Increased eryptosis in patients with primary antiphospholipid syndrome (APS): a new actor in the pathogenesis of APS

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Abstract

Objective

Antiphospholipid syndrome (APS) is an autoimmune disease characterised by a hypercoagulable state and the presence of antiphospholipid antibodies (aPL). During the mechanism of red blood cells (RBCs) death, called eryptosis, RBCs can adhere to vascular wall participating in the development of a pro-thrombotic state. It is known that enhanced eryptosis contributes to several pathological conditions but the role of this process in APS has not been investigated yet. We analysed spontaneous eryptosis in a cohort of APS patients and aPL carriers (asymptomatic subjects with positive aPL tests). The effect on eryptosis of antibodies (Abs) purified from serum of APS patients and aPL carriers was also investigated.

Methods

In this study, 30 patients with primary APS (PAPS) and 17 aPL carriers were recruited. Twenty healthy donors (HD) and 13 patients affected by autoimmune haemolytic anaemia (AHIA) were also recruited. RBCs were incubated with PAPS and aPL carriers Abs, purified by ammonium sulfate precipitation. Levels of eryptosis were analysed by flow cytometry.

Results

In vitro Abs from APS patients induced eryptosis in RBCs isolated from HD after 4 h of culture. On the contrary, Abs from aPL carriers had no effect on the percentage of phosphatidylserine-exposing RBCs. Ex vivo, APS patients showed higher levels of spontaneous eryptosis compared to HD and aPL carriers.

Conclusion

In this study, we demonstrated a potential new aspect of APS pathogenesis based on the ability of Abs isolated from APS patients, not identified in aPL carriers, to stimulate eryptosis suggesting a possible contribution of this process in the clinical manifestations of APS.

Key words

antiphospholipid syndrome, eryptosis, red blood cells, antibodies, thrombosis

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Received on May 14, 2020; accepted in revised form on July 6, 2020.

Competing interests: none declared.

Introduction

Red blood cells (RBCs) are involved in the transport of O_2 and in the maintenance of vascular tone. Despite the absence of nucleus and the lack of crucial elements in the machinery of apoptosis, they have developed a rapid self-destruction process called eryptosis. During this process, the externalisation of phosphatidylserine (PS) activates the correct elimination of RBCs by phagocytes, preventing inflammation and intravascular haemolysis (1). Eryptosis is stimulated by calcium entry through Ca-permeable PGE2 activated cation channels, by hyperosmotic shock, energy depletion, oxidative stress and other stimuli. Enhanced eryptosis is observed in mice with deficient annexin 7, cGMP-dependent protein kinase type I (cGKI), AMP-activated protein kinase (AMPK), anion exchanger 1 (AE1), adenomatous polyposis coli (APC), as well as in mouse models of sickle cell anaemia and thalassaemia. Moreover, it is inhibited by nitric oxide, catecholamines and a variety of further small molecules (1, 2).

Data from the literature have demonstrated the implication of eryptosis in the pathogenesis of several conditions like diabetes, neoplasia, heart failure, end stage renal disease, haemolytic-uraemic syndrome, sepsis, malaria, thalassaemia, Wilson disease (3, 4). Few pilot studies have been conducted about eryptosis in patients affected by autoimmune diseases, such as systemic sclerosis (SS), systemic lupus erythematosus (SLE) and autoimmune haemolytic anaemia (AIHA) (5-7). Giovannetti and colleagues found RBCs alterations in patients affected by SS. The authors found not only increased levels of reactive oxygen species in patients compared to controls, an also altered expression of some markers of senescence such as glycophorin A and CD47 in RBCs from SS subjects (5).

It was recently hypothesised an involvement of eryptosis in SLE-associated anaemia (6). In fact, anaemic SLE patients showed higher percentage of PS-exposing RBCs compared to healthy controls. In addition, plasma of patients affected by SLE is able to enhance eryptosis in RBCs from healthy subjects, suggesting a role of plasma components in the stimulation of this process *in vitro* (6).

Concerning AHIA, autoantibodies against antigens on the RBCs surface are responsible for the destruction of these cells. Bartolmäs and co-authors demonstrated that patients affected by warm type of AHIA in complete remission showed levels of eryptosis comparable to those of healthy subjects (7). Interestingly, erythropoietin treatment, a strong inhibitor of eryptosis, seems to improve anaemia in a small group of patients (7).

Some studies highlighted that eryptosis could be involved in blood clots formation and consequently in thrombophilic conditions. Eryptotic erythrocytes exposing PS adhere largely to the vascular wall, stimulate blood clotting and thus foster thrombosis (8). Therefore, eryptosis stimulation could lead to microcirculation alterations.

Vascular thrombosis (arterial and/or venous) and/or recurrent pregnancy loss are characteristic of antiphospholipid syndrome (APS), an autoimmune disease driven by antiphospholipid antibodies (aPL). The aPL family includes several antibodies directed against anionic phospholipids, phospholipid-binding plasma proteins, and phospholipid-protein complexes (9, 10). Although aPL have a clinical relevance in APS, some subjects show serum positivity for aPL in the absence of disease manifestations. They are defined as aPL carriers, and they represent a valuable population to explore the mechanisms involved in APS pathogenesis. Patients with APS display several signs of endothelial dysfunction (11) but some of the processes involved remain to be clarified. As eryptotic erythrocytes may adhere to the vascular wall and thus modify the microcirculation, in the present study we aimed at exploring eryptosis in APS patients to evaluate its possible role in APS pathogenesis.

Materials and methods

Study population

Consecutive patients affected by primary APS (PAPS) and aPL carriers

were recruited at the Lupus Clinic, Sapienza University of Rome, after written informed consent. The local ethics committee approved the study (Prot. n. 99/199).

PAPS patients with severe heart and hepatic failure, chronic renal failure, sepsis, diabetes and malignancy were excluded. At each visit, clinical and laboratory assessments were performed. Regarding autoantibodies, anticardiolipin (aCL, IgG and IgM isotype), and anti-β2-glycoprotein I (anti-β2GPI, IgG and IgM isotype) antibodies were measured by ELISA (Diamedix, Miami, FL, USA), while lupus anticoagulant (LA) was assessed according to the guidelines of the International Society on Thrombosis and Hemostasis (12). As age-matched control population, healthy donors (HD) were also recruited. Moreover, patients affected by autoimmune haemolytic anaemia (AHIA) were enrolled by the Center of Haematology, Sapienza University of Rome.

Purification of antibodies

Antibodies were purified by precipitation in saturated ammonium sulphate (SAS). Sera from 5 PAPS patients and 5 aPL carriers with triple antiphospholipid antibody positivity were centrifuged and the supernatant was exposed to SAS drop-wise overnight in ice. After a centrifugation at 3000 x g for 30 min at 4°C, the pellet was resuspended in phosphate buffered saline (PBS) and dialysed against >20 volumes of PBS for 48h at 4°C. IgG concentration was measured by Bradford assay.

Erythrocytes isolation and cell culture conditions

To isolate RBCs, fresh anticoagulated blood samples were centrifuged for 20 min at 120g, and after removal of plasma and buffy coat, RBCs were collected. Spontaneous eryptosis was analysed by flow cytometry (see below), while the remaining RBCs were *in vitro* cultured at a haematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 N-2- hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES; pH 7.4), 5 glucose, one $CaCl_2$, at 37°C for 4 h (13).

Table I. Clinical and serological characteristics of study subjects.

Characteristics	PAPS patients n=30	aPL carriers n=17
Age (years) mean ± SD	50.7 (8.1)	49.9 (11.3)
Males/females	9/21	3/14
Venous thrombosis n (%)	10 (33.3)	–
Arterial thrombosis n (%)	16 (53.3)	–
Pregnancy morbidity n (%)	5 (23.8)	–
Haemoglobin (g/dL) mean ± SD	13.3 (1.1)	14.1 (1.6)
MCV	85.6 (9.3)	86.7 (4)
aCL n (%)	17 (56.6)	10 (58.8)
aB2GP1 n (%)	14 (46.6)	8 (47)
LA n (%)	12 (40)	12 (70.5)
Triple positivity n (%)	3 (10)	4 (23.5)
ASA n (%)	14 (46.6)	–
TAO n (%)	14 (46.6)	–
ASA/TAO n (%)	1 (3.3)	–
NAO n (%)	1 (3.3)	–

aCL: anti-cardiolipin; aB2GP1: anti-b2glycoprotein I; ASA: acetylsalicylic acid; LA: Lupus anticoagulant; MCV: mean corpuscular volume; NAO: novel oral anticoagulants; TAO: oral anticoagulant therapy.

Evaluation of eryptosis by flow cytometry

Eryptosis was measured immediately after erythrocyte purification and after 4 h of culture with antibodies using Annexin V (AV) probe (ebioscence). AV binds to negatively charged phospholipids such as PS, a molecule that is exposed to the outer leaflet of plasma membrane in erythrocytes undergoing eryptosis (13).

RBCs were resuspended in AV binding buffer (ebioscence) and incubated with fluorescein isothiocyanate (FITC)-AV (1:100 dilution) for 15 min at room temperature in the dark. A non-marked sample was included in each experiment. Then 50,000 events for each sample were run on a FACS Calibur cytometer (BD BioSciences), and the data were analysed using the CellQuest Pro software (BD BioSciences). The reported data refer to AV binding cells.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism Software Inc. version 6 (San Diego, CA, USA). Normal distribution of variables was assessed using the Kolmogorov-Smirnov test. Differences between groups were analysed using the Mann-Whitney test, and statistical correlations were examined using the Pearson rank correlation coefficient. p-values <0.05 were considered statistically significant.

Results

Population study

We enrolled:

- 30 PAPS patients (M/F 9/21, mean age±SD 50.7±8.1)
- 17 aPL carriers (M/F 3/14, mean age ±SD 49.9±11.3)
- 20 HD (M/F 2/18, mean age±SD 36.4±10.6)
- 13 AHIA (M/F 9/4, mean age±SD 57.3±17.9)

The clinical and laboratory data about PAPS and aPL carriers are reported in Table I. We defined aPL carriers as healthy subjects persistently positive for aPL in at least two determinations, with an interval of at least 12 weeks. These subjects underwent the aPL test due to infertility (6 subjects) and hormonal treatment (6 subjects); in the remaining 5 aPL carriers due to family history of autoimmune diseases. Of note, serum positivity for LAC was detected in 13 PAPS patients, while 14 and 17 patients were positive for anticardiolipin (aCL) and anti-β2-GPI antibodies, respectively. Three PAPS patients had a triple positivity for aPL. RBCs autoantibodies of the IgG class were found in 5 AHIA patients, while 7 subjects presented IgM autoantibodies. Auto-antibodies of IgA class were present in only 1 patient.

No statistically significant difference was found in haemoglobin levels between PAPS patients, aPL carriers and

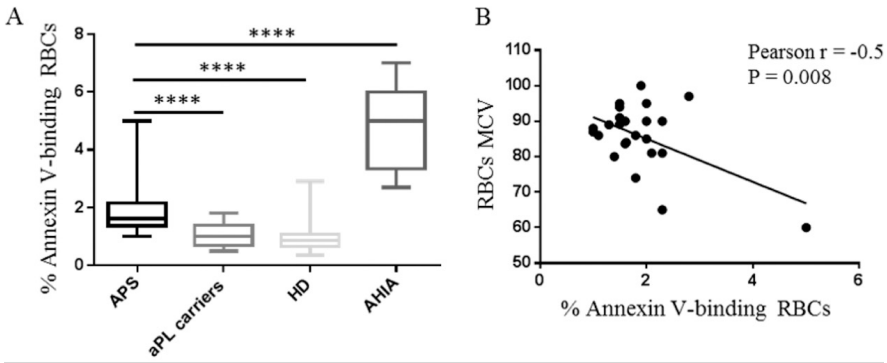

Fig. 1. Enhanced eryptosis in PAPS patients.
A: Levels of spontaneous eryptosis in patients with PAPS. A representative histogram showing percentages of Annexin V-binding erythrocytes (RBCs) analysed by flow cytometry in primary APS patients (n=30), aPL carriers (n=17), healthy donors (n=20) and patients affected by autoimmune haemolytic anaemia (AHIA, n=13). **** $p<0.0001$, Mann-Whitney test.
B: Correlation analysis between percentage of eryptotic erythrocytes and mean corpuscular volume (MCV) in patients with APS. Pearson correlation (Pearson's r) and linear regression coefficients are displayed.
AV: Annexin V; HD: healthy donors; MCV: mean corpuscular volume.

subjects affected by AHIA (mean ± SD 11 g/dl ±3.461).

Spontaneous eryptosis is upregulated in APS patients

Firstly, we analysed spontaneous eryptosis levels in PAPS patients and in aPL carriers. The percentage of AV binding RBCs was significantly increased in PAPS patients compared to aPL carriers ($p<0.0001$, Fig. 1A). In addition, RBCs from HD showed reduced eryptosis levels compared to PAPS patients ($p\leq0.0001$, Fig. 1A). As expected, in patients affected by AHIA, pathological condition associated with an excessive haemolysis mediated by antibodies directed against self-erythrocytes, the percentage of RBCs undergoing eryptosis was increased compared to all studied populations ($p<0.0001$, Fig. 1A). Furthermore, in our cohort of PAPS patients the percentage of PS-exposing RBCs inversely correlated with the mean corpuscular volume (MCV) of RBCs (Fig. 1B). There was no significant correlation with other clinical features, including haemoglobin, and levels of eryptosis (data not shown).

Antibodies from APS patients induce eryptosis

In order to analyse the potential involvement of antibodies on the enhanced eryptosis demonstrated in patients affected by PAPS, RBCs were exposed to Abs purified from PAPS and aPL carriers. After 4 h of culture, Abs from PAPS patients induced a significant increase in the percentage of RBCs undergoing eryptosis in HD untreated *versus* treated with PAPS Abs ($p=0.02$, Fig. 2). Conversely, the treatment with Abs from aPL carriers and intravenous immunoglobulins (IVIG) did not cause relevant changes in PS externalisation at the RBCs surface. Performing the experiments also in RBCs isolated from both PAPS patients and aPL carriers, we confirmed the ability of Abs from patients, and not from aPL carriers, to stimulate eryptosis *in vitro* (Fig. 3A-B). Interestingly, both in untreated

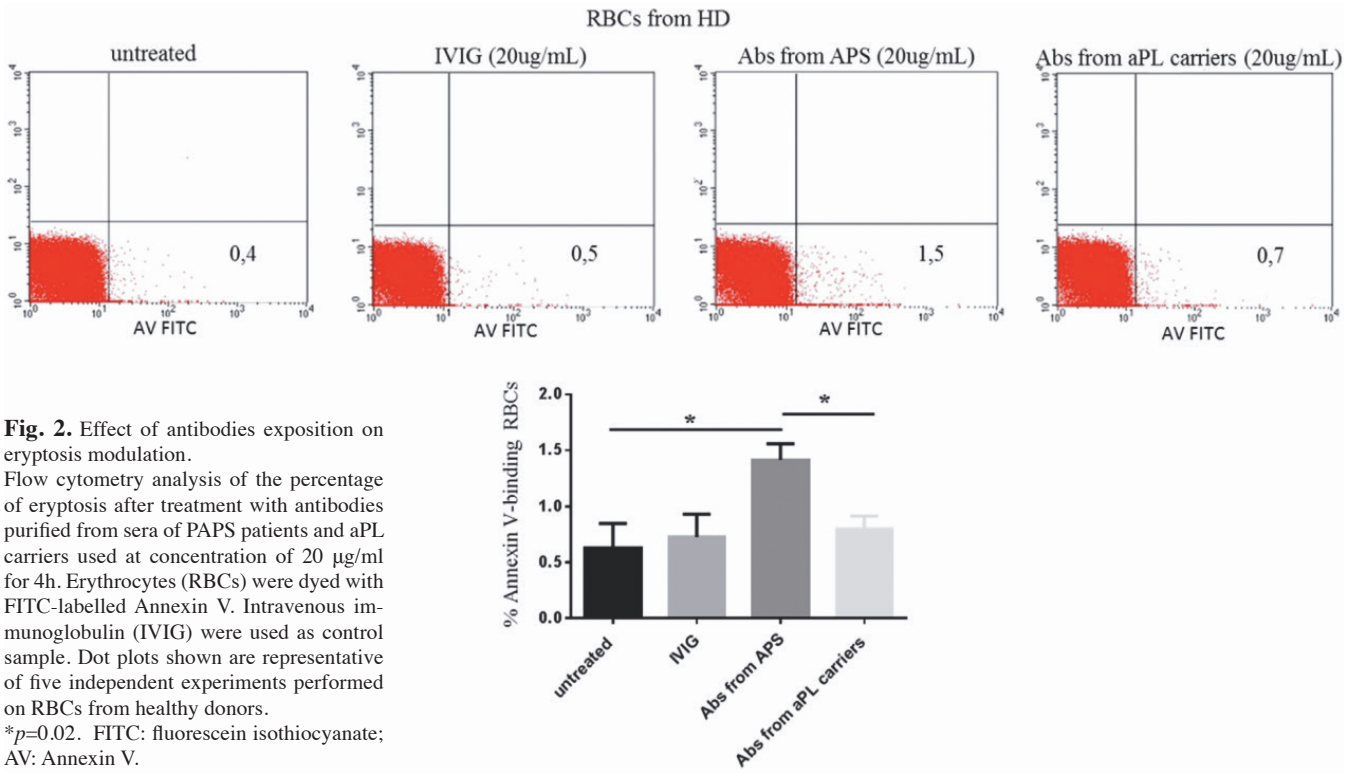

Fig. 2. Effect of antibodies exposition on eryptosis modulation.
Flow cytometry analysis of the percentage of eryptosis after treatment with antibodies purified from sera of PAPS patients and aPL carriers used at concentration of 20 µg/ml for 4h. Erythrocytes (RBCs) were dyed with FITC-labelled Annexin V. Intravenous immunoglobulin (IVIG) were used as control sample. Dot plots shown are representative of five independent experiments performed on RBCs from healthy donors.
*$p=0.02$. FITC: fluorescein isothiocyanate; AV: Annexin V.

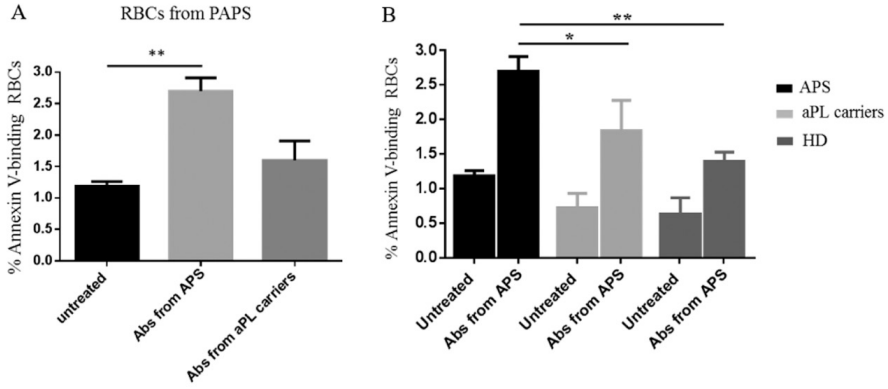

Fig. 3. Induction of eryptosis after antibodies exposition in RBCs from primary APS patients compared to RBCs from aPL carriers and HD.
A: Representative histogram of eryptosis levels following exposure to antibodies from PAPS patients and aPL carriers at the concentration of 20 μg/ml for 4h. Results are expressed as percentage of Annexin V (AV)-binding erythrocytes (RBCs). Data are referred to five independent experiments performed on RBCs from different PAPS patients.
B: Histogram displaying differences in eryptosis activation after treatment with antibodies purified from patients affected by primary APS in RBCs from PAPS patients, aPL carriers and HD as described above.
**p*=0.03, ***p*=0.007.

and in Abs-stimulated condition, the percentage of PS exposing cells was higher in RBCs from PAPS compared to those from HD and aPL carriers, suggesting that eryptosis was upregulated in APS patients both *in vivo* and *in vitro* (Fig. 3B).

Discussion

In this study, we hypothesised that eryptosis might contribute to the pathogenesis of APS. Our data showed that Abs purified from PAPS patients, and not from aPL carriers, were able to induce eryptosis, a process involved in the altered adhesion of RBCs to vascular endothelium and in procoagulant phenotype. Eryptotic erythrocytes, by exposing PE, interact with surface molecules on endothelial cells such as CXCL16, CD36 and thrombospondin, enhancing the adherence to vascular wall (14, 15). Increasing experimental evidences suggest that the presence of only aPLs seems incapable of triggering the thrombogenic effects in APS patients. In fact, inflammatory stimuli or infections seem to be necessary to induce the disease manifestations ("second hit hypothesis") (16, 17). A second explanation of the absence of symptoms in aPL carriers includes the possibility that the antibodies have different antigenic targets (17). Our results suggest that antibodies from PAPS patients and aPL carriers differ in their ability to induce eryptosis, and this phenomenon could have a role in the onset of the clinical manifestations of the disease. Jiang and colleagues previously demonstrated that the incubation with plasma of SLE patients activated eryptosis (6); in this study, the significant increase in eryptosis upon stimulation with Abs from PAPS patients, and not from aPL carriers, could explain, at least in part, the pro-thrombotic state of PAPS patients.
The difference in the capacity of aPL from PAPS patients and aPL carriers to induce eryptosis found in this study supports the hypothesis that antibodies from asymptomatic carriers and PAPS patients might be biologically and structurally different. It cannot be excluded that a specific aPL profile or a different aetiology of aPL was responsible for the effect of autoantibodies on eryptosis; however, further experimental investigations are needed to analyse possible factors implicated in this aspect and to deepen the relationship between eryptosis and vascular damage in the syndrome.
The current study demonstrates, for the first time, that patients with PAPS are characterised by increased spontaneous eryptosis levels compared to both HD and aPL carriers. As expected, and in agreement with a previous study (7), patients affected by AHIA showed higher percentage of PS-exposing RBCs. Interestingly, in aPL carriers the levels of eryptosis were comparable to those observed in HD, despite the presence of aPLs. This result suggests that antibodies from APS patients and from aPL carriers could exert distinct pathological functions related to different disease aspects. Since activation of eryptosis is followed by cell shrinkage, the inverse correlation between the MCV of RBCs and the percentage of PS-exposing cells detected in PAPS patients could represent a confirmation of hyperactivated eryptosis *in vivo*.
In conclusion, the positivity for aPLs in subjects without any clinical manifestations is considered a risk factor for the development of thrombosis, but the mechanisms involved are largely unknown. Our results suggest that the induction of eryptosis mediated by Abs could have a role in this scenario. However, additional experimental evidences are required in order to demonstrate the direct involvement of eryptosis on thrombosis events typical of APS. Characterisation of the molecular basis of the different effects of Abs from APS and aPL carries on eryptosis induction might offer a new perspective for possible therapeutic targets for APS.

References

1. LANG E, LANG F: Triggers, inhibitors, mechanisms, and significance of eryptosis: the suicidal erythrocyte death. *Biomed Res Int* 2015; 2015: 513518.
2. LANG E, QADRI SM, LANG F: Killing me softly - suicidal erythrocyte death. *Int J Biochem Cell Biol* 2012; 44: 1236-43.
3. LANG F, QADRI SM: Mechanisms and significance of eryptosis, the suicidal death of erythrocytes. *Blood Purif* 2012; 33: 125-30.
4. LANG F, JILANI K, LANG E: Therapeutic potential of manipulating suicidal erythrocyte death. *Expert Opin Ther Targets* 2015; 19: 1219-27.
5. GIOVANNETTI A, GAMBARDELLA L, PIETRAFORTE D *et al*.: Red blood cell alterations in systemic sclerosis: a pilot study. *Cell Physiol Biochem* 2012; 30: 418-27.
6. JIANG P, BIAN M, MA W *et al*.: Eryptosis as an underlying mechanism in systemic lupus erythematosus-related anemia. *Cell Physiol Biochem* 2016; 40:1391-400.
7. BARTOLMÄS T, MAYER B, BALOLA AH, SALAMA A: Eryptosis in autoimmune haemolytic anaemia. Eur *J Haematol* 2018; 100: 36-44.
8. BORST O, ABED M, ALESUTAN I *et al*.: Dynamic adhesion of eryptotic erythrocytes to endothelial cells via CXCL16/SR-PSOX. *Am*

J Physiol Cell Physiol 2012; 302: C644-51.
9. ALESSANDRI C, CONTI F, PENDOLINO M, MANCINI R, VALESINI G: New autoantigens in the antiphospholipid syndrome. *Autoimmun Rev* 2011; 10: 609-16.
10. NOURELDINE MHA, NOUR-ELDINE W, KHAMASHTA MA, UTHMAN I: Insights into the diagnosis and pathogenesis of the antiphospholipid syndrome. *Semin Arthritis Rheum* 2019; 48: 860-6.
11. VELÁSQUEZ M, ROJAS M, ABRAHAMS VM, ESCUDERO C, CADAVID ÁP: Mechanisms of endothelial dysfunction in antiphospholipid syndrome: association with clinical manifestations. *Front Physiol* 2018; 9: 1840.
12. PENGO V, TRIPODI A, REBER G: Update of the guidelines for lupus anticoagulant detection. Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis. *J Thromb Haemost* 2009; 7: 1737-40.
13. JEMAÀ M, FEZAI M, BISSINGER R, LANG F: Methods employed in cytofluorometric assessment of eryptosis, the suicidal erythrocyte death. *Cell Physiol Biochem* 2017; 43: 431-44.
14. ABED M, TOWHID ST, MIA S *et al*.: Sphingomyelinase-induced adhesion of eryptotic erythrocytes to endothelial cells. *Am J Physiol Cell Physiol* 2012; 303: C991-9.
15. QADRI SM, BISSINGER R, SOLH Z, OLDENBORG PA: Eryptosis in health and disease: A paradigm shift towards understanding the (patho)physiological implications of programmed cell death of erythrocytes. *Blood Rev* 2017; 31: 349-61.
16. RUIZ-IRASTORZA G, CROWTHER M, BRANCH W, KHAMASHTA MA: Antiphospholipid syndrome. *Lancet* 2010; 376: 1498-509.
17. MERONI PL, BORGHI MO, RASCHI E, TEDESCO F: Pathogenesis of antiphospholipid syndrome: understanding the antibodies. *Nat Rev Rheumatol* 2011; 7: 330-9.