

ORIGINAL PAPER

IVIg treatment increases thrombin activation of platelets and thrombin generation in paediatric patients with immune thrombocytopenia

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Summary

Clinical manifestations and laboratory parameters of haemostasis were investigated in 23 children with newly diagnosed immune thrombocytopenia (ITP) before and after intravenous immunoglobulin (IVIg) treatment. ITP patients with platelet counts of less than $20 \times 10^9/L$ and mild bleeding symptoms, graded by a standardized bleeding score (BS), were compared with healthy children with normal platelet counts and children with chemotherapy-related thrombocytopenia. Markers of platelet activation and platelet apoptosis in the absence and presence of platelet activators were analysed by flow cytometry; thrombin generation in plasma was determined. ITP patients at diagnosis presented with increased proportions of platelets expressing CD62P and CD63 and activated caspases, and with decreased thrombin generation. Thrombin-induced activation of platelets was reduced in ITP compared with controls, while increased proportions of platelets with activated caspases were observed. Children with a higher BS had lower proportions of CD62P-expressing platelets compared with those with a lower BS. IVIg treatment increased the number of reticulated platelets, the platelet count to more than $20 \times 10^9/L$ and improved bleeding in all patients. Decreased thrombin-induced platelet activation, as well as thrombin generation, were ameliorated. Our results indicate that IVIg treatment helps to counteract diminished platelet function and coagulation in children with newly diagnosed ITP.

KEYWORDS

bleeding, caspase-activation, endogenous thrombin generation, IVIg, paediatric ITP, platelet-activation

INTRODUCTION

Primary paediatric immune thrombocytopenia (ITP) is a common and mostly benign illness. Children with ITP usually have a brief course of symptoms and recover

spontaneously. However, severe bleeding and even fatal intracranial haemorrhage can occur in rare occasions.^{1,2} Treatment is required in only a proportion of children, and first-line therapy includes intravenous immunoglobulins (IVIg) and/or corticosteroids or anti-D antibodies.^{3–5} The

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pooled antibody blood product IVIg can improve platelet count in ITP patients.⁶ At least in part, this is caused by Fc gamma RIIb signalling and by increasing the expression of inhibitory Fc gamma receptors on splenic macrophages, thus reducing platelet consumption.⁷

Traditionally, it was considered that ITP is caused by binding of autoantibodies to the glycoprotein (GP) complexes GPIIb/IIIa or GPIb/IX/V on platelets, resulting in their enhanced clearance.^{8–10} As some studies suggest, also T-cell-mediated cytotoxicity and abnormalities in regulatory T-cells may be possible mechanisms for enhanced platelet destruction in ITP; however, the exact mechanisms underlying these associations have not yet been fully demonstrated.^{11,12} An increased number and activation of cytotoxic T-cells and abnormalities in T-helper cells could contribute to impaired platelet production as well as enhanced platelet destruction.^{13,14} Such mechanisms mainly involve Fc receptor-mediated cell clearance by the reticuloendothelial system. However, some autoantibodies to GPIIb of the GPIb-IX-V complex result in its desialylation, thus leading to an immune response to megakaryocytes and increased platelet clearance.^{15,16}

It is now well accepted that antibodies can alter platelet function; the majority of studies have reported that such antibodies induce platelet activation.^{17–19} However, in ITP, limited and conflicting information exists on the effect of autoantibodies in activating or inhibiting platelets.^{20–22} In the case of activating autoantibodies, increased surface expression of the activation markers P-selectin (CD62P), CD63 and phosphatidylserine (PS) has been reported.²² Examination of the correlation of platelet activation marker expression in ITP with bleeding symptoms has yielded conflicting results. An earlier study did not find a correlation between platelet CD62P expression at baseline and the bleeding score (BS) in ITP patients.²³ A subsequent publication from a larger cohort reported higher baseline CD62P expression in patients with a higher BS. As well, higher levels of CD62P-expressing and PAC-1-positive platelets after thrombin receptor-activating peptide (TRAP) stimulation were observed in ITP patients with a lower versus a higher BS.²⁴

Platelets, although anucleate, are now well recognized to undergo apoptosis, displaying mitochondrial membrane depolarization, activation of caspases, formation of extracellular vesicles (EVs) and PS exposure; these apoptotic-like events also occur during platelet activation (reviewed in Reddy and Rand²⁵). An early study showed apoptotic features in megakaryocytes from ITP patients that were associated with factors in ITP plasma,²⁶ and we subsequently demonstrated increased proportions of platelets from paediatric ITP patients at initial presentation with activated caspase-3 and -9 and PS exposure, and enhanced formation of EVs.²⁷ Most recently, it was reported that (auto)antibodies to GPIIb elicit platelet apoptotic-like events via Akt strain transforming (Akt) signaling pathway activation.²⁸ Whether apoptotic-like events in ITP platelets at presentation are further enhanced by platelet activation has not been previously examined.

Thrombin is one of the key inducers of the coagulation signalling cascade and platelets are involved in the generation of thrombin.²⁹ It is of interest to investigate the influence of the very low platelet count in ITP on the generation of thrombin, but conflicting results had been obtained in the past. A negative influence of a very low platelet count on thrombin generation as determined by the endogenous thrombin potential (ETP) had been reported and a low ETP in thrombocytopenic adult patients with more pronounced bleeding in haematological malignancies was found.^{30,31} In contrast, recently an enhanced ETP in platelet-poor plasma (PPP) was found in adult chronic ITP and the authors speculated about the existence of a procoagulant state in ITP patients.³²

In the present study, we investigated the platelet activation markers CD62P, CD63, and activated GPIIb-IIIa pre and post stimulation, as well as ETP in PPP in children with ITP at diagnosis and after IVIg treatment in comparison with healthy paediatric controls and paediatric chemotherapy patients (CIT). We demonstrated, in this clinically well-defined ITP cohort at diagnosis, increased baseline platelet activation with a reduced response to stimulation, and increased proportions of platelets with activated caspases with an increased response to stimulation, while a reduced ETP in PPP was observed. The large majority of these findings ameliorated after treatment with IVIg.

MATERIALS AND METHODS

Patients

In this prospective study, approved by the Ethics Committee of the Canton of Zurich, Switzerland, 23 children with a platelet count of less than $20 \times 10^9/L$, fulfilling the criteria for primary ITP⁴ were investigated after obtaining written informed parental consent (Table 1).

The severity of bleeding symptoms in the 24 h preceding diagnosis was graded with a standardized BS on a scale of 0–4, as published.^{27,33,34} To correlate the laboratory findings with the clinical presentation, that is, bleeding symptoms, of our patients, thrombin generation and platelet activation in the eight patients with the lowest BSs of 1 and 2 were compared with that in the eight patients with a high BS of 3 (note that no patient had a BS of 4). In the high-BS group, overt mucosal bleeding occurred.

All ITP patients were treated with IVIg at a dose of 0.8 g/kg within 3–4 h of diagnosis and were re-evaluated 12–24 h after IVIg; if platelet counts remained below $20 \times 10^9/L$, patients received an additional IVIg dose. Maximally, three IVIg doses were administered ($n = 2$). Patients were followed for 1 year. Sixteen patients had normalized platelet counts after 6 months, two patients after 9 and 12 months and five patients developed chronic ITP.

Twenty-two healthy control children, 11 females and 11 males, median age of 7.6 (0.8–19) years, median platelet count $300.4 \times 10^9/L$, with no history of autoimmune

TABLE 1 Clinical and laboratory characteristics of children with newly diagnosed ITP ($n = 23$)

Patient #	Age (years)	Sex	Bleeding score	Platelet count at diagnosis ($\times 10^9/L$)	# of IVIg doses (0.8 g/kg)	Platelet count after last IVIg dose ($\times 10^9/L$)
1	2.4	F	3	7	1	40
2	4.0	M	2	10	1	39
3	6.5	F	2.5	3	1	54
4	4.2	F	3	1	1	22
5	5.8	M	2.5	1	1	47
6	2.1	M	2	<1	1	31
7	1.6	F	2	2	1	38
8	2.8	M	3	14	1	28
9	2.4	F	2.5	<1	3	29
10	14.2	F	3	<1	2	52
11	6.6	M	3	<1	3	42
12	4.8	F	3	7	1	59
13	4.8	M	2.5	3	2	57
14	14.4	F	2	4	1	59
15	13.9	M	2	<1	1	98
16	2.7	F	3	<1	2	20
17	7.8	F	2	4	1	123
18	3.5	F	2	2	2	45
19	2.5	M	2.5	6	1	24
20	3.8	M	2.5	8	1	24
21	12.0	M	3	7	1	41
22	10.1	F	1	17	1	39
23	3.5	F	2.5	9	1	46
Median	4.2	—	2.5	6	1	41

Note: Bleeding scores at diagnosis, platelet counts before and after IVIg treatment and number of IVIg doses given are presented for each individual patient. The following signs of infection were noted at time of and preceding the diagnosis of ITP: respiratory tract infection without fever ($n = 3$), respiratory tract infection in the preceding 1–2 weeks ($n = 4$), antibiotic treatment because of streptococcal angina in the week before diagnosis ($n = 2$), gastroenteritis 2–4 weeks preceding ($n = 4$) and varicella infection 2 weeks preceding the diagnosis ($n = 1$). In nine patients, there was no history of infection.

Abbreviations: ITP, immune thrombocytopenia; IVIg, intravenous immunoglobulin.

disease, coagulopathy or blood transfusion, and, to control for the effects of thrombocytopenia itself, 12 patients with CIT, median platelet count $16 \times 10^9/L$ (range: $3\text{--}53 \times 10^9/L$), were recruited after obtaining written informed parental consent (for clinical characteristics see [Tables S1](#) and [S2](#)).

Blood sampling and routine testing

Venous blood samples were drawn from ITP patients at diagnosis prior to treatment, 12–24 h after the last IVIg treatment, and from healthy controls during routine laboratory testing prior to a minor surgical intervention. In children with CIT, blood was drawn prior to transfusion of a platelet concentrate. Samples were collected into citrate anticoagulant (final concentration, 10.5 mM). Blood counts were measured using an automated blood analyser (Sysmex XE-2100; Sysmex Digitana).

Flow cytometry

PE-conjugated anti-CD41a (GPIIb, clone HIP8), Per CP-conjugated anti-CD42a (GPIX, clone BEB1), PE-conjugated anti-CD42b (GPIb, clone: HIP1), APC-conjugated anti-CD62P (clone AK-4), PE-conjugated anti-CD63 (clone H5C6), as well as FITC-conjugated PAC-1 (clone PAC-1) were purchased from BD Biosciences (Rotkreuz). Fluorochrome inhibitors of caspases (FLICA) FAM-DEVD-FMK, specific to active caspase-3, and FAM-LEHD-FMK, specific to active caspase-9, were from Millipore.

Platelet-rich plasma (PRP) was obtained from citrated whole blood by centrifugation at 140g at 22°C for 10 min. To analyse markers of platelet activation, PRP was diluted 10-fold with isotonic Hepes-buffered saline with Ca^{2+} (HBS- Ca^{2+} ; 150 mM NaCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 2 mM GPRP, 2 mM Hepes, pH 7.4), containing PAC1-FITC, anti-CD42a-PerCP, anti-CD63-PE and anti-CD62P-APC. For analysis of activated caspase-3

and -9, FAM-DEVD-FMK and FAM-LEHD-FMK respectively were used in combination with anti-CD42b-PE. Measurements were done without and with thrombin (1 U/mL) or Ca^{2+} -ionophore A23187 (3 μM) stimulation of platelets for 1 h. Platelet size was determined by forward scatter. Platelet-derived EVs and reticulated platelets (RPs) were determined as described previously.^{35,36} For each sample, 10 000 platelets, identified as CD42a+, CD42b+ or CD41+ events, were acquired using a FACSCalibur flow cytometer (BD Biosciences). Data were analysed with FCS Express (De Novo Software).

Thrombin generation

Platelet-poor plasma, obtained by centrifugation of citrated blood at 13000g at 22°C, was stored at -80°C. Thrombin generation was measured in thawed PPP after activation with recombinant tissue factor using the Technothrombin TGA kit (Technoclone) on an Infinite M200 microplate fluorescence reader (Tecan; software: Technoclone) as previously described.³⁷ Generated thrombin is reported as the ETP (the integrated area under the thrombogram curve).

Statistical analysis

GraphPad Prism Software Version 8.00 was used and data were analysed using *t*-tests and for analysis between the four groups (ITP; ITP after IVIg; healthy controls; CIT), ANOVA with the Bonferroni test for comparison of means or Wilcoxon signed-rank test between pairs of medians. Correlation was assessed by the Spearman correlation coefficient. Data were considered statistically significant at $p < 0.05$ and are presented as means \pm standard deviation, standard error of the mean (SEM), or as medians with minimum to maximum or with interquartile range (IQR).

RESULTS

Bleeding, platelet counts in ITP patients before and after IVIg

The patients presented with mild to moderate bleeding symptoms: the majority of children suffered from large haematomas and multiple petechiae, six patients had mild epistaxis and gum bleeding, and two children presented with severe epistaxis and haematemesis. After IVIg therapy, platelet counts increased to $20 \times 10^9/\text{L}$ or more in 17 patients after one dose, in four after a second and in two patients, after a third dose. After the last IVIg dose, all showed more than a twofold increase in their platelet count (median platelet count: 41; range: $20\text{--}123 \times 10^9/\text{L}$). Bleeding symptoms improved in all patients except for one who continued to have epistaxis after IVIg (Table 1).

Platelet activation in ITP patients before and after IVIg compared with controls with CIT and healthy controls

We observed an increased percentage of CD62P+ (mean \pm SEM: $20.1 \pm 4.3\%$) and CD63+ ($34.4 \pm 5.5\%$) platelets in paediatric ITP patients at diagnosis compared to healthy children (CD62P+: $3.8 \pm 0.8\%$; CD63+: $10.6 \pm 2.1\%$). In CIT patients, compared to healthy children, a borderline increase of CD62P+ but no CD63+ increase was observed (CD62P+: $4.7 \pm 1.3\%$; CD63+: $8.8 \pm 2.1\%$) (Figure 1A,B). Thrombin stimulation increased the percentage of CD62P+ as well as of CD63+ platelets in ITP patients (CD62P+: $63.8 \pm 6.2\%$; CD63+: $64.0 \pm 6.5\%$) and in CIT patients (CD62P+: $54.2 \pm 9.6\%$; CD63+: $51.2 \pm 6.8\%$). Still, the values from both ITP and CIT patients were lower compared to healthy children where the highest results had been observed (CD62P: $91.6 \pm 1.6\%$; CD63: $85.2 \pm 3.8\%$).

After IVIg treatment, without thrombin stimulation, the percentages of CD62P+ and CD63+ platelets decreased only non-significantly. However, after thrombin stimulation, the percentage of CD62P+ platelets in ITP patient after IVIg therapy increased from $18.0 \pm 4.9\%$ to $86.9 \pm 5.1\%$, and the percentage of CD63+ platelets increased from $24.4 \pm 5.1\%$ to $86.3 \pm 4.6\%$. (Figure 1A,B).

The percentages of PAC-1-binding resting platelets in ITP ($2.6 \pm 0.9\%$) and after IVIg therapy ($3.5 \pm 2.8\%$) were neither significantly different from healthy control levels ($1.0 \pm 0.4\%$) nor from those in CIT patients ($0.5 \pm 0.1\%$). After thrombin stimulation the increase of PAC-1-binding was not as great in ITP patients ($33.5 \pm 6.3\%$) or in CIT patients ($16.6 \pm 4.9\%$) compared with healthy controls ($67.3 \pm 6.5\%$) (Figure 1C). After treatment with IVIg, thrombin-stimulated platelets showed an increased binding of PAC-1 ($53.9 \pm 7.3\%$), similar to healthy control levels (Figure 1C).

Results of percentage CD62+ and CD63+ and percentage PAC1-binding platelets at baseline and after thrombin stimulation of platelets from ITP patients are depicted in Figure 2A–F, with results for each patient connected by lines, to indicate the individual increases by thrombin stimulation. Thrombin stimulation increased the proportion of CD62P+ and CD63+ platelets to more than 50% in 72% of newly diagnosed patients (for both CD62P and CD63) and for PAC1-binding platelets, in 22% of patients. After IVIg, thrombin stimulation increased proportion of CD62P+, CD63+ and PAC1-binding platelets to more than 50% in 95%, 91% and 68% of patients respectively.

Thrombin generation in ITP patients before and after IVIg compared with healthy controls and controls with CIT

The median ETP was significantly lower in patients at diagnosis (146; range: 18–401 nM/min) than in healthy children (366; 85–673 nM/min) and CIT (242; 185–786 nM/min). After IVIg treatment, even though the platelet count did not completely normalize, the median ETP increased significantly to 253 (120–539) nM/min (Figure 3). We observed a significant

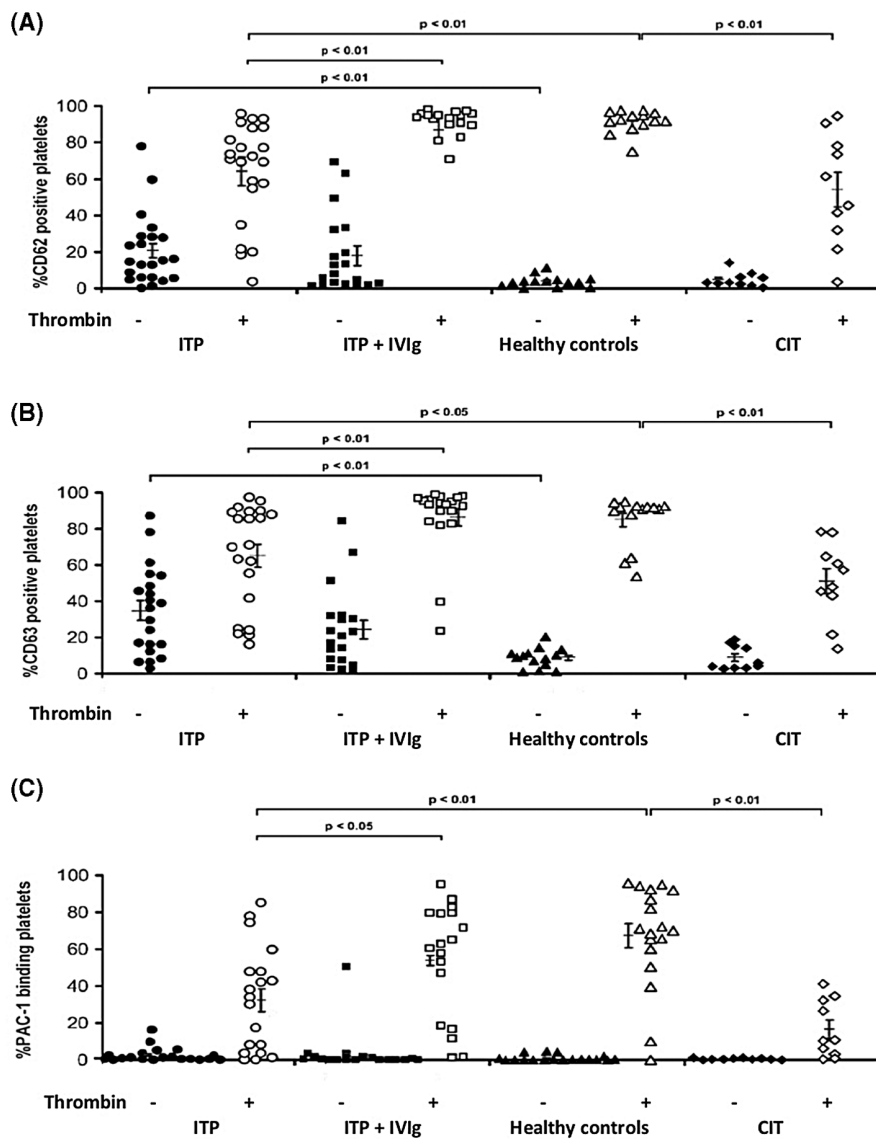


FIGURE 1 Percentage CD62P+ (A), and CD63+ (B) platelets and PAC-1-binding platelets (C) in children with immune thrombocytopenia at diagnosis (ITP; $n = 22$) and after intravenous immunoglobulin (IVIg) treatment (ITP + IVIg; $n = 22$), compared with controls: chemotherapy-related thrombocytopenia (CIT; $n = 12$) and healthy controls (healthy controls; $n = 22$). Measurements were performed by flow cytometry in the absence (baseline, filled symbols) and presence (+ thrombin, open symbols) of platelet stimulation by thrombin. Data are presented as mean \pm SEM and p -values are as indicated (statistical analysis by ANOVA with multiple comparison of means). Circles indicate ITP patients at diagnosis; squares, ITP patients after IVIg treatment; triangles, healthy children; and diamonds, patients with CIT.

correlation ($p < 0.001$) between ETP and platelet count (all study subjects included: $r = 0.45$; $n = 46$). No correlation was observed between ETP and the percentage of EVs ($r = 0.11$; $p > 0.1$).

Thrombin generation and platelet activation in ITP patients at diagnosis according to high and low BSs compared with healthy controls

Results in children with a BS of 1 or 2 (low BS, $n = 8$) were compared to those in children with a BS of 3 (high BS, $n = 8$).

The median ETP was significantly lower in the low-BS group (median 152; IQR: 108–200 nM/min) than in the high-BS group (192; 28–401 nM/min) (Figure 4A).

The mean percentages of CD62P+ platelets at baseline and after thrombin stimulation were significantly lower in patients in the high-BS group compared with the low-BS group (at baseline: $11.5 \pm 4.1\%$ versus $21.7 \pm 6.9\%$; after thrombin stimulation: $55.4 \pm 9.5\%$ versus $72.3 \pm 7.2\%$; Figure 4B).

Proportion of young RPs and platelet-derived EVs before and after IVIg compared with healthy controls and controls with CIT

Percentage RPs (median 13.6%; range: 2.4–27.9%; $n = 22$), as well as median platelet size (32.8 forward scatter [FSC]; 13–109 FSC; $n = 20$), were significantly elevated at ITP diagnosis

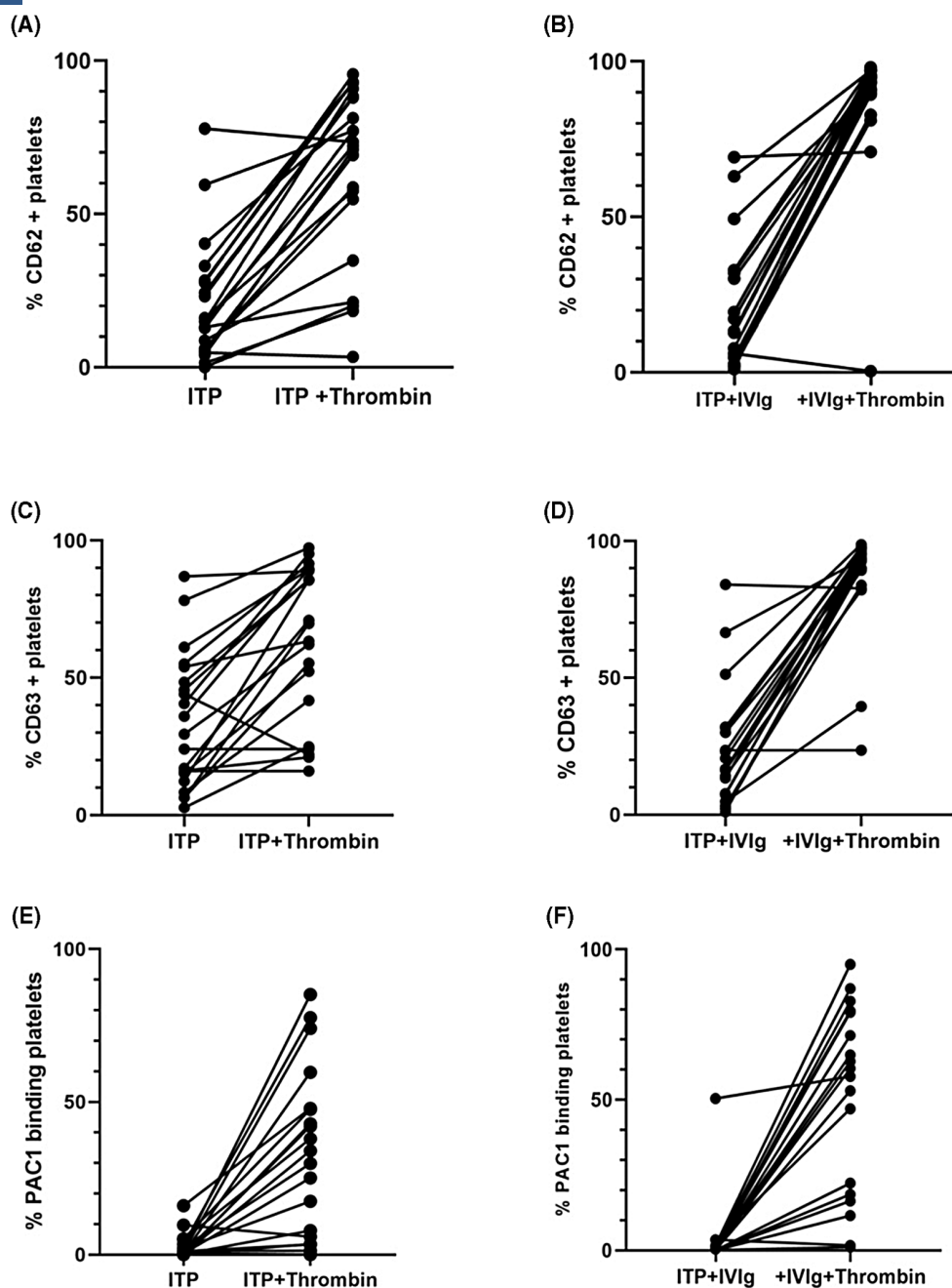


FIGURE 2 In ITP patients ($n = 22$), percentage CD62+ platelets in the absence and presence of thrombin at time of diagnosis (A) and after intravenous immunoglobulin (IVIg; B); percentage CD63+ platelets in the absence and presence of thrombin at time of diagnosis (C) and after IVIg (D); and percentage PAC-1-binding platelets in the absence and presence of thrombin at time of diagnosis (E) and after IVIg treatment (F). The results are connected with a line for individual patients to indicate the trend within the same patient.

compared to those in healthy children (RPs: 1.5%; 0.6%–2.9%; and size: 19.0 FSC; 13.6–24.1 FSC; $n = 19$; $p < 0.001$). After IVIg therapy, percentage RPs and FSC decreased significantly, to 3.5% (0.7–5.4%; $n = 21$, $p < 0.001$) and 29.0 FSC (12.0–42.2 FSC; $n = 18$) respectively (Figure 5A; data for platelet size not shown). The absolute numbers of RPs (median 0.53; range: 0.002 – 3.6×10^9 ; $n = 22$) were significantly decreased at ITP diagnosis compared to healthy children (2.11 ; 0.82 – 6.73×10^9 ; $p < 0.0001$). After IVIg therapy,

RPs increased significantly, to 1.44; 0.27–7.77; $p < 0.001$ (Figure 5B).

The percentage EVs was significantly elevated at diagnosis in ITP patients (9.1%; 3.4%–23.1%; $n = 23$), compared with that in healthy controls (5.6%; 1.8%–13.2%; $n = 22$). The difference of the increase of percentage EVs in CIT (8.3%; 3.7%–19.3%; $n = 9$) compared to ITP at diagnosis did not reach statistical significance. In ITP patients, after IVIg, percentage EVs decreased significantly (5.5%; 1.5%–34.6%; $n = 20$,

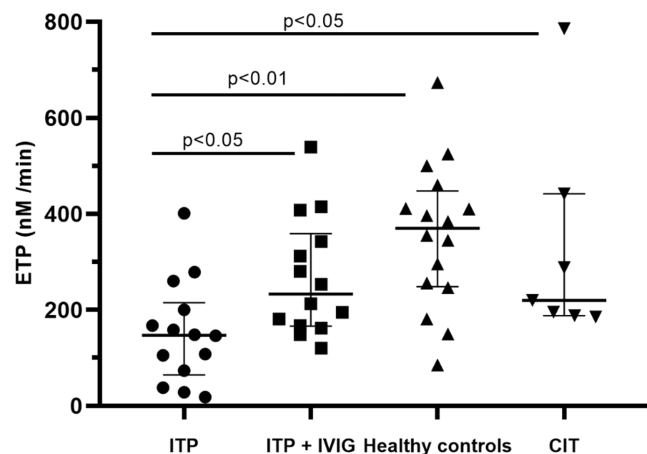


FIGURE 3 Thrombin generation [endogenous thrombin potential (ETP)] in plasma of children with immune thrombocytopenia at diagnosis (ITP) and post intravenous immunoglobulin (IVIg) therapy (ITP + IVIg), compared with controls: chemotherapy-related thrombocytopenia (CIT) and healthy controls (Healthy controls). ETP was measured in platelet-poor plasma after addition of tissue factor. Medians and interquartile range (IQR; $n = 14$ for ITP; $n = 14$ for ITP + IVIg; $n = 16$ for control; $n = 7$ for CIT) and p -values are as indicated (statistical analysis by Wilcoxon signed-rank test between pairs of medians).

$p < 0.01$), similar to that of healthy controls (Figure 5C). The absolute number of EVs was significantly lower at ITP diagnosis (median 0.39 ; range 0.05 – 7.85×10^9) compared to that in healthy controls (15.96 ; 1.84 – 42.11×10^9 ; $p < 0.0001$). It increased after IVIg (1.70 ; 1.21 – 10.59×10^9 ; $p < 0.05$), but remained significantly lower compared to that in healthy controls ($p < 0.001$; Figure 5D).

Activated caspase-3 and -9 in platelets from ITP patients compared with healthy controls

Platelets from patients ($n = 10$) at presentation and after *in vitro* activation of caspases by the Ca^{2+} -ionophore A23187 were measured.

The percentages of resting, unstimulated platelets (median; IQR) with activated caspase-3 and -9 in patients with ITP (7.2%; 6.0%–19.3% and 13.3%; 7.7%–20%) were significantly increased compared with those in healthy controls (0.4%; 0.2%–0.7% and 0.7%; 0.7%–1.6%). Upon stimulation with thrombin or A23187, increased percentages of ITP platelets with activated caspase-3 and -9 were observed (caspase-3: 15.4%; 4.5%–37.9% and 24.4%; 11.0%–51.0%; caspase-9: 22.3%; 6.8%–68.0% and 31.7%; 18.3%–45.2%) compared with those in healthy controls (caspase-3: 1.6%; 0.6%–4.3% and 12.0%; 4.5%–18.3%; caspase-9: 1.0%; 0.9%–3.1% and 7.5%; 3.1%–13.3%) (Figure S1). A comparison between unstimulated and thrombin- or A23187-stimulated ITP platelets showed the greatest median caspase-3 and caspase-9 expression after A23187 stimulation (24.4% and 31.7%), but the differences between the stimulated ITP cohorts were not significant.

DISCUSSION

In this study, we compared results from a clinically well-defined group of children with newly diagnosed ITP to those from healthy children and children with CIT. We demonstrated increased proportions of platelets with surface-expressed CD62P as well as CD63 from ITP patients at presentation compared with both healthy children and children with CIT. Further, we observed that thrombin-induced activation of platelets from ITP patients is reduced compared to that of those from healthy controls, similar to that observed in platelets from CIT patients (Figure 1). Our results indicated that platelet activation as measured by percentage CD62P+ platelets is lower at baseline and after thrombin stimulation in ITP patients with a higher BS compared to those with a lower BS (Figure 4B). Finally, our study demonstrated not only a benefit of IVIg treatment for platelet count and RP count, but also an improved platelet activation and, as measured by ETP, enhanced thrombin generation (Figures 1–3).²⁷

The present study was able to confirm our previous results regarding an increase of absolute numbers of RPs and a decrease of the percentage of RPs after IVIg.²⁷ A previous study of 24 adult and paediatric ITP patients reported a corresponding response of immature platelet fractions (IPF) results and platelet counts after treatment with thrombopoietic agents or immunoglobulins (IVIg and anti-D).³⁸ In this study, only a portion of the patients included were treated with IVIg and of those, only a minority responded with an increase of platelet count and absolute numbers of the IPFs in parallel. Further studies need to investigate if and how IVIg could interfere with platelet destruction or production.

In this study, we confirmed our earlier findings of elevated levels of percentage EVs in ITP patients at diagnosis.²⁷ Post IVIg, percentage EVs decreased significantly to levels similar to that of controls (Figure 5C). Absolute EVs increased after IVIg in parallel with a platelet count increase, but still, absolute numbers of EVs did not reach the amount that is seen in healthy controls (Figure 5D). On this point, the effect of IVIg on the proportion of EV levels was not investigated and cannot be explained by this study any further. We can only speculate as to the mechanism involved in this: as EV formation by activated platelets is closely linked to the scrambling of plasma membrane phospholipids leading to the exposure of PS on the platelet surface, and IVIg treatment reduces platelet PS exposure, percentage EV formation may concomitantly be reduced.^{25,27,39}

Regarding the association of platelet activation with BSs in ITP, it was previously reported in a large paediatric study that higher platelet surface CD62P expression was associated with a higher BS, while higher levels of CD62P and activated GPIIb-IIIa on platelets stimulated with TRAP were associated with a lower BS.²⁴ While our study also found higher CD62P expression after thrombin stimulation in patients with a lower BS, baseline CD62P expression in our subgroup with a low BS was lower compared to that in children with a higher BS. The differences could be related to the small

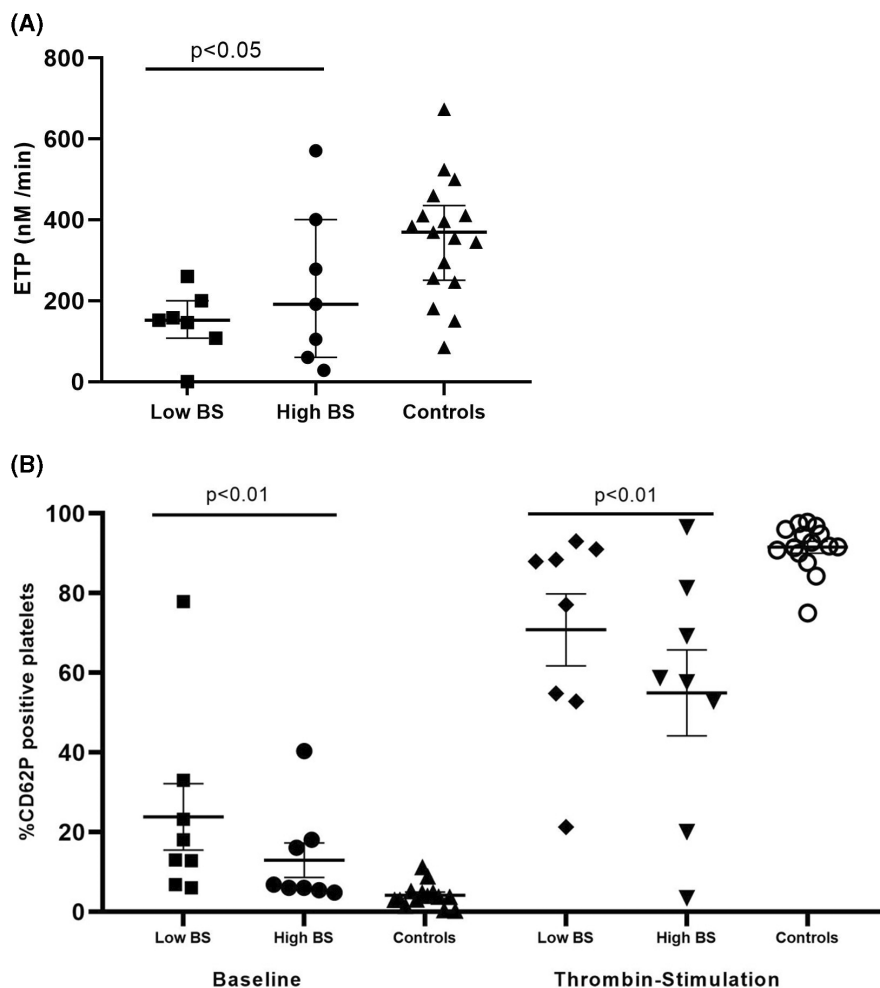


FIGURE 4 Thrombin generation [endogenous thrombin potential (ETP)] in plasma and CD62P expression on platelets from children with immune thrombocytopenia (ITP) at diagnosis grouped according to low and high bleeding score (BS). ETP [medians and interquartile range (IQR), $n = 7$ each group] in ITP patients at diagnosis (A) and percentage CD62P-positive platelets (mean \pm SEM, $n = 8$ each group) (B) grouped according to BS (low BS: BS of 1 or 2; high BS: BS of 3; $n = 8$ each group) compared with healthy children (Controls, $n = 16$). p -Values are as indicated (statistical analysis by Wilcoxon signed-rank test between pairs of medians and Student's t -test for means and SEM).

number of patients in our cohort and as well as the different grouping and BSs used, but cannot currently be further explained by our study results.

In children with CIT, we found that the percentages of CD62P-expressing platelets at baseline were significantly lower compared with children with newly diagnosed ITP (Figure 1A). A study in adults also showed lower *in* and *ex vivo* platelet activation in thrombocytopenia due to chemotherapy and in haematological malignancies compared to ITP patients.²

Another important finding of this study was the observation of reduced thrombin generation in PPP of ITP patients at diagnosis. ETP results correlated with the platelet count and interestingly ETP was higher in patients with a higher BS (Figure 4A). After IVIg, ETP increased but was still lower than in healthy controls (Figure 3). The effect of IVIg on ETP could be explained by the contribution of several factors, besides a rise in platelet count alone. As IVIg preparations can contain small amounts of activated coagulation factors,

e.g., FIXa and FXIa that could increase their thrombogenicity, we speculate that also this can be an explanation for the observed increase of ETP in our cohort after IVIg administration.^{40,41} Certainly, this needs to be addressed in future studies.

The majority of laboratory parameters studied in our cohort demonstrated an improvement, that is, in haemostasis, in platelet count and function, and in thrombin generation, after IVIg treatment. Such an improvement is beneficial in an ITP patient with overt bleeding or an increased risk for bleeding, that is, after trauma, and also in young children, due to their risk according to their motoric or behavioural developmental state. In the phase-3 TIKa trial, normalization of platelet count was significantly higher at 1–12 weeks in children treated with IVIg compared to observation only, while the proportion that developed chronic ITP did not differ.⁶ Other studies have indicated that IVIg alone, or in combination with high-dose methylprednisolone, is superior to oral corticosteroids alone for the time taken to achieve a

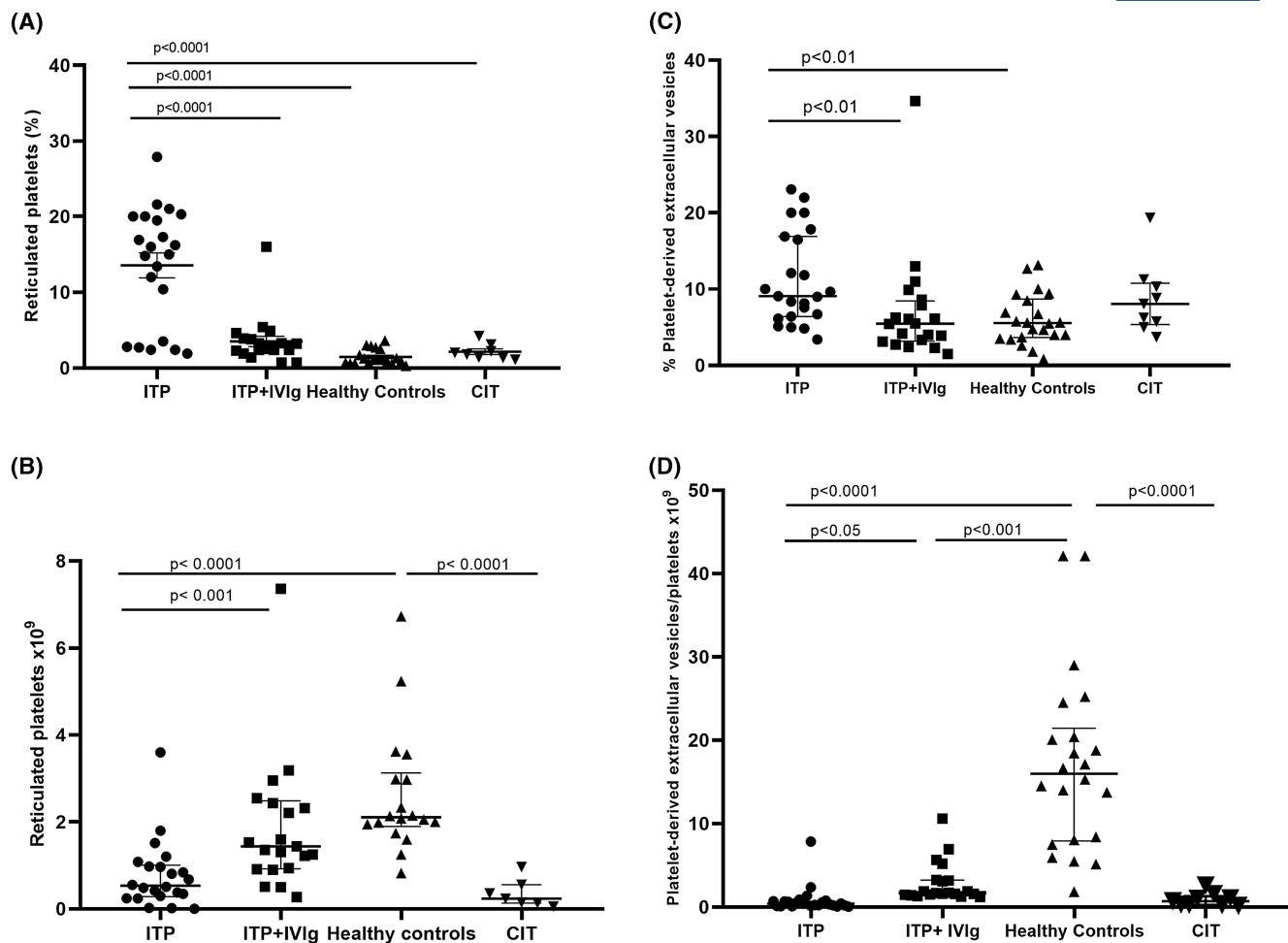


FIGURE 5 Percentage and absolute number of reticulated platelets (RPs) and platelet-derived extracellular vesicles (EVs) in children with immune thrombocytopenia at diagnosis (ITP, $n = 23$ for EVs and 22 for RPs) and post intravenous immunoglobulin (IVIg) therapy (ITP+IVIg, $n = 20$ for EVs and 21 for RPs) compared with controls: chemotherapy-related thrombocytopenia (CIT, $n = 9$ for EVs and 8 for RPs) and healthy controls (Healthy controls, $n = 22$ for EVs and 19 for RPs). Percentage reticulated platelets (A), absolute number of reticulated platelets (B) as determined by thiazole orange staining and percentage EVs, defined as the proportion of CD42a-positive events $< 0.9 \mu\text{m}$ (C) and absolute number of EVs (D) as determined by flow cytometry. Data are presented as medians with interquartile range (statistical analysis by ANOVA followed by Wilcoxon signed-rank test between pairs of medians). Data were published in part previously and were updated for this study; see Winkler *et al.*²⁷

platelet count of more than $50 \times 10^9/\text{L}$.⁴² Thus, we conclude that IVIg is an efficient treatment in children with ITP and enhanced bleeding signs or an increased risk for bleeding. Although safety studies or additional cost/benefit assessments were not included in this study, the use of IVIg might be beneficial for such a group of paediatric ITP patients.

It must be considered that the higher baseline CD62P and CD63 expression on platelets from ITP patients at diagnosis could have arisen from pre-analytical platelet activation during blood sampling from the young children and/or preparation of PRP for the flow cytometric analyses.^{35,43} However, careful and standardized measures were taken to avoid pre-analytical activation, and it should be noted that CD62P and CD63 expression on platelets from both groups of controls, that is, healthy children and children with CIT, was low.

Several limitations of this study need to be acknowledged: the small size of the study cohort may reduce the power of the study and the analysis of subgroups that we performed.

Also, unfortunately, not all laboratory tests were performed in all patients, due to the small amount of blood that we were able to draw from some of our younger patients. The ITP BS used was only able to divide patients into rather broad categories. In addition, no children with severe and life-threatening bleeds had been included.

Apoptotic-like events such as activation of caspases, formation of EVs, and PS exposure are well recognized to occur as a result of platelet activation (reviewed in Reddy and Rand²⁵). Building on our previous findings that platelets from paediatric ITP patients at initial presentation have higher proportions with activated caspase-3 and -9 compared with those from healthy children,²⁷ we demonstrated here that compared with controls, thrombin and A23187 stimulation result in further increases. Interestingly, while compared to controls, CD62P and CD63 expression on thrombin-stimulated ITP platelets was reduced, caspase activation after stimulation reached higher levels in ITP platelets compared to controls (Figure S1). While it was not

the focus of this study, our earlier study had indicated that after IVIg, the proportion of caspase-3 and -9 decreased significantly.²⁷

Overall, our results indicate that platelets of paediatric ITP patients at diagnosis have signs of platelet activation and further stimulation with thrombin is impaired. While such findings were more pronounced in children with a higher BS, IVIg treatment is able to reverse such abnormalities and increase thrombin generation, making it an efficacious therapeutic option for children with ITP and more intense bleeding.

AUTHOR CONTRIBUTIONS

Oliver Speer, Margaret L. Rand and Markus Schmugge designed the study; Jeannine Winkler, Francesca Daniela Franzoso and Oliver Speer performed the experiments, Jeannine Winkler, Oliver Speer, Francesca Daniela Franzoso, Sabine Kroiss and Markus Schmugge analysed data; Sabine Kroiss, Markus Schmugge and Monika Seiler: attended and enrolled the patients and collected clinical data; and Jeannine Winkler, Francesca Daniela Franzoso, Markus Schmugge and Margaret L. Rand wrote the manuscript.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

We agree with data sharing, according to the journal recommendations and Wiley's data sharing policy.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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