CD4^+ CD28^{null} in cases of polycystic ovary syndrome
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Background
The immunopathogenesis of polycystic ovary syndrome (PCOS) is established. However, the role of CD4^+ CD28^{null} in such patients is underinvestigated.

Aim
The aim of this study was to evaluate and compare CD4^+ CD28^{null} T cells in patients with polycystic ovary (PCO) (with high androgen level and with normal androgen level) and non-PCO patients.

Patients and methods
This study was carried out at Benha University Hospital. It included 100 PCO patients and 50 controls selected from the gynecology and obstetrics outpatient clinics. All included female patients were subjected to history taking and clinical examination. Transvaginal ultrasound was performed to confirm the ultrasonic criteria of PCOS. Hormonal profile included the evaluation of thyroid-stimulating hormone, prolactin, dehydroepiandrostendione-S total testosterone, lipid profile, and fasting blood glucose. Finally, total lymphocytic count, CD4 T cell, and CD4^+ CD28^{null} frequency were evaluated.

Results
Ovarian volume was significantly increased in higher and normal androgen subgroups (11.18±1.31 and 10.53±0.84) when compared with the control group (7.15±1.66). Immunological profile revealed that there was a significant increase in total lymphocyte count and CD4^+ CD28^{null} in the study group when compared with the control group. In addition, there was a significant increase in total lymphocyte count and CD4^+ CD28^{null} in both higher and lower androgen subgroups when compared with the control group. Finally, there was a significant increase in CD4^+ CD28^{null} in higher androgen when compared with lower androgen subgroup (3.15 vs. 2.73, respectively).

Conclusion
There was a higher expression of CD4^+ CD28^{null} T cells in PCOS, especially with hyperandrogenic state.

Keywords:
CD4^+ CD28^{null}, hyperandrogenism, immunological profile, polycystic ovary

Introduction
Polycystic ovary syndrome (PCOS) is a very common endocrine disorder among women in reproductive age and affects ~4–12% [1]. PCOS is defined by National Institute of Health as an androgen excess (hyperandrogenism), oligo-ovulation, with exclusion of known disorders resulting in hypersecretion of androgen. In 2003, Rotterdam criteria for definition of PCOS were the presence of two of the following three features: oligo-ovulation or anovulation, androgen excess (clinical and/or biochemical), and polycystic ovaries diagnosed using sonography [2].

It is a state of anovulation characterized by inappropriate gonadotropin secretion, leading to alteration of gonadotropin-releasing hormone pulsatility with subsequent increase in luteinizing hormone (LH) secretion compared with follicle-stimulating hormone (FSH), and hence LH : FSH reaches 2 : 1 in 60% of PCOS women [1].

Moreover, patients with PCOS have a higher degree of insulin resistance with compensatory hyperinsulinemia. Several medical disorders were associated with insulin resistance; it included type-2 diabetes mellitus, hypertension, dyslipidemia, and cardiovascular complications. Androgen production was stimulated by both high insulin and elevated LH leading to increased testosterone and androstenedione. In addition, high insulin, corticoids, and growth hormone suppress sex hormone-binding globulin production in the liver. This leads to increased free testosterone, which causes: hirsutism, acne, acanthosis nigricans, follicular atresia, and anovulation [1].

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PCOS is heterogeneous with several symptoms and clinical signs related to reproduction, cardiometabolic, and psychologic disorders [3]. PCOS patients complain of dysfunctional uterine bleeding extending from amenorrhea and oligomenorrhea to episodic menometrorrhagia. Amenorrhea is due to anovulation, failed progesterone production, or high androgen. Unpredictable heavy bleeding in polycystic ovary (PCO) patients is due to exhaustion of thick endometrium after prolonged unopposed estrogen. Infertility or subinfertility is a common complaint in women with PCOS [1]. In addition, early and recurrent miscarriage may occur in 30–50% of PCO patients [4].

There are studies that showed that PCO patients have a high risk for cardiovascular complications induced by dyslipidemia, hypertension, oxidative stress, and inflammatory changes. In addition, patients with PCO showed an increase in unusual T-cell population, which mediates vascular damage named CD4+ CD28null T-cell lymphocytes. These cells exert preinflammatory changes by producing high levels of interferon-γ, tumor necrosis factor-α, interleukin-2, and cytolytic enzymes [5].

These T cells (CD4+ CD28null) have specific characters that make them susceptible to immunoregulation. These cells are rarely found in healthy people. However, it may slightly increase in elderly people, and their presence indicates preclinical atherosclerosis, inducing arterial endothelial dysfunction [3].

**Aim**
The study aimed to evaluate and compare CD4+ CD28null T cells in patients with PCO (with high androgen level and with normal androgen level) and non-PCO patients.

**Patients and methods**
This study was carried out at Benha University Hospital. It included 100 PCO patients and 50 control individuals, selected from the gynecology and obstetrics outpatient clinics. Patients with the following criteria were included in the study: (i) age 18–35 years; (ii) BMI less than 25 kg/m²; (iii) regular menses; (iv) normal androgen level; (v) normal ovarian morphology using ultrasound; and (vi) absence of hirsutism and acne. In addition, all PCO patients were diagnosed according to Rotterdam-ESHRE criteria: two of the following three criteria should be present: oligo-ovulation or anovulation, hyperandrogenism (clinical and/or biochemical), and/or PCO diagnosed by ultrasonography. The study protocol was approved by local ethics committee and an informed consent was obtained from each patient.

However, patients with one or more of the following criteria were excluded from the study: (i) absence of inclusion criteria; (ii) chronic or acute inflammatory diseases; (iii) neoplasms; (iv) drugs [e.g., insulin-sensitizing drugs (metformin, pioglitazone, and rosiglitazone), clomiphene citrates, oral contraceptive pills, antiandrogen drugs in the last 6 months before evaluation]; and (v) diabetes mellitus, major surgery 3 months before inclusion, or other hormonal dysfunction.

Serum androgen levels (in the form of total testosterone) were evaluated for all patients on day 3 of menstrual cycle. Out of the PCO patients, we selected the first 50 patients who had elevated androgen level (testosterone>3 ng/ml), and were assigned as study group 1, and then we selected another 50 patients with normal androgen level and were assigned as the study group 2.

All women included in this study were subjected to history taking, clinical examination, and hirsutism evaluation using Ferriman–Gallwey (FG) map scoring system (hirsutism was diagnosed if FG>8) [6]. Height, weight, BMI, and waist/hip ratio were measured and documented. Transvaginal ultrasound was performed to confirm the ultrasonic criteria of PCOS using transvaginal probe 10 mHz (Toshiba, Neoamio, Japan) in our study, and PCOS criteria at ovaries are 12 or more follicles measuring 2–9 mm and/or an increased ovarian volume of >10 cm³). Hormonal profile included thyroid-stimulating hormone, prolactin, dehydroepiandrosterone-S (DHEAS), and total testosterone. Thereafter, lipid profile and fasting blood glucose were measured. Fresh blood samples were collected using sterile tubes containing EDTA and sent to laboratory for measurement of total lymphocytic count, CD4 T cell, and CD4+ CD28null frequency. CD28null cells were evaluated using CD28 fluorochrome-conjugated antibody, and CD4+ cells using CD4 fluorochrome-conjugated antibody, which permits the identification and numeration of these cells in human biological sample using flow cytometry. Flow cytometry was performed using Axetris Impedance Flow Cytometer (Leister Process Technologies, Axetris Division, Germany). Quantitative determination of CD4+ CD28null in human blood using monoclonal antibody, fluorescein isothiocyanate conjugated.

**Statistical analysis**
The collected data were coded, organized, tabulated, and statistically analyzed using statistical package for
the social science (SPSS, version 16; SPSS Inc., Chicago, USA), running on IBM compatible computer. Qualitative data were represented as relative frequency and percent distribution, and for comparison between groups the $\chi^2$ or the Mann–Whitney test was used. Quantitative data were represented as mean and SD, minimum and maximum, and for comparison between two means, the unpaired Student’s $t$ test was used, whereas for comparison between more than two means, the one-way analysis of variance $F$ test was used. For correlation between two variables both Pearson’s and Spearman’s correlation coefficients were calculated. The correlation was mild if less than 0.3; moderate from 0.3 to 0.7, and powerful if greater than 0.7, either proportional (positive) or inverse (negative). For interpretation of results, $P$-value less than 0.05 was considered significant.

**Results**

In the present work, age ranged from 19 to 33 years, BMI ranged from 21.30 to 26.77, and waist/hip ratio ranged from 0.70 to 0.83, and there was no significant difference between studied groups as regards demographic data. FG score ranged from 4 to 8 with a mean of 6.43±0.97 and there was a significant increase in the study group when compared with the control group (6.86±0.61 vs. 5.58±1.01, respectively). There was a significant increase in FG score in higher and normal androgen subgroups in comparison with the control group, but the difference between the two subgroups is statistically nonsignificant (Table 1).

As regards hormonal profile, there was a statistically significant difference between studied groups as regards total testosterone, DHEAS, and serum fasting glucose level (Table 2).

As regards lipid profile, there was a significant increase in total cholesterol, triglycerides, and low-density lipoprotein (LDL) and a significant decrease in high-density lipoprotein (HDL) in the study group when compared with the control group. In addition, there was a significant increase in total cholesterol, triglycerides, and LDL and a significant decrease in HDL in higher and normal androgen subgroups when compared with the control group. Finally, there was a significant increase in LDL and a decrease in HDL in higher androgen subgroup when compared with normal androgen subgroup (Table 3).

As regards ovarian volume, there was a significant increase in ovarian volume in higher and normal androgen subgroups (11.18±1.31, 10.53±0.84) when compared with the control group (7.15±1.66). Finally, there was a significant increase in ovarian volume in higher androgen subgroup when compared with normal androgen subgroup.

As regards immunological profile, there was a significant increase in total lymphocyte count and CD4+ CD28null in the study group when compared with the control group. In addition, there was a significant increase in total lymphocyte count and CD4+ CD28null in both higher and normal androgen subgroups when compared with the control group. Finally, there was a significant increase inCD4+ CD28null in higher androgen when compared with normal androgen subgroup (3.15 vs. 2.73, respectively) (Table 4).

**Discussion**

The present study was designed to evaluate and compare CD4+ CD28null T cells in patients with PCO (with high androgen level and with normal androgen level) and non-PCO patients.
The results of the present study showed no significant difference between cases with PCOS whether normal or higher androgen levels as regards patient characteristics, hypertension, thyroid-stimulating hormone, serum prolactin levels, and serum fasting glucose. Comparable results were reported by Moro et al. [7]. The importance of these findings in the present study related to removal of the effects of such factors on the CD4+CD28null cell expansion. The remaining effects related to the nature of PCOS. However, results of the present study showed increased FG score, androgens, and DHEAS levels in study groups when compared with the control group.

### Table 2 Comparison between study subgroups and control groups as regards hormonal profile, FBS and ovarian volume

<table>
<thead>
<tr>
<th>Items</th>
<th>High androgens (n=50)</th>
<th>Normal androgen (n=50)</th>
<th>Control group (n=50)</th>
<th>F-test</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total testoster (ng/ml)</td>
<td>Mean±SD 3.4±0.40</td>
<td>1.08±0.21</td>
<td>0.52±0.15</td>
<td>181.68</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>Range 3.1–5.6</td>
<td>0.78–1.59</td>
<td>0.32–0.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSH (μIU/ml)</td>
<td>Mean±SD 1.14±0.21</td>
<td>1.15±0.21</td>
<td>1.14±0.21</td>
<td>0.034</td>
<td>0.967</td>
</tr>
<tr>
<td></td>
<td>Range 0.50–1.52</td>
<td>0.74–1.52</td>
<td>0.74–1.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prolactin (ng/ml)</td>
<td>Mean±SD 9.23±0.72</td>
<td>9.42±0.70</td>
<td>9.21±0.63</td>
<td>1.433</td>
<td>0.242</td>
</tr>
<tr>
<td></td>
<td>Range 7.98–11.24</td>
<td>7.98–11.20</td>
<td>7.89–11.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHEAS (μg/ml)</td>
<td>Mean±SD 25.16±3.66</td>
<td>25.57±1.83</td>
<td>14.69±3.40</td>
<td>201.073</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Fasting glucose (mg/dl)</td>
<td>Mean±SD 97.56±9.55</td>
<td>100.30±7.96</td>
<td>103.70±5.12</td>
<td>7.816</td>
<td>0.001*</td>
</tr>
<tr>
<td></td>
<td>Range 74.00–110.00</td>
<td>82.00–110.00</td>
<td>93.00–112.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovarian volume</td>
<td>Mean±SD 11.18±1.31</td>
<td>10.53±0.84</td>
<td>7.15±1.66</td>
<td>134.23</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>Range 8.90–15.20</td>
<td>8.90–12.36</td>
<td>4.90–11.59</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DHEAS, dehydroepiandrostendione-S; TSH, thyroid-stimulating hormone. * < 0.05; ** < 0.001.

### Table 3 Comparison between study subgroups and control groups as regards lipid profile

<table>
<thead>
<tr>
<th>Items</th>
<th>High androgens (n=50)</th>
<th>Normal androgen (n=50)</th>
<th>Control group (n=50)</th>
<th>F-test</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>Mean±SD 176.68±15.93</td>
<td>174.82±18.64</td>
<td>149.38±11.75</td>
<td>47.181</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>Range 142.0–210.0</td>
<td>136.0–206.0</td>
<td>131.00–183.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>Mean±SD 97.5±25.4</td>
<td>97.1±24.9</td>
<td>60.26±26.53</td>
<td>34.924</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>Range 43.0–145.0</td>
<td>54.0–145.0</td>
<td>32.00–129.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>Mean±SD 53.76±7.76</td>
<td>62.7±7.3</td>
<td>70.54±13.33</td>
<td>38.202</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>Range 45.00–80.00</td>
<td>47.00–75.00</td>
<td>45.00–95.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>Mean±SD 116.60±8.12</td>
<td>89.62±17.32</td>
<td>70.14±1.38</td>
<td>146.067</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>Range 102.0–130.0</td>
<td>65.0–124.0</td>
<td>49.0–105.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglycerides. ** < 0.001.

### Table 4 Immunological profile (total lymphocytic count, CD4 T cells, CD4+ CD28null frequency)

<table>
<thead>
<tr>
<th>Higher androgen</th>
<th>Normal androgen</th>
<th>Control</th>
<th>F</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lymphocyte count×10⁹/l</td>
<td>Mean±SD 2.36±0.49</td>
<td>2.38±0.42</td>
<td>1.82±0.62</td>
<td>18.03</td>
</tr>
<tr>
<td></td>
<td>Range 1.36–3.69</td>
<td>1.56–3.69</td>
<td>1.10–3.39</td>
<td></td>
</tr>
<tr>
<td>Total CD frequency (%)</td>
<td>Mean 45.64</td>
<td>45.59</td>
<td>45.43</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>Range 38.40–53.10</td>
<td>39.0–53.10</td>
<td>39.0–53.10</td>
<td></td>
</tr>
<tr>
<td>CD4+ CD28null frequency (%)</td>
<td>Mean 3.15</td>
<td>2.73</td>
<td>0.27</td>
<td>24.38</td>
</tr>
<tr>
<td></td>
<td>Range 1.59–7.20</td>
<td>1.59–3.56</td>
<td>0.10–0.42</td>
<td></td>
</tr>
</tbody>
</table>

* < 0.001.
Total testosterone ranged from 0.32 to 5.6, and there was a significant increase in high and normal androgen groups when compared with the control group (3.40 ±0.40, 1.08±0.21 vs. 0.52±0.15, respectively), and there was a significant increase in the higher androgen group when compared with normal androgen subgroup. These results are in agreement with ACOG Committee on Practice Bulletins – Gynecology [8], which found that androgen excess presents as hirsutism. The degree of hirsutism was evaluated using the modified FG score. In addition, androgen excess is a characteristic feature of PCOS because it is strongly implicated in the genesis of the disorder [9]. A proposed mechanism for anovulation and hyperandrogenism suggested that, under the increased stimulatory effect of LH, stimulation of the ovarian theca cells is increased. These cells increase the production of androgens. Because of decreased level of FSH relative to LH, the ovarian granulosa cells cannot aromatize the androgens to estrogens, which lead to decreased estrogen levels and consequent anovulation [10].

In the present work, there was a significant increase in ovarian volume in high androgen when compared with the normal androgen group or the control group (11.18 ±1.31 vs. 10.53±0.84 and 7.15±1.66, respectively). Moreover, there was a significant increase in ovarian volume in normal androgen when compared with the control group. These results are in agreement with previous studies, in which ovarian volume is increased in PCOS and non-PCOS ovaries. Observations showed that, in the unstimulated, natural cycling state, the distribution of most leukocytes was similar between PCOS and non-PCOS ovaries, and there are no differences in terms of total lymphocyte count between women and those with PCOS and controls. These results are in agreement with the present study in terms of total CD4+ count, whereas it is contradicted with current results as regards total lymphocyte count.

Comparable results were reported by Moro et al. [3], who reported a significant increase in CD4+ CD28null in PCOS when compared with controls. Moreover, these results are consistent with those reported by Niccoli et al. [5], who reported that, in women with PCOS, there is an expansion of T-cell population that mediates vascular damage, identified by CD4+ CD28null T lymphocytes and postulated that CD4+ CD28null cells might be involved in the increased long-term cardiovascular comorbidity observed in those women. They added that neither hyperinsulinemia nor high-sensitivity C-reactive protein levels were associated with CD4+ CD28null. However, the small sample size of their study may have underpowered a possible role of androgens in CD4+ CD28null expansion. This role was clearly demonstrated in the present study.

In addition, the same authors demonstrated that there are no differences in terms of total lymphocyte count and total CD4+ T-cell frequency between women and those with PCOS and controls. These results are in agreement with the present study in terms of total CD4+ count, whereas it is contradicted with current results as regards total lymphocyte count.

Moro et al. [7] reported that the CD4+ CD28null lymphocyte clonal expansion in patients with PCOS seems to be an isolated feature in the T-cell population. The reason for expansion in young women with PCOS is not known. PCOS is a state of inflammatory activation [9]. It has been suggested that chronic inflammation and persistent infection can induce loss of CD28 from the cell surface [12].

Results of the present study are also in agreement with Wu et al. [13], who examined leukocyte distribution in follicular phase ovaries in stimulated follicular cells in PCOS and non-PCOS patients. Observations showed that, in the unstimulated, natural cycling state, the distribution of most leukocytes was similar between PCOS and non-PCOS ovaries, apart from T lymphocytes and in particular the subset of CD45 RO-positive cells.

With strict inclusion criteria in patients of the present work, the possible effects of diabetes, obesity, or other factors on the levels of CD4+ CD28null cells was avoided. The remaining explanation of expansion of CD4+ CD28null cells in PCOS patients is the autoimmune nature and chronic inflammatory nature of the PCOS. This explanation can be supported by several reports that described the expansion of CD4+ CD28null T cells in the peripheral circulation of patients with various immune disorders, including...
autoimmune diseases, chronic inflammatory diseases, and immune deficiency [14–16].

In summary, results of the present study showed that there was higher expression of CD4+ CD28null T cells in PCOS, especially with hyperandrogenic state. The importance of these findings can be expressed in the development of drugs targeting these cells.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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