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## Evaluation of platelet aggregation and platelet derived microparticles in acute leukemia patients

### Evaluarea agregării plachetare și a microparticulelor cu origine trombocitară la pacienți cu leucemie acută

Andra Costache<sup>1\*</sup>, Silvana Angelescu<sup>1,2</sup>, Doina Barbu<sup>2</sup>, Elena Popescu<sup>3</sup>,  
Delia Ileana Mut Popescu<sup>1</sup>, Anca Roxana Lupu<sup>1,2</sup>

1. “Carol Davila” University of Medicine and Pharmacy

2. “Coltea” Clinical Hospital, Hematology Department

3. “Medcenter” Central Laboratory – Center for Excellence in Laboratory Medicine and Pathology

#### Abstract

*Patients with acute leukemia develop abnormalities of haemostasis, leading not only to bleeding, but also to thrombotic complications. The pathogenesis of these complications is complex and multifactorial. Because platelets and platelet derived microparticles are key players in haemostasis and thrombosis, we presumed their roles in the prediction of bleeding and thrombotic complications. Our study groups included 24 patients with acute leukemia and 16 healthy volunteers. Platelet aggregation evaluation was performed by impedance whole blood aggregometry and the enumeration of platelet derived microparticles was done by means of flow cytometry. Eight patients developed hemorrhagic complications associated with reduced platelet aggregation response at hospital admission. Major thrombotic events occurred in 5 patients, being preceded by increased platelet aggregation in 3 cases and high level of platelet derived microparticles in 2 cases. Our findings reveal that whole blood platelet aggregometry could be a valuable tool especially in the detection of platelet hyperreactivity and in the prediction of thrombotic events. A high level of platelet derived microparticles could also predict thrombosis. These hypotheses need further evaluation and confirmation on larger number of patients.*

**Key words:** acute leukemia, platelet aggregation, platelet-derived-microparticles, bleeding, thrombosis

#### Rezumat

*Pacienții cu leucemie acută dezvoltă anomalii ale hemostazei care determină apariția de complicații hemoragice și chiar trombotice. Patogeneza acestor complicații este complexă și multifactorială. Deoarece trombocitele și microparticulele trombocitare reprezintă elemente cheie în cadrul proceselor de hemostază și tromboză, am încercat să evidențiem rolul lor în predicția complicațiilor hemoragice și trombotice. Grupurile studiate au inclus 24 de pacienți cu leucemie acută și 16 voluntari sănătoși. Evaluarea agregării plachetare s-a realizat prin agregometrie prin impedanță în sânge integral, iar pentru determinarea microparticulelor trombocitare s-a utilizat citometria în flux. Opt pacienți au dezvoltat complicații hemoragice, care au fost asociate cu agregabilitate*

**\*Corresponding author:** Andra Costache, “Carol Davila” University of Medicine and Pharmacy, Bucharest, România, e-mail: andracostache@gmail.com

trombocitară diminuată la internare. Evenimentele trombotice majore au complicat evoluția a 5 pacienți și au fost precedate de agregare trombocitară crescută la 3 cazuri și nivel crescut de microparticule trombocitare la 2 cazuri. Rezultatele noastre evidențiază faptul că agregometria plachetară în sânge integral poate fi un instrument valoros în special în identificarea hiperreactivității plachetare și în predicția evenimentelor trombotice. Un nivel crescut de microparticule trombocitare poate, de asemenea, să anticipeze apariția trombozei. Aceste ipoteze necesită evaluare și confirmare pe un număr mai mare de pacienți.

**Cuvinte cheie:** leucemie acută, agregare plachetară, microparticule cu origine trombocitară, hemoragie, tromboză

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## Introduction

Acute leukemias (AL) are characterized by uncontrolled clonal proliferation of immature hematopoietic precursors leading to impaired hematopoiesis and its accompanying complications – bleeding, infection and organ infiltration (1). Even though, in terms of haemostasis, the clinical picture is mostly dominated by the bleeding episodes, thrombosis can also complicate the course of the disease (2-6). These complications have a significantly negative impact on the morbidity and mortality and their prediction is often difficult because of the lack of laboratory markers.

Although the pathogenesis of haemostatic defects is complex, a central role is played by the platelets (7), and possibly by the platelet derived microparticles (PMPs) (8). Understanding these defects may contribute to the prediction of these complications as well as to the design of appropriate measures for intervention to prevent them.

Platelets are key players in physiological haemostasis as well as in pathologic bleeding and thrombosis (9). In AL platelets present alterations both in term of number and function. Although thrombocytopenia is the major cause of bleeding, acquired platelet defects have been reported too (10). On the other hand, hyperfunction of platelets is associated with an increased risk of thrombosis and was reported in a number of diseases including cancers (11).

PMPs are vesicles smaller than 1  $\mu\text{m}$  released from the surface of activated or apoptotic

platelets as a result of membrane remodeling. PMPs are the largest population of circulating microparticles (MPs), representing between 70% and 90% of the total number of MPs. PMPs are highly procoagulant due to phosphatidylserine (PS) and tissue factor (TF) expression on their outer membrane, which are the main initiators of the coagulation cascade (12). Elevated levels of PMPs were reported in a wide range of diseases with thrombotic tendency, including cardiovascular diseases and neoplastic diseases (13-18).

In contrast, patients with decreased numbers of PMPs are prone to bleeding diathesis as revealed by Castaman syndrome and Scott syndrome (19).

Our study aimed to evaluate platelet aggregation characteristics by whole blood aggregometry (WBA) and to assess the circulating PMPs levels in patients with AL and their possible role in hemorrhagic and thrombotic complication prediction.

## Material and methods

A number of 24 patients with newly diagnosed AL from the Department of Hematology of Coltea Clinical Hospital, Bucharest and 16 healthy volunteers who had not taken any drugs were recruited for study participation over a 10 months period, between February and November 2012. The patients were diagnosed essentially based on French-American-British (FAB) guidelines by morpho-cytochemical and

immunophenotypic criteria: 21 of the patients had acute myeloid leukemia (AML) (AML0 – 1 case, AML1 – 1 case, AML2 – 7 cases, AML4 – 10 cases, AML5 – 1 case, AML6 – 1 case) and 3 acute lymphocytic leukemia (ALL). This study was approved by the Ethics Committee of Coltea Clinical Hospital and written informed consent was obtained from every participant of the study in accordance with the Declaration of Helsinki. All analyses were performed using peripheral venous blood sample collected at the time of diagnosis prior to chemotherapy treatment. The patients were followed-up prospectively for the occurrence of either bleeding or thrombotic manifestation during the first month of hospitalization.

Hematologic parameters were determined with a Sysmex XT 1800i hematology analyzer using ethylene diamine tetraacetic acid (EDTA) sample tubes and plasma coagulation parameters were evaluated with an ACL 9000 (Instrumentation Laboratory) coagulation analyzer from 3.2% trisodium citrate sample tubes. Coagulation parameters included: prothrombin time (RecombiPlasTin 2G kit, HemosIL), activated partial thromboplastin time (APTT) (APTT-SP kit, HemosIL) and fibrinogen (Fbg) (Fibrinogen-C kit, HemosIL).

Platelet aggregation was assessed in whole blood by multiple electrode aggregometry (MEA) or impedance aggregometry (Multiplate analyzer, Roche). Venous blood was collected by clean venopuncture on hirudin tubes (Hirudin Blood Tube for Multiplate analysis, Roche) after discarding at least first three mL. The final concentration of hirudin was 15 µg/mL. The samples were maintained at room temperature avoiding agitation and were analyzed within 3 hours post drawing.

Hirudin-anticoagulated whole blood was diluted 1:1 with physiological saline in single-use test cells and then preheated at 37 C° for three minutes. Addition of activating agonist induc-

es platelets' activation, adhesion and aggregation on the surface of silver-coated electrodes within each test cell. A Teflon-coated stirring bar rotating at 1000 rpm was used for continual sample mixing. As platelet adhere to the electrode surfaces, the impedance of an alternating current applied across electrodes increases. This change in electrical impedance was recorded continuously for 6 minutes and was expressed by the area under the aggregation curve (AUC) - arbitrary units (AU) plotted against the time (AU\*min). Platelets were stimulated by the following agonists: adenosine diphosphate (ADP) (ADPtest, Roche) with a final concentration of 6.5 µM, collagen (COLtest, Roche) with a final concentration of 3.2 µg/mL and thrombin receptor activating peptide-6 (TRAPtest, Roche) with a final concentration of 32 µM.

PMPs enumeration was performed by flow cytometry. Venous blood was drawn into 3.2% trisodium citrate tubes. Samples were kept at room temperature and processed in an interval of maximum two hours post-drawing. Platelet free plasma (PFP) was obtained by a serial centrifugation protocol (15 min at 1500g, 5 min at 13 000g), frozen as 500 µL aliquots and stored at -80C until use. For PMPs labeling we used monoclonal antibody to CD41 allophycocyanin (APC) –conjugated, clone MEM-06 (Exbio Praha) and Annexin V fluorescein isothiocyanate (FITC) – conjugated (Annexin V – FITC kit, Beckmann Coulter).

CD41-APC and AnnexinV-FITC pre-diluted in Binding Buffer were added to PFP and incubated for 20 minutes at room temperature, in the dark. Then samples were diluted in Annexin V kit Binding Buffer. Analyses were performed on BD FACSCantoII flow cytometer using FACSDiva software. The setting up of the MP analysis region (0,5-1µm) was done utilizing a blend of calibrated fluorescent beads of three diameters (0.5, 0.9 and 3 µm) (Megamix, Bio-Cytex). Single staining controls were used to check fluorescence compensation settings and

to set up positive regions. PMPs were defined as dual positive PS+/CD41+ events, as seen in dual-color fluorescence plots after staining with Annexin V-FITC and CD41-APC. Counting beads with an established concentration close to 1000 beads/ $\mu\text{L}$  (Flow Count Fluorospheres, Beckman-Coulter) were added to each sample in order to express MP counts as absolute numbers per  $\mu\text{L}$  of PFP. The identification of PMP was based on size ( $<1 \mu\text{m}$ ) and the binding of fluorescent-labeled antibodies.

For statistical analysis, data were evaluated with Microsoft Excel 2007 software. Values were expressed as mean  $\pm$  SD.

## Results

The main hematologic and coagulation parameters of the 24 patients and 16 controls are displayed in *Table I*.

The platelet aggregation parameters and PMPs levels of patients and healthy subjects are summarized in *Table II*.

Of the 24 patients, 8 developed hemorrhagic complication (one patient with AML0, 3 patients with AML2 and 4 patients with AML4. Major thrombotic events (myocardial infarction or stroke) occurred in 5 patients: 2 patients with AML2 and 3 patients with AML4.

**Table I. Characteristics of the study groups**

Variables	Control group	AL Patients
n	16	24
Male / Female	5/11	13/11
Age (years)	44 $\pm$ 7.9	53.7 $\pm$ 18.4
Platelets (x 10 <sup>9</sup> /L)	282.94 $\pm$ 18.38	109.46 $\pm$ 128.18
White Blood Cells (x 10 <sup>9</sup> /L)	6.87 $\pm$ 1.68	26.65 $\pm$ 33.76
Red Blood Cells (x 10 <sup>12</sup> /L)	4.59 $\pm$ 0.376	2.5 $\pm$ 0.63
Hemoglobin (g/dL)	13.73 $\pm$ 1.69	8.08 $\pm$ 1.4
PT (sec.)	10.61 $\pm$ 0.61	13.31 $\pm$ 2.32
INR	0.98 $\pm$ 0.056	1.22 $\pm$ 0.21
APTT (sec.)	25.95 $\pm$ 2.43	27.42 $\pm$ 5.55
Fibrinogen (g/L)	2.84 $\pm$ 0.51	4.02 $\pm$ 0.99

PT – prothrombin time

APTT - activated partial thromboplastin time

Values are expressed as mean  $\pm$  standard deviation.

**Table II. Aggregation parameters and PMPs levels of study groups**

Parameters	Control group	AL Patients	P values (for Patients compared to Control)
ADP aggregation (AUC)	742 $\pm$ 38.52	293 $\pm$ 83.98	P = 0.000122
Collagen aggregation (AUC)	743 $\pm$ 38.23	403 $\pm$ 101.58	P = 0.00983
TRAP aggregation (AUC)	1064 $\pm$ 39.30	486 $\pm$ 105.99	P = 0.0000754
PMPs/ $\mu\text{L}$	353 $\pm$ 197.70	1302 $\pm$ 821.59	P = 0.239

AUC – area under aggregation curve

Values are expressed as mean  $\pm$  standard deviation.

Our results showed that compared with the control group, patients with AL displayed reduced platelet aggregation response in 19 cases (79%), increased response in 3 cases (13%) and similar response for all agonists in 2 cases (8%). The reduced platelet aggregation response was associated with hemorrhagic complications development in 8 patients (42%) and the increased response to agonists was associated with major thrombotic events (myocardial infarction and stroke) occurrence in all the 3 cases (100%).

The three subgroups are too small for a statistical evaluation of the differences.

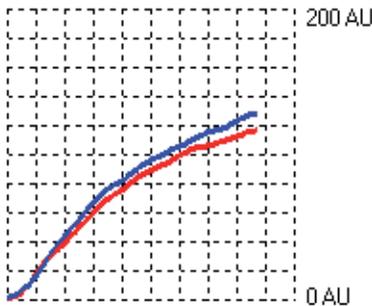
Figure 1 a,b,c shows a normal aggregation profile in a healthy volunteer.

Figure 2 a,b,c shows impaired aggregation profile in a patient with AL who developed bleeding complications.

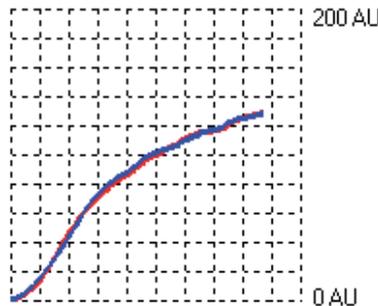
Figure 3 a,b,c shows platelet hyperaggregability profile in a patient with AL who developed ischemic stroke.

The level of PMPs was similar to the control group in 21 out of the 24 patients (88%) and much higher in 3 patients (12%), 2 of which developed major thrombotic events (67%). These subgroups are too small for a statistical evaluation of the differences, too.

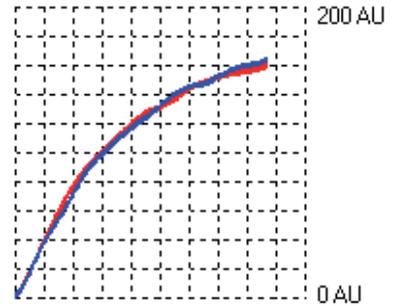
Figure 4 represent a flow cytometric dot plot displaying a high level of PMPs in a patient with AL who developed myocardial infarction in the course of disease.



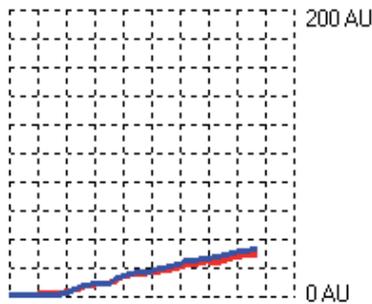
**Figure 1a. Normal ADP Aggregation Curve (impedance aggregometry) in a healthy volunteer. AUC=682 AU\*min**



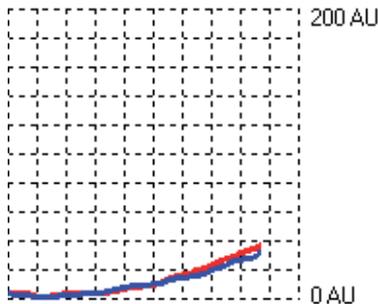
**Figure 1b. Normal Collagen Aggregation Curve (impedance aggregometry) in a healthy volunteer. AUC=732 AU\*min**



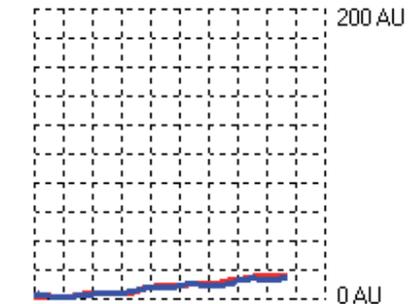
**Figure 1c. Normal TRAP Aggregation Curve (impedance aggregometry) in a healthy volunteer. AUC=1000 AU\*min**



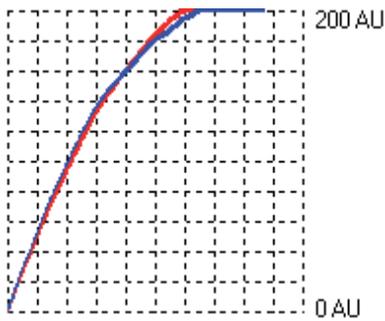
**Figure 2a. Impaired ADP Aggregation Curve (impedance aggregometry) in a patient with AL. AUC=126 AU\*min**



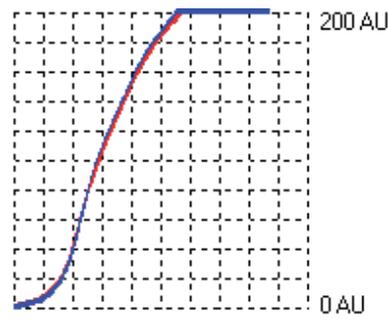
**Figure 2b. Impaired Collagen Aggregation Curve (impedance aggregometry) in a patient with AL. AUC=95 AU\*min**



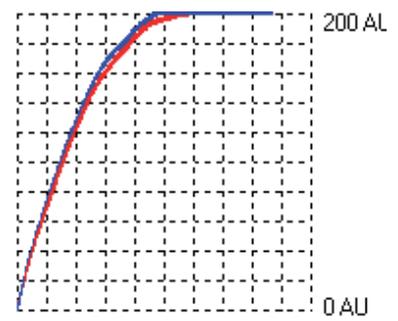
**Figure 2c. Impaired TRAP Aggregation Curve (impedance aggregometry) in a patient with AL. AUC=61 AU\*min**



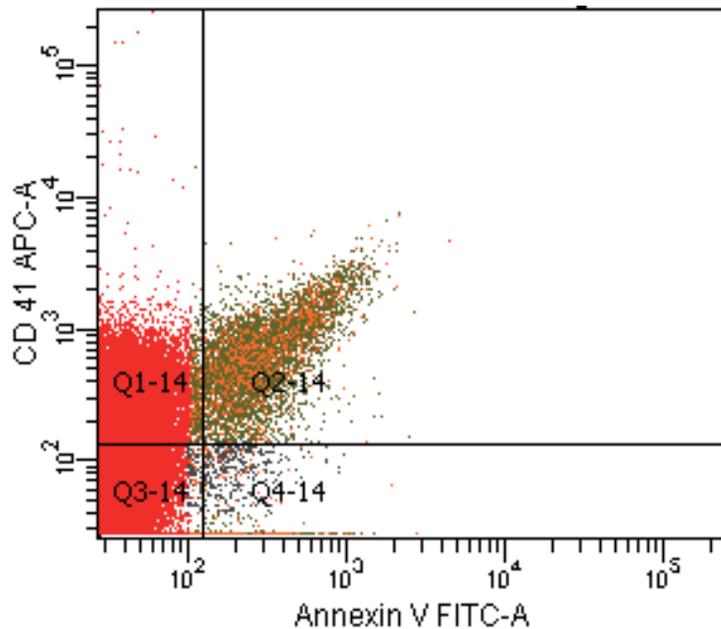
**Figure 3a. ADP Aggregation Curve (impedance aggregometry) displaying platelet hyperreactivity in a patient with AL. AUC=1374 AU\*min**



**Figure 3b. Collagen Aggregation Curve (impedance aggregometry) displaying platelet hyperreactivity in a patient with AL. AUC=1295 AU\*min**



**Figure 3c. TRAP Aggregation Curve (impedance aggregometry) displaying platelet hyperreactivity in a patient with AL. AUC=1496 AU\*min**



**Figure 4. Flow cytometry dot plot displaying a high level of PMPs. PMPs are dual positive PS+/CD41+ events, stained with Annexin V-FITC and CD41-APC.**

## Discussion

A number of factors that prone to bleeding have been reported in patients with AL: thrombocytopenia, endothelial cell injury, disseminated intravascular coagulation, excessive fibrinolysis, acquired hemophilia, acquired von

Willebrand syndrome, drugs (e.g., antiplatelet drugs and anticoagulant therapy and associated comorbidities (e.g., infections, impaired liver function, impaired kidney function, malnutrition) (20). However, the primary determinant of the bleeding risk in these patients is the degree

of thrombocytopenia (21). The relationship between bleeding and the platelet count has been well documented in the literature, major hemorrhage being more likely at thrombocytopenia below 10 000/ $\mu$ l (22). Notwithstanding, bleeding may happen in some patients with higher platelet counts and numerous other patients with even severe thrombocytopenia do not experience spontaneous hemorrhage. Therefore, platelet count alone is not suitable in bleeding occurrence prediction, platelet function being also significant (23). In our study group, bleeding occurred in patients with platelet counts between 12 000 and 33 000/ $\mu$ l. As expected, in these patients, platelet aggregation curve was collapsed and in two of them aggregation was absent. However platelet aggregation assay cannot discriminate between the effects of impaired platelet function and the effects of thrombocytopenia. WBA results cumulate the interactions between thrombocytes, leukocytes, and erythrocytes to reproduce the complex *in vivo* setting (24).

In our study PMPs levels seem to have no clinical relevance in acute leukemia patients with bleeding complications.

The pathogenesis of the prothrombotic state in AL patients is complex, involving the interplay of multiple factors: hyperleukocytosis, increased expression of molecules of adhesion by endothelial cells and the homologous receptors in blast cells, increased expression of TF, cancer procoagulant presence, cell derived microparticle expressing PS and TF, adverse effects of therapeutic agents, vascular access catheters, comorbid conditions (e.g., thrombophilia, infections) (20, 25-27). Although studies report the platelet hyperreactivity as a risk factor for thrombosis in solid neoplasms (11), scarce data are available concerning platelet role in thrombotic complications occurrence during the course of leukemic disease. Our findings suggest that platelet hyperreactivity may play a crucial role in major thrombotic events onset in patients with AL,

however further studies are needed to confirm this hypothesis and to prove its impact in predicting and preventing these complications.

Circulating PMPs are important procoagulant factors (14, 18) and the hypercoagulable state in patients developing thrombotic events could be partially explained by the elevated levels of PMPs.

However, a limitation of this study is the small number of patients enrolled.

## Conclusions

Our study confirms that the low platelet count remains the main predictor of hemorrhagic complications. Platelet hypoaggregability could also precede bleeding complications, while platelet hyperreactivity could predict major thrombotic events in leukemic patients. To our knowledge, this is the first study pointing out platelet hyperreactivity in AL patients.

Our data suggests that PMPs enumeration could offer significant information on the haemostatic status of patients with AL, increased levels of PMPs suggesting a procoagulant status and an increased risk of thrombotic events.

Notwithstanding our study group was too small, this hypothesis requiring further evaluation and validation.

## Acknowledgments

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## Disclosure

The authors state that they have no conflict of interest.

## Abbreviations

ADP - adenosine diphosphate

AL - acute leukemia

ALL - acute lymphocytic leukemia

AML - acute myeloid leukemia  
 APC - allophycocyanin  
 APTT - activated partial thromboplastin time  
 AU - arbitrary units  
 AUC - area under the aggregation curve  
 Fbg - fibrinogen  
 FITC - fluorescein isothiocyanate  
 MEA - multiple electrode aggregometry  
 MPs - microparticles  
 PFP - platelet free plasma  
 PMPs - platelet derived microparticles  
 PS - phosphatidylserine  
 TF - tissue factor  
 TRAP - thrombin receptor activating peptide-6  
 WBA - whole blood aggregometry

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