

Circulating platelet-derived microparticles are elevated in women with polycystic ovary syndrome diagnosed with the 1990 criteria and correlate with serum testosterone levels

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**Running title:** Platelet-derived microparticles in PCOS

**Word count:** 4350

**Abstract**

**Objective:** Women with polycystic ovary syndrome (PCOS) appear to have higher cardiovascular risk than healthy population. Patients diagnosed with PCOS according to the 1990 criteria have more adverse metabolic profile than those diagnosed with the 2003 criteria. Platelet-derived microparticles (PMPs) appear to contribute to atherosclerosis but have not been assessed in PCOS. The aim of this study was to determine plasma PMPs in PCOS. **Design:** A cross-sectional study. **Methods:** We assessed plasma PMPs in 76 normal weight women with PCOS [39 belonging to the phenotypes 1 and 2 (Group I) and 37 belonging to the phenotypes 3 and 4 (Group II)] and in 21 healthy normal weight women. **Results:** Markers of obesity and insulin resistance did not differ between women with PCOS and controls. Serum testosterone levels and the free androgen index (FAI) were higher in Group I than in Group II and controls ( $p < 0.001$  for all comparisons), but did not differ between the latter two groups. Plasma PMPs were higher in Group I than in controls ( $p = 0.018$ ), but did not differ between Group II and controls or between Groups I and II. In the total study population ( $n = 97$ ), plasma PMPs correlated with serum testosterone levels ( $r = 0.207$ ,  $p = 0.042$ ) and the FAI ( $r = 0.207$ ,  $p = 0.042$ ). **Conclusions:** Plasma PMPs are elevated in women with phenotypes 1 and 2 of PCOS compared with healthy controls, but not in women with phenotypes 3 and 4. Hyperandrogenemia, which is more pronounced in phenotypes 1 and 2, appears to be implicated in the increase in plasma PMPs.

**Keywords:** polycystic ovary syndrome, platelet-derived microparticles, obesity, hyperandrogenemia, insulin resistance

## Introduction

The polycystic ovary syndrome (PCOS) is characterized by 3 cardinal features, which include chronic oligo- or anovulation, biochemical hyperandrogenemia or clinical manifestations of hyperandrogenemia and polycystic ovarian morphology on ultrasound [1]. It should be emphasized that PCOS is diagnosed after the exclusion of other states that mimic or cause these 3 abnormalities [2].

Currently, there are two main definitions of PCOS, and this is a subject of major controversy [3,4]. According to the criteria proposed by the National Institutes of Health (NIH) in 1990, the diagnosis of PCOS requires the presence of chronic oligo- or anovulation and biochemical or clinical hyperandrogenemia [5]. On the other hand, according to the criteria proposed by the European Society for Human Reproduction and Embryology and the American Society for Reproductive Medicine-Sponsored Consensus Group in Rotterdam in 2003, PCOS is diagnosed when at least two of the following three features are present: a) chronic oligo- or anovulation, b) biochemical or clinical hyperandrogenemia, and c) polycystic ovarian morphology on ultrasound [1]. By adding a third diagnostic criterion to the NIH definition, i.e. polycystic ovarian morphology on ultrasound, 4 phenotypes of PCOS emerge (Table 1).

Accumulating data suggest that women with PCOS have greater atherosclerotic burden and higher risk for cardiovascular events [6]. Several cardiovascular risk factors, including dyslipidemia [7,8], impaired glucose metabolism [9], abdominal obesity [10] and the metabolic syndrome [11] are more prevalent in PCOS than in the general population and possibly contribute to the

greater cardiovascular morbidity and mortality in PCOS. Of note, women diagnosed with PCOS according to the 1990 criteria have more adverse metabolic profile than those diagnosed according to the additional 2003 criteria [12,13]. However, it is unclear whether cardiovascular event rates differ between these two groups.

During the last decade, the role of microparticles in atherothrombosis is being intensively investigated [14,15]. Microparticles are submicron vesicles that emerge by budding of the plasma membrane of several cell types, including endothelial cells, monocytes and platelets, upon activation [14,15]. Platelet-derived microparticles (PMPs) are the most abundant subtype of microparticles and exert procoagulant effects, mainly due to the presence of negatively charged phospholipids on their surface [14,15]. They also have pro-inflammatory actions and were shown to correlate with serum interleukin-6 levels, a proinflammatory cytokine that is frequently elevated in obesity and in the metabolic syndrome [13,16]. In addition, PMPs might also induce apoptosis of endothelial and vascular smooth muscle cells, vasoconstriction and endothelial dysfunction, which plays an important role in the development of atherosclerosis [13,17]. Several lines of evidence suggest that PMPs contribute to the pathogenesis of atherothrombosis. Indeed, plasma PMPs are elevated in patients with established cardiovascular disease compared with healthy controls [18,19] and are positively correlated with the atherosclerotic burden [20]. More importantly, preliminary results suggest that higher PMPs are associated with greater risk for cardiovascular events [18].

Given the increased cardiovascular risk of patients with PCOS and the role of PMPs in the pathogenesis of atherothrombosis, the aim of the present study was to assess plasma PMPs in women with PCOS and to evaluate the presence of correlations between plasma PMPs and anthropometric, metabolic, hormonal and

ultrasonographic features of PCOS. In addition, given the differences in the metabolic profile between women diagnosed with PCOS according to the 1990 or 2003 criteria, we aimed to compare plasma PMPs between these two groups. To our knowledge, there are no other studies that evaluated plasma PMPs in women with PCOS.

## **Patients and methods**

### **Patients**

We studied 76 normal weight [i.e. with body mass index (BMI)  $<25.0 \text{ kg/m}^2$ ] women with PCOS, 39 belonging to the phenotypes 1 and 2 (age  $24.2 \pm 3.9$  years, BMI  $21.6 \pm 2.1 \text{ kg/m}^2$ ) and 37 belonging to the phenotypes 3 and 4 (age  $25.7 \pm 6.2$  years, BMI  $22.1 \pm 2.1 \text{ kg/m}^2$ ). We also studied 21 healthy normal weight women (age  $25.9 \pm 3.6$  years, BMI  $22.3 \pm 2.4 \text{ kg/m}^2$ ) with normal ovulating cycles ( $28 \pm 2$  days, blood progesterone levels  $>10 \text{ ng/ml}$  in 2 consecutive cycles), no signs of hyperandrogenism, and normal sonographic appearance of the ovaries (controls). All women with PCOS were outpatients at the Gynecological Endocrinology Infirmary of the Second Department of Obstetrics and Gynecology, Aristotle University of Thessaloniki. Women of the control group were healthy volunteers.

Diagnosis of PCOS was based on the revised criteria of Rotterdam [1](see study protocol). None of the women studied had galactorrhea or any endocrine or systemic disease that could possibly affect reproductive physiology. A Synachten test was performed with tetracosactide (Synachten 0.25 mg/1ml; Novartis Pharma, Rueil-Malmaison, France) in each woman with a basal  $17\alpha$ -hydroxyprogesterone ( $17\alpha$ -OHP) plasma level  $>1.5 \text{ ng/ml}$  to exclude congenital adrenal hyperplasia.

No woman reported use of any medication that could interfere with the normal function of the hypothalamic-pituitary-gonadal axis during the last semester. Informed

consent was obtained from all women, and the study was approved by the Institutional Review Board; the study met the requirements of the 1975 Helsinki guidelines.

### **Study protocol**

In all women, weight, height, and waist circumference (W) were measured. Body weight was measured with analog scales and in light clothing; height was measured barefoot with a stadiometer. BMI ( $\text{kg/m}^2$ ) was calculated by dividing weight (in kg) by height squared (in m) to assess obesity. W was obtained as the smallest circumference at the level of the umbilicus.

Baseline blood samples were collected between days 3 and 7 of the menstrual cycle in the control group and after a spontaneous bleeding episode in the PCOS group, after an overnight fast. The circulating levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), total testosterone (T),  $\Delta_4$ -androstenedione ( $\Delta_4$ -A), dehydroepiandrosterone-sulfate (DHEA-S),  $17\alpha$ -OHP, sex hormone-binding globulin (SHBG), glucose, insulin, thyroid stimulating hormone (TSH) and free thyroxin (FT4) were measured. Immediately after baseline blood sampling an oral glucose tolerance test (OGTT) was performed; 75 g of glucose were administered orally and serum glucose levels were determined after 30, 60, 90 and 120 min. At the same day transvaginal ultrasonography was performed and the volume of each ovary was determined, as well as the number of small follicles (measuring 2-9 mm in diameter) in each ovary.

Women with PCOS were divided in two groups. The first group (Group I) included 39 women who were diagnosed with PCOS according to the 1990 criteria (phenotypes 1 and 2, Table 1)[5]. These women had oligo- or anovulation (<8 spontaneous hemorrhagic episodes/yr), biochemical hyperandrogenemia (early follicular phase testosterone >60 ng/dl, corresponding to the mean $\pm$ 2 SD of 200

control subjects measured in our laboratory), and normal sonographic appearance of the ovaries (phenotype 2, Table 1) or polycystic ovaries on ultrasound ( $\geq 12$  small follicles in at least one ovary and/or ovarian volume  $> 10\text{cm}^3$ ; phenotype 1, “severe PCOS”, Table 1). The second group (Group II) included 37 women with the additional PCOS phenotypes introduced by the 2003 criteria (phenotypes 3 and 4, Table 1)[1]. These women had either biochemical hyperandrogenemia and polycystic ovaries on ultrasound with ovulation (phenotype 3, “ovulatory” PCOS, Table 1) or had oligo- or anovulation and polycystic ovaries on ultrasound, without biochemical hyperandrogenemia or clinical manifestations of hyperandrogenemia (phenotype 4, “mild” PCOS, Table 1).

## Methods

Serum glucose, insulin, LH, FSH, PRL, androgen,  $17\alpha\text{-OHP}$ , SHBG, TSH and FT4 concentrations were measured as previously described [21]. The intra-assay coefficients of variation (CV) were 3.8% for insulin, 0.7% for LH, 1.5% for FSH, 2.7% for PRL, 1.3% for T, 5.9% for  $\Delta_4\text{-A}$ , 9.4% for DHEA-S and 5.8% for SHBG. The inter-assay CV were 4.4% for insulin, 1.7% for LH, 3.2% for FSH, 3.4% for PRL, 2.2% for T, 9.2% for  $\Delta_4\text{-A}$ , 12.1% for DHEA-S and 7.8% for SHBG. Free androgen index (FAI) was determined as follows:  $\text{FAI} = \text{T (nmol/l)} \times 100 / \text{SHBG (nmol/l)}$  (values  $> 5.0$  define hyperandrogenemia) [22]. The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated as follows:  $\text{HOMA-IR} = \text{fasting insulin } (\mu\text{IU/ml}) \times \text{fasting glucose (mg/dl)} / 405$  [23]. The Quantitative insulin sensitivity check index (QUICKI) was calculated according to the following formula:  $\text{QUICKI} = 1 / [\log\text{Insulin } (\mu\text{IU/ml}) + \log\text{Glucose (mg/dl)}]$  [24].

Plasma PMPs were determined with flow cytometry using the BD FACScan flow cytometer system (Becton Dickinson Biosciences, San Jose, CA). Samples were

prepared as follows: First, whole blood was centrifuged for 15 min at 1,500g to separate plasma. The supernatant was then centrifuged for 2 min at 13,000g to remove platelet debris. The supernatant was then centrifuged for 20 min at 18,000g. Then, the supernatant was removed and the PMPs pellet was collected. After resuspension with 1 ml binding buffer from the Annexin V FITC kit, the sample was incubated in the dark at room temperature for 15 min. Afterwards, the sample was centrifuged for 5 min at 1,500g and the supernatant was removed. Then, the pellet was resuspended with 500µl PBS and sample analysis was performed. PMPs were measured in a pellet volume of 20 µl after adding 10µl Annexin V FITC, 20µl CD14 PeRCP clone 47-3D6 (mouse IgG1) and 20µl CD41α PE clone HIP8 (mouse IgG1). All reagents were from Immunostep S.L. (Salamanca, Spain). Absolute numbers of PMPs were determined using CYTOCOUNT beads (Zebra Biosciences B.V., Enschede, Netherlands). The CYTOCOUNT beads are provided in a solution of standardized concentration (specific number of beads / µl), and this is taken into account in the determination of PMPs, according to the following formula:  $MPs/\mu l = (\text{events MPs} \times \text{total number of beads}) / [\text{events cyto count} \times \text{sample volume (i.e. 20}\mu\text{l)}]$ . The flow cytometer system was adjusted for the measurement of PMPs using the Megamix beads (Biocytex, Marseille, France). The flow cytometer system was set to detect microparticles measuring 0.5, 0.9 and 3 µm.

### **Transvaginal ultrasonography**

Transvaginal ultrasound scans of the ovaries were performed by an experienced sonographer in women who participated in the study. Ovarian volume was calculated by the formula:  $V = (\pi/6) \times D_{\text{length}} \times D_{\text{width}} \times D_{\text{thickness}}$ , where  $D$  is dimension. The presence of polycystic ovaries was diagnosed by the presence of 12 or

more follicles in each ovary measuring 2-9 mm in diameter and/or increased ovarian volume ( $>10 \text{ cm}^3$ ).

### **Statistical analysis**

Data analysis was performed with the statistical package SPSS (version 17.0; SPSS Inc., Chicago, IL). Data are reported as mean $\pm$ SD. Because most parameters did not follow normal distribution as assessed with the Kolmogorov-Smirnov test, comparisons between groups were performed with the Kruskal-Wallis and Mann-Whitney tests for comparisons of all 3 groups and pair-wise comparisons, respectively. Correlations between plasma PMPs and other parameters were assessed with Spearman Rank Order correlation. In all cases, a *p* value  $<0.05$  was considered significant.

### **Results**

The anthropometric, hormonal, metabolic and ultrasonographic characteristics of the two groups of women with PCOS and of controls are shown in Table 2. Age, BMI, W and W to hip ratio (W/H) did not differ between the two groups of women with PCOS and controls.

Serum FSH, LH, PRL,  $17\alpha$ -OHP and SHBG levels did not differ between the two groups of women with PCOS and controls. Serum T,  $\Delta_4$ -A and DHEA-S levels as well as the FAI were higher in Group I of women with PCOS (phenotypes 1 and 2) than in Group II of women with PCOS (phenotypes 3 and 4) and controls ( $p < 0.001$  for all comparisons), but did not differ between the latter two groups (Table 2).

Serum glucose and insulin levels, the glucose/insulin ratio, the HOMA-IR and QUICKI indices and the area under the OGTT curve did not differ between the two groups of women with PCOS and controls.

The mean ovarian volume and the mean number of follicles in the ovaries were higher in the two groups of women with PCOS than in controls, but did not differ between Groups I and II of women with PCOS.

Plasma PMPs were higher in Group I of women with PCOS than in controls ( $p = 0.018$ ), but did not differ between Group II of women with PCOS and controls. In addition, plasma PMPs did not differ between Groups I and II of women with PCOS.

In the total study population ( $n = 97$ ), plasma PMPs correlated with serum T levels ( $r = 0.207$ ,  $p = 0.042$ ; Figure 1) and the FAI ( $r = 0.207$ ,  $p = 0.042$ ; Figure 2).

## **Discussion**

The main finding of the present study is that plasma PMPs are higher in women with phenotypes 1 and 2 of PCOS than in healthy controls, but do not differ between women with phenotypes 3 and 4 of PCOS and controls. This is the first study that assessed plasma PMPs in PCOS. Previous reports showed that cardiovascular risk factors that are frequent in women with PCOS, including obesity, the metabolic syndrome and type 2 diabetes mellitus, are also associated with elevated plasma PMPs [17,25-27]. A correlation between plasma PMPs and BMI, W and serum glucose levels has also been reported [25,26]. However, in the present study, women with PCOS did not differ with controls in markers of adiposity (BMI, W and W/H) or in indices of insulin resistance and impaired glucose metabolism (serum glucose and insulin levels, glucose/insulin ratio, HOMA-IR, QUICKI, and area under the OGTT curve). Therefore, neither adiposity nor impaired glucose metabolism can explain the difference in plasma PMPs between women with PCOS and controls. On the other hand, markers of hyperandrogenemia (serum T,  $\Delta_4$ -A and DHEA-S levels as well as the FAI) were higher in Group I of women with PCOS (phenotypes 1 and 2) than in controls. In addition, plasma PMPs correlated with serum T levels and the FAI.

Therefore, our study suggests that hyperandrogenemia is primarily responsible for the elevated plasma PMPs in women with PCOS. This is also supported by our finding that women with phenotypes 3 and 4 of PCOS, who did not differ in markers of hyperandrogenemia with controls, had comparable PMPs with controls. The association between serum T levels and PMPs has not been previously evaluated. However, men have higher PMPs than women and it is possible that the greater serum T levels in men might contribute to this difference [16,27].

The introduction of the 2003 criteria for the diagnosis of PCOS caused major controversy and has affected clinical diagnosis of PCOS and the design of clinical studies in this population. Recent guidelines issued by the Androgen Excess and PCOS Society state that PCOS should be primarily considered a disorder of androgen synthesis, turnover and catabolism [28]. Therefore, ovulating women with biochemical or clinical hyperandrogenemia and polycystic ovarian morphology on ultrasound (phenotype 3, Table 1) have a mild form of PCOS [29]. On the other hand, preliminary observations suggest that women with oligo- or anovulation and polycystic ovaries (phenotype 4, Table 1) also manifest mild endocrine and metabolic abnormalities that resemble mild PCOS [29]. However, the metabolic abnormalities of these women are currently considered very mild and they do not appear to be associated with increased risk for development of the metabolic disorder that characterizes PCOS [30,31].

Our study further supports previous observations that women with phenotypes 1 and 2 of PCOS have more pronounced hormonal abnormalities than those with phenotypes 3 and 4 [12,13]. Indeed, markers of androgen excess (serum T,  $\Delta_4$ -A and DHEA-S levels as well as the FAI) were higher in phenotypes 1 and 2 of PCOS (Group I) than in phenotypes 3 and 4 (Group II). Interestingly, plasma PMPs were

higher in phenotypes 1 and 2 of PCOS than in controls, but did not differ between phenotypes 3 and 4 of PCOS and controls. This finding also suggests a more adverse cardiovascular risk profile in phenotypes 1 and 2 than in 3 and 4. We did not observe a significant difference in plasma PMPs between the two groups of women with PCOS, but this is probably due to the relatively small number of patients and the large variation in plasma PMPs in our population.

Given the association between elevated plasma PMPs and atherosclerosis, our finding that the increase in plasma PMPs in PCOS is related to hyperandrogenemia has several potential implications. First, it is possible that women with PCOS but without hyperandrogenemia (i.e. with phenotype 4) might have lower cardiovascular risk compared with the other 3 phenotypes [28]. Second, targeting hyperandrogenemia in women with PCOS (e.g. with metformin) might lower plasma PMPs and this might also reduce the cardiovascular risk of these women.

In conclusion, plasma PMPs are elevated in women with phenotypes 1 and 2 with PCOS compared with healthy controls but not in women with phenotypes 3 and 4. Hyperandrogenemia, which is more pronounced in phenotypes 1 and 2 of the syndrome, appears to be implicated in the increase in plasma PMPs. Prospective studies are needed to evaluate the potential impact of the elevated plasma PMPs in women with PCOS on cardiovascular events.

**Declaration of interest**

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

**Funding**

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

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**Table 1.** Definition of the 3 study groups.

Study groups	ANOV	HA	PCO	PCOS phenotype
<b>PCOS phenotype 1</b>	+	+	+	Severe PCOS
<b>PCOS phenotype 2</b>				
<b>Group I (n=39)</b>				
PCOS phenotype 3	-	+	+	Ovulatory PCOS
PCOS phenotype 4				
<b>Group II (n=37)</b>				
PCOS phenotype 4	+	-	+	Mild PCOS
Controls (n=21)	-	-	-	

ANOV, anovulation; HA, hyperandrogenemia; PCO, polycystic ovaries in transvaginal ultrasonography.

Groups in bold were also classified as PCOS before 2003 according to the National Institutes of Health criteria [6].

**Table 2.** Characteristics of the study population.

	Women with PCOS		Controls (n=21)	p (overall)	p (for pair-wise comparisons)		
	Group I (phenotypes 1 and 2) (n=39)	Group II (phenotypes 3 and 4) (n=37)			Group I vs. controls	Group II vs. controls	Group I vs. Group II
Age (years)	24.2±3.9	25.7±6.2	25.9±3.6	NS	NA	NA	NA
BMI (kg/m <sup>2</sup> )	21.6±2.1	22.1±2.1	22.3±2.4	NS	NA	NA	NA
Waist (cm)	72.3±5.9	73.8±5.8	74.1±6.5	NS	NA	NA	NA
W/H	0.74±0.04	0.74±0.05	0.75±0.05	NS	NA	NA	NA
FSH (mIU/ml)	6.7±1.3	7.4±1.9	7.6±2.6	NS	NA	NA	NA
LH (mIU/ml)	9.6±6.0	7.9±5.5	6.7±3.4	NS	NA	NA	NA
Prolactin (ng/ml)	13.2±5.9	14.2±7.0	12.2±3.3	NS	NA	NA	NA

Testosterone (ng/dl)	85.3±20.7	50.6±26.1	40.6±23.1	< 0.001	< 0.001	NS	< 0.001
Δ <sub>4</sub> -A (ng/ml)	3.5±1.9	2.2±0.8	1.9±0.6	< 0.001	< 0.001	NS	< 0.001
DHEA-S (ng/ml)	3567.7±1329.4	2380.9±1108.0	2131.2±871.3	< 0.001	< 0.001	NS	< 0.001
FAI	6.05±3.2	3.66±3.0	2.55±3.03	< 0.001	< 0.001	NS	< 0.001
17α-OHP (ng/ml)	1.0±0.5	0.9±0.4	0.8±0.4	NS	NA	NA	NA
SHBG (nmol/ml)	62.5±32.4	64.8±32.4	74.1±31.9	NS	NA	NA	NA
Glucose (mg/dl)	93.7±7.7	94.3±8.3	96.1±8.0	NS	NA	NA	NA
Insulin (μIU/ml)	7.5±3.2	8.1±4.7	6.8±2.7	NS	NA	NA	NA
Glucose/insulin	14.46±5.62	13.49±4.6	16.82±7.85	NS	NA	NA	NA
AUC OGTT	14192.7±2802.9	13921.2±2709.0	13392.1±2852.6	NS	NA	NA	NA
HOMA-IR	1.74±0.73	1.91±1.18	1.62±0.69	NS	NA	NA	NA
QUICKI	0.36±0.02	0.35±0.02	0.36±0.03	NS	NA	NA	NA
Mean ovarian	8.5±4.6	9.7±6.2	5.4±1.9	0.002	0.004	< 0.001	NS

volume (cm <sup>3</sup> )							
Mean number of ovarian follicles	10.3±5.0	12.0±4.5	6.7±3.3	< 0.001	0.006	< 0.001	NS
Microparticles (/μl)	133.1±140.2	93.2±89.9	80.0±106.7	0.045	0.018	NS	NS

PCOS, polycystic ovary syndrome; NS, not significant; NA, not applicable; BMI, body mass index; W/H, waist to hip ratio; FSH, follicle stimulating hormone; LH, luteinizing hormone;  $\Delta_4$ -A,  $\Delta_4$ -androstenedione; DHEA-S, dehydroepiandrosterone sulfate; FAI, free androgen index;  $17\alpha$ -OHP,  $17\alpha$ -hydroxyprogesterone; SHBG, sex hormone-binding globulin; AUC OGTT, area under the oral glucose tolerance test curve; HOMA-IR, homeostasis model assessment of insulin resistance; QUICKI, quantitative insulin sensitivity check index.

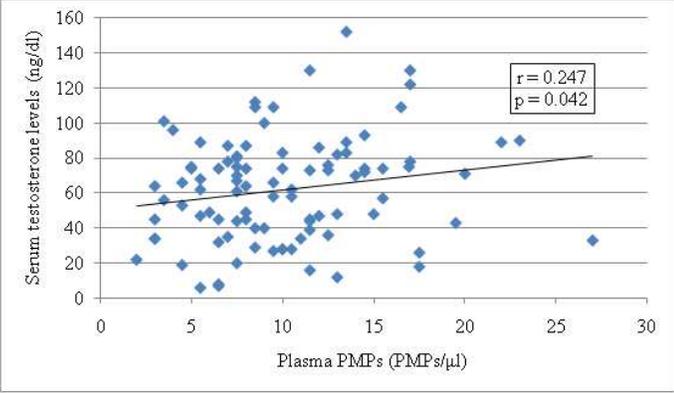


Figure 1.  
Correlation between plasma levels of platelet microparticles (PMPs) and serum testosterone levels in the total study population (n = 97).  
216x121mm (96 x 96 DPI)

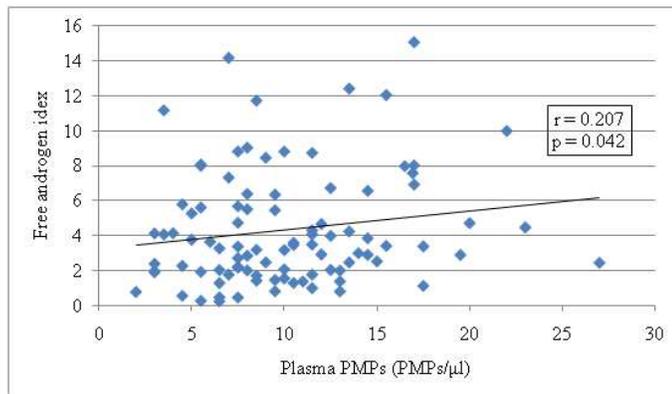


Figure 2.  
Correlation between plasma levels of platelet microparticles (PMPs) and the free androgen index in the total study population (n = 97).  
216x121mm (96 x 96 DPI)