

Original Article

Immune Thrombocytopenia in Older Adults: Age-Related and Disease-Specific Alterations in Platelets

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ABSTRACT: Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder with a higher incidence among older adults. Aging per se alters platelet characteristics, including reduced reactivity and increased caspase activity, changes also observed in ITP. The aim of this study was to assess the impact of aging on the hemostatic characteristics of patients with ITP and its potential contribution to the increased burden of the disease. Additionally, the study sought to identify characteristics of the disease itself, independent of age. Patients with ITP over 65 years of age (ITP>65) and patients aged 65 or under (ITP≤65) were included, as well as age-matched healthy controls (HC). Basal P-selectin exposure was higher in the ITP≤65 than in the HC≤65 group but no difference was observed between the ITP>65 and HC>65 groups. This may be due to the synergistic effect of disease and aging, because P-selectin exposure was higher in the HC>65 group than in the HC≤65 group. The ITP>65 patients exhibited reduced platelet responsiveness to agonists and increased caspase-3/7 activity compared with the ITP≤65 patients. Their clots were more resistant to lysis, likely due to elevated plasma levels of circulating cell-free DNA (cfDNA) and plasminogen activator inhibitor-1 (PAI-1). Comparisons between the ITP≤65 and HC≤65 groups as well as the ITP>65 and HC>65 groups revealed ITP-specific features, such as reduced sialic acid residues due to elevated neuraminidase-1 (NEU1) exposure. These findings showed that age-related changes in haemostasis can overlap with disease-specific features, highlighting the need for age-stratified approaches.

Keywords: Immune thrombocytopenia, aging; platelets, coagulation, desialylation

INTRODUCTION

Immune thrombocytopenia (ITP) is an acquired, immune-mediated disorder characterised by a platelet count of

<100×10⁹/L in peripheral blood, with an increased risk of bleeding [1].

A number of mechanisms contribute to lower platelet counts in ITP. Loss of immune tolerance results in a

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hyperactive adaptive immune system, in which a regulatory T-cell deficiency allows T follicular helper cells to stimulate autoreactive splenic B-cells that contribute to the production of autoantibodies against platelets [2].

Platelet-glycoprotein antibodies and/or CD8+ T-cells targeting megakaryocytes and platelets mediate increased apoptosis, cytotoxic platelet destruction, Fc receptor-mediated phagocytosis by macrophages, and complement-mediated destruction of platelets or platelet desialylation followed by clearance in the liver [3, 4].

The incidence of ITP increases with age, reaching its peak in individuals over 80 years old [5]. This is possibly because aging is a significant risk factor for autoimmunity due to ongoing adaptations of the immune system to environmental exposures over a lifetime.

In addition, aging affects the platelet phenotype. This results in a reduced platelet response to thrombin receptors PAR-1 and PAR-4, accompanied by an increased exposure of P-selectin and CD63 on the surface of quiescent platelets [6]. This may explain the increased coagulability observed in elderly individuals. These effects of aging on platelet characteristics are similar to those observed in platelets from adult patients with ITP [7]. However, the specific characteristics of older patients with ITP have not been thoroughly documented.

In this scenario and according to the conventional definition of “elderly” as individuals over 65 years of age [8], the aim of this study was to examine haemostasis in patients with ITP over 65 years of age (ITP>65) compared to ITP patients aged 65 or under (ITP≤65). A comparison was also made between the ITP groups and age-matched

healthy controls (HC). This study aimed to evaluate how aging influences the hemostatic profile of patients with ITP and its possible role in increasing disease burden. It also aimed to identify disease-specific characteristics that are independent of age.

METHODS

Study design

This was an observational, and prospective study that included adult patients (>18 years) with chronic primary ITP, stratified by age into two groups: ≤65 and >65 years, with an inclusion period between September 2019 and June 2024, in the Haematology units of our hospitals.

Clinical and treatment characteristics of these patients were collected from electronic medical records. HCs were also recruited at the same hospitals to provide two age- and sex-matched control groups (≤65 and >65 years) for the two cohorts of patients with ITP.

Patients who had undergone B-cell depletion therapy within three years of the study, or who were taking anticoagulant (e.g. heparins or direct oral anticoagulants) and/or antiplatelet (e.g. aspirin or P2Y12 inhibitors) medications, or who had undergone a platelet or blood transfusion within 15 days of the study, were excluded from the study.

This study was authorized by the local medical Ethics Committee of La Paz University Hospital Hospital Universitario La Paz (PI-5524), and it was conducted in accordance with the Declaration of Helsinki. Signed informed consent was obtained from the participants.

Table 1. The demographic characteristics of the healthy controls (HC) and patients with immune thrombocytopenia (ITP) included in the study are outlined below.

	Aged ≤65		Aged >65	
	HC	ITP	HC	ITP
N	150	125	60	65
Age (Mean ± SD)	42 ± 12	44 ± 14	78 ± 9	76 ± 7
Female (N, %)	70 (46.7)	73 (58.4)	33 (55)	42 (64.6)
Platelet count (Mean x10 ⁹ /L ± SD)	241±61	137±113*	243±113	141±118*
Platelet volume (Mean fL ± SD)	7.1±0.9	8.0±1.2*	8.0±0.8	8.2±1.5

The Mann-Whitney test was performed to compare the HC≤65 and ITP≤65 groups and the HC>65 and ITP>65 groups. A p-value of less than 0.05 was considered significant. *Significant difference between each ITP group and its age-matched HC group.

Demographics of patients with ITP and clinical data

During the inclusion period, 144 patients ≤65 years (ITP≤65 yo) and 101 patients >65 years (ITP>65 yo) were treated in our unit. Of these, 19 from the ITP≤65 yo group and 36 from the ITP>65 yo group were excluded because they were treated with antiplatelet/anticoagulant therapy and/or B-cell depletion therapy (Fig. 1). As observed,

more individuals had to be excluded from the ITP>65 yo group because they were on antiplatelet/anticoagulant therapy. HC≤65 yo (n=150) and >65 yo (n=60) were also included for sake of comparison. The healthy control groups aged ≤65 and >65 years were recruited either at the Blood Donor Unit or at the Geriatrics Department of La Paz University Hospital. The healthy control participants had no known conditions that could affect the study

outcomes. Table 1 presents the age, sex distribution, and platelet count and volume data for the HC and ITP cohorts included in the study. The mean platelet volume of

platelets from all ITP patients (ITP $\leq$ 65 yo and ITP $>$ 65 yo) and from HC $>$ 65 yo were larger than that from HC $\leq$ 65 yo.

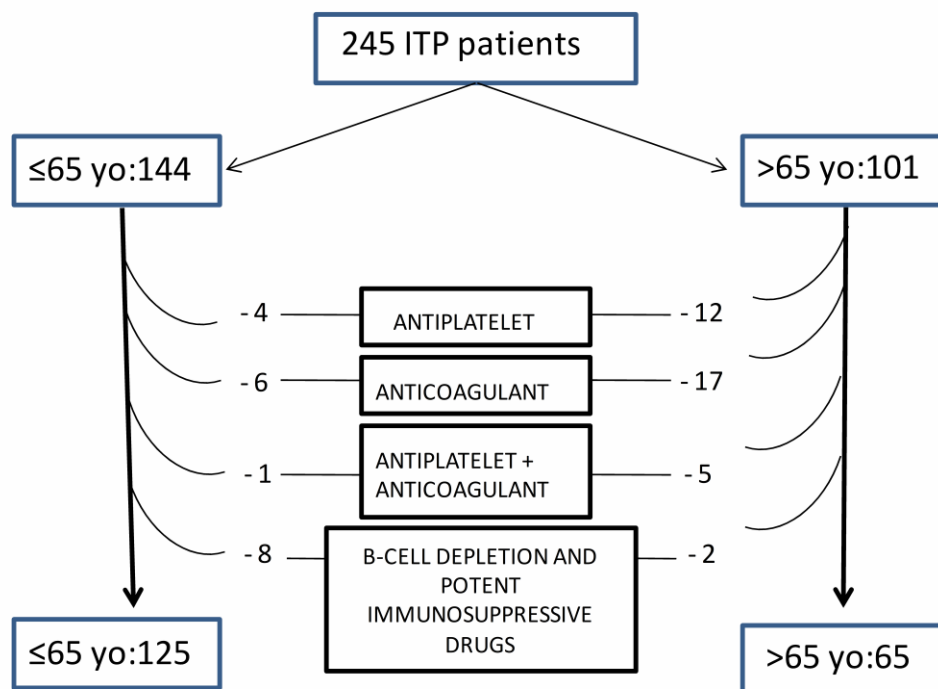


Figure 1. Distribution of patients with ITP treated during the inclusion period (September 2019 and June 2024), in the Hematology units of La Paz University Hospital and Severo Ochoa University Hospital.

Sample collection and preparation

We collected human peripheral blood samples in 3.8% sodium citrate tubes. Platelet-free plasma (PFP) was obtained by centrifugation at 1,500 g for 15 min at 23 °C, followed by two additional centrifugations (first: 15 min at 1,500 g; second: 2 min at 13,000 g), and platelet-rich plasma (PRP) was obtained by centrifugation of whole blood (150 g for 10 min at 23 °C). To obtain washed platelets, the upper two-thirds volume of the PRP was collected and centrifuged (650 g for 10 min at 23 °C) after the addition of acid-citrate-dextrose (1:10). The pellet was resuspended in an equal volume of HEPES buffer (10 mM HEPES, 145 mM sodium chloride, 5 mM potassium chloride and 1 mM magnesium sulphate, pH 7.4).

For serum preparation, peripheral blood was collected in serum tubes (BD Vacutainer, Plymouth, UK) and separated by centrifugation of clotted blood (2,500 g for 15 min at 23°C). Plasma and serum aliquots were stored at -80°C until analysis.

Determination of platelet characteristics

The activation state of the fibrinogen receptor, and P-selectin and CD63 exposure were determined using FITC-

PAC1 and the corresponding fluorescent monoclonal antibodies (mAbs), respectively. Sialic acid residues on the platelet surface were indirectly determined by incubating washed platelets (50×10^3 platelets/ μ L) with FITC-Ricinus communis agglutinin (RCA; Vector Laboratories, Barcelona, Spain) [9, 10].

Neuraminidase 1 (NEU1) was determined by incubating diluted PRP with Alexa Fluor® 546-anti-NEU1 mAb (Santa Cruz Biotechnology, Inc., USA; SC-166824_AF546). Samples were analysed by flow cytometry [11].

Active caspase-3/7, -8 or -9 were determined using Millipore kits (Madrid, Spain). All the samples were analysed by flow cytometry within two hours of blood collection.

Refer to Table 2 for details of the reagents used for flow cytometry and the platelet activator employed.

Rotational thromboelastometry

Rotational thromboelastometry is a viscoelastometric coagulation test that measures the kinetics of clot formation and fibrinolysis. This test was performed on patients and controls for whom sufficient blood was available. Hence, we performed ROTEM® analyses on 35

people in the HC $\leq$ 65 group (60% female, mean age 43.5 years; platelet count (range) 231 (141-380) $\times 10^3$ platelets/ μ L); 40 ITP $\leq$ 65 patients (55.8% female, mean age 41.3 years; platelet count (range) 134 (20-469) $\times 10^3$ platelets/ μ L); 30 people in the HC $>$ 65 group (48.5%

female, mean age 75.4 years; platelet count(range) 280.9 (108-609) $\times 10^3$ platelets/ μ L); and 35 ITP $>$ 65 patients (53.3% female, mean age 72.9 years; platelet count (range) 155 (6-537) $\times 10^3$ platelets/ μ L).

Table 2. Description of the reagents used for flow cytometry and platelet activator employed.

Reagent Cat #		Fluorophore	Clone, RRID	Provider
Antibody 340507	Mouse-anti human PAC1 (binds to the activated glycoprotein IIb/IIIa)	FITC	PAC-1, AB_2230769	BD Biosciences
Antibody 555523	Mouse Anti-Human CD62P	FITC	AK-4, AB_395909	BD Pharmingen™
Antibody 557288	Mouse Anti-Human CD63	FITC	H5C6, AB_396617	BD Pharmingen™
Antibody sc-166824	Mouse antihuman Neuraminidase 1	Alexa Fluo 546	H7, AB_2149061	Santa Cruz Biotechnology
VC-FL-1081	Ricinus communis agglutinin I (RCA I)	FITC		Vector Laboratories
Kit APT403	CaspaTag™ Caspase-3/7 In Situ Assay Kit	FITC	FLICA with (FAM-DEVD-FMK) reagent	Millipore
Kit APT408	Caspatag™ Caspase 8 in situ Assay kit	FITC	FLICA with (FAM-LETD-FMK) reagent	Millipore
Kit APT409	Caspatag™ Caspase 9 in situ Assay kit	FITC	FLICA with (FAM-LEHD-FMK) reagent	Millipore
Platelet activator SP-5061-25	TRAP-6 (Thrombin Receptor Activator Peptide 6) (SFLLRN)	None		Innovagen

RRID: Research Resource Identifier. FLICA: Fluorochrome Inhibitors of Caspases. FITC: Fluorescein Isothiocyanate

Rotational thromboelastometry (ROTEM®, Werfen, Madrid, Spain) was performed on fresh PRP adjusted to 25×10^9 platelets/L with the subjects' own PFP within two hours of blood collection. Recalcification (NATEM) was used as a trigger.

We recorded the clotting time (CT, time from the start of measurement to the start of clotting, in seconds); clot formation time (CFT, time from the start of clotting to 20 mm of amplitude, in seconds, which reflects the speed of the clotting process); α angle (tangent to the curve at 2 mm amplitude, in degrees, which reflects the rate of fibrin polymerization); maximum clot firmness (MCF, in mm, which reflects the maximum tensile strength of the thrombus); and lysis at 60 minutes (Ly60, in %; residual clot firmness 60 minutes after CT).

PAI-1 and cell-free DNA

Plasminogen activator inhibitor-1 (PAI-1; eBioscience, Hatfield, UK) and cell-free DNA (cfDNA; Quant-iT-PicoGreen dsDNA assay, Thermo Fisher Scientific, Waltham, MA, USA) were determined in PFP, following the manufacturer's instructions.

Anti-platelet antibodies

Anti-platelet antibodies (anti-GPIIb/IIIa, anti-GPV and anti-GPIb/IX) were measured in serum samples using indirect MAIPA (monoclonal antibody immobilisation of

platelet antigens) assay [12]. This test was performed on patients for whom sufficient blood was available.

Statistical analyses

The sample size required to detect differences between the ITP $\leq$ 65 and ITP $>$ 65 groups was calculated using G*Power (version 3.1.9.7) with an alpha level of 0.05 and a power of 0.80. The size effect was calculated based on the means and standard deviations obtained in preliminary experiments. Thus, 42 individuals per group were needed for platelet function analysis, and 24 for the ROTEM® Ly60 parameter analysis.

The data were analysed by GraphPad Prism software 6.0. Mann-Whitney or Student's t-tests were performed, and the data were expressed as median (percentile 25%–percentile 75%) or mean $\pm$ standard deviation (SD) depending on the sample distribution.

Multiple comparisons (ITP $\leq$ 65 vs ITP $>$ 65, ITP $\leq$ 65 vs HC $\leq$ 65, ITP $>$ 65 vs HC $>$ 65, and HC $\leq$ 65 vs HC $>$ 65) were performed by 2-way ANOVA followed by Bonferroni adjustments.

The correlation analysis was performed using Pearson's or Spearman's test. All tests were two-tailed and statistical significance was set at $p < 0.05$.

RESULTS

Demographics of patients with ITP and clinical data

At the time of sampling, a higher proportion of the ITP $\leq$ 65 group were not receiving treatment for ITP compared with the ITP $>$ 65 group (Table 3). Among the ITP $\leq$ 65 patients receiving treatment, the most commonly used drugs were

thrombopoietin receptor agonists (TPO-RAs) alone (60.0%) or in combination with other drugs (15.7%). Among the ITP $>$ 65 receiving treatment, the proportions were, respectively, 66.0% and 22.0%.

There was no difference between the two ITP groups in terms of either the number of previous lines of treatment or the percentage of patients who had undergone splenectomy (Table 4).

Table 3. Treatment received by patients with ITP at the time of sample collection in each cohort of ITP patients.

TREATMENT AT SAMPLING		+IVIg	+CORT	+ROMI	+AVA	CORT+ROMI+IVIg
ITP $\leq$ 65						
WITHOUT, n (%)	55 (44%)					
IVIg, n (%)	4 (3.2%)					
CORT, n (%)	5 (4.0%)	3 (2.4%)				
ETP, n (%)	22 (17.6%)	1 (0.8%)				
ROMI, n (%)	16 (12.8%)	3 (2.4%)	2 (1.6%)			
AVA, n (%)	4 (3.2%)					
FOST, n (%)	5 (4.0%)			4 (3.2%)		1 (0.8%)
ITP $>$ 65						
WITHOUT, n (%)	15 (23.1%)					
IVIg, n (%)	1 (1.5%)					
CORT, n (%)						
ETP, n (%)	13 (20.0%)		2 (3.1%)			
ROMI, n (%)	17 (26.2%)		5 (7.7%)			
AVA, n (%)	3 (4.6%)		2 (3.1%)			
FOST, n (%)	1 (1.5%)		4 (6.2%)	1 (1.5%)	1 (1.5%)	

IVIg: intravenous immunoglobulins; CORT: corticosteroids; ETP: eltrombopag; ROMI: romiplostim; AVA: avatrombopag; FOST: fostamatinib; Spl: splenectomized.

Increased baseline P-selectin and reduced response to platelet stimulation in ITP $>$ 65 patients compared to ITP $\leq$ 65 patients

No significant differences were observed in basal fibrinogen receptor activation or CD63 exposure between the ITP $\leq$ 65 and ITP $>$ 65 (Fig. 2A and 2B). However, both ITP groups had elevated CD63 surface exposure compared to their respective HC groups (Fig. 2B). P-

selectin exposure levels were higher in the ITP $\leq$ 65 group than in the HC $\leq$ 65 group. However, no significant difference was observed between the ITP $>$ 65 and HC $>$ 65 groups. This finding may reflect a synergistic effect of disease and aging, as P-selectin exposure was already elevated in the HC $>$ 65 group compared with the HC $\leq$ 65 group (see Fig. 2C). There were no significant differences observed between patients with ITP $\leq$ 65 and those with ITP $>$ 65.

Table 4. Number of previous lines of treatment received for ITP and splenectomized patients in each cohort of ITP patients. Spl: splenectomized.

Number of Previous lines of Treatment for ITP		
	ITP $\leq$ 65	ITP $>$ 65
1, n (%)	39 (31.2%)	15 (23.1%)
2, n (%)	22 (17.6%)	8 (12.3%)
3, n (%)	26 (20.8%)	20 (30.8%)
4, n (%)	22 (17.6%)	8 (12.3%)
>4, n (%)	16 (12.8%)	14 (21.5%)
Spl, n (%)	19 (15.2%)	10 (15.4%)

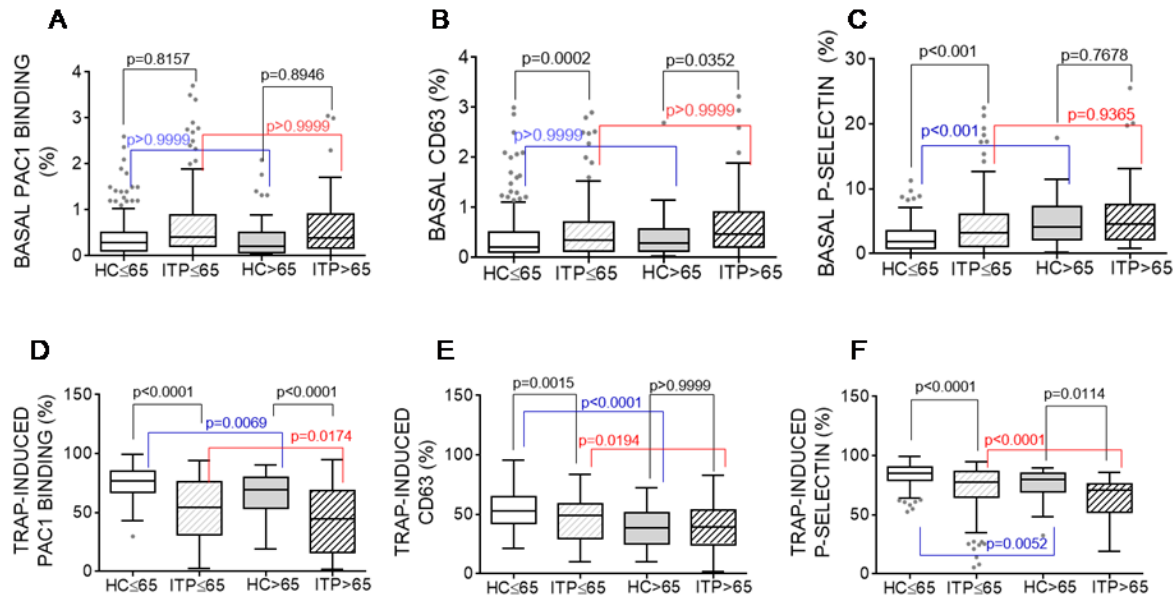


Figure 2. Platelet activation markers. Basal (A, B, C) and TRAP-induced (D, E, F) platelet activation markers were assessed by flow cytometry and are expressed as the percentage of positive cells. Figures represent median with interquartile (25th-75th) percentile; points outside the whiskers represent outliers and were included in the statistical analyses. Sample sizes were as follows: ITP≤65, $n = 125$; ITP>65, $n = 65$; HC≤65, $n = 150$; and HC>65, $n = 60$. Statistical comparisons were performed using two-way ANOVA followed by Bonferroni correction for multiple comparisons. A corrected $P < 0.05$ was considered statistically significant. Significant differences between ITP and their age-matched HC groups are indicated in black; between ITP≤65 and ITP>65 in red; and between HC≤65 and HC>65 in blue. ITP, immune thrombocytopenia; HC, healthy controls; TRAP, thrombin receptor-activating peptide; PAC1, antibody recognizing the activated fibrinogen receptor.

After stimulation with thrombin receptor-activating peptide (TRAP), platelets from ITP>65 patients exhibited reduced binding of PAC1 (Fig. 2D) and decreased exposure of CD63 (Fig. 2E) and P-selectin when compared with platelets from the ITP≤65 group (Fig. 2F). Platelets from both ITP groups showed reduced TRAP-induced fibrinogen receptor activation (Fig. 2D), and P-selectin exposure than their HC groups (Fig. 2F), whereas a reduced CD63 exposure was only evident in the ITP≤65 group compared to HC≤65 group (Fig. 2E).

Increased caspase-3/7 activity in platelets from ITP>65 patients compared to ITP≤65 patients

The caspase-3/7 activity of platelets from ITP>65 patients was higher than from ITP≤65 patients (Fig. 3A), whereas caspase-8 and -9 activity was comparable between the two ITP groups (Fig. 3B and 3C). Additionally, caspase activity in platelets from all ITP patients was elevated compared with the age-matched HC group even though aging in itself led to an increase in platelet caspase activity (Fig. 3A, 3B, and 3C).

We observed an inverse correlation between platelet count and caspase-3/7, -8 and -9 activity in ITP patients (Fig. 3D, 3E, 3F).

Comparable loss of sialic acid on the platelet surface in ITP≤65 and ITP >65 patients

Platelet clearance due to exposure of β -galactose, may be a contributing factor to the etiopathogenesis of ITP. The exposure of β -galactose, resulting from the loss of sialic acid from the carbohydrate side chains of platelet glycoproteins, was evaluated through RCA binding. Thus, a higher level of RCA binding corresponded to a lower level of sialic acid content. As shown in Figures 4A and 4B, platelet surface RCA binding and NEU1 exposure (a sialidase involved in the removal of sialic acid residues) were similar in patients with ITP ≤ 65 and ITP > 65. These platelets exhibited lower sialic acid content and higher levels of NEU1 exposure compared to platelets from age-matched HC (Fig. 4A and 4B). Notably, aging in the control group (HC > 65) was associated with decreased RCA binding, i.e., increased sialic acid exposure, accompanied by reduced NEU1 on platelet surfaces.

In addition, an inverse correlation was observed between platelet count and binding of RCA in ITP patients (Fig. 4C), giving support to the involvement of desialylation as a mechanism of platelet's clearance in ITP. Conversely, no significant correlation was found between RCA binding and caspase activities (RCA binding vs. caspase-3/7: Spearman's $r = -0.08773$,

$p=0.6509$; vs. caspase-8: Spearman's $r=-0.3056$,
 $p=0.6509$; and vs. caspase-9: Spearman's $r=-0.1119$,
 $p=0.5634$).

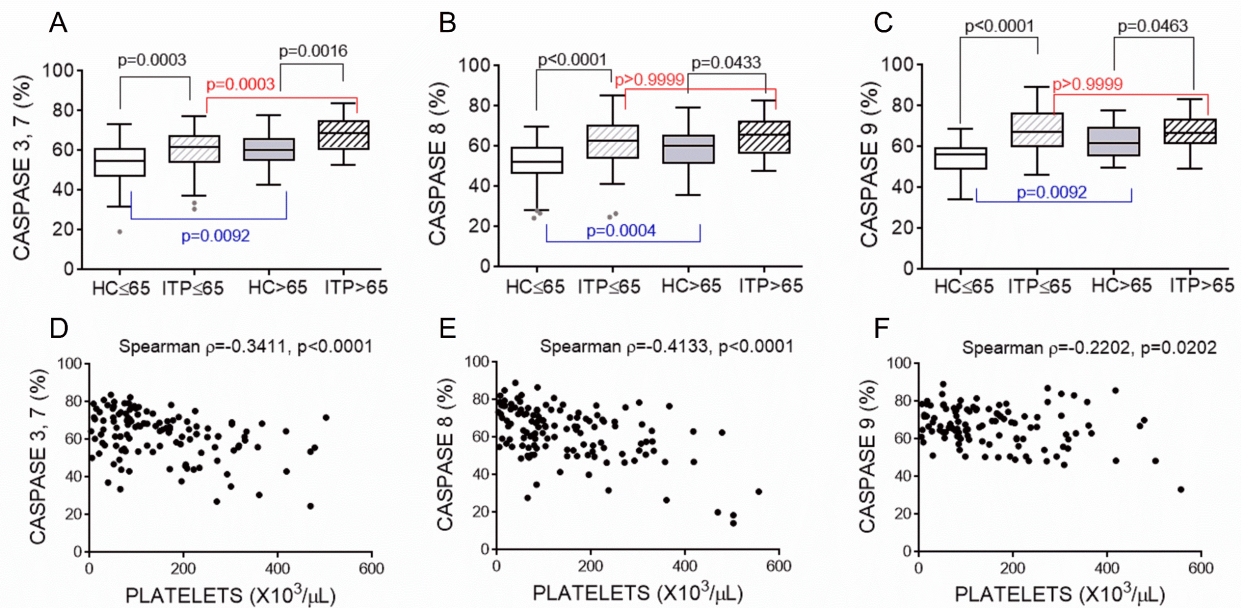


Figure 3. Caspase activity in quiescent platelets and its association with platelet count. (A, B and C) Activities of caspases-3/-7, -8, and -9 were measured in quiescent platelets by flow cytometry and are expressed as the percentage of positive cells. Data are presented as the median with interquartile range (25th–75th percentiles). Sample sizes were as follows: ITP≤65, $n = 90$; ITP>65, $n = 50$; HC≤65, $n = 100$; and HC>65, $n = 50$. Statistical comparisons were performed using two-way ANOVA followed by Bonferroni correction for multiple comparisons. A corrected $P < 0.05$ was considered statistically significant. Significant differences between ITP and their age-matched HC groups are indicated in black; between ITP≤65 and ITP>65 in red; and between HC≤65 and HC>65 in blue. (D, E and F) Correlations between platelet count and the activities of caspases-3/-7, -8, and -9 were assessed using Spearman's rank correlation test. A $P < 0.05$ was considered statistically significant. ITP, immune thrombocytopenia; HC, healthy controls.

The effect of anti-platelet antibodies on caspase activities and desialylation in platelets

The presence of antiplatelet antibodies has been associated with increased desialylation and apoptosis of platelets [13]. We analyzed the presence of these antibodies in plasma of patients with ITP when blood samples were available. Of the 59 patients with ITP≤65,

25 had detectable antibodies, and of the 52 patients with ITP>65, 16 had detectable antibodies. Figure 5A shows the specificity and distribution of antiplatelet antibodies in each age group. Whilst the inability to determine the presence of antiplatelet glycoprotein antibodies is a limitation of this study, the fact that the positivity rate is similar to that reported by other authors [14] validates the representativeness of these results.

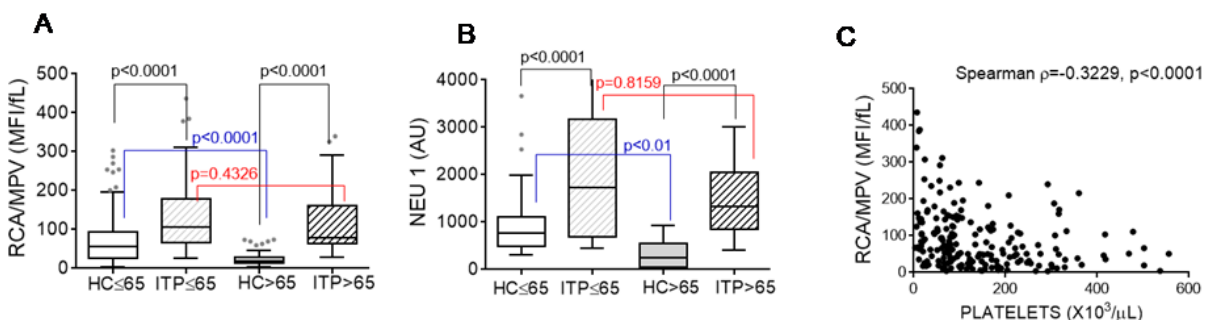


Figure 4. Platelet desialylation and neuraminidase surface expression. (A) Binding of *Ricinus communis* agglutinin (RCA), expressed as the ratio of mean fluorescence intensity (MFI) to mean platelet volume (MPV; fL), and (B) surface expression of neuraminidase 1 (NEU1), expressed as arbitrary units (AU), were evaluated by flow cytometry. Data are presented as the median with interquartile range (25th–75th percentiles); points outside the whiskers represent outliers and were included in the statistical

analyses. Sample sizes were as follows: ITP $\leq$ 65, $n = 125$; ITP $>$ 65, $n = 65$; HC $\leq$ 65, $n = 150$; and HC $>$ 65, $n = 60$. Statistical comparisons were performed using two-way ANOVA followed by Bonferroni correction for multiple comparisons. A corrected $P < 0.05$ was considered statistically significant. Significant differences between ITP and their age-matched HC groups are indicated in black; between ITP $\leq$ 65 and ITP $>$ 65 in red; and between HC $\leq$ 65 and HC $>$ 65 in blue. (C) The correlation between RCA/MPV and platelet count was assessed using Spearman's rank correlation test. A $P < 0.05$ was considered statistically significant. ITP, immune thrombocytopenia; HC, healthy controls.

We examined whether the presence of antiplatelet antibodies in our cohorts altered caspase activity and desialylation without stratifying by age to increase the statistical power of the analysis. No difference in caspase activity was observed between ITP patients with and without antiplatelet antibodies (Fig. 5B). However, platelets from ITP patients with antibodies showed reduced surface sialic acid content compared with platelets from ITP patients without antibodies (Fig. 5C).

Reduced clot lysis in ITP $>$ 65 patients compared with ITP $\leq$ 65 patients

The dynamics of clot formation revealed no significant differences in CFT and α -angle (data not shown), or MCF (Fig. 6A) between patients with ITP $\leq$ 65 and ITP $>$ 65.

However, clots from the ITP $>$ 65 group exhibited a reduced lysis after 60 minutes compared with the ITP $\leq$ 65 group (Figure 6B). This increased resistance to clot lysis might be due to the higher levels of cfDNA (Fig. 6C) and PAI-1 (Fig. 6D) found in plasma from ITP $>$ 65 patients compared with ITP $\leq$ 65 patients.

It is important to note that the ITP $\leq$ 65 patients formed clots with a higher MCF and displayed greater resistance to lysis than the HC $\leq$ 65 (Figure 6A and 6B). This may be due to their higher plasma levels of cfDNA (Fig. 6C), but not of PAI-1 (Fig. 6D). Conversely, clots formed by samples from ITP $>$ 65 patients exhibited equivalent MCF to their HC $>$ 65 (Figure 6A), but showed heightened resistance to lysis (Fig. 6B) and elevated plasma PAI-1 levels (Fig. 6D).

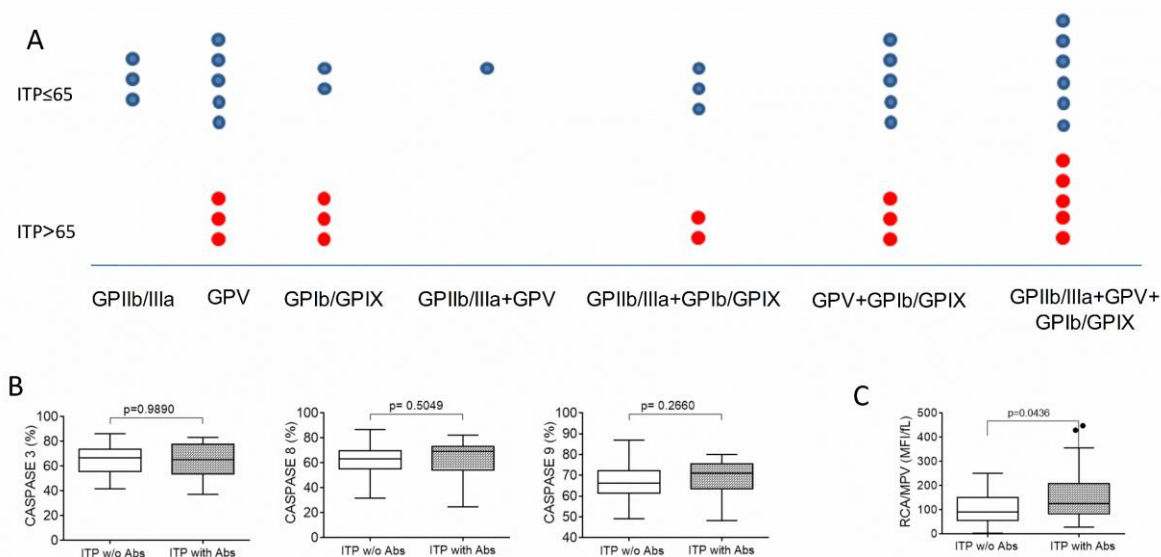


Figure 5. Association between antiplatelet antibodies, caspase activity, and platelet desialylation in patients with immune thrombocytopenia (ITP). (A) Distribution and glycoprotein specificity of antiplatelet antibodies in the ITP $\leq$ 65 and ITP $>$ 65 groups. Antiplatelet antibodies were evaluated in 59 patients with ITP $\leq$ 65 and in 52 patients with ITP $>$ 65. Each point represents one patient positive for antiplatelet antibodies. (B) Activities of caspases-3/-7, -8, and -9, expressed as the percentage of positive cells, and (C) binding of *Ricinus communis* agglutinin (RCA), expressed as the ratio of mean fluorescence intensity (MFI) to mean platelet volume (MPV; fL), were measured in quiescent platelets by flow cytometry in patients with ITP who were tested for the presence of antiplatelet glycoprotein antibodies. Data are presented as the median with interquartile range (25th–75th percentiles); points outside the whiskers represent outliers and were included in the statistical analyses. Comparisons between groups were performed using the Mann–Whitney U test. A $P < 0.05$ was considered statistically significant.

DISCUSSION

This study identified differences in haemostasis between ITP $>$ 65 and ITP $\leq$ 65 patients. Platelets from the ITP $>$ 65

group showed reduced activation by agonists than those from ITP $\leq$ 65. The impairment in platelet stimulation in the ITP $>$ 65 group seemed to be a synergic effect of

disease and aging because HC>65 had a reduced response to agonist's stimulation when compared to HC≤65.

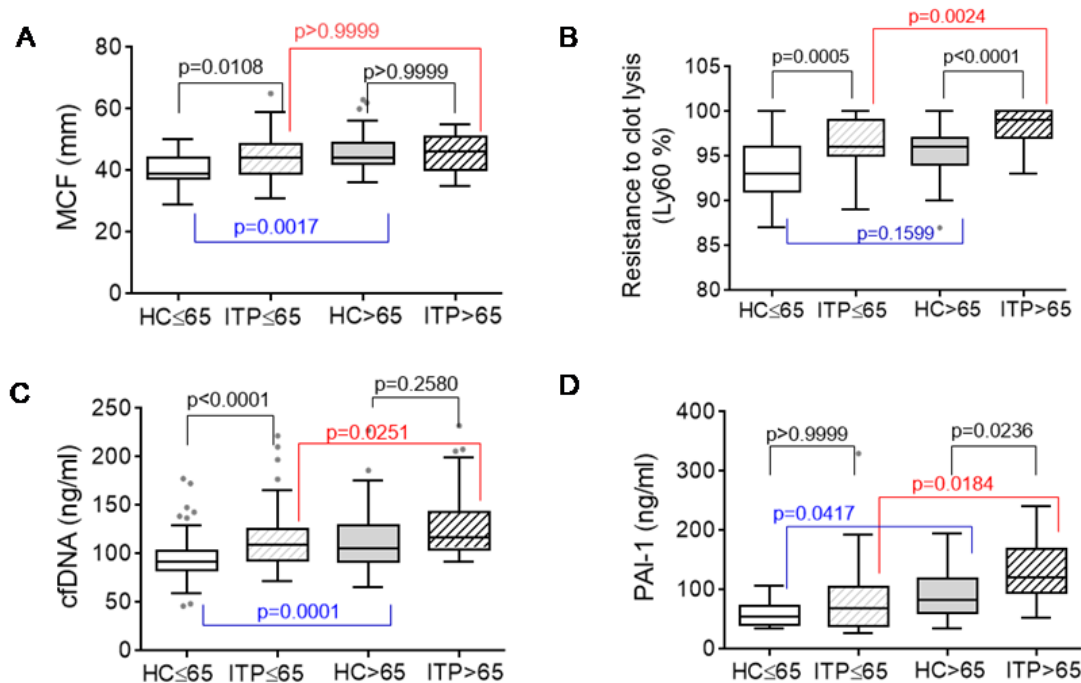


Figure 6. Clot formation kinetics and plasma cell-free nucleic acid DNA (cfDNA) and Plasma plasminogen activator inhibitor-1 (PAI-1) in patients with immune thrombocytopenia (ITP) and healthy controls (HC). Rotational thromboelastometry was performed in platelet-rich plasma adjusted to 25×10^9 platelets/L. Data are presented as the median with interquartile range (25th–75th percentiles); points outside the whiskers represent outliers and were included in the statistical analyses. Sample sizes for these determinations were as follows: ITP≤65, n = 40; ITP>65, n = 35; HC≤65, n = 35; and HC>65, n = 30. **(A)** Maximum clot firmness (MCF, mm). **(B)** Resistance to clot lysis (Ly60, %). **(C)** cfDNA levels (ng/mL). **(D)** PAI-1 levels (ng/mL). Statistical comparisons were performed using two-way ANOVA followed by Bonferroni correction for multiple comparisons. A corrected $P < 0.05$ was considered statistically significant. Significant differences between ITP and their age-matched HC groups are indicated in black; between ITP≤65 and ITP>65 in red; and between HC≤65 and HC>65 in blue.

Platelet samples from both ITP groups exhibited a higher activity of caspases in comparison to their corresponding control groups. However, it is important to note that aging itself also increased platelet's caspase activity. Furthermore, platelets from patients with ITP>65 exhibited a significantly higher level of caspase-3/7 activity compared to those with ITP≤65.

Our research also revealed that ITP>65 clots exhibited higher resistance to lysis compared to ITP≤65 and HC>65 clots. This finding is likely attributable to the elevated levels of cfDNA and PAI-1 in the ITP>65 group. A higher proportion of ITP>65 patients received pharmacological treatment than those in the ITP≤65 group (77% vs 56%), which matches previous findings [15].

TPO-RAs emerged as the predominant second-line option in older ITP patients (64.7% of ITP>65 vs. 38.4% of ITP≤65 patients), despite the potential thrombotic risks associated with them [16–18].

During the five-year inclusion period, 33.7% of ITP>65 patients were excluded due to prior thrombotic

events requiring anticoagulant and/or antiplatelet therapy, compared to 7.6% of ITP≤65 patients. These findings provide further evidence of the prothrombotic nature of ITP [16, 19, 20] and the age-related increase in thrombotic risk [21, 22].

One potential factor contributing to the observed elevated risk of thrombosis in patients with ITP is an altered fibrinolysis. Thrombelastography indicates the presence of prothrombotic mechanisms in ITP, revealing reduced fibrinolysis in both ITP≤65 and ITP>65 patients compared with their age-matched controls. This clot resistance to lysis was more pronounced in the ITP>65 group, possibly due to the higher levels of cfDNA and of PAI-1. Previous studies have reported increased PAI-1 levels and reduced tPA activity in ITP patients [23–25]. tPA-PAI-1 complex levels were positively associated with clot lysis time, whereas plasmin generation parameters were not [25]. In support to the lack of relationship between plasmin activity and clot lysis time, Sang et al. demonstrated that loss or suppression of

plasmin activity does not increase venous thrombus formation in mice and that plasminogen levels or activity were not associated with venous thromboembolism in humans [26]. With regard to the involvement of cfDNA in fibrinolysis, it has been reported that cfDNA suppresses fibrinolysis by accelerating the inactivation of tPA by PAI-1 [27]. Elevated cfDNA levels may be the result of endothelial activation [28] and of NET formation [29].

Basal platelet activation may also contribute to the procoagulant profile in ITP. Quiescent platelets from ITP $\leq$ 65 patients showed higher exposure of P-selectin from α - granules than those from HC $\leq$ 65. There are no significant differences between ITP $>$ 65 patients and HC $>$ 65 because aging per se elevated exposure of P-selectin on platelet's surface. This is consistent with the previously reported age-related increase in platelet degranulation, as evidenced by proteomic analysis [30].

The release of CD63 from dense granules in resting platelets was higher in all ITP patients than in their age-matched HCs. The difference in the release behaviour of α - and dense granules can be explained by the tight regulation of the release mechanism in space and time [31, 32].

Platelets from all the ITP patients showed a reduced response to TRAP, likely due to elevated caspase activity compared to the age-matched HCs, as shown by an inverse correlation between caspase activity and platelet activation [7].

Caspase-3/7 activity was higher in platelets from ITP $>$ 65 than from ITP $\leq$ 65 patients, possibly explaining their more pronounced functional impairment.

Our research indicates increased caspase activity associated with a reduced platelet count. Increased susceptibility to platelet apoptosis in ITP patients has been explained by the formation of aggregates with CD8 $^{+}$ T-cells, which trigger platelet activation and apoptosis via the T-cell receptors [33].

Sun et al. have proposed that anti-platelet antibodies cause increased platelet apoptosis in ITP patients [34]. However, we did not observe differences in platelet caspase activity between patients with and without anti-platelet antibodies. This may be due to the limited sensitivity of the methods used to detect anti-platelet antibodies [35] or the poorly studied functional role of various types of platelet-antibodies.

As reported previously [7, 36], we observed that platelets from all patients with ITP exhibited a reduced level of sialic acid compared to platelets from their corresponding age-matched HCs. We observed a relationship between sialic acid loss and platelet count, but not with caspase activity. This supports previous findings suggesting that apoptosis and sialic acid loss are not necessarily related [37, 38].

Zheng et al. described how anti-GPIIb/IIIa antibodies appeared to be more capable of causing NEU1 surface exposure than anti-GPIb/IX antibodies [13], Li et al. showed that anti-GPIb α , but not anti-GPIIb/IIIa antibodies induced desialylation [39], and Amini et al. that GPIb/IX antibodies were associated with hepatic sequestration [40]. However, Marini et al. showed that both these anti-GPs were capable of desialylating platelets [38]. We also observed a significant increase in platelet desialylation among patients with anti-platelet antibodies. Clearly, the association between anti-platelet antibodies and desialylation is poorly studied and merits further investigation. However, we should also consider the potential involvement of CD8 $^{+}$ T-cells in platelet desialylation [41].

Our results showed that some of the differences observed between HC and ITP patients aged $\leq$ 65 years disappeared when comparing HC and ITP groups aged $>$ 65 years. This is because aging triggers events in platelets that are similar to those caused by the disease. In other cases, the pathogenic effects of ITP and aging may be additive. For example, when compared to ITP $\leq$ 65 and HC $>$ 65 groups, platelets from the ITP $>$ 65 group were more resistant to agonist stimulation. These platelets exhibited increased caspase -3/7 activity, and the clots they formed were more resistant to lysis. These effects potentially exacerbate ITP severity in the ITP $>$ 65 group, affecting platelet biology and increasing disease burden.

Moreover, we can also conclude that increased platelet desialylation is a mechanism involved in ITP pathophysiology independently of age. Our findings underscore the importance of validating this phenotypic profile in larger patient cohorts and of conducting mechanistic studies. Additionally, our results highlight the importance of age-based stratification in patients with ITP. Managing older patients is particularly challenging due to the increased risk of bleeding, thrombotic events, and treatment-related complications associated with aging. Personalized treatment approaches may improve safety, maintain quality of life, and reduce morbidity in these patients.

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Author contributions

NB and MTAR designed the research study. EMM, CHC, PA, LdPM, LP and EGAS performed the research. CHC, AM, MMS, MIRP, MGK and RRM contributed to patient's recruitment and to collection and interpretation

of clinical data from patients' medical records. EMM, MTAR and NB analysed the data. NB wrote the paper. ELG, RK and VJY provided a critical feedback on the manuscript. All authors reviewed and approved the submitted final version.

The raw data supporting the conclusions of this article will be made available by the authors to those who request it. During the preparation of this work the author(s) used free versions of DeepL Write to improve the quality of the English writing. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Conflicts of interest

The authors report no conflicts of interest related to this work

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