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Article

Circulating microparticles and thrombin generation in patients with Chronic Lymphocytic Leukemia

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Chronic Lymphocytic Leukemia (CLL) has shown great biological heterogeneity, with a variable prognosis, and a short survival in some patients. In this light, the present study focused on the prognostic utility of circulating microparticles (MPs) and a Thrombin Generation (TG) Profile for thrombotic risk and disease progression in CLL patients. Circulating microparticles and TG were evaluated in 35 patients with CLL and 35 healthy individuals. For circulating microparticles, significant differences were observed among the following groups: MPs derived from endothelial cells ($p = 0.002$), B lymphocytes ($p < 0.001$), platelets ($p = 0.003$), and Tissue Factor MPs in monocytes ($p < 0.001$). In all cases, MP values were higher for the CLL group. When compared to the controls, CLL patients presented a decrease in TG, characterized by a reduced endogen thrombin potential (ETP) ($p = 0.031$). When the results were analyzed according to the Binet stage, as compared to the controls, the Binet B+C group also presented lower ETP values ($p = 0.009$). No significant differences were observed between the control and the Binet A groups or between the Binet A and the Binet B + C groups. Although hemostatic alterations may occur in patients with CLL, these parameters do not seem to be useful to indicate disease progression.

Keywords: Chronic lymphocytic leukemia. Prognosis. Microparticles. Thrombin Generation.

INTRODUCTION

Chronic lymphocytic leukemia (CLL), characterized by the accumulation of B lymphocytes in the body, is a disease that is more prevalent in the elderly, with a mean age between 64 and 70 years of age. CLL is derived from a $CD5^+$ B cell population, and these cells have a high longevity, in most cases determined by apoptosis inhibition (Krause *et al.*, 2016). The survival rate of patients with CLL is approximately 10 years; however, this prognosis is highly variable, with and some patients surviving for only a short period of time (Wiestner, 2015). Thus, it is important to identify those patients whose disease will progress rapidly so as to offer early diagnoses and more aggressive treatment protocols.

In symptomatic patients, the most common findings include lymphadenopathy, weight loss, fatigue and bacterial infections, and commonly pneumonia. As the disease progresses, patients may develop severe anemia and bleeding due to thrombocytopenia and other clinical events (Palanca-Wessels *et al.*, 2015).

Classical systems of disease staging according to Rai (1975) and Binet (1981), are based on clinical and hematological characteristics. These systems define early (Rai 0, Binet A), intermediate (Rai I/II, Binet B), and advanced (Rai III/IV, Binet C) stages, with > 10 years, 5-7

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years, and 1-3 years of survival, respectively (Lemanne *et al.*, 2015; Martinez-Torres *et al.*, 2015).

In recent years, many researchers have conducted studies focusing on the role of microparticles (MPs) from different sources in the pathogenesis of many diseases (Helbing *et al.*, 2014; Yuana, Sturk, Nieuwland, 2013). As reviewed by Helbing *et al.* (2014), MPs consist of fragments of the membrane or cytoplasm of their origin cells and are capable of transferring cell components from those cells to the target cells, which often results in altered cell functions. The number of MPs, mainly platelet-derived, is elevated in thromboembolic disorders (Domnikova *et al.*, 2013). In light of the knowledge that malignancies follow a path with varying degrees of hypercoagulability, according to the type of tumor, it is possible that MPs play a role in the pathogenesis of leukemia, thus predisposing the MPs to thrombotic events.

Tumor cells lead to the activation of coagulation cascades, resulting in the increased generation of thrombin and fibrin. The activation of platelets, leukocytes, and endothelial cells also occurs, which exposes pro-coagulant factors (Vasilatou *et al.*, 2013; Duarte *et al.*, 2015). Although the increased risk of venous thromboembolism (VTE) has been reported in many hematological malignancies (Wu, Tang, Wang, 2017; Gade *et al.*, 2017), data on VTE in CLL are quite scarce.

Therefore, studies focusing on various aspects of CLL, such as diagnosis, progression, and treatment, are highly relevant when considering the need for more precise knowledge in order to improve the management of these patients. This study aimed to evaluate the hemostatic profile of patients with CLL through the plasma quantification of circulating microparticles and the potential of thrombin generation, together with their correlation with disease progression, and the clinical and demographic data of the patients. It is believed that the development of this study may add some knowledge to pre-existing data on the pathophysiology of CLL and aid professionals in generating further insight into the importance of microparticles and the potential of thrombin generation as prognostic biomarkers in CLL.

MATERIAL AND METHODS

Patients

Patients were selected by hematologists from the Hematology Unit of the Clinical Hospital, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil. Patients' clinical data were obtained from their medical records. Sample size was calculated considering the variables and evaluated groups, applied treatments, and test power of 0.8 and alpha 0.05, through the SigmaSTAT® software, version 2.03. A sample of 28 individuals was calculated for each group.

A total of 35 patients with confirmed CLL were included according to criteria established by the World Health Organization (WHO), Matutes Scoring System, and International Working Group Classification (Binet System): 18 with a low risk and 17 with moderate and high risks. As a control group, 35 clinically healthy individuals with normal blood counts and no history of blood disorders were enrolled in the study. The institutional Ethics Committee of the Federal University of Minas Gerais (UFMG) approved this study, and informed consent was obtained from all participants. This study was carried out in accordance with the Declaration of Helsinki.

Blood samples

Whole blood samples (4.0 mL) were obtained by venipuncture using sodium citrate vacuum system tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Samples were processed to obtain platelet-poor plasma (PPP) by double centrifugation at 1,500 g for 15 minutes in an Excelsa centrifuge, Mod 206BL (BD Biosciences, California, EUA). After the second centrifugation, the upper two thirds of the plasma were removed, referring to the poor portion of platelets. The obtained plasma was stored at -80°C until further analysis.

Analysis of circulating microparticles (MPs)

The analysis of MPs was performed according to Campos *et al.* (2010) by Flow Cytometry. Antibodies

used in this reaction were those markers of platelets (CD41), B lymphocytes (CD19), tissue factors (CD142), monocytes (CD14), and endothelial cells (CD51/61). It should be noted that the expression of tissue factor MPs in the monocytes was also evaluated. These antibodies are labeled with the fluorochromes PECy5 (CD41), PE (CD19, CD51/61 and CD142), and APC (CD14). In addition, the Annexin V-FITC, a classic marker of MPs, directed against phosphatidylserine found on its surface, was used.

After obtaining the PPP, the supernatant was aspirated and diluted 1:3 in citrate phosphate buffered saline (PBS) containing heparin. The diluted sample was centrifuged at 15,000xg for 90 min at 15°C in a Laborzentrifugen 2K15 centrifuge (Sigma-Aldrich, St. Louis, Missouri, EUA). Carefully, the supernatant was removed and the MP pellet was resuspended in 100 µl of annexin buffer (binding buffer). Separately, 5 µl of each antibody (CD51/61, CD19, CD41, CD142, and CD14) and 2.5 µl of Annexin V-FITC were added in tubes suitable for cytometer reading. To these tubes, 100 µl of the resuspended sample in 1x annexin buffer was added, as obtained above. These concentrations were based on an experiment previously undertaken for the titration of antibodies. The tubes were incubated for 30 minutes at room temperature (25°C) in the dark. To the incubated material, 300 µl of annexin buffer was added, and analysis was performed using the cytometer.

The cytometer was adjusted to read the samples at a high speed (60 µl/second) for each sample. The number of MPs/µl of plasma was calculated as described by Campos *et al.* (2010): MPs/µl = (Nx400)/(60x100), where N is the number of events acquired in the MP region, 400 is the total volume in each tube before analysis, 60 is the sample volume analyzed in this study, and 100 is the original volume of the MP suspension.

Isolated MPs were gated based on their forward (FSC) and side (SSC) scatter distribution when compared to the distribution of synthetic 0.7-0.9 µm SPHERO™ Amino Fluorescent Particles (Spherotech Inc., US) (Figure 1A). The presence of phosphatidylserine residues on the surface of MPs was assessed for their positive staining with monoclonal antibodies against annexin V FITC. The specific monoclonal antibody was corrected by isotype-matched control antibodies mouse-FITC (Figure 1B) to eliminate nonspecific labeling. Cell-specific monoclonal antibodies were used to identify the source of the MPs (Figure 1C).

The samples were analyzed in an LSR Fortessa Cytometer (BD Biosciences, California, EUA), using FACSDiva™ 6.2 software (BD Biosciences, California, EUA) for data acquisition, followed by analysis performed on the FlowJo® software x10.0.7.

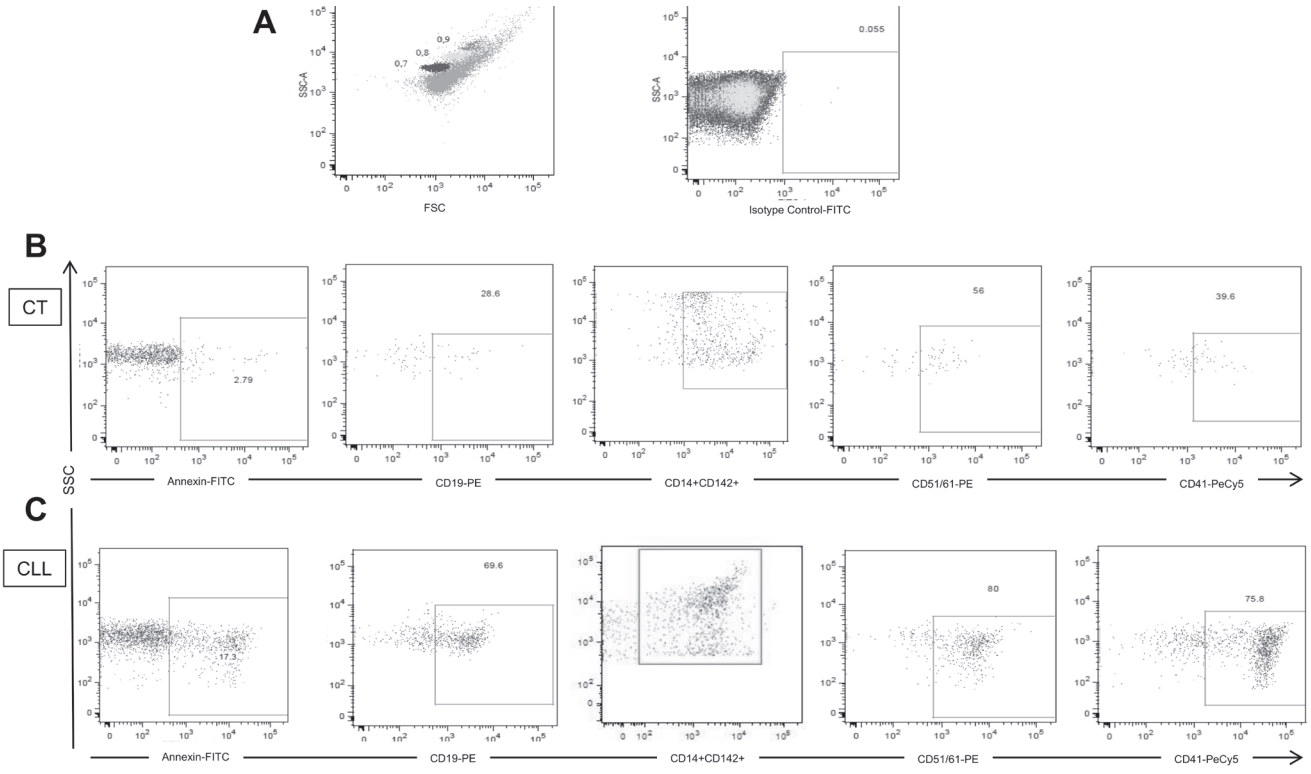

FIGURE 1 - Isolated MPs were gated based on their forward (FSC) and side (SSC) scatter distribution as compared to 0.7-0.9 µm synthetic MPs and Mouse IgG FITC-conjugated isotype control (A). Events present on the gate were assessed for their annexin V positive staining, using FITC-conjugated monoclonal antibodies and subsequent identification of the source of MPs, using cell-specific monoclonal antibodies (CD19-PE for lymphocyte, CD41-PeCy5.5 for platelets, CD51/61-PE for endhotelial cells) in control groups (B) and CLL patients (C).

Thrombin Generation Test

Thrombin generation (TG) was determined according to Hemker *et al*. (2003) and Castoldi & Rosing (2011) in platelet-poor plasma (PPP), using the Calibrated Automated Thrombogram (CAT) method (Thrombinoscope BV, Maastricht, The Netherlands). In summary, TGT was determined by using 80 µl of PPP and 5 µl of HNBSA (Hepes/NaCl/BSA) buffer mixed with 20 µl of triggering reagent (PPP Reagent High – Stago, France) in a 96-well plate. For each sample, one calibrator well was run, consisting of 80 µl of PPP, 5 µl of HNBSA buffer, and 20 µl of Thrombin calibrator (Stago, France). Finally, the reaction was started by adding 20 µl of the fluorogenic substrate and calcium chloride (FLUCA kit - Stago, France), and the plate was read for 60 minutes. All wells contained a final volume of 125 µl. Fluorescent substrate is cleaved by the thrombin formed and the intensity of fluorescence is read in a Fluorimeter (Fluoroscan Ascent Reader -Thermo LabSystems), which is then converted into thrombin concentrations (nM), using a reference curve prepared by measuring the rate of the substrate conversion by a thrombin calibrator (Stago, France).

The Thrombinoscope software (Thrombinoscope, Maastricht, The Netherlands) was used to construct the curve time (min) versus thrombin concentration (nM) and to calculate TG parameters, such as Endogenous Thrombin Potential (ETP), lag-time, peak, and time to peak.

Statistical analyses

The results were analyzed using the SigmaSTAT® program, version 2.03, and presented as a mean and standard deviation in the case of normal distribution, while the results that did not follow normal distribution

were presented as median and interquartile ranges. For the variables with normal distribution, analysis of variance (ANOVA) was used, followed by a Tukey multiple comparison test to evaluate three groups, while the Student's t-test was used to evaluate two groups. For those variables that did not present a normal distribution, the nonparametric Mann Whitney test was used for to compare two groups, while the Kruskal-Wallis non-parametric test, followed by the Dunn's multiple comparison test, was used for to compare more than two groups. Investigation of the correlation between parameters was performed by the Pearson correlation for quantitative variables, and the Spearman test was applied to evaluate qualitative variables. A value of $p < 0.05$ was considered significant.

RESULTS

Characteristics of the study population are presented in Table I.

TABLE I - Variables in patients with CLL and in matched healthy control individuals

Variables	Controls n = 35	CLL n = 35	*P*
M/F	11/24	19/16	$P = 0.090$
Age* (years)	69.5 (66.0-76.0)	66.2 (55.0-80.1)	$P = 0.267$
Hemoglobin* (g/dL)	13.5 (13.1-14.6)	12.3 (10.9-14.2)	$P = 0.004$
Leukocytes* (10^3/mm³)	5.7 (4.3 – 6.5)	17.9 (6.2-41.8)	$P < 0.001$
Platelets* (10^3/mm³)	202.0 (176.3 – 239.0)	136.0 (106.0-182.0)	$P < 0.001$
Binet A	NA	18	
Binet B	NA	7	
Binet C	NA	10	

M: male; F: female; NA: not applicable; CLL= chronic lymphocytic leukemia. *median and interquartile range.

No significant difference was observed when comparing age and gender between CLL patients and controls ($p = 0.267$ and 0.090, respectively).

Patients and controls were also compared for hemoglobin levels, and total leukocyte and platelet counts. For these three parameters, with $p < 0.05$, significant differences were observed between the CLL and control groups. Platelets number and hemoglobin levels were lower in CLL patients than in the control group. However, the number of leukocytes was higher in CLL patients than in the controls (Table I).

When patients were analyzed according to the Binet stage, significant differences were observed for platelet counts between Binet A (164.889 ± 42.397/mm³) and Binet B + C (111.294 ± 50.575/mm³) ($p = 0.002$) groups. Significant differences were also observed for platelet counts between Binet A and the control group [202.00 (176.25-239.00/ mm³)] ($p = 0.004$) and between Binet B + C and the control group ($p < 0.001$), with the lowest results being those of the Binet B + C group, followed by the Binet A group, and the highest scores being found in the control group. The comparison of total leukocyte counts showed a significant difference between the Binet A group [16.350 (5.875-42.700/ mm³)] and the control group [5.650 (4.300-6.450/ mm³)] ($p < 0.001$), as well as between the Binet B + C group [18.600 (7.750-45.200)] and the control group ($p < 0.001$). However, no significant difference was observed between the Binet A and the Binet B + C groups ($p = 0.704$). Moreover, no significant differences were observed between the lymphocyte counts for Binet A [10.47 (3.11-35.8/ mm³)] and those for Binet B + C [16.51 (5.10-36.37/ mm³)] ($p = 0.656$), although absolute lymphocyte median values were higher in the more advanced stage of the disease.

Significant differences were observed between patients and controls for the four MPs analyzed in this study: MPs derived from B lymphocytes ($p = 0.0004$, figure 2A), endothelial cells ($P = 0.0014$, figure 2B), platelets ($p = 0.0011$, figure 2C), and tissue factor MPs in monocytes ($p = 0.0011$, figure 2D). In all cases, MPs values were higher in the CLL group.

When patients were analyzed according to the Binet stage, significant differences ($p < 0.05$) were also found for the Binet A versus control groups regarding the

four analyzed MPs (MPs from lymphocytes, $p = 0.003$; endothelial cells, $p = 0.001$; platelets, $p = 0.0087$; and TF in monocytes, $p < 0.0001$) with the highest MP values found in the Binet A group. No significant difference was observed between the Binet A and the Binet B + C groups, although the results did prove to be higher for the Binet A group. Likewise, no significant difference was found between the Binet B + C group versus the control group for any of the analyzed microparticles (Figures 3A, 3B, and 3C), except for TF in monocyte MPs ($p < 0.0001$) (Figure 3D). Medians and interquartile ranges of MP analysis are shown in Table II.

TABLE II - Medians and interquartile range values for circulating microparticles in plasma samples from patients with CLL and controls

Microparticles	Controls n = 35	CLL n = 35	Binet A n = 18	Binet B+C n = 17	*P*
Endothelial cells* (MPs/μL)	81.6 (53.1-121.0)	132.1 (78.6-199.1)	149.8 (75.3-212.5)	132.1 (70.4-187.8)	<0.005a, b
B lymphocytes* (MPs/μL)	85.2 (63.4-115.9)	142.1 (93.7-203.1)	145.2 (91.9-235.6)	142.33 (83.1-169.3)	<0.005a,b
Platelets* (MPs/μL)	81.2 (52.8-118.9)	134.8 (85.7-183.1)	148.1 (82.2-202.1)	134.8 (73.1-173.0)	<0.005a,b
TF monocytes* (MPs/μL)	8.2 (6.4-11.4)	37.37 (23.4-57.1)	36.4 (23.2-59.7)	38.33 (21.8-53.7)	<0.005a,b,c

CLL= Chronic Lymphocytic Leukemia. *Median and interquartile range.a: CLL versus Control group; b: Binet A versus Control group; c: Binet B+C versus Control group

Concerning the TG tests, the four main parameters of the TG curve were evaluated, i.e., endogenous thrombin potential (ETP), lag-time, peak, and time to peak. Significant differences were observed only for ETP when comparing CLL patients [1453.00 (1176.33-1602.57 nM)] to control groups [1577.38 (1326.20-1816.95 nM)] ($p = 0.0312$). Patients presented decreased median and interquartile range values when compared to the control group (Figure 4A).

When ETP results were analyzed according to the Binet stage, a significant difference was observed between the controls [1577.38 (1326.20-1816.95 nM)] and the Binet B + C group [1216.54 (1130.47-1540.15 nM)] ($p = 0.0093$). It should be noted that the group of CLL patients presented decreased values when compared to the control group.

No significant differences were observed between the controls and the Binet A group [1511.60 (1393.66-1706.26)], and between the Binet A and the Binet B + C groups ($p > 0.05$, Figure 4B).

No correlation could be observed between MPs or TG values and hemoglobin levels, global leukocytes, B lymphocytes, and platelet counts. Likewise, no correlation was found between MP values and TG results.

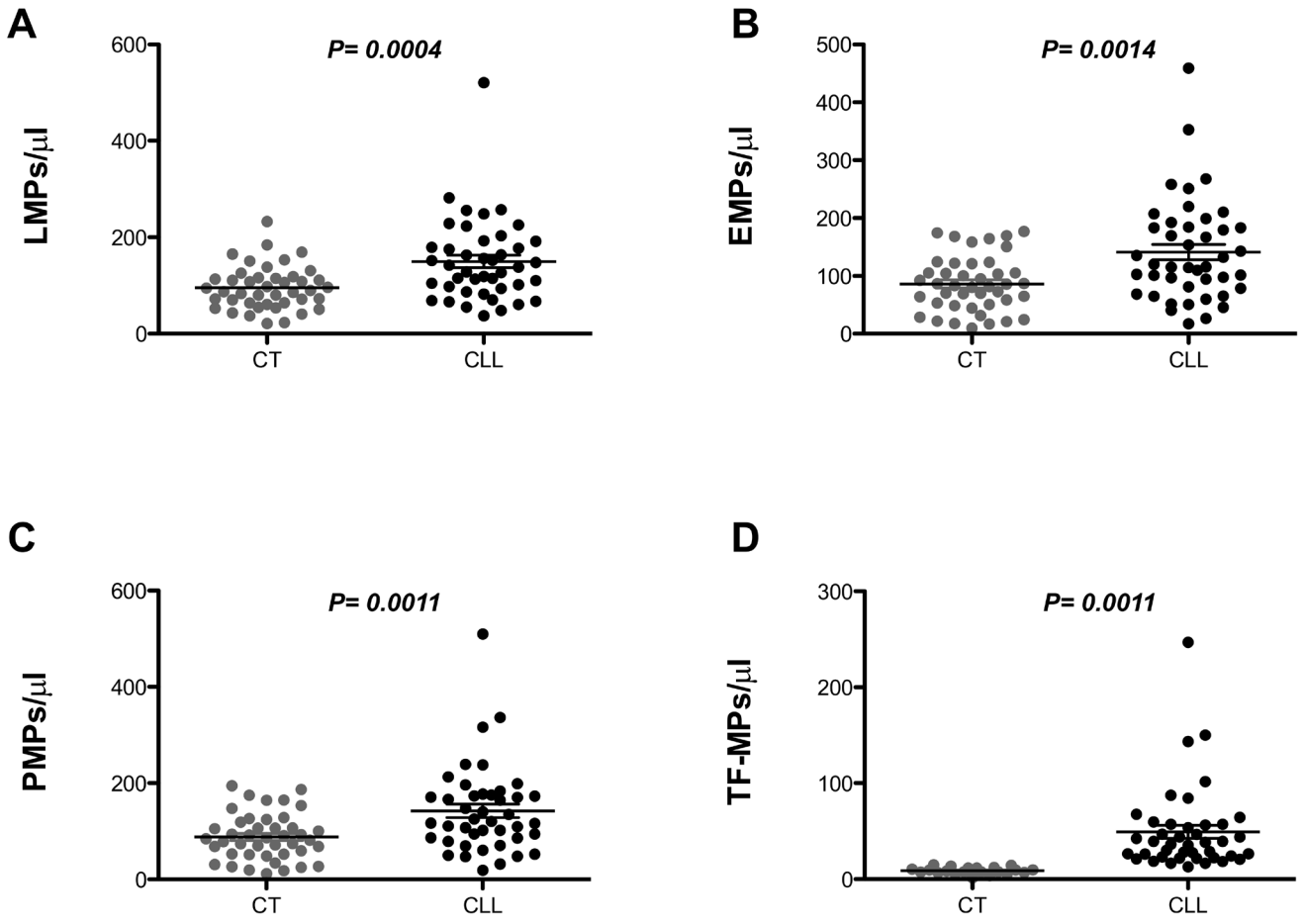

FIGURE 2 - Analysis of circulating microparticles derived from B lymphocytes (A), endothelial cells (B), platelets (C), and TF (D) in plasma samples from patients with CLL and controls (X axis).

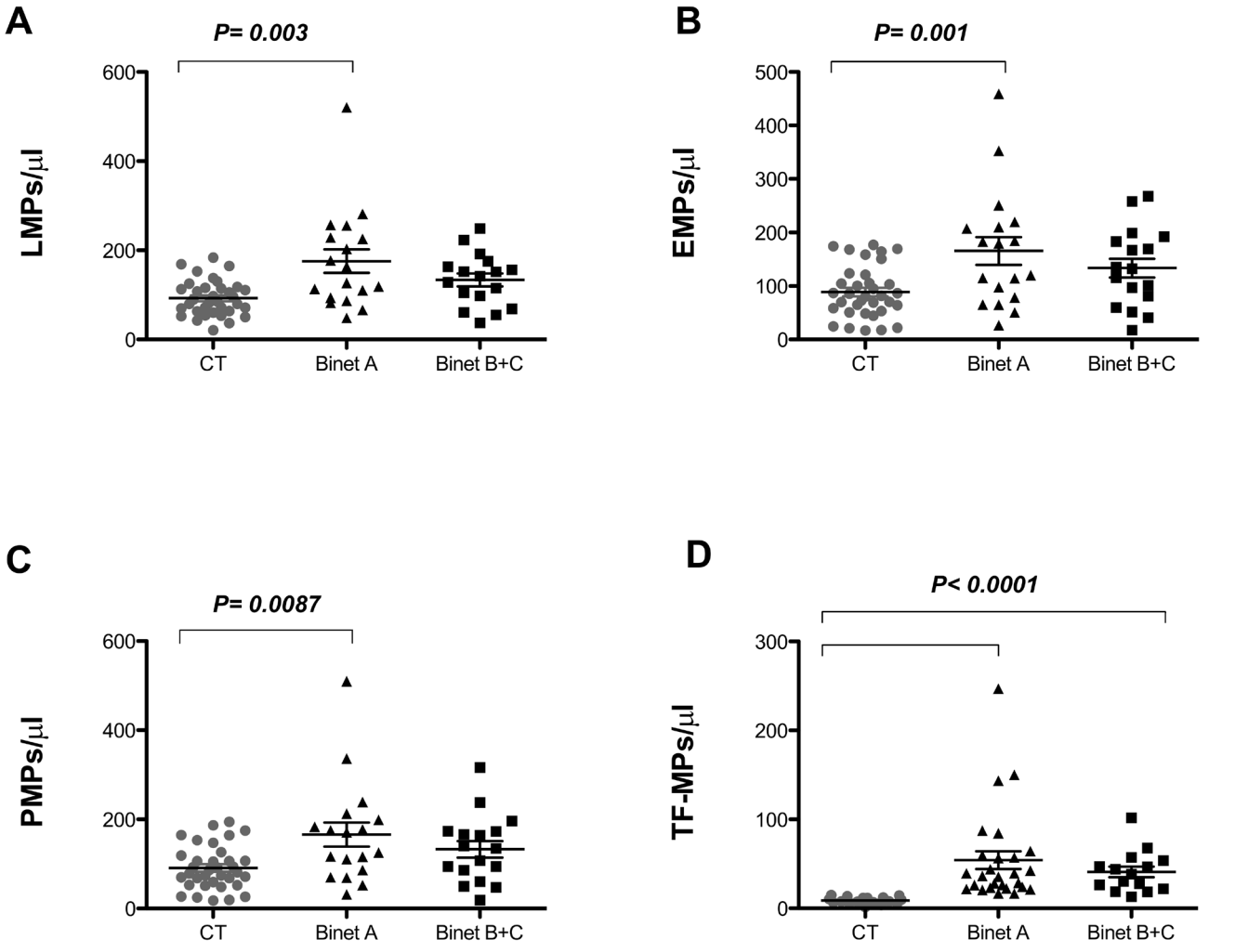

FIGURE 3 - Analysis of circulating microparticles derived from B lymphocytes (A), endothelial cells (B), platelets (C), and TF (D) in plasma samples from patients with CLL and controls (X axis), according to the staging of the disease (Binet A, B+C stages). The Y axis represents the amount of MPs/µl.

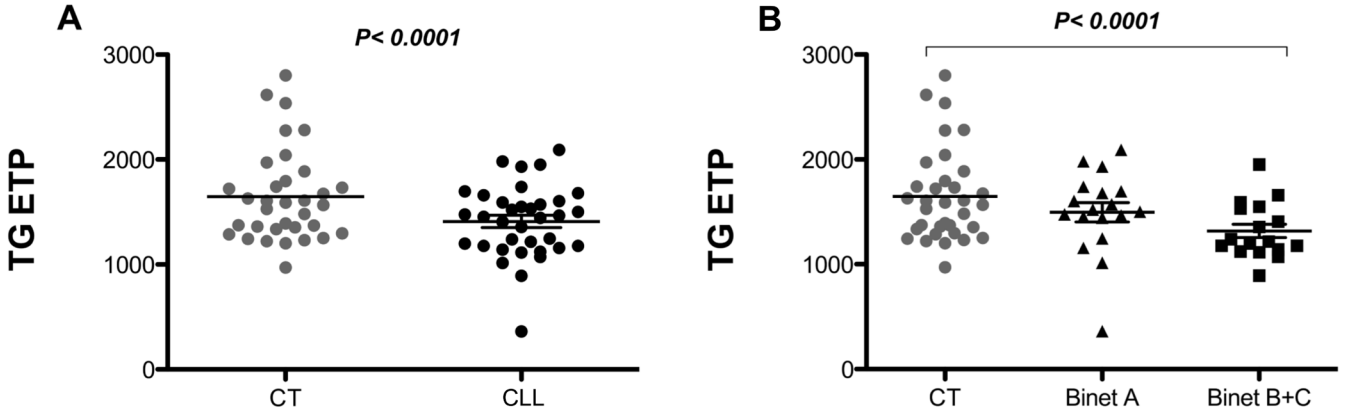

FIGURE 4 - ETP in plasma samples from patients with CLL and controls (A). In B, the patients were stratified according to the Binet stage (1981). The Y axis corresponds to the ETP.

DISCUSSION

It is known that CLL is a disease with a higher prevalence in the elderly, with a mean of 64-70 years of age; however, it may, in exceptional cases, occur in youth and adults (Aguirre Palma *et al.*, 2016). In addition, CLL is known to affect males more than females. As expected, in this study, an increased prevalence of males was observed in the group of patients.

The disease itself is characterized by anemia and/or thrombocytopenia. This was also observed in our study, since a significant drop in hemoglobin levels and platelet counts number was observed in patients as compared to the control group. In addition, platelet values were significantly lower in the Binet B + C group than in the Binet A group. In fact, thrombocytopenia can be more pronounced in advanced stages of the disease, leading to hemorrhagic events (Dighiero, 2005; Sagatys, Zhang, 2012).

As CLL is a disease characterized by the accumulation of small monoclonal B lymphocytes in the blood, bone marrow, and lymphoid tissue (Martinez-Torres *et al.*, 2015), absolute and relative lymphocytosis often occurs, leading to leukocytosis. This was also observed in our study, since that group of patients presented a significant increase in the number of leukocytes ($17.9x10^3/mm^3$) when compared to the control group ($5.7x10^3/mm^3$). It was expected that this leukocytosis would be higher in advanced stages of the disease. However, in our study, it was not possible to observe a significant difference when the Binet A (16.35 x 10^9/L) and Binet B + C (18.60 x 10^9/L) groups were compared, although the overall leukocyte count was slightly increased in the Binet B + C group. When the presence of circulating microparticles was evaluated in the plasma of patients and controls, a significant difference was observed between these two groups for all of the evaluated MPs. The increase in MPs derived from endothelial cells, platelets, and B lymphocytes in our study confirms the results of Ghosh *et al.* (2010), and suggests that the malignant process is characterized by an increase in these MPs, possibly associated with the pathogenesis of the disease and tumor proliferation.

Increased levels of TF MPs were also observed in CLL patients when compared to the control group. In addition to the pro-thrombotic potential of MPs, it is well-known that these present pro-angiogenic activity, since MPs have agents that stimulate angiogenesis. In addition, these express cell adhesion molecules on their surface and have the ability to induce the release of pro-angiogenic factors by tumor cells. TF expression by MPs also represents an important mechanism involving MPs and cancer progression (Young *et al.*, 2012).

The FT/FVII complex, besides being the main trigger of coagulation, is also responsible for the activation of PAR-2 receptors, which are mainly expressed in cancer cells, leading to an up-regulation of transcriptional programs, controlling survival and angiogenesis. TF is also considered a transcriptional target of oncogenic pathways (controlled by RAS, p53 or PTEN), which are commonly associated with the onset and progression of cancer (Boccaccio, Comoglio, 2009; Falanga, Marchetti, Vignoli, 2013).

According to Ghosh *et al.* (2010), angiogenesis plays an important role in the pathogenesis of CLL, especially in tumor proliferation. One of the proposed mechanisms is the increase in neovascularization in the bone marrow and extramedullary tissues. Tumor, platelet, and endothelial cell MPs have proven to express adhesion molecules, growth factors, and matrix metalloproteinases, which are essential for local and systemic angiogenesis (Yu *et al.*, 2005; Voloshin, Fremder, Shaked, 2014). Considering the role of angiogenesis in CLL, and the importance of MPs and TF its pathogenesis, it is possible that the increase in these biomarkers, as shown in our study, is not directly related to thrombosis, but to the process of angiogenesis in this disease.

In the present study, it was not possible to prove the relationship between the increase in B lymphocytes and endothelial cell MPs and the stage of disease, since no significant differences were observed between the Binet A and Binet B + C groups for the two analyzed MPs. It may therefore be suggested that the disease itself causes the microvesiculation of lymphocytes and endothelial cells, and that this is not necessarily related to the staging of this disease. It is also worth mentioning the use of chemotherapy by some patients in the Binet B + C group, since this class of drugs decreases cell proliferation and, consequently, the progression of disease.

Regarding platelet-derived MPs, it is interesting to note that there was a significant difference in platelet count between all groups analyzed and, as the disease worsened, the number of platelets decreased compared to controls. This fact suggests that the disease itself causes considerable thrombocytopenia, particularly in the more severe forms of the disease, unlike PMPs. An analysis of the PMPs data revealed an inverse relationship between the number of platelets/uL and the number of PMPs. Thus, our data indicated that the subgroup B + C presented proportionally greater number of PMPs in relation to the number of platelets / microliter of blood, which suggests a possible greater platelet hyperactivation and consequent microvesiculation as the disease progresses. In order to be clearer, subgroup B + C presented 106,000 platelets/uL and 134.8 PMPs/uL, while subgroup A presented 154,500 platelets/uL and 148.1 PMPs/uL. In view of our findings, more robust studies focusing on PMPs would be desirable to investigate whether there is in fact an association between the number of platelets, PMPs and the pathogenesis of CLL.

Regarding the TG test, it was possible to observe a significant difference between the individuals in the control group and the patients for the ETP parameter. When the CLL group was stratified according to the Binet classification, a significant difference was observed only between the control group and the Binet B + C group. An analysis of these results points to the idea that the ETP would be slightly reduced in the more advanced stage of the disease (Binet B + C) as compared to both the Binet A and control groups. A search in the literature revealed no similar report regarding hemostatic evaluation though the application of TG tests in patients with CLL. However, the present study's findings suggest an imbalance in the hemostatic system of these patients, with a tendency towards hypocoagulability.

Since ETP, represented by the area under the curve (Figure 4), characterizes the potential of TG as a function of the balance between coagulation and natural anticoagulation, it may be suggested that there is a qualitative or quantitative deficiency of coagulation factors. Thrombocytopenia, therefore, may well be contributing to the lower TG in these patients, since the activation of clotting factors occurs on the surface of activated platelets. However, this issue warrants further study in order to confirm these findings. According to Šimkovič *et al.* (2015), in a cohort study conducted in 2015, thrombotic events were observed in 37% of the patients on CLL treatment. However, most of these patients were being treated with high doses of glucocorticoids, which is a known risk factor for deep vein thrombosis, mainly due to the increased levels of coagulation factors. Still, according to the same authors, there are other specific risk factors in patients with CLL that could play a role in the development of thrombotic events, such as hyperleukocytosis (overall leukocyte count above 100,000/mm3), leading to leukostasis.

However, according to reports from hematologists at the UFMG Clinical Hospital, most of the evaluated patients do not use glucocorticoids. No intense leukocytosis (> 100,000/mm³) was observed in any of the evaluated patients, which may contribute to the non-thrombotic tendency observed in our patients.

Regarding hypocoagulability, there are no reports in the literature that explain the mechanisms involved in hemorrhagic events in these patients, when they exist. These events are usually related to severe thrombocytopenia, common in this group of individuals. The use of chemotherapeutics is also an important factor that should be considered as interfering to justify the results found in the Binet B + C group, both for microparticles and for thrombin generation. The treatment for CLL has undergone extensive changes in recent decades. Since alkylating agents such as chlorambucil and glucocorticoids were inserted into the treatment of patients with CLL in the 1950s, much has been developed in the treatment of this leukemia and a significant increase in the survival of patients has been observed (Yu *et al.*, 2016). In the 1980s, purine analogs were introduced into the treatment, including cladribine, fludarabine, and pentastatin (Danilov, 2006; Holowiecki *et al.*, 2012; Lukenbill, Kalaycio, 2013).

According to reports from hematologists at the UFMG Clinical Hospital, the use of protocols including chlorambucil, cyclophosphamide, and fludarabine are the most frequent to date. However, these drugs are known to have several side effects.

According to Lukenbill and Kalaycio (2013), severe myelosuppression leading to leukopenia, severe anemia,

and thrombocytopenia, as well as frequent bleeding events resulting from these, are among the most devastating consequences of fludarabine toxicity. This drug works by inhibiting the synthesis of DNA and proteins, leading to the apoptosis of leukemic cells. However, this action is not very specific, and the drug can also reach normal cells. Similarly, cyclophosphamide and chlorambucil act to inhibit DNA synthesis and have cytotoxic and myelosuppressive effects (Bracci *et al.*, 2014; Goede, Klein, Stilgenbauer, 2015). Thus, it is probable that the cytotoxic effects of these chemotherapeutics may interfere in the microvesiculation of the cells, reducing the amount of MPs in the group of treated patients (Binet B + C) when compared to the Binet A group, contrary to that which was expected. Likewise, the action of these drugs may interfere with hemostasis, predisposing these patients to hemorrhagic events and decreasing their potential for TG.

As for the lack of a correlation between the number of MPs of any origin and the results of the TG tests, and considering that MPs contain numerous proteins and lipids derived from the cells of origin, it is possible that the content of these did not have a considerable impact on the biological function of hemostasis. In this context, Trappenburg *et al.* (2009) reported that not only the quantity but also the characteristics of circulating MPs are associated with an increased risk of arterial and venous thrombosis in several diseases, including several types of cancer. Clearly, a considerable increase in circulating microparticles was observed in patients with CLL as was a reduction in the potential of TG. Considering the multifactorial nature involved in hemostatic alterations, more studies assessing prognostic factors in a larger group of patients with CLL are necessary in order to corroborate the results found by the TG technique and to better clarify the mechanisms involved in the hemostatic system of these patients. In addition, it is important to clarify the mechanisms involved in microvesiculation associated with the pathogenesis and progression of the disease.

Finally, analyzing the data as a whole allows us to conclude that, although hemostatic alterations configured by a higher number of MPs found and a lower potential of thrombin in patients with CLL were observed in relation to the control group, these parameters do not seem to be useful enough to indicate disease progression or severity. The lack of a significant difference for the MPs and TG between the Binet A and B + C stages supports this assertion. Within this context, increased number of MPs in CLL and decreased thrombin generation in CLL are not necessarily related to disease progression, since such parameters may be affected by therapeutic schemes. So, this condition precludes any inference about the role of these variables in the clinical heterogeneity in patients with CLL.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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