Platelet apoptosis in pediatric immune thrombocytopenia

Osama B. Sedeek, Ashraf E. Abdelsalam, Heba A. Abd El Hafeez, Zeinab A. Zahran

Department of Clinical Pathology, Faculty of Medicine, Assiut University, Assiut, Egypt

Correspondence to Zeinab A. Zahran, Department of Clinical Pathology, Faculty of Medicine, Assiut University, Assiut, Egypt
Tel: +20 109 565 3919;
e-mail: zeinab_albadry@yahoo.com

Received 05 September 2017
Accepted 30 October 2017

Journal of Current Medical Research and Practice
September-December 2017, 2:192–198

Introduction
Immune thrombocytopenia (ITP) is a disorder characterized by immune-mediated accelerated destruction and suppressed production of platelets. The etiology of ITP is not yet known, and the diagnosis continues to be one of exclusion. Apoptosis is the physiologically most common form of programmed cell death. The anucleated platelets and megakaryocytes both possess a functional apoptotic machinery that controls their survival and dictates their lifespan.

Aim
We aimed to assess platelets apoptosis in pediatric patients with ITP and to correlate it with the clinical outcome of the disease.

Patients and methods
The study included 40 pediatric patients with acute ITP and 20 controls. Detection of platelet apoptosis was done with annexin V and CD41 by flow cytometry.

Results
Platelet count was significantly decreased in patients with ITP, and mean platelets volume was significantly increased in patients with ITP. The percentage of total apoptotic platelets and early and late apoptosis of platelets were significantly increased in patients with ITP than the controls. The patients with brief duration of thrombocytopenia were younger than patients with prolonged duration of thrombocytopenia. Platelets count and mean platelets volume were significantly increased in the patients with prolonged duration than patients with brief duration of thrombocytopenia. Moreover, the percentage of total apoptotic platelets and early apoptosis of platelets were significantly decreased in patients with brief duration than patients with prolonged duration of thrombocytopenia.

Conclusion
This study demonstrates an increase in platelets apoptosis in pediatric ITP.

Keywords:
apoptosis, caspase, immune thrombocytopenia, platelets

J Curr Med Res Pract 2:192–198

2357-0121

Introduction

Immune thrombocytopenia (ITP) is an immune-mediated acquired disease of children and adults, characterized by transient or persistent decrease of the platelet count and, depending on the degree of thrombocytopenia, increased risk of bleeding [1,2]. ITP is classified as primary or secondary. Primary thrombocytopenia (idiopathic) indicates the absence of any obvious initiating and/or underlying cause, and secondary ITP is caused by drugs, vaccination, infections, and so on [3–5].

Based on the duration of thrombocytopenia, ITP can also be classified as 'acute' versus 'chronic'. Thrombocytopenia resolving before 6 months is called acute and more than 6 months is called chronic [6].

Bleeding is the most common clinical manifestation of ITP in the form of purpura, epistaxis, menorrhagia, gingival, and gastrointestinal bleeding [7].

Apoptosis is a process of programmed cell death that occurs in multicellular organisms [8]. The mechanisms of apoptosis are highly complicated, involving an energy-dependent cascade of molecular events. Although platelets are anucleate cells, they express at least part of the apoptotic machinery known from nucleated cells. In addition, they have been observed to undergo apoptotic-like events [9,10].

There are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway [11]. There is an additional pathway that involves T-cell-mediated cytotoxicity and perforin/granzyme-dependent killing of the cell [12].

One of the major apoptotic pathways is the intrinsic (mitochondrial) apoptotic pathway. The intrinsic pathway of apoptosis is tightly regulated by

members of the B-cell lymphoma protein 2 family, which contains both prodeath and prosurvival proteins. The changes in the mitochondrial outer membrane result in the release of cytochrome c and other apoptogenic proteins into the cytosol. Once released, cytochrome c assembles with apoptotic protease-activating factor 1 and the initiator caspase, procaspase-9, to form the apoptosome, which activates caspase-9 and downstream effector caspases [13].

The extrinsic pathway of apoptosis is triggered by ligation of 'death receptors' belonging to the tumor necrosis factor receptor superfamily that contains an intracellular death domain, which can recruit and activate the initiator caspase, procaspase-8. This leads to direct activation of downstream effector caspases by caspase-8, such as caspase-3, caspase-6, or caspase-7 [13].

Perforin/granzyme pathway, mediated by cytotoxic T lymphocytes, which are able to kill target cells through the extrinsic pathway, and fatty acid synthetase ligand (FasL)/fatty acid synthetase receptor (FasR) interaction, is the predominant method of cytotoxic T lymphocyte-induced apoptosis [12].

During apoptosis, phosphatidylserine (PS) is translocated from the inner to the outer leaflet of plasma membrane, which is an essential characteristic that leads to recognition of the apoptotic cell by phagocytes [14,15].

Other studies have been carried out to determine platelets apoptosis in adult patients with ITP. They described increased platelets apoptosis involving loss of mitochondrial membrane potential, caspase-3 activation, and PS externalization [16]. Deng *et al.* [17] reported that the enhancement of platelets apoptosis observed in patients with chronic ITP may be one of the pathogenic mechanisms of chronic ITP. In our study, we assessed platelets apoptosis in our pediatric patients with acute ITP.

Aim

The aim of this work was to assess platelet apoptosis by flow cytometric detection of annexin V expression on the platelets in pediatric patients with ITP and to correlate it with the clinical outcome of the disease.

Patients and methods

This was a case-control study. The study included 40 pediatric patients with acute ITP. The patients were recruited from the Pediatric Clinical Hematology Unit of Children Hospital, Assiut University. In addition, 20 healthy children of comparable age and sex were taken as controls. The study was approved by the institutional review board, and informed consents were obtained from children's parents.

At diagnosis, all patients and controls were subjected to thorough history and physical examination with stress upon disease duration, drug intake, preceding viral infection, bleeding manifestations, organomegaly, and lymphadenopathy. In addition, the following investigations were conducted: full blood picture (Celltac E automated hematology analyzer' Nihon Kohden Corporation, Tokyo, Japan), bone marrow examination (if needed), coagulation profile, and detection of platelet apoptosis done using annexin V and CD41a by flow cytometry. The patients were managed according to grade of severity [18]. According to the response of the treatment, the patients were then classified into two group: patients with short duration, who recovered before 6 months, and patients with long duration, who did not recover within 6 months [5].

Inclusion criteria

The following were the inclusion criteria:

- Children aged from 6 months to 16 years, presenting with newly diagnosed acute ITP
- Platelet count less than 50×10^9/l
- No prior immunomodulating (intravenous immunoglobulin and corticosteroids) treatment before diagnosis.

Exclusion criteria

The following were the exclusion criteria:

- Clinical features that are not compatible with diagnosis of acute ITP, such as presence of organomegaly, other cytopenias besides thrombocytopenia, other autoimmune phenomena, or features suggestive of infectious disease like hepatitis and Epstein–Barr virus
- Patients with chronic ITP (chronic ITP is characterized by a thrombocytopenia persisting for >6 months) [19,20]
- Immunomodulating treatment within 4 weeks before diagnosis
- Severe or life-threatening bleeding at presentation.

Principle

Type of sample

A volume of 5 ml of peripheral blood from patients and controls was collected by venipuncture into 3.8% trisodium citrate solution.

Reagents

For detection of platelet apoptosis, we used fluorescein isothiocyanate-conjugated annexin V apoptosis detection kit (Biolegend headquarters is San Diego, California, U.S.) and peridinin–chlorophyll–protein (Per-CP)-conjugated CD41 [Becton Dickinson (BD) Biosciences, San Jose, California, USA].

Flow cytometry

Apoptosis detection was done according to manufacturer's instruction. Platelet-rich plasma (PRP) was separated from the citrated blood samples by centrifugation at 1000 rpm for 10 min at room temperature and placed in a separate tube. The PRP was then centrifuged once again at 2000 rpm for 10 min to further concentrate platelets.

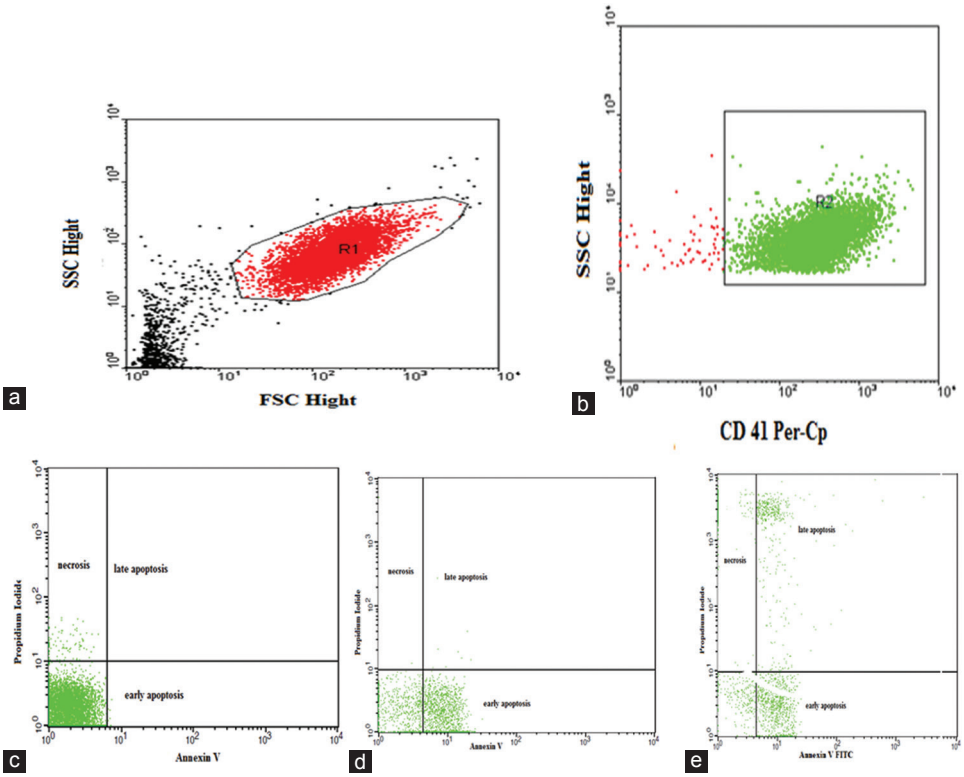
Plastic Falcon tubes of 5 ml were labeled with Laboratory number and staining antibody, including controls. The cells were washed twice with (Biolegend, GmbH, Germany), Biolegend headquarters is San Diego, California, U.S Cell Staining Buffer, and then resuspended in annexin V binding buffer at a concentration of 0.25–1.0 × 10^7 cells/ml. A volume of 100 µl of cell suspension was transferred into a 5-ml test tube. A volume of 10 µl of CD41 along with 10 µl of annexin V was then added to the cell suspension. A volume of 10 µl of propidium iodide solution was added. The cells were gently vortexed and incubated for 15 min at room temperature (25°C) in the dark. A volume of 400 µl of annexin V binding buffer was added to each tube. Flow cytometric analysis was done by FACS Calibur flow cytometry with Cell Quest Software (BD Biosciences). Antihuman immunoglobulin G was used as an isotype-matched negative control for each sample.

Forward and side scatter histogram was used to define the platelet population and expression of the markers (Fig. 1).

Statistical analysis

All statistical analyses were performed using statistical package for the social sciences software, version 18 (SPSS v18 software; SPSS Inc., Chicago, Illinois, USA). Qualitative data were expressed number and percentage; quantitative data were expressed by mean ± SEM. Mann–Whitney analysis was used to detect the statistical differences between the study groups. Spearman's correlation was used to correlate the studied parameters. *P* value less than 0.05 denoted significant difference.

Figure 1



Flow cytometric detection of platelets apoptosis. (a) Forward and side scatter histogram was used to define the platelets population (R1). (b) The expression of CD41 was assessed in R1 to confirm platelets population (R2). (c–e) Then, the expression of annexin V and propidium iodide on $CD41^+$ platelet population was detected. (c) One representative case showing no apoptosis of platelets (the platelets are negative for both propidium iodide and annexin V). (d) One case showing increased frequency of early apoptosis (expression of annexin V was detected in some platelets). (e) One case showing increased frequency of early and late apoptosis. Expression of annexin V was detected in some platelets (early apoptosis) and coexpression of both propidium iodide and annexin V in some platelets (late apoptosis). SSC=side scatter, FSC=forward scatter.

Results

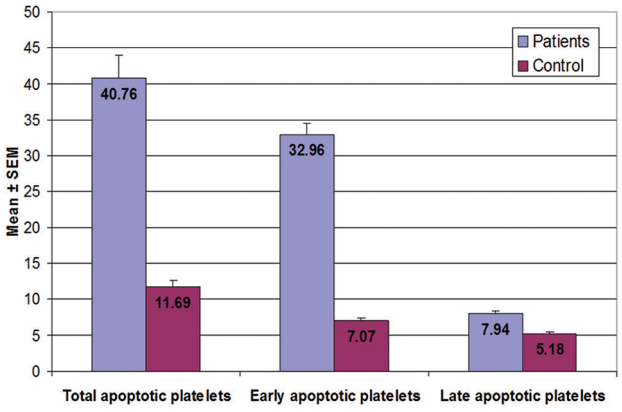
The study included 40 children with age range from 1 to 13.9 years (5.8 ± 0.62 years). It comprised 22 males and 18 females. Cutaneous manifestations were present in all patients. None of our patients presented with enlarged liver or spleen or significant lymphadenopathy. Twenty-seven (67.5%) patients recovered within 6 months and 13 (32.5%) patients had duration of thrombocytopenia of more than 6 months (Table 1).

Platelet count was significantly decreased in patients with ITP (12.69 ± 1.09 × 10^9/l) than the controls (281.45 ± 17.18 × 10^9/l), with *P* value less than 0.001. The mean platelet volume (MPV) was significantly increased in the patients (11.10 ± 0.44 fl) than the controls (8.21 ± 0.26 fl), with *P* value less than 0.001. There were no significant differences in white blood cell count and hemoglobin concentration between patients with ITP and the controls (Table 2).

There were significant differences in platelet apoptosis between patients with ITP and the controls. The percentage of total apoptotic platelets was significantly increased in patients with ITP (40.76 ± 3.13%) than controls (11.69 ± 0.96%), with *P* value less than 0.001. The percentage of early apoptosis of platelets was significantly increased in patients with ITP (32.96 ± 1.51%) than the controls (7.07 ± 0.31%), with *P* value less than 0.001. The percentage of late apoptosis of platelets was significantly increased in patients with ITP (7.94 ± 0.39%) than the controls (5.18 ± 0.32%), with *P* value of 0.007 (Fig. 2).

There was a negative correlation between the percentage of apoptotic platelets and the platelet count in patients with ITP (Fig. 3).

Figure 2



Platelet apoptosis in patients with immune thrombocytopenia and controls.

The patients with brief duration of thrombocytopenia were younger than patients with prolonged duration of thrombocytopenia, with *P* value of 0.032.

Platelet count was significantly decreased in the patients with ITP with brief duration of thrombocytopenia (10.76 ± 1.04 × 10^9/l) than patients with prolonged duration of thrombocytopenia (14.28 ± 1.08 × 10^9/l), with *P* value of 0.012. The MPV was significantly increased in the patients with prolonged duration of thrombocytopenia (13.08 ± 0.73 fl) than patients with brief duration of thrombocytopenia (10.26 ± 0.46 fl), with *P* value of 0.003. There were

Table 1 Baseline clinical characteristics of patients with immune thrombocytopenia and controls

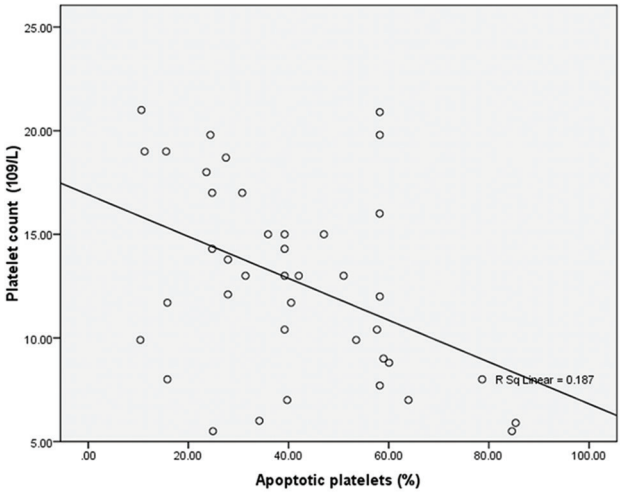
	Patients (*n*=40)	Controls (*n*=20)
Age (years)	5.8±0.62	6.02±0.85
Sex (male/female)	22/18	14/6
Petechia	40/40	0/20
Purpura	40/40	0/20
Echymosis	40/40	0/20
Epistaxis (mild)	9/40	0/20
Splenomegaly	0/40	0/20
Hepatomegaly	0/40	0/20
Preceding febrile illness	26/40	0/20
Medication	Supportive, steroid, and immunoglobulin	–

Table 2 Baseline laboratory characteristics of patients with immune thrombocytopenia and controls

	Patients (*n*=40)	Controls (*n*=20)	*P*
Platelets (10^9/l)	12.69±1.09	281.45±17.18	<0.001
MPV (fl)	11.10±0.44	8.21±0.26	<0.001
WBCs (10^9/l)	6.56±0.28	7.51±0.44	0.098
Hemoglobin (g/dl)	11.52±0.19	12.69±0.26	0.593

Data represented as mean±SEM. MPV, mean platelets volume; WBC, white blood cells; $P \leq 0.05$, significant.

Figure 3



Negative correlation between the percentage of apoptotic platelets and the platelet count in patients with immune thrombocytopenia.

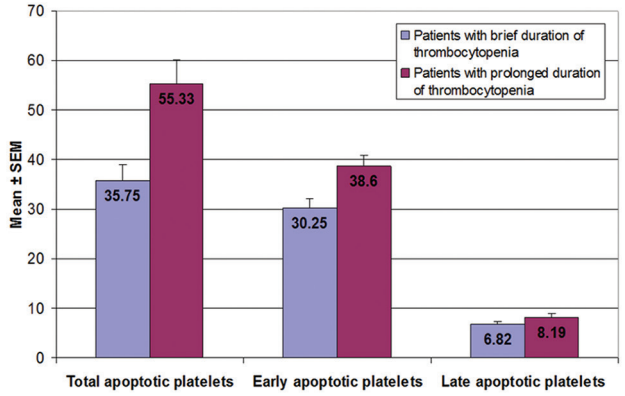
no significant difference in white blood cell count and hemoglobin concentration between patients with brief duration and patients with prolonged duration of thrombocytopenia (Table 3).

The percentage of total apoptotic platelets was significantly decreased in patients with ITP with brief duration of thrombocytopenia (35.75 ± 3.28%) than patients with prolonged duration of thrombocytopenia (55.33 ± 4.79%), with *P* value of 0.001. The percentage of early apoptosis of platelets was significantly decreased in patients with brief duration of thrombocytopenia (30.25 ± 1.76%) than patients with prolonged duration of thrombocytopenia (38.60 ± 2.23%), with *P* value of 0.007, but there was no significant difference in late apoptosis of platelets between patients with brief duration and patients with prolonged duration of thrombocytopenia (Table 3 and Fig. 4).

Discussion

In this study, we studied 40 pediatric patients with ITP. All of them presented with bleeding symptoms. Boys were affected more than girls. The mean age of affected children was found to be normally about 4–5 years in most studies, which is similar to our results [4,21]. Female were more affected than male in previous studies [22], which is against our finding. The patients with ITP presented with lower platelet count than controls. Moreover, the MPV was significantly increased in patients than controls. Our results match those reported in previous studies [23–25].

Figure 4



Laboratory data in patients with brief duration and patients with prolonged duration of thrombocytopenia.

ITP is a heterogeneous autoimmune disease, characterized by accelerated platelets destruction and impaired platelet production [26]. Apoptosis is the main mechanism that regulates cell lifespan and the elimination of damaged or infected nucleated cells. Similarly to nucleated cells, platelet lifespan is also controlled by an apoptotic program that triggers collapse of the mitochondrial inner membrane potential; activation of caspases-3, caspases-8, and caspases-9; PS externalization; and microparticle shedding [27–29]. Externalization of PS on the surface of cells undergoing apoptosis represents the most universal and best characterized 'eat me' signal that leads to recognition as 'unwanted self' and clearance of the apoptotic cell by phagocytes [14,15]. The translocation of PS from the inner to the outer leaflet of the lipid bilayer occurs early in the apoptotic process and involves a nonspecific bidirectional phospholipid flip-flop along with the blockade of an aminophospholipid translocase that normally confines and reverts PS to the cytosolic side of the cell membrane [14].

In our study, there was increased frequency of platelet apoptosis. Both early apoptosis and late apoptosis were detected in our patients with ITP. The observed apoptotic events in platelets from patients with ITP could indicate that platelet apoptosis might contribute to thrombocytopenia. The increased PS exposure on platelets in ITP may lead to their clearance from the circulation by the reticuloendothelial system and cause or aggravate prevailing thrombocytopenia. Previous studies in patients with ITP found an increase in platelet apoptosis. Abnormally increased PS exposure on platelets in ITP has been reported for pediatric patients with acute ITP and for adult patients with chronic ITP [27,30–33].

Winkler *et al.* [33] reported that the frequency of platelets exposing PS was significantly higher in

Table 3 Clinical and laboratory data in patients with brief duration and patients with prolonged duration of thrombocytopenia

	Patients with brief duration of thrombocytopenia (*n*=27)	Patients with prolonged duration of thrombocytopenia (*n*=13)	*P*
Age (years)	4.54±0.55	7.89±0.84	0.032
Platelets (10^9/l)	10.76±1.04	14.28±1.08	0.012
WBCs (10^9/l)	6.48±0.32	6.62±0.44	0.819
Hemoglobin (g/dl)	11.92±0.32	11.04±0.21	0.073
MPV (fl)	10.26±0.46	13.08±0.73	0.003
Total apoptotic platelets (%)	35.75±3.28	55.33±4.79	0.001
Early apoptotic platelets (%)	30.25±1.76	38.60±2.23	0.007

Data represented as mean±SEM. MPV, mean platelets volume; WBC, white blood cell; $P \leq 0.05$, significant.

children with acute ITP and a significantly higher proportion of ITP platelets contained activated capases-3, caspases-8, and caspases-9 compared with controls. They suggest activation of the extrinsic, in conjunction with the intrinsic, pathway of apoptosis. Catani *et al*. [30] reported that both fresh and aged platelets from patients with chronic ITP exhibited significantly higher surface PS levels compared with healthy donor platelets, as assessed by flow cytometric annexin V staining. The pathway that initiates the apoptotic events in ITP platelets is unknown; it may be because of an apoptosis-dependent pathway leading to activation of caspase-3, caspases-8, and caspases-9 or PS exposure [28,29,34]. Platelet apoptosis may result from an increased proplatelets formation in response to the peripheral platelets destruction [35].

During the follow-up of our patients, we found that the patients were divided into two groups. One group included those patients (67.5%) who had brief duration of thrombocytopenia that lasts for less than 6 months. The other group included patients (32.5%) who had prolonged duration of thrombocytopenia that lasts for more than 6 months. These results were reported in Badrawy *et al*. [23] and Zahran and Elsayh [36].

In our study, patients having thrombocytopenia for a brief duration had lower age and platelet count than those with prolonged duration. This is in consistent with result of Edslev *et al*. [37], where the MPV was significantly lower in those having thrombocytopenia for a brief duration than those with prolonged duration of thrombocytopenia. This is also consistent with the results of Ahmed *et al*. [38] and Zahran and Elsayh [36].

Platelet apoptosis was more provoked in patients with long duration of thrombocytopenia than those with brief duration of thrombocytopenia, and this finding was more evident in early apoptosis of platelets. This result in addition to the negative correlation between platelets count and the percentage of apoptotic platelet may indicate that platelet apoptosis may have a role in the prognosis of pediatric ITP. Moreover, the modulation of apoptotic cell death and the enhancement of apoptotic cell removal is considered as novel therapies for autoimmunity. One approach would be to prevent or induce cell death by targeting the molecules involved in the apoptotic machinery [39,40].

Conclusion

This study demonstrates an increase in platelets apoptosis in pediatric ITP. The results could suggest the role of platelets apoptosis in pathogenesis and prognosis of pediatric ITP especially at early diagnosis. The effect of different lines of treatment on the level of apoptotic platelets in these patients should be investigated in further research. Moreover, future studies of the intervention in the pathways of apoptosis with possibilities to modulate this process are recommended for effective treatment of ITP and other autoimmune disorders.

Acknowledgements

The financial support was provided by Grants Unit, Faculty of Medicine, Assiut University.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1 Cooper N, Bussel J. The pathogenesis of immune thrombocytopaenic purpura. Br J Haematol 2006; 133:364–374.

2 Rodeghiero F, Stasi R, Gernsheimer T, Michel M, Provan D, Arnold DM, *et al*. Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. Blood 2009; 113:2386–2393.

3 Ansari S, Shirali A, Khalili N, Daneshfar R, Arefi H. Neutropenia following intravenous immunoglobulin therapy in pediatric patients with idiopathic thrombocytopenic purpura. Iranian J Blood Cancer 2014; 6:81–85.

4 Bertuola F, Morando C, Menniti-Ippolito F, Da Cas R, Capuano A, Perilongo G, *et al*. Association between drug and vaccine use and acute immune thrombocytopenia in childhood. Drug Safety 2010; 33:65–72.

5 Nazari S, Abdollah Gorji F, Sadeghi-Koupai M. Epidemiology of idiopathic thrombocytopenic purpura in children. Iran J Pediat Hematol Oncol 2012; 2:35–39.

6 Blanchette VS, Carcao M. Childhood acute immune thrombocytopenic purpura: 20 years later. Semin Thromb Hemost 2003; 29:606–618.

7 Neylon AJ, Saunders PW, Howard MR, Proctor SJ, Taylor PR. Clinically significant newly presenting autoimmune thrombocytopenic purpura in adults: a prospective study of a population-based cohort of 245 patients. Br J Haematol 2003; 122:966–974.

8 Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol 2007; 35:495–516.

9 Böing AN, Hau CM, Sturk A, Nieuwland R. Platelet microparticles contain active caspase 3. Platelets 2008; 19:96–103.

10 Lopez J, Salido G, Pariente J, Rosado J. Thrombin induces activation and translocation of Bid, Bax and Bak to the mitochondria in human platelets. J Thromb Haemost 2008; 6:1780–1788.

11 Igney FH, Krammer PH. Death and anti-death: tumour resistance to apoptosis. Nat Rev Cancer 2002; 2:277–288.

12 Brunner T, Wasem C, Torgler R, Cima I, Jakob S, Corazza N. Fas (CD95/Apo-1) ligand regulation in T cell homeostasis, cell-mediated cytotoxicity and immune pathology. Semin Immunol 2003; 15:167–176.

13 Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol 2008; 9:47–59.

14 Erwig L, Henson P. Clearance of apoptotic cells by phagocytes. Cell Death Differ 2008; 15:243–250.

15 Savill, J, Fadok V. Corpse clearance defines the meaning of cell death. Nature 2000; 407:784–788.

16 Goette NP, Glembotsky AC, Lev PR, Grodzielski M, Contrufo G, Pierdominici MS, *et al*. Platelet apoptosis in adult immune thrombocytopenia: insights into the mechanism of damage triggered by auto-antibodies. PLoS One 2016; 11:e0160563.

17 Deng G, Yu S, Li Q, He Y, Liang W Yu L, *et al*. Investigation of platelet

apoptosis in adult patients with chronic immune thrombocytopenia. Hematology 2017; 22:155–161.

18 Provan D, Stasi R, Newland AC, Blanchette VS, Bolton-Maggs P, Bussel JB, *et al.* International consensus report on the investigation and management of primary immune thrombocytopenia. Blood 2010; 115:168–186.

19 Alvina A. Idiopathic thrombocytopenic purpura: laboratory diagnosis and management. Univ Med 2016; 30:126–134.

20 Levine S. Thrombotic thrombocytopenic purpura and other forms of nonimmunologic platelet destruction. Wintrobe Clin Hematol 2004; 2:1555–1564.

21 Watts RG. Idiopathic thrombocytopenic purpura: a 10-year natural history study at the childrens hospital of alabama. Clin Pediatr (Phila) 2004; 43:691–702.

22 Marieke Schoonen W, Kucera G, Coalson J, Li L, Rutstein M, Mowat F, *et al.* Epidemiology of immune thrombocytopenic purpura in the General Practice Research Database. Br J Haematol 2009; 145:235–244.

23 Badrawy H, Elsayh KI, Zahran AM, EL-Ghazali MH. Platelet antibodies, activated platelets and serum leptin in childhood immune thrombocytopenic purpura. Acta Haematol 2013; 130:312–318.

24 Imbach P, Kühne T. Immune thrombocytopenic purpura ITP. Vox Sang 1998; 74:309–314.

25 Negash M, Tsegaye A. Diagnostic predictive value of platelet indices for discriminating hypo productive versus immune thrombocytopenia purpura in patients attending a tertiary care teaching hospital in Addis Ababa, Ethiopia. BMC Hematol 2016; 16:18.

26 Qiao J, Liu Y, Li D, Wu Y, Li X, Yao Y, *et al.* Imbalanced expression of Bcl-xL and Bax in platelets treated with plasma from immune thrombocytopenia. Immunol Res 2016; 64:604–609.

27 Contrufo G, Glembotsky AC, Goette NP, Lev PR, Grodzielski M, Pierdominici MS, *et al.* Platelet apoptosis in adult immune thrombocytopenia. Relationship with auto-antibodies, platelet function and treatment. Blood 2014; 124:2792.

28 Schoenwaelder SM, Yuan Y, Josefsson EC, White MJ, Yao Y, Mason KD, *et al.* Two distinct pathways regulate platelet phosphatidylserine exposure and procoagulant function. Blood 2009; 114:663–666.

29 Vogler M, Hamali HA, Sun X-M, Bampton ET, Dinsdale D, Snowden RT, *et al.* BCL2/BCL-XL inhibition induces apoptosis, disrupts cellular calcium homeostasis, and prevents platelet activation. Blood 2011; 117:7145–7154.

30 Catani L, Fagioli ME, Tazzari PL, Ricci F, Curti A, Rovito M, *et al.* Dendritic cells of immune thrombocytopenic purpura (ITP) show increased capacity to present apoptotic platelets to T lymphocytes. Exp Hematol 2006; 34:879–887.

31 Galluzzi L, Aaronson S, Abrams J, Alnemri E, Andrews D, Baehrecke E, *et al.* Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. Cell Death Differen 2009; 16:1093–1107.

32 Speer O, Schmugge M. Investigating caspases and other markers of apoptosis in ITP. Ann Hematol 2010; 89:45–46.

33 Winkler J, Kroiss S, Rand ML, Azzouzi I, Annie Bang K, Speer O, *et al.* Platelet apoptosis in paediatric immune thrombocytopenia is ameliorated by intravenous immunoglobulin. Br J Haematol 2012; 156:508–515.

34 Leytin V, Allen DJ, Mutlu A, Mykhaylov S, Lyubimov E, Freedman J. Platelet activation and apoptosis are different phenomena: evidence from the sequential dynamics and the magnitude of responses during platelet storage. Br J Haematol 2008; 142:494–497.

35 de Botton S, Sabri S, Daugas E, Zermati Y, Guidotti JE, Hermine O, *et al.* Platelet formation is the consequence of caspase activation within megakaryocytes. Blood 2002; 100:1310–1317.

36 Zahran AM, Elsayh KI. CD4+ CD25+ High Foxp3+ regulatory T cells, B lymphocytes, and T lymphocytes in patients with acute ITP in Assiut Children Hospital. Clin Appl Thromb Hemost 2014; 20:61–67.

37 Edslev PW, Rosthøj S, Treutiger I, Rajantie J, Zeller B, Jonsson OG. A clinical score predicting a brief and uneventful course of newly diagnosed idiopathic trombocytopenic purpura in children. Br J Haematol 2007; 138:513–516.

38 Ahmed S, Siddiqui AK, Shahid RK, Kimpo M, Sison CP, Hoffman MA. Prognostic variables in newly diagnosed childhood immune thrombocytopenia. Am J Hematol 2004; 77:358–362.

39 Walensky LD, Kung AL, Escher I, Malia TJ, Barbuto S, Wright RD, *et al.* Activation of apoptosis *in vivo* by a hydrocarbon-stapled BH3 helix. Science 2004; 305:1466–1470.

40 Xie J, Awad KS, Guo Q. RNAi knockdown of Par-4 inhibits neurosynaptic degeneration in ALS-linked mice. J Neurochem 2005; 92:59–71.