polycystic ovary syndrome: an endocrine and metabolic disease. Gynecol Endocrinol 2000;14:392–5.
6. Legro RS. Diabetes prevalence and risk factors in polycystic ovarian syndrome. Obstet Gynecol Clin North Am 2001;28:99–109.
7. Dunaif A, Segal KR, Futterweit W, Dobrjansky A. Profound peripheral insulin resistance, independent of obesity, in polycystic ovary syndrome. Diabetes 1989;38:1165–74.
8. Kelly CC, Lyall H, Petrie JR, Gould GW, Connell JM, Sattar N. Low-grade chronic inflammation in women with polycystic ovarian syndrome. J Clin Endocrinol Metab 2001;86:2453–5.
9. Schachter M, Raziel A, Friedler S, Strassburger D, Bern O, Ron-El R. Insulin resistance in patients with polycystic ovary syndrome is associated with elevated plasma homocysteine. Hum Reprod 2003;18:721–7.

10. Legro RS, Kunselman AR, Dodson WC, Dunaif A. Prevalence and predictors of risk for type 2 diabetes mellitus and impaired glucose tolerance in polycystic ovary syndrome: a prospective controlled study in 254 affected women. J Clin Endocrinol Metab 1999;84:165–9.
11. Apridonidze T, Essah PA, Iuorno MJ, Nestler JE. Prevalence and characteristics of the metabolic syndrome in women with polycystic ovary syndrome. J Clin Endocrinol Metab 2005;90: 1929–35.
12. Kelly CJ, Speirs A, Gould GW, Petrie JR, Lyall H, Connell JM. Altered vascular function in young women with polycystic ovary syndrome. J Clin Endocrinol Metab 2002;87:742–6.
13. Talbott EO, Guzick DS, Sutton-Tyrrell K, McHugh-Pemu KP, Zborowski JV, Remsberg KE, et al. Evidence for association between polycystic ovary syndrome and premature carotid atherosclerosis in middle-aged women. Arterioscler Thromb Vasc Biol 2000;20:2414–21.
14. Battaglia C, Mancini F, Cianciosi A, Busacchi P, Persico N, Paradisi R, et al. Cardiovascular risk in normal weight, eumenorrheic, nonhirsute daughters of patients with polycystic ovary syndrome: a pilot study. Fertil Steril 2009;92: 240–9.
15. Giubilato S, Brugaletta S, Smaldone C, Pazzano V, Pitocco D, Ghirlanda G, et al. Cardiovascular risk in T2DM: The role of an unusual T cell population. 10-A-12489- December 2009, ACC Abstract).
16. Weyand CM, Brandes JC, Schmidt D, Fulbright JW, Goronzy JJ. Functional properties of $CD4^{+}CD28^{-}$ T cells in the aging immune system. Mech Ageing Dev 1998;102:131–47.
17. Nakajima T, Schulte S, Warrington KJ, Kopecky SL, Frye RL, Goronzy JJ, et al. T-cell-mediated lysis of endothelial cells in acute coronary syndromes. Circulation 2002;105:570–5.
18. Namekawa T, Wagner UG, Goronzy JJ, Weyand CM. Functional subsets of CD4 T cells in rheumatoid synovitis. Arthritis Rheum 1998;41: 2108–16.
19. Liuzzo G, Kopecky SL, Frye RL, O'Fallon WM, Maseri A, Goronzy JJ, et al. Perturbation of the T-cell repertoire in patients with unstable angina. Circulation 1999;100:2135–9.
20. Liuzzo G, Biasucci LM, Trotta G, Brugaletta S, Pinnelli M, Digianuario G, et al. Unusual CD4+CD28null T lymphocytes and recurrence of acute coronary events. J Am Coll Cardiol 2007;50: 1450–8.
21. Liuzzo G, Goronzy JJ, Yang H, Kopecky SL, Holmes DR, Frye RL, et al. Monoclonal T-cell proliferation and plaque instability in acute coronary syndromes. Circulation 2000;101:2883–8.
22. Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. Fertil Steril 2004;81:19–25.
23. Third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III) Final Report. Circulation 2002;106:3143–421.
24. Ciampelli M, Leoni F, Cucinelli F, Mancuso S, Panunzi S, De Gaetano A, et al. Assessment of insulin sensitivity from measurements in the fasting state and during an oral glucose tolerance test in polycystic ovary syndrome and menopausal patients. J Clin Endocrinol Metab 2005;90: 1398–406.
25. Schirmer M, Vallejo AN, Weyand CM, Goronzy JJ. Resistance to apoptosis and elevated expression of Bcl-2 in clonally expanded CD4+CD28-T cells from rheumatoid arthritis patients. J Immunol 1998;161:1018–25.
26. Alber HF, Duftner C, Wanitschek M, Dörler J, Schirmer M, Suessenbacher A, et al. CD4+CD28-lymphocytes and the extent and severity of coronary artery disease. Int J Cardiol 2009;135: 27–35.
27. McMahona M, Hahna BH. Atherosclerosis and systemic lupus erythematosus: mechanistic basis of the association. Curr Opin Immunol 2007;19: 633–9.
28. Tanasescu C, Jurcut C, Jurcut R, Ginghina C. Vascular disease in rheumatoid arthritis: from subclinical lesions to cardiovascular risk. Eur J Intern Med 2009;20:348–54.
29. Weyand CM, Goronzy JJ. T-cell responses in rheumatoid arthritis: systemic abnormalities-local disease. Curr Opin Rheumatol 1999;11: 210–7.
30. Kuller LH, Tracy RP, Shaten J, Meilahn EN. Relation of C-reactive protein and coronary heart disease in the MRFIT nested case-control study. Multiple Risk Factor Intervention Trial. Am J Epidemiol 1996;144:537–47.
31. Biasucci LM, Liuzzo G, Grillo RL, Caligiuri G, Rebuzzi AG, Buffon A, et al. Elevated levels of C-reactive protein at discharge in patients with unstable angina predict recurrent instability. Circulation 1999;99:855–60.