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Original Research Article

Menstrual Cycle: Evaluation of Hematological Changes

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ABSTRACT

Introduction: Literature suggests that menstrual cycle is associated with inflammatory process and with complete blood count (CBC) and hemostatic changes.The aim of this study was to investigate whether CBC, surface platelet markers, platelets-leukocytes aggregates (PLA) and plasma levels of D-Di, PAI-1 and FVIII are able to clarify the hematological changes during menstruation and in the middle day of the menstrual cycle, as well as the influences of the use of combined oral contraceptives (OC) and the age of the woman on this process.
Methods: 46 women were evaluated, including young non-combined OC users (Young = 16), young combined OC-users (Young-OC = 20) and >42 years-old non-combined OC users (Woman = 10). CBC was carried out semi-automatically, surface platelet markers and PLA were analyzed by flow cytometry and coagulation and fibrinolysis markers by ELISA.
Results: Neutrophil count, platelet activation and plasma levels of D-Di have variation depending on the menstrual cycle. Neutrophil count, platelet activation, PLA, plasma levels of D-Di, and PAI-1 are influenced by the use of combined OC. Platelet count and PAI-1 serum levels are influenced by age of the woman. There are positive correlations among platelet count, platelet surface markers, PLA, coagulation cascade and fibrinolysis whose intensity depends on menstrual cycle phase, combined OC use and woman's age.
Conclusions: Menstrual cycle was associated to modifications in hematological components due to hormonal participation. OC use and age seem to be associated to hypercoagulable *status*.

Keywords: Menstrual cycle; Blood coagulation; Hemostasis, Platelet glycoproteins; Platelet activation

1. INTRODUCTION

Menstrual cycle is a complex process in which estrogen and progesterone are responsible for the changes that occur in the endometrium, uterine cervix and vagina, as well as feedback regulation of follicle stimulating hormone secretion (FSH) and luteinizing hormone (LH). [1]

The human endometrial tissue is subjected to mediated growth hormones, followed by enzymatic breakdown, bleeding, bleeding cessation and tissue remodeling. [2] Bleeding interruption is accomplished by local hemostatic mechanisms *via* platelet aggregation, fibrin deposition and thrombus formation. On the other hand, fibrinolysis prevents clot

formation within the uterine cavity governed by plasminogen activator inhibitors (PAI). [3]

Literature suggests that menstrual cycle could change complete blood count (CBC), promoting fluctuations in platelet count [4] and leukocytosis, giving it some resemblance to inflammatory processes. [5,6] During the follicular phase, estrogen, in addition to inducing endometrial proliferation, stimulates angiogenesis through activation of human endometrial stromal cells. [7] In luteal phase, progesterone induces decidualization around blood vessels and increases the expression of angiopoietin-1, which is able to stabilize newly formed blood vessels, and stimulates the synthesis of procoagulants tissue factor (TF) and PAI-1, which reduce the chance of bleeding in case of implantation of the blastocyst. [7,8] There are evidences that platelet activation varies throughout the menstrual cycle, suggesting hypercoagulable stage. [9-12] It is assumed that platelet-leukocyte aggregates play an important role in blood coagulation-inflammation interface, contributing to increased susceptibility to inflammatory disease and thromboembolic events during ovulation. [13]

Combined oral contraceptives (OC), introduced in the 1950s, are combinations between estrogen and progesterone analogs. [14] They are used by more than 100 million women worldwide as safe and low cost method of contraception, although there is evidence of increased risk of cardiovascular, thromboembolic, and cancer associated with its use since the 1960s. [15,16] These pills are classified into first, second, third and fourth generations, according to the type of progestogen used. [17]

There are evidences in literature that the combined use of estrogen and progesterone, even at low doses, increases levels of procoagulants (coagulation factors II, VII, VIII IX and XI and fibrinogen); increases activity of PAI-1 and PAI-2; and reduces the activity of natural anticoagulants (anti-thrombin-AT, tissue factor pathway inhibitor-TFPI, C and S proteins), predisposing to hypercoagulable state.However, its consequences in women's health remain inconclusive. [18]

The aim of the present paper is to investigate whether CBC, platelet activation surface markers, frequency of platelet-leukocyte aggregates, and the concentration of D-Dimer (D-Di), PAI-1 and FVIII are ableto clarify the hematological changes during menstruation and in the middle day of the menstrual cycle, as well as the influences of the use of combined OC and woman's age on this process.

2. MATERIALS AND METHODS

2.1 Study Population

The present study included 46women in childbearing age, with regular menstrual cycles, organized on three groups: Young women aged between 18 and 30 years-old, subdivided in non-oral contraceptives (OC)users or any other presentation form for at least 5 month/Young (N=16) and monophasic combined oral contraceptives users for at least 5 month/Young-OC(N=20); and above 42 years-old women, non-OC users or any other presentation form for at least 5 month/Woman (N=10).Members of groups were selected in the Federal University of Minas Gerais (UFMG/ *Campi* Pampulha and Health) and in the Functional Unit, Gynecology, Obstetrics and Neonatology of Clinical Hospital of UFMG.

Clinical data were collected from an interview with the participant by telephone or in person. Blood samples were collected on menstruation's second day and on the middle day of the same menstrual cycle. Collecting date of the second sample was determined based on the previous menstrual cycles (at least two month), aiming identify, for Young and Woman groups, ovulatory phase.

All Young-OC participants used combined monophasic OC of second (levonorgestrel+ ethinylestradiol; N=2), third (desogestrel/gestodene+ ethinylestradiol; N=10), or fourth (drospirenone/

chloromadinoneacetate+ ethinylestradiol; N=8) generation. As there are no estrogen nor progestogen dose variations in each of the 21 pills, the second moment of sample collection correspond to mathematical middle of the menstrual cycle.

Common inclusion criterion was to have regular menstrual cycles. Common exclusion criteria were chronic hypertension, hemostatic abnormalities; cancer; diabetes; gynecological, cardiovascular, autoimmune, infectious contagious, renal and hepatic diseases; polycystic ovary syndrome; obesity (BMI >30Kg/m^2); inflammatory/infectious process for at least 3 weeks, breast-feeding and anticoagulant or corticosteroids therapy. This study was approved by the Ethics Committee at Federal University of Minas Gerais-Brazil and informed consent was obtained from all participants.

2.2 Blood sampling

Blood samples (2mL) were taken in EDTA-K_3 1.8mg/mL (Vacuette®) and (3,5mL) in sodium citrate (0.129mol/L) in 9:1 volume ratio. EDTA blood samples were used for complete blood cell count (Coulter T890®- Beckman Coulter, United States) and for triplicate manual counting of the blood film. Citrated blood samples were used for platelet surface markers analysis, platelet-leukocytes aggregates analysis assessed by flow cytometry and to determine D-Di, FVIII and PAI-1 concentrations by enzyme-linked immunosorbent assay (ELISA). Samples were collected using vacuum system and disposable material. They were processed within two hours after venipuncture and flow cytometry analysis was performed within 24 hours.

2.3 Analysis of activation platelet surface markers

Platelet phenotypic features were assessed according to original protocol. [19] Briefly, citrated blood samples were centrifuged at 246*xg* for 10 minutes to obtain platelet rich plasma (PRP).Subsequently, 200µL of PRP were added, under vortex agitation, to 800µL of fixative solution (paraformaldehyde at10g/L, sodium cacodylate at 10.2g/L, and sodium chloride at6.63g/L, with a pH equal to 7.2-7.4) and kept overnight at 4-8ºC. Platelet suspension was washed once with phosphate buffer solution-PBS (0.015M, pH 7.2) and centrifuged for 10 minutes at 677*xg*. The supernatant was discarded and the pellet was suspended gently in PBS. The platelet count was adjusted to $5x10^6$ platelets/mL (Coulter T890®). 100µL aliquots (500,000 platelets) were incubated with monoclonal antibodies-mAbs (Pharmingen Becton Dickinson®, United States) directed against platelet surface glycoproteins labeled with fluoresce in isothiocyanate/FITC (anti-CD42a/clone ALMA.16) or with phycoerythrin/PE (anti-CD41a/clone HIP8; anti-CD61/clone VI-PL2 and anti- CD62P/clone AK-4). The samples were vortex homogenized and then incubated in the dark for 30 minutes at room temperature.

Following incubation, the stained platelets were washed once with1mL of PBS and centrifuged for 10 minutes at 677*xg*. The supernatant was discarded and the pellet was suspended in200µL of PBS. Aninternal control for auto-fluorescence (background) was carried out in parallel for each blood sample, in which a platelet suspension (100µL) was incubated in the absence of mAbs.

A total of 50,000 events were acquired per sample using flow cytometer BD LSRFortessa® (Becton Dickinson, United States). The software FlowJo® (FlowJo Inc., United States) was used for acquisition and data analysis. Different strategies were employed for analysis of activation platelet surface markers as shown in Fig 1A.

Platelets were first selected on forward scatter (FSC) *versus* side scatter (SSC), followed by one-dimensional fluorescence his to grams to determine the mean fluorescence intensity (MFI) for CD41a, CD42a, CD61 and CD62P.

Activated platelets percentage ($CD62P^+$population) was calculated by quadrant statistical analysis in dot plotsanti-CD42a FITC*versus*anti-CD62P PE. The results, corresponding to the upper-right double-positive quadrant, were expressed as percentage of$CD42a^+/CD62P^+$platelets.

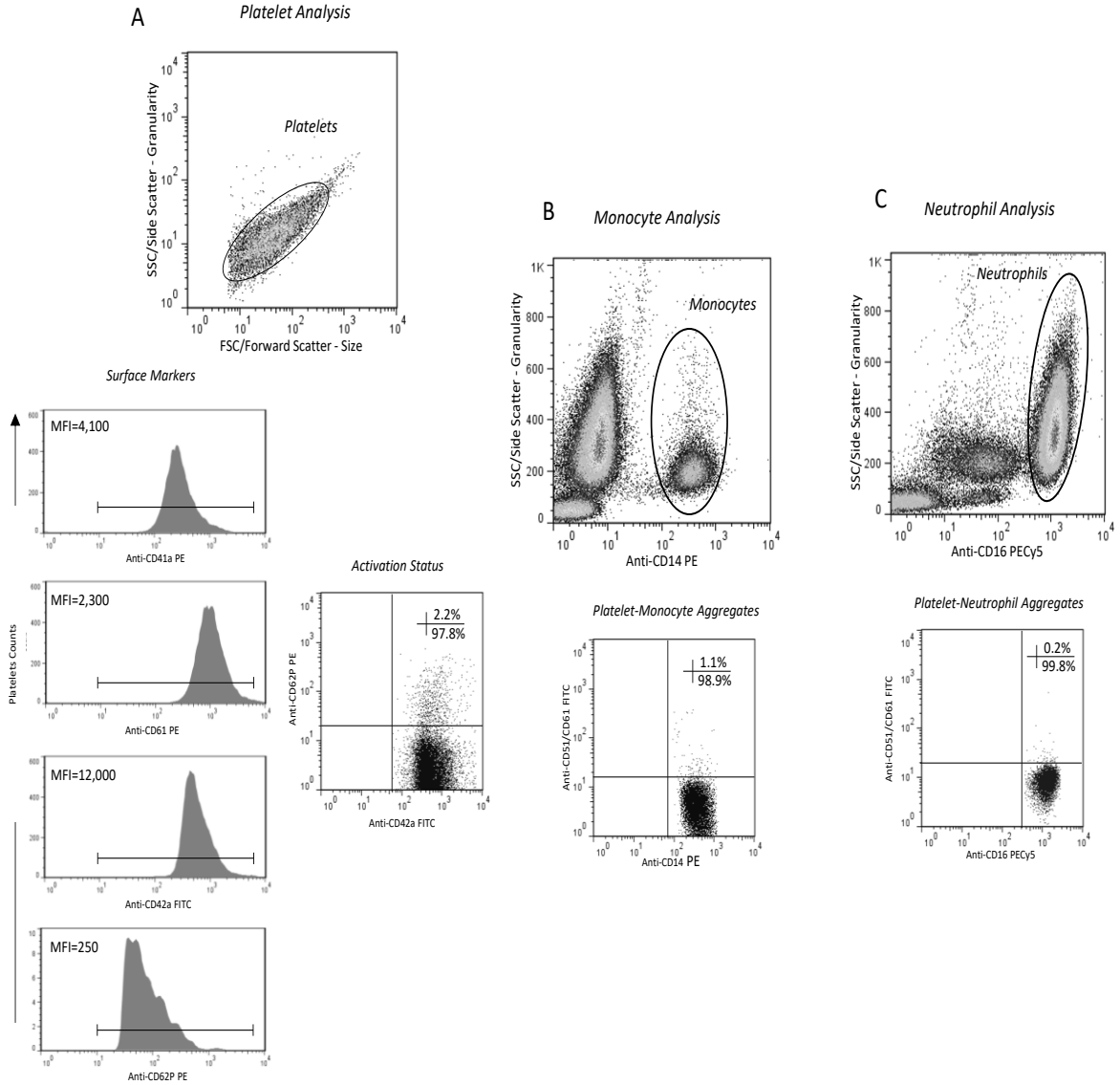

Figure 1. Representative flow cytometry charts used for platelet (A), monocyte (B) and neutrophil (C) analysis.

Analysis of platelet-leukocytes aggregates

Assessment of platelet-monocyte (PMA) and platelet-neutrophil aggregates (PNA) were performed according to the original protocol. [19] Briefly, 100µLofcitrated blood were added into two separated tubes in the presence of anti-CD51/CD61 glycoprotein complex mAb(clone 23C6), labeled with FITC, combined with anti-CD14 mAb(clone MϕP9), labeled with PE, or anti-CD16 mAb (clone 3G8),labeled with PE-cyanine-5/PE-Cy5. The samples were vortex homogenized and incubated in the dark, for 30 minutes at room temperature.

Following incubation, erythrocytes were lysatedusing2mL of lysing solution (dehydrated sodium citrate 25g/L, commercial sodium heparin 5000UI/L, formaldehyde 54v/v, diethylene glycol 30v/v, with pH 7.85) diluted 10times in distilled water. After homogenization by vortex, the preparations were incubated and protected in the dark for 10 minutes at room temperature. Samples were centrifuged for 7 minutesat400*xg*, the supernatant was

discarded and the pellet was suspended. The stained leukocytes were washed once with 2mLPBS. Centrifugation and discarding of supernatant steps were repeated. The pellet was then suspended in200µLPBS. Aninternal control for auto-fluorescence (background) was carried out in parallel for each blood sample, in which a blood aliquot (100µL) was incubated in the absence of mAbs.

A total of 50,000 events were acquired per sample using the flow cytometer BD LSRFortessa®. The software FlowJo® was used for acquisition and data analysis.

Different strategies were employed for analysis of the PMA and PNA profiles are shown in Fig1B and C, respectively. The selective analysis of Monocytes was determined combining PE anti-CD14*versus* SSC and for neutrophils analysisPECy5 anti-CD16 *versus* SSC combination was used. Agate selecting the target population and subsequent combining PE anti-CD14 *versus*FITCanti-CD51/CD61was used to define the percentage of PMA and PECy5 anti-CD16 *versus* FITC anti-CD51/CD61were used to define PNA.

2.4 D-Dimer (D-Di), factor VIII (FVIII) and plasminogen activator inhibitor type-1 (PAI-1)

Specific commercially available enzyme-linked immunosorbent assay (ELISA) Kit Imuclone® D-Dimer, Kit IMUBIND® Factor FVIII and Kit IMUBIND® Plasma PAI-1 all of them from Sekisui Diagnostics (Germany) were used according to manufacturer's instructions.

2.5 Data analysis

2.5.1 Statistical analysis

Statistical analysis was carried out using Minitab®17.0 software (Minitab Inc., United States). Data normality was confirmed by Shapiro-Wilk test.

Comparison of means between the two moments of the collections in the same group was taken by paired T-test for continuous variables with normal distribution. For data with non-normal distribution, Wilcoxon test was used to compare the medians.

Comparison of means between pairs of different groups (Young *versus* Young-OC and Woman) was performed by Student's T-test for continuous variables with normal distribution. For data with non-normal distribution, Mann Whitney test was used to compare the medians. P values ≤0.05 were considered statistically significant.

2.5.2 Spearman correlation analysis

To evaluate the association among platelet count, cytometric biomarkers and parameters assessed by ELISA, Spearman correlation coefficient was used. The correlation index (ρ) was used to categorize the correlation strength as negative (ρ<0), moderate (0.36<ρ<0.67) and strong (ρ>0.68), as proposed by Richard Taylor. [20] Minitab®17.0 software was used for data analysis.Correlations were classified as negative, with inversely proportional association, if ρ <0.0 and positive, with directly proportional association, if ρ> 0.0. Significance was defined as P≤0.05.

3. RESULTS

3.1 Complete blood count

Due to high number of data evaluated, as well as to complexity of the comparisons performed within the same group and between the groups we opted to present and discuss the results which were statistically significant. The others were considered within the reference intervals established in the literature and with no statistical difference for all the comparisons evaluated.

Our data showed that relative (%) neutrophil count was increased during menstruation in Young and Young-OC groups as compared tomiddle day of the cycle. However, in Woman, a lower % neutrophil count was observed comparing these moments (P<0.001 in all cases) (Fig 2A).

We observed that platelet count was higher in Young, in themiddle day of the

cycle, than Young-OC and Woman (P=0.005 and P=0.015, respectively) (Fig 2B). Absolute neutrophil count was higher, during menstruation, in Young than Young-OC (P<0.001) (Fig 2B). Relatives tab count was higher in Young-OC, in themiddle day of the cycle, than in Young (P=0.037) (Fig 2B).

No significant differences were observed for the analysis of the others complete blood count parameters amongst groups.

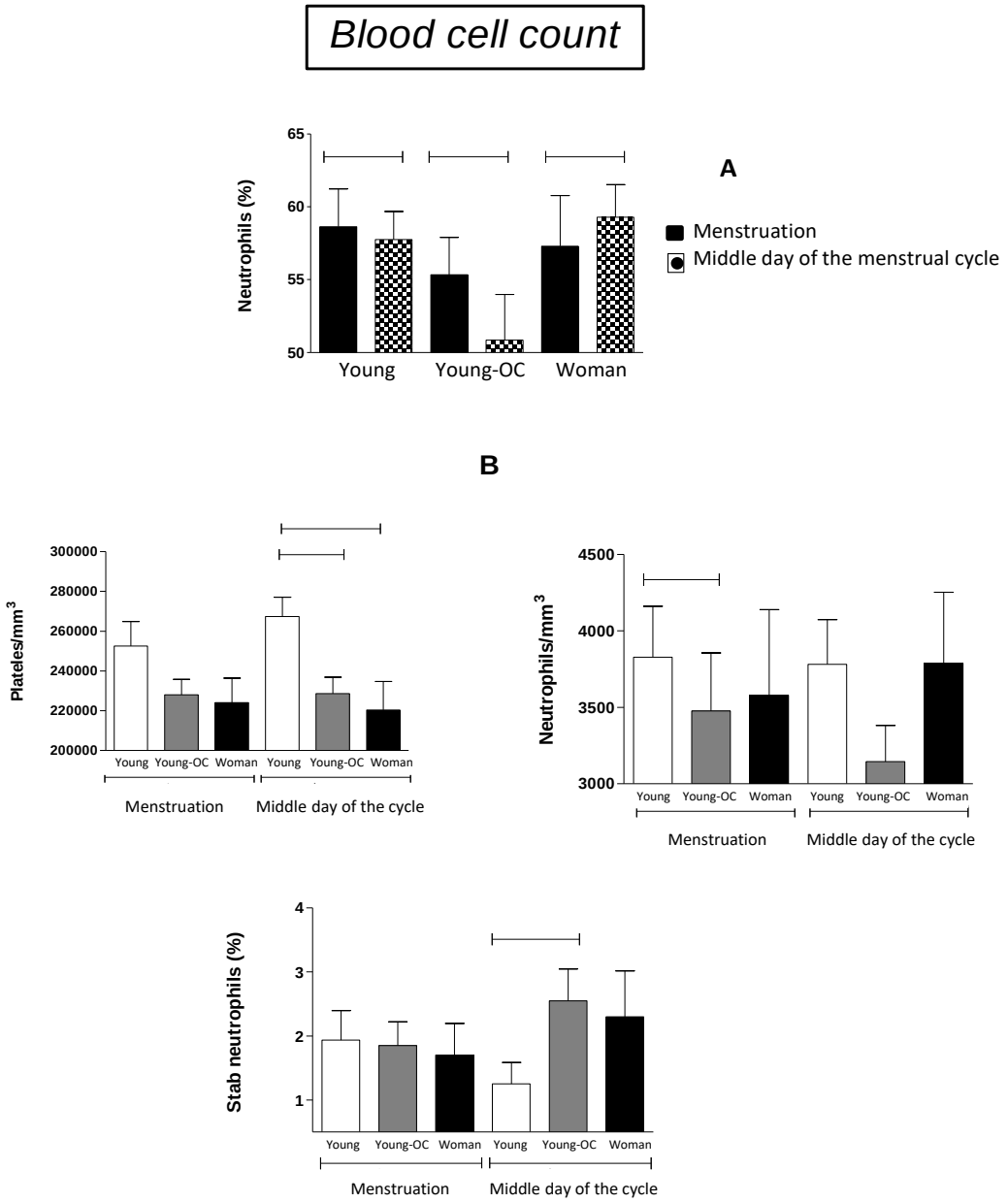

Figure 2. Neutrophil counts, platelets counts and stab neutrophils count in (A) menstrual phase as compared to middle day of cycle; (B) and among Young, Young-OC and Woman. The results are expressed as mean value (bars) and scattering distribution of individual values. Significant differences at P<0.05 are highlighted by connecting lines.

3.2 Activation platelet surface markers

We observed that the expression of GPIIIa-CD61 was higher in Young-OC, in the middle day of the cycle, as compared to menstruation (P=0.040) (Fig 3A). GPIIb/IIIa-CD41a expression was also increased, in this moment, in Young-OC than Young (P=0.005) (Fig 3B). No significant differences were found in the analysis of CD42a and CD62P expressions.

3.3 Platelet-leukocyte aggregates

We founded higher frequency of PMA and PNA, in the middle day of the cycle, in Young-OC as compared to Young (P=0.001, P=0.015, respectively) and a higher frequency of PNA, during menstruation, in Young-OC than Young (P=0.040) (Fig 3C).

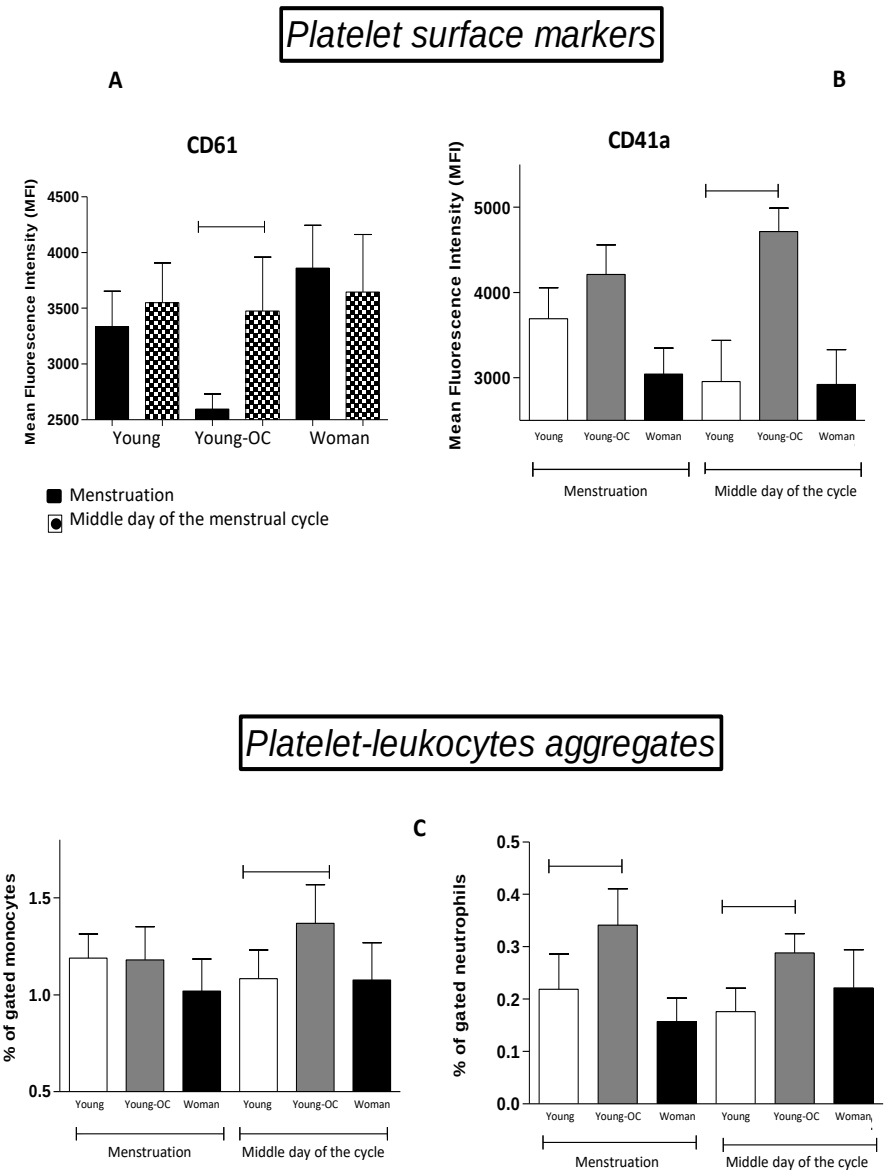

Figure 3. Mean fluorescence intensity (MFI) of CD61 in (A) menstrual phase as compared to the middle day of the cycle and MFI of CD41a (B)among Young, Young-OC and Woman. Frequency of platelet-leukocytes aggregates (C) amongst these groups. The results are expressed as mean value (bars) and scattering distribution of individual values. Significant differences at P<0.05 are highlighted by connecting lines.

3.4 D-Dimer (D-Di), plasminogen activator inhibitor type-1 (PAI-1) and factor VIII (FVIII) measures

Our data showed that D-Di concentration was increased, during menstruation, in Young and Woman groups as compared to middle day of the cycle (P<0.001, P=0.022, respectively) (Fig 4A). Higher D-Di concentration values was observed, in the middle day of the cycle, in Young-OC as compared to Young (P=0.040) (Fig 4B).

For PAI-1 concentration, during menstruation, Young showed higher value as compared to Young-OC (P=0.023) and Woman (P=0.040) (Fig 4C). The same behavior was seen, in the middle day of the cycle (P=0.001 and P=0.040, respectively)

(Fig 4C). Data analysis did not demonstrate any difference in the concentration of FVIII amongst the groups.

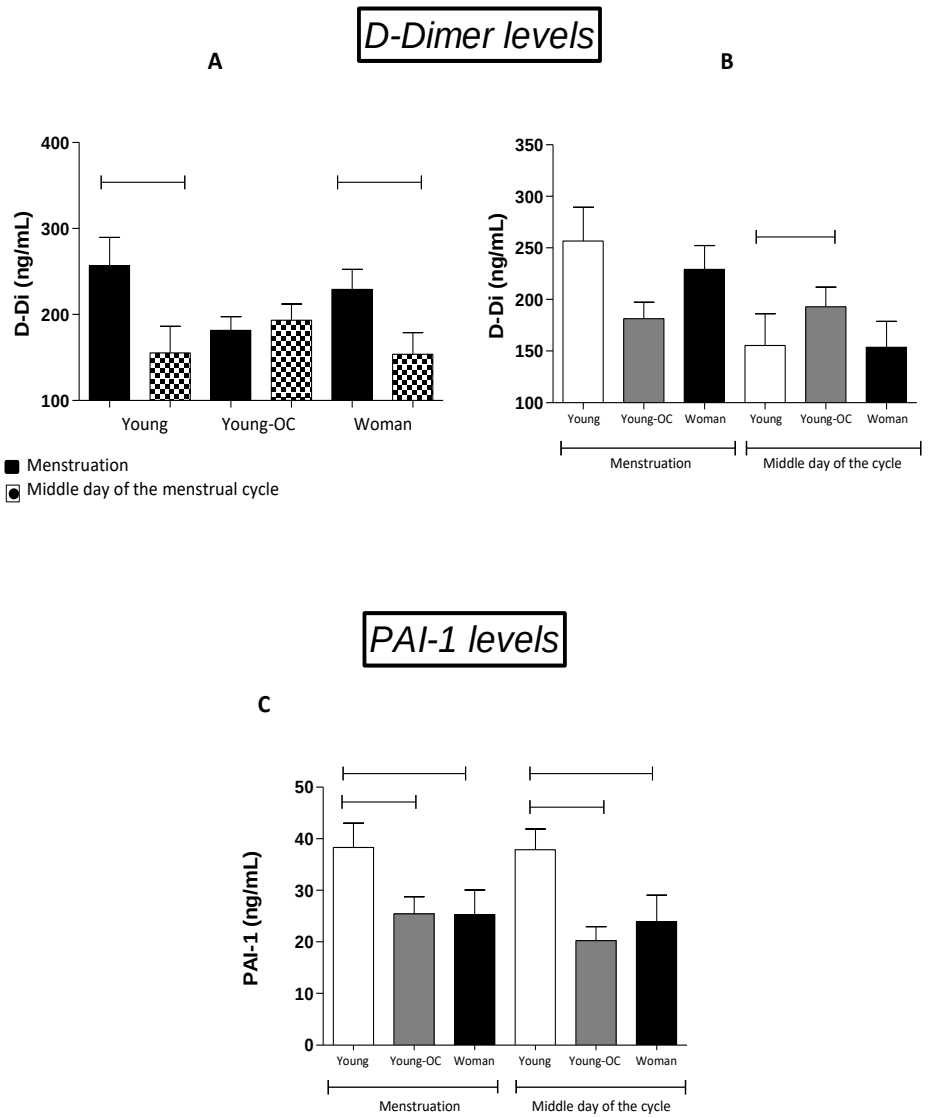

Figure 4. D-Dimer levels (ng/mL) in (A) menstrual phase as compared to the middle day of the cycle and (B) among Young, Young-OC and Woman. PAI-1 levels (ng/mL) amongst these groups (C). The results are expressed as mean value (bars) and scattering distribution of individual values. Significant differences at $P<0.05$ are highlighted by connecting lines.

3.5 *Platelet count and hemostatic biomarkers Spearman correlation analyses*

During menstruation, we found out that Young presents five moderate associations among platelet count and platelet activation biomarkers. For Young-OC, the same number of significant associations was detected, but with D-Di association and the emergence of a strong association between (MFI) CD62-%CD62. Finally, Three strong associations in Woman involving platelet activation biomarkers, platelet count and D-Di (Fig 5A).All correlations found were positive.

For the middle day of the cycle, we observed that Young presents four moderate associations among platelet count, platelet activation biomarkers, D-Di and PAI-1. Besides, there was an emergence of two strong associations between (MFI) CD62-%CD62 and PAI-1-%CD62. For Young-OC, we verified moderate associations involving platelet activation biomarkers and FVIII. Finally, five strong associations in Woman involving platelet activation

biomarkers and PAI-1 (Fig 5B). All correlations found were positive.

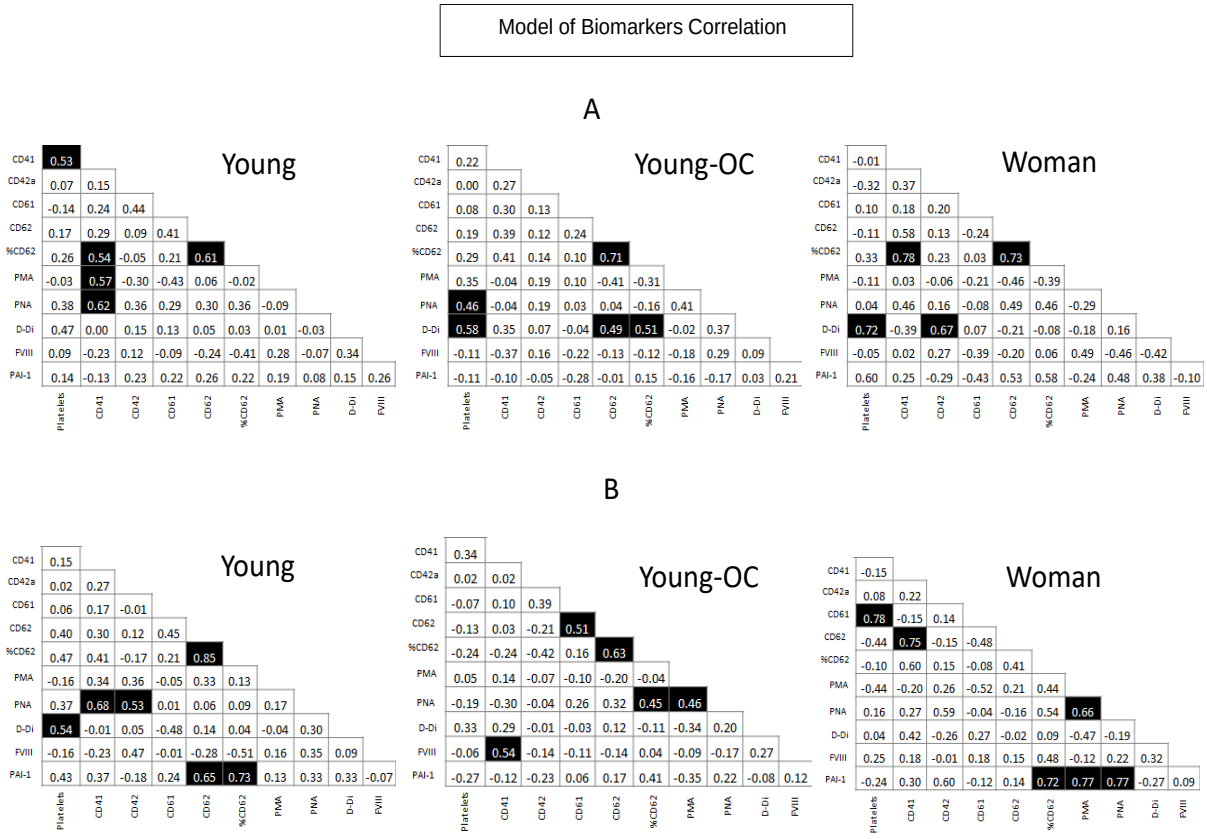

Figure 5. Spearman correlation indexes (ρ), in squares, for platelet count, platelet biomarkers, coagulation and fibrinolysis components in (A) menstruation and (B) in the middle day of the cycle for all study groups. In black, significant ρ values (P≤0.05).

DISCUSSION

Present study involved two groups of women with physiological fluctuations in hormone levels (Young and Woman) and Young-OC group, in which hormone levels are almost constant. In Young-OC, the synthetic progestogens and estrogens present in the combined OC inhibit the hormones (LH and FSH) that trigger ovulation by negative feedback. Steroid concentrations are maintained above the physiological levels throughout the period of use, except for seven days in which the woman stops use and, for hormonal deprivation, bleeding occurs. [21] Combined monophasic OC is characterized by having the same hormonal concentrations in each of the 21 pills. Thus, the changes observed in this women group could be explained by a cumulative effect of this medication.

Proving the hypothesis that leukocyte count can be altered when occurs a physiological change inestrogen and/or progesteronehormone concentration, [22] in the present study we found that the relative neutrophil count increased during menstruation in Young and Young-OC groups. Maybin & Critcheley [23] consider the menstruation as classic inflammatory moment in which endometrium displays inflammation hallmarks, including tissue edema and influx of immune cells, justifying our data. However, these data are in disagreement with Bain & England [22] that verified, in women between 20 and 35 years-old, a fall in the neutrophil count at menstruation. In Woman group, there was a decrease in the relative neutrophil count during menstruation. No literature reports were found to justify this observation, which may be due to the small sample size of this group.

For the neutrophil absolute count, we verified higher count, during menstruation, in Young than Young-OC, suggesting that in OC non-users, the

physiological oscillations of estrogen concentrations play a relevant role. Contradictorily, this observation was not confirmed in the middle day of the cycle to the same group, because the stab leukocyte relative count decrease in Young compared to Young-OC. Probably, the cumulative effect of the hormones present in OC, maintained at constant and higher concentrations than the physiological ones.

Our data showed a lower platelets count, in the middle day of the cycle, in Young-OC and Woman comparing to Young, differently from [24] that verified higher count in first/second generations OC users than in non-users. We did not observed any significant difference between platelet count during menstrual cycle for any evaluated group, although literature reports an increased count in the ovulatory phase [4,13] and a reduction in menstruation [25,26] compared to both proliferative and secretory phases. [26] However, Bain & England observed only minor variations, being the highest during and after menstruation and the lowest in the second and third weeks. [22] In second generation OC users, Buchan and Macdonald [24] found out that platelet count remained steady throughout the cycle, exactly the same that was observed on this study.

A hypothesis for the lower platelet count observed in Young-OC and Woman groups would be that, in the first case, the use of these drugs could activate platelets that would be consumed in hemostatic processes. [27] The same would occur naturally throughout the woman's life. [23,28]

Our data also differ from Segal & Moliterno [29] that verified that platelet count varies according to age, being higher in the range of 40 to 49 years-old compared to 20 to 29 years-old. It is important to state that this study evaluated only North-America population and did not consider the menstrual cycle phase, which may be the cause of the divergence between the results. In addition, Brazilian population, due to the great miscegenation, makes it difficult to compare it with a defined ethnic group.

Considering that platelet activation is associated with conformational change of GPIIb/III a surface glycoprotein (recognized by CD61 and CD41a), that releases its granules to favor platelet aggregation, we have observed that CD61expression increased in Young-OC in the middle day of cycle as compared to menstruation. For CD41a, the data have showed higher expression in Young-OC, in the same moment, as compared to Young. Thus, combined OC would act favoring platelet aggregation by increasing homocysteine levels (a marker of hypercoagulability) and reducing NO levels (an important platelet aggregation inhibitor) [30]

However, literatures related to platelet activation during menstrual cycle are contradictory. There are studies that were observedboth an increase number of platelet micro particles (MP)expressing CD61during luteal phase, [9] a reduction of platelet aggregation in this phase [12] and during the peri-ovulatory period, [31] and an exacerbated platelet aggregation induced during the follicular phase. [32] In accordance with our data, Repina *et al* [33] did not observe variation of platelet aggregation parameters in OC non-users during menstrual cycle.

However, special care must be taken to interpret platelet activation. It should be highlighted that the reaction between fibrinogen and CD41a can be affected by *in vitro* sample handling or by the use of fixative and platelet inhibitors, which may hide the expected levels of platelet activation. [34] Experiments *in vitro* have demonstrated that the maximal CD62P expression may require extra stimulation. [34] It is possible that the use of *in vitro* stimulation systems would be relevant to identify difference in the CD62P expression amongst the groups. Besides, a critical analysis of literature revealed that the results did not show standardized distribution, which make difficult to establish the cut-off for platelet activation. Konijnenberg *et al* [35] admitted that normally only a subpopulation of platelets seems to be

activated and they are rapidly cleared from the circulation. It should be emphasized that CD62P is unstable and is released from the platelet surface rapidly remaining soluble and functional in plasma, but it is undetectable by flow cytometry. [36,37] It is also necessary to mention that the platelets fixation immediately after blood collection seems to be crucial to avoid the platelet activation *in vitro.* [38] On the other hand, it is important to highlight that fixation can also interfere in the analysis, concealing the expected levels of platelet activation. [34]

About frequency of PNA and PMA, our data demonstrated a higher frequency of these markers, in the middle day of cycle, in Young-OC as compared to Young and of PNA, during menstruation, in Young-OC compared to Young, suggesting that OC use results in interaction between blood coagulation and inflammation.In fact, Larsen *et al* [39] found an increase in serum C-reactive protein concentrations in women using combined OC compared to non-users. Thus, it can be deduced that the use of these drugs induces an inflammatory process, which would be contribute to the increased risk of thromboembolic event, by hemostatic system activation.

Differently from our data and considering only OC non-users, Rosin *et al* [13] verified that PLA frequency was higher in ovulation compared to the other phases of the menstrual cycle, suggesting strong estrogen effects on platelet-leukocyte interactions and a variation in platelet function during specific phases of the menstrual cycle. It is important to clarify that these investigators performed a previous platelet activation, being able to overestimate the obtained results. Another hypothesis to justify the disagreement between results is that the PLA formation is Ca^{2+} dependent and can be inhibited by anticoagulants sequestering this cation, such as sodium citrate, used in the present study. [40] It should be also highlighted that the technique for determining the PLA percentage is variable, including different marking protocols, [13,40-42] limiting a safe comparison between results.

In agreement with our results, Robb *et al* [40] did not verify any change on these markers or significant correlation between them and the concentration of estrogen or progesterone during menstrual cycle in OC non-users.

Our data showed higher D-Di (one of the fragments resulting from fibrin degradation) levels, during menstruation, in Young and Woman compared to the middle day of the cycle, in agreement with Koh *et al.* [43] This observation can be justified by the occurrence of endometrial microvasculature lesion, followed by platelet and coagulation factors activation, with subsequent formation and dissolution of the clot that occur during this phase.

Increased D-Di levels, in the middle day of the cycle, in Young-OC compared to Young observed in our study also suggest an important cumulative contribution of OC, for hemostasis imbalance, favoring a hypercoagulable state as observed in literature. [27,44] Besides, estradiol, present in OC, induces a change in the fibrin structure, making it more resistant to the fibrinolytic system action, which predisposes women to thromboembolic events. [15]

In this context, decreased PAI-1 (afibrinolysis inhibitor) levels, during menstruation and in the middle day of the cycle, in Young-OC and Woman compared to Young, can be justified by increase in fibrinolytic potential in those women. First, because OC activates procoagulant mechanisms and induces the fibrinolytic system, aiming to dissolve formed clots. [45] Second, over the years, there is a natural tendency to increasement of procoagulant factors compared to natural anticoagulants, resulting in a greater susceptibility to hypercoagulable state. [23, 46]

No differences were observed in PAI-1 levels during menstrual cycle by Onundarson *et al* [47] and Koh *et al* [43] according our data.However, there are reports of reduced fibrinolysis in the follicular [48] and luteal phases; [43] and

activation during ovulation [49] in endometrial fluid samples. Assuming that there is a cyclic variation in the fibrinolytic potential, it can be inferred that the determination of PAI-1 in the peripheral blood would not present sufficient sensitivity to detect changes within the normal range, which would justify the results obtained in the present study.

Nocycle differences were observed for FVIII (an important activator of coagulation cascade) levels, in accordance with Tchaikoviski *et al.* [50] Nevertheless, the studies investigating this parameter during menstrual cycle are limited and conflicting. In this sense, there are reports that showboth reduced [4] and increased [10] FVIII levels. Contradictorily, Onundarson *et al* [47] did not find any association between levels of estradiol or progesterone and this factor. Probably, FVIII levels would be higher if our measures were made in the end of the menstruation, since the coagulation cascade was further activated to stop the bleeding. [3]

An important limitation to consider in relation to the literature is the variety of sampling moments during the menstrual cycle, making it difficult to compare the data. Besides, the studies did not observe cyclical variations or, when they are present, were considered discrete and restricted to follicular or luteal phases, no analyzed by us. [51]

Although Tchaikovski *et al* [18] and Tchaikovski & Rosing [50] verified the presence of procoagulant changes in several hemostatic parameters, including FVIII during combined OC use, these changes were not observed in the present study. There were also no changes in Woman group, although it is known that there is an increase in clotting factors over the years. [23,28,46] Probably, the small sample size of the studied population underestimated possible changes in plasma levels of FVIII.

Finally, we showed that there are significant positive associations among platelet count, platelet activation biomarkers, coagulation cascade and fibrinolysis during menstrual cycle. These associations change in intensity depending on the phase of the menstrual cycle and the studied group. During menstruation, they have moderate nature, involving platelet elements and PLA in Young, suggesting that in this phase, these women would be physiologically less exposed to thromboembolic events as compared to other evaluated groups. For Young-OC and Woman, there is D-Di participation, simultaneously involved in the coagulation cascade and fibrinolysis. Strong correlations were also observed (one for Young-OC and three for Woman), providing these groups pronounced hypercoagulable *status* due to combined OC use and age, respectively.

Considering middle day of the cycle results, associations are gradually stronger, involving PAI-1, D-Di and FVIII, resulting in an increasing stimulus to hypercoagulability probably due to the estrogen levels. The most evident changes are observed in Woman, in which were verified five strong correlations, involving platelet elements, PLA and PAI-1. These data suggest that, in this phase of the menstrual cycle, these women are physiologically more prone to thromboembolic events compared to the other groups.

It should be noted that despite these correlations and differences among studied parameters, we cannot draw definitive conclusions about the magnitude and consequences of hematologic changes during the menstrual cycle, considering hormonal differences and age of the women. This is due, in part, to the small number of study participants. Another relevant point to be considered is the variability of platelet biomarkers profiles observed in the population, requiring more studies in this cytometry markers and standardization of the pre-analytical conditions and analytical methods. Besides, we also must consider the absence of hormonal measures in this study, which would be able to determine with more accurately the phase of the menstrual cycle (for Young and Woman groups).

In summary, menstrual cycle was associated to modifications in neutrophil count, platelet activation and D-Di levels. Besides these parameters, PLA and PAI-1 levels are influenced by combined OC use. The age of the women influences platelet count and PAI-1 levels. Finally, there are positive correlations among platelet count, activation platelet markers, coagulation cascade and fibrinolysis, whose intensity depends on the phase of menstrual cycle, combined OC use and women age.

Conflict of interest

The authors declare that they have no conflicts of interest regarding this article.

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