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## Anti- $\beta$ 2 Glycoprotein I in Childhood Immune Thrombocytopenic Purpura

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The antibodies could be an epiphenomena or surrogate marker or directly involved in the cause-and-effect relationship of childhood immune thrombocytopenic purpura disease and study the predictive value of either elevated anti- $\beta$ 2glycoprotein (anti-  $\beta$ 2GP1) or Anticardiolipin Antibodies (ACA) concentrations for secondary ITP detection and compare their levels to the steroid therapy responsiveness. The study was conducted on 3 groups of children and adolescents. Group I consisted of 15 children with acute ITP (8 males, 7 females) with mean age at examination  $9.7\pm 4.25$  years, this group was sub classified into: I a acute ITP in active disease ( $N^{\circ} = 4$  cases) at the time of diagnosis, Ib acute ITP in remission stage ( $N^{\circ} = 11$  cases), Group II consisted of 27 children with chronic ITP (12 males, 15 females), with mean age at examination  $12.2\pm 5.07$  years, this group was sub classified according to response of steroid therapy into: II a: chronic and steroid dependent ITP ( $N^{\circ} = 17$  cases) II b: chronic and steroid resistant ITP ( $N^{\circ} = 10$  cases), Group III: 28 healthy controls(13 males and 15 females) matched in age and sex to patients groups. All children had thorough medical history and examination, CBC, liver, renal function and bone marrow aspirate was performed. Antinuclear antibodies (ANA), ACA (Ig M, G) and anti-  $\beta$ 2GP1 (IgG) were assessed in all studied children and adolescents. There was a significant higher mean concentrations of ACA (IgM) ( $6.7\pm 4.75$  MPLU), ACA (IgG) ( $11.4\pm 5.52$  GPLU) and anti-  $\beta$ 2GP1 (IgG) ( $79.5\pm 62.0$  u L<sup>-1</sup>) in chronic ITP cases when compared to their corresponding levels in acute or control cases ( $p = 0.000$ ). About 77.8% of chronic ITP cases showed elevated serum concentrations of IgG ACA while increased serum levels of IgG anti-  $\beta$ 2GP1 in all (100%) chronic ITP cases was observed. A significant positive correlation between increased levels of IgG anti- $\beta$ 2GP1 and increased serum concentrations of IgG ACA was determined ( $r = 0.42$ ,  $p < 0.01$ ). The detection of increased concentrations of IgG isotype of both ACA and anti- $\beta$ 2GP1 was significantly correlated to steroid therapy resistance ( $r = 0.54$ ,  $p = 0.000$ ). About 76.1% (32/42) of ITP cases had positive APA including all chronic and only 5 cases from acute studied ITP children. Splenectomy was done in 28.1% (9/32) of ITP children with positive APA. Elevated serum concentrations of IgG isotype of either ACA or anti- $\beta$ 2GP1 in all 9 splenectomized ITP children with positive APA was observed, only 3 cases of them showed increased levels of IgM ACA.

**Key words:** Immune Thrombocytopenic Purpura, anti- $\beta$  GPI or ACA, steroid therapy, children

## INTRODUCTION

Immune Thrombocytopenic Purpura (ITP) is an autoimmune disorder characterized by a low circulating platelets count ( $<150 \times 10^9 \text{ L}^{-1}$ ) due to autoantibody or immune complexes binding to platelets surface antigen. This leads to premature platelets destruction by the reticuloendothelial system (Provan and Newland, 2003). In children, the disease occurs in two forms, an acute and chronic forms based on the disease duration. An acute ITP is generally self limiting illness that accounts for over 80% of all cases. Chronic ITP is conventionally defined as persistence of thrombocytopenia for longer than six months duration from the onset of illness (Blanchette and Price, 2003). Chronic ITP occurs also in children in approximately 20-30% of patients with acute ITP (Kuhne, 2003); this classification of ITP needs to be evaluated based on new findings of the pathophysiology of the disease as well as the clinical aspects; because it is not known whether a child has acute ITP or the child has or develops chronic form at the onset of the disease. Pediatric chronic ITP is different from adult chronic ITP in that 30-80% of children with chronic ITP experience a spontaneous or therapy-induced remission. The development of chronic ITP seems to describe the pathogenesis of the chronic disease as Antiphospholipids Syndrome (APS) or autoimmune disorders. Antibodies against platelets are assumed to be crucial to the mechanism of immune-mediated platelets destruction because they have been demonstrated in up to 90% of ITP patients (Stasi *et al.*, 1994).

It has been reported that elevated concentrations of Anticardiolipin Antibodies (ACA) were present in about 30% of the sera of ITP patients at the time of diagnosis (Dash *et al.*, 2004). ACA belong to a much larger class of antibodies directed towards negatively charged phospholipid antigen that include the Lupus Anticoagulant (LA) and ACA. APA are dependent upon a serum cofactor; a phospholipid binding protein known as  $\beta 2$  Glycoprotein I ( $\beta 2$ GP1) or apolipoprotein H to recognize the phospholipid. This 54-KD serum glycoprotein appears to be the major, but not the only, cofactor for the recognition of antigenic phospholipid (Rand, 1998).  $\beta 2$ GP1 is a physiological anticoagulant found in plasma in a concentration of  $200 \text{ Ug mL}^{-1}$ . Approximately 40% of  $\beta 2$ GP1 is associated with lipoproteins of various classes.  $\beta 2$ GP1 inhibits the contact phase of coagulation, prothrombinase reaction and ADP-induced platelet aggregation. Although  $\beta 2$ GP1 is an anticoagulant, hereditary deficiency of this protein does not lead to venous or arterial thromboembolic events

(Triplett, 2000). Thrombocytopenia is identified as one of well-documented clinical complications of APS. ITP may be secondary to underlying disorders as SLE or APS. Positive anti- $\beta 2$ GP1 is more specific in identifying patients with APS (Wilson *et al.*, 1999; Chi, 2002).

The clinical data are still lacking regarding the relevance and prognostic significance of anti- $\beta 2$ GP1 in ITP patients. In this study, we investigate ANA, ACA (IgM, G) and anti- $\beta 2$ GP1 (IgG) in childhood ITP to keep an open mind about these antibodies could be an epiphenomena or surrogate marker or directly involved in the cause-and-effect relationship of this disease process. In addition, we study the predictive value of either elevated anti- $\beta 2$ GP1 or ACA concentrations for secondary ITP detection and compare their levels to the steroid therapy responsiveness and clinical outcomes.

## MATERIALS AND METHODS

The present study was a case-control study and was carried out from 1st March to end of August 2000. The study conducted on 42 children and adolescents with immune Thrombocytopenic Purpura (ITP) and 28 age and sex matched healthy control children. A follow up study of all ITP cases was carried out during the period from 2000 to 2004 to evaluate the clinical outcomes of these increased APA in initially diagnosed ITP to clarify whether it is primary or secondary to other underlying disease as APS or SLE. They were recruited from regularly treated patients in the Pediatric Hematology Unit, Children's Hospital, Ain Shams University. The diagnosis of ITP was made by exclusion of other causes of thrombocytopenia. Disorders known to cause shortened survival or decreased production of platelets were ruled out, including multi system autoimmune disease, lymphoproliferative disorders, drug-induced thrombocytopenia, myelodysplastic syndrome, hepatitis A, B, C and other viral as well as bacterial infection. Bone marrow aspirate was performed in all patients prior to steroid therapy. The examination of marrow specimen was considered to be consistent with the diagnosis of ITP if the cellularity was normal with normal or increase in the number of megakaryocytes.

The study included three groups, classification criteria for the studied ITP cases were summarized in Table 1:

**GP 1:** Included 15 patients with acute ITP, 8 males and 7 females (M/F ratio 8/7) with mean age at examination  $9.7 \pm 4.25$  years (ranged 4-18 years), this group was sub classified into:

**Table 1: Criteria classification for studies of ITP cases**

Classification criteria of ITP cases			
		Chronic (N° = 27)	
Steroid therapy phase	Acute (N° = 15)	Dependent (N° = 17)	Resistant (N° = 10)
Steroid therapy	Increased platelets count within 7-10 days	Increased platelets count within 7-10 days	No Increased platelets count within 7-10
Steroid tapering	Sustained increased platelets count	Decreased platelets count (relapse)	No change, even more decreased platelets count

- 1a acute ITP in active disease (N° = 4 cases) at the time of diagnosis
- 1b acute ITP in remission stage (N° = 11 cases)

**GP II:** Included 27 patients with chronic ITP (defined by the persistence of thrombocytopenia for >6 months duration), 12 males and 15 females (M/F ratio 12/15) with mean age at examination 12.2±5.07 years (ranged 4-20 years), this group was sub classified into:

- 2a: chronic and steroid dependent ITP (N° = 17 cases)
- 2b: chronic and steroid resistant ITP (N° = 10 cases)

**GP III:** Controls group consisted 28 children, 13 males and 15 females (M/F ratio 13/15) with mean age at examination 8.8±3.90 years (ranged 3-18 years). They were healthy subjects without history of thrombosis or autoimmune disorders.

The following parameters were assessed for all included children:

- Full medical history was taken, considering history of drug intake, preceding viral infections, bleeding manifestations and history suggestive of collagen vascular diseases including musculoskeletal complains (arthritis and muscle pains), skin rash, photosensitivity, renal, neurological, psychosis.
- Thorough clinical examination.
- All patients and controls underwent a panel of laboratory tests

Four milliliter blood sample was withdrawn from each patient and divided into two tubes; the first on EDTA for complete blood picture, second tube centrifuged and serum was collected and divided into aliquots and stored at -20°C until used in assessment of anticardiolipin antibodies of Ig G and Ig M isotypes (ACA IgG, IgM), anti-β2glycoproteinI (anti-β2GP1(IgG)) and antinuclear antibody (ANA).

- Complete blood picture using an automated hematology analyzer (Cell Dyne)
- Serum auto antibodies assessment of anticardiolipin antibodies of IgG and IgM isotypes was performed

using a quantitative solid phase enzyme linked immunosorbent assay (REAADSR) that uses cardiolipin-coated micro wells and incorporates Horseradish Peroxidase (HRP) labeled antihuman IgG and IgM anticardiolipin concentration (Wilson *et al.*, 1999; Chi, 2002). Samples below or equal 10 GPL or MPL units (measuring ACA IgG and IgM autoantibodies, respectively) were regarded as negative and above 10 units were regarded as positive (elevated).

- Antinuclear Antibodies (ANA) were detected by means of indirect immunofluorescent using air dried cryostat section (IMMCO Diagnostics, Inc.) as substrate, the rest of these antibodies was considered positive when the titer was equal or higher than 1/20.
- Measurement of anti-β2GP1 of IgG isotype was performed using micro titer plate (Nune-Immuno plate, Maxi Sorp, Kamstrup, Roskilde, Denmark) irradiated electron beam at 100 KGY (Triplett, 2000) Samples below 8 unit/L (measuring anti-β2GP1 of IgG isotype) were regarded as negative and above or equal 8 unit/L were regarded as positive (elevated).

**Statistical analysis:** Data entry and verification was done using SPSS version 9. Chi square test of significance was done for comparing categorical variables between the studied groups. for comparing continuous variables between groups, Mann Whitney test was used instead of t test as the data was not normally distributed. Bivariate Pearson correlation analysis was done to detect the significance of the relation between level of ACA antibodies and anti-β2GP1 of IgG.

**RESULTS**

Table 2 shows descriptive data of the studied ITP cases. Table 3 shows the mean serum concentrations of

**Table 2: Descriptive features of the studied ITP cases**

Variable	Acute	Chronic
Total No. (42)	15	27
Male/Female (M/F)	8/7	12/15
Age/diagnosis (years)	7.0±4.6	8.7±4.9
Age/examination (years)	9.7±4.3	12.2±5.1
Splenectomy	none	9(33.3%)
ANA positivity	none	5(18.5%)

**Table 3: Mean serum concentrations of laboratory parameters among the studied ITP cases (acute and chronic) versus the control**

Laboratory parameter	Acute (n = 15)	Chronic (n = 27)	Controls (n = 28)	p	p*	p**
ACA (IgM) (MPLU)	2.4±2.54	6.7±4.75	0.99±0.32	NS	<0.000	<0.000
ACA (IgG) (GPLU)	4.4±2.96	11.4±5.52	2.60±0.49	NS	<0.000	<0.000
Antiβ2GPI (IgG) (U L <sup>-1</sup> )	6.5±1.74	79.5±62.0	2.50±0.90	NS	<0.000	<0.000

p represents the significance difference between acute and control while p\* is the significance difference between chronic and control and p\*\* between acute and chronic ITP cases

**Table 4: The detection of elevated ACA (IgM), ACA (IgG) and anti-β2GPI(IgG) serum concentrations in acute versus chronic ITP cases**

Variable		Chronic (n = 27)	Acute (n = 15)	p-value
ACA (IgM) (MPLU)	Increase	1 (6.7%)	4 (14.8%)	NS
	Normal	14 (93.3%)	23 (85.8%)	
ACA (IgG) (GPLU)	Increase	4 (26.7%)	21 (77.8%)	p<0.001
	Normal	11 (73.3%)	6 (22.2%)	
Antiβ2GPI (IgG) (U L <sup>-1</sup> )	Increase	2 (13.3%)	27 (100%)	p = 0.000
	Normal	13 (36.7%)	none	

**Table 5: The relationship between the percentage of detection of elevated serum concentrations of ACA(IgM), ACA(IgG) and anti-β2GPI(IgG) among studied ITP patients with different steroid therapy response**

Variable	ITP cases			p	p*	p**
	Acute and remission Responsive (n = 11)	Chronic ITP				
		Dependent (n = 17)	Resistant (n = 10)			
ACA (IgM) (MPLU)	None	17.6% (3)	10% (1)	NS	NS	NS
ACA (IgG) (GPLU)	None	70.6% (12)	90% (9)	0.000	0.000	NS
Antiβ2GPI (IgG) (U L <sup>-1</sup> )	9.1% (1)	100% (17)	100% (10)	0.000	0.000	NS

p represents the significance difference between responsive and dependent while p\* is the significance difference between responsive and resistant and p\*\* between dependent and resistant ITP cases

ACA (IgM), ACA (IgG) and anti-β2GPI (IgG) in either acute or chronic ITP cases in comparison to their levels in controls. There was a significant serum elevation of the 3 studied laboratory tests in chronic ITP cases than that detected in either acute cases or controls (p = 0.000), but no significant difference of their serum concentrations between acute cases and controls.

Table 4 shows that about 78% of chronic ITP cases had elevated ACA (IgG) versus 26.7% in acute ITP cases (p<0.001). While, increased serum concentrations of anti-β2GPI (IgG) in all chronic ITP (27) versus 13.3% from acute cases was detected (p<0.000).

Table 5 demonstrates the relationship between the elevated ACA (IgM), ACA (IgG) and anti-β2GPI (IgG) serum concentrations and steroid therapy response of ITP cases. The percentages of patients with elevated ACA IgG and anti-β2GPI (IgG) were significantly higher among chronic ITP, either steroid dependent (70.6 and 100%) or resistant cases (90 and 100%), respectively when compared to that detected among acute steroid responsive ITP group (none, 9.1%), respectively (p<0.000). No significant difference of the percentage of detection of elevated ACA (IgM), ACA (IgG) and anti-β2GPI (IgG) in steroid dependent versus steroid resistant ITP cases.

There was a significant positive correlation between the elevated concentrations of anti-β2GPI (IgG) and increased ACA (IgG) levels (r = 0.42, p<0.01) but not

significantly related to elevated concentrations of IgM ACA. The detection of increased concentrations of IgG isotype of both ACA and anti-β2GPI is significantly correlated to resistance to steroid therapy (r = 0.54, p = 0.000). But neither increased levels of IgG ACA nor IgG anti-β2GPI related to steroid therapy response (r = 0.11, p = 0.4).

The Antiphospholipid Antibodies (APA) positivity is considered when either or both elevated concentrations of IgG isotype ACA or anti-β2GPI is detected. Present study revealed that 76.1% (32/42) of the studied ITP cases showed positive APA, including 5 cases from acute and all chronic cases (27). No significant difference of the platelet count in ITP cases with positive APA (106.7±118.7×10<sup>9</sup> L<sup>-1</sup>) and those with negative APA(113.7±144.3×10<sup>9</sup> L<sup>-1</sup>) (p = .89) was found. Splenectomy was done in 28.1% (9/32) of ITP children with positive APA. Elevated concentrations of IgG isotype of either ACA and anti-β2GPI was found in all 9 splenectomized ITP children while increased levels of IgM ACA was detected in 3 cases of them.

Follow up of the studied ITP children during the period from year 2000-2004 clarify that 16.7% (7/42) cases proved to develop clinical and laboratory criteria of Systemic lupus Erythematosus (SLE). Figure 1 shows descriptive data of initially diagnosed ITP cases who developed SLE disease. One (9.1%) (1/11) case of acute and in remission stage while 6 cases of chronic

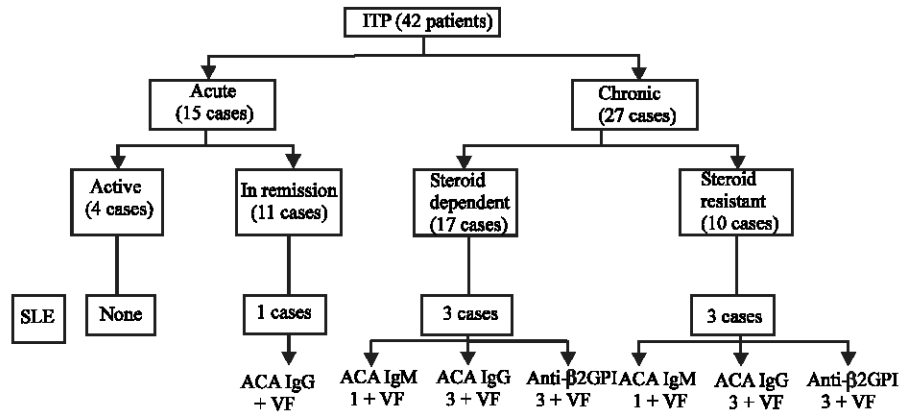


Fig. 1: Results of follow up study for initially presented ITP patients to show those who developed SLE and their antiphospholipid antibodies status at start of the study

(3(17.6%) steroid dependent and 3 (30%) steroid resistant) cases were discovered to develop clinical and laboratory evidence of SLE. Elevated concentrations of IgG isotype ACA was found in all 7 cases (100%) who proved to have SLE disease by follow up of previously diagnosed as ITP at the start of study. While 6 cases (85.8%) of them have increased levels of IgG anti-β2GPI. IgM ACA is found in only 2 cases (28.6%) of diagnosed SLE cases.

### DISCUSSION

Immune Thrombocytopenic Purpura (ITP) is a syndrome of primary acquired thrombocytopenia in the absence of marrow failure, in which platelets are destroyed by the host immune system (Wang and Shen, 1997). ITP is referred to as autoimmune thrombocytopenic purpura because the etiology of the disease is not clarified and thrombocytopenia is caused primarily by the generation of antibodies against platelets antigens. Antibodies against Phospholipid Antigen (APA) have been demonstrated in ITP patients (Ravelli and Martini, 1997), but the precise role played by increased serum concentrations of APA in the mechanism of thrombocytopenia has remained elusive (Rand, 1998).

The present study revealed a significant higher concentrations of ACA (IgM) (6.7±4.75 MPLU), ACA (IgG) (11.4±5.52 GPLU) and anti-β2GPI (IgG) (79.5±62.0 u L<sup>-1</sup>) in chronic ITP cases when compared to their corresponding levels in acute or control cases (p = 0.000). Elevated concentrations of IgG ACA were found in 26.7% of acute studied ITP versus 77.8% from chronic cases (p<0.001). Similarly, Harris *et al.* (1985) detected positive IgM ACA in 28.1% and IgG ACA in 14.6% in all studied ITP cases. While 46.3% of newly diagnosed ITP cases was found to have elevated

concentrations of ACA by Stasi *et al.* (1994) and IgG ACA was detected in 23.2% in children with ITP (Normura *et al.*, 1992). The variability of ACA detection may be attributed to either different sensitivity and specificity of assay used for detection.

Increased levels of IgG anti-β2GPI in all chronic (100%) versus 13.3% of acute ITP children was detected (p = 0.000). 77.8% of chronic ITP cases showed elevated serum concentrations of IgG ACA while increased serum levels of IgG anti-β2GPI in all (100%) chronic ITP cases was observed. This observation indicated significant proportion of chronic ITP cases had elevated levels of either or both IgG class of ACA and anti-β2GPI which reflects ongoing chronic process.

Moreover, a significant positive correlation between increased levels of IgG anti-β2GPI and increased serum concentrations of IgG ACA was determined (r = 0.42, p<0.01), but not related to elevated ACA (IgM) levels. This result agree with Nash *et al.* (2004) study that reported that patients with ACA (IgG) levels over 60 GPLU were found to be positive also for IgG anti-β2GPI.

Because of the clinical suspicion of autoimmune diseases in ITP cases, evaluation of Antiphospholipid Antibody (APA) status was done. This study revealed that 76.1% (32/42) of ITP cases had positive APA including all chronic and only 5 cases from acute studied ITP children. This result supports Diz-Kucukkaya *et al.* (2001) study who indicates that significant proportion of patients initially presented with ITP had positive APA. Splenectomy was done in 28.1% (9/32) of ITP children with positive APA. Elevated serum concentrations of IgG isotype of either ACA or anti-β2GPI in all 9 splenectomized ITP children with positive APA was observed, only 3 cases of them showed increased levels of IgM ACA. This observation consistent

with hypothesis that the plasma protein  $\beta$ 2GP1 is a major antigenic target of anticardiolipin autoantibodies in patients with antiphospholipid syndrome (Ravelli *et al.*, 1996; Harper *et al.*, 1998; Ong *et al.*, 2001). The platelet count is not significantly different between positive-APA versus negative-APA ITP cases. This result agreed with Normura *et al.* (1992), Stasi *et al.* (1994) and Sakai *et al.* (2002). They reported although it is common to find positive APA in ITP patients, but its presence is not associated with age, sex, platelet count decrease or bleeding severity. It confirms that the finding of positive APA in ITP patients not relevant to thrombocytopenia pathogenesis. Concerning the steroid therapy in our studied ITP cases, no significant different of the percentage of detection of increased of IgM ACA levels and the response of steroid therapy. Ninety percent of chronic steroid resistant and 70.6% of chronic steroid dependent ITP cases versus none of acute steroid responsive cases were found to have increased levels of IgG ACA levels ( $p = 0.000$ ). While elevated serum concentrations of anti- $\beta$ 2GP1 was detected in all chronic whether steroid dependent or resistant ITP cases versus 9.1% of acute patients in remission ( $p = 0.000$ ). The detection of increased concentrations of IgG isotype of both ACA and anti- $\beta$ 2GP1 is significantly correlated to steroid therapy resistance ( $r = 0.54$ ,  $p = 0.000$ ). This result is contradictory to reports that indicate no different response to steroid therapy in ITP cases with positive APA (Stasi *et al.*, 1994).

Furthermore, follow up of our studied ITP children during the period from 2000-2004 revealed that 16.7% (7 cases) proved to develop clinical and laboratory criteria of Systemic Lupus Erythematosus (SLE). While 83.3% (35/42) neither showed clinical reports of other autoimmune diseases nor history of thrombosis. These observation matches with Stasi *et al.* (1994) and may be attributed to relative short period of observation. The study clarified that the discovered SLE cases consisted of one case (9.1%) of acute and in remission stage and 6 chronic (3 cases (17.6%) steroid dependant and 3 cases (30%) steroid resistant) ITP cases. This finding support the statement of autoimmune thrombocytopenia is a frequent accompanying problem in children with APS (Ravelli and Martini, 1997). Elevated serum concentrations of IgG ACA was found in all 7 cases who proved to have SLE disease by fellow up of initially diagnosed ITP children at the start of study while 6 cases (85.7%) from them have increased levels of anti- $\beta$ 2GP1. Only 2 cases of them (28.6%) showed increased serum concentrations of IgM ACA. These findings go in parallel with Ravelli *et al.* (1996). Study who detected ACA and anti- $\beta$ 2GP1 in all studied SLE patients. It confirms the high prevalence of

ACA in pediatric SLE was observed in 30-87% (Ravelli and Martini, 1997; Palomo *et al.*, 2002; Cervera and Asherson, 2003).

In the light of the present results, it is emphasized that the assay of IgG class of both ACA and anti- $\beta$ 2GP1 may be considered as determinant cofactors for the developing risk of APS or autoimmune diseases in ITP patients. Moreover, great attention should paid to both assay as predictors for steroid therapy response. The presence of APA in ITP patients not relevant to the pathogenesis of thrombocytopenia. Nevertheless, further study are needed to address this important issue because of the small number of patients in our series.

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