Antiplatelet antibody predicts platelet desialylation and apoptosis in immune thrombocytopenia

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Antiplatelet antibody predicts platelet desialylation and apoptosis in immune thrombocytopenia

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Data that support the findings of this study are available from the corresponding author upon reasonable request. All data generated or analysed are included in this published article.
Abstract
Immune thrombocytopenia (ITP) is a bleeding disorder caused by dysregulated B and T cell functions, which lead to platelet destruction. A well-recognised mechanism of ITP pathogenesis involves anti-platelet and anti-megakaryocyte antibodies recognising membrane glycoprotein (GP) complexes, mainly GPIb/IX and GPIIb/IIIa. In addition to the current view of phagocytosis of the opsonised platelets by splenic and hepatic macrophages via their Fc gamma receptors, antibody-induced platelet desialylation and apoptosis have also been reported to contribute to the ITP pathogenesis. Nevertheless, the relationship between the specific thrombocytopenic mechanisms and various types of antiplatelet antibodies has not been established. To ascertain such association, we used sera from 61 ITP patients and assessed the capacity of antiplatelet antibodies to induce neuraminidase 1 (NEU1) surface expression, RCA-1 lectin binding and loss of mitochondrial inner membrane potential on donors’ platelets. Sera from ITP patients with detectable antibodies caused significant platelet desialylation and apoptosis. Anti-GPIIb/IIIa antibodies appeared more capable of causing NEU1 surface translocation while anti-GPIb/IX complex antibodies resulted in a higher degree of platelet apoptosis. In ITP patients with anti-GPIIb/IIIa antibodies, both desialylation and apoptosis were dependent on Fc-gamma RIIa signalling rather than platelet activation. Finally, we confirmed in a murine model of ITP that destruction of human platelets induced by anti-GPIIb/IIIa antibodies can be prevented with the NEU1 inhibitor oseltamivir. A collaborative clinical trial is warranted to investigate the utility of oseltamivir in the treatment of ITP.

Article Summary
- Immune thrombocytopenia is a bleeding disorder and antiplatelet antibodies are major contributors to its pathogenesis, however, the clear association between antibody specificity and various thrombocytopenic mechanisms is unclear.
- This study demonstrates that sera with detectable anti-platelet antibodies caused significant platelet desialylation and apoptosis, with anti-GPIIb/IIIa antibodies triggering more platelet desialylation, and anti-GPIb/IX antibodies inducing a higher degree of platelet apoptosis.
Introduction
Immune thrombocytopenia (ITP) is an acquired autoimmune disease characterised by enhanced platelet destruction and impaired platelet production from megakaryocytes.\(^1\) ITP patients can present with no predisposing condition (hence, primary ITP) or with a variety of associated disorders (secondary ITP),\(^2\) such as autoimmune diseases (especially systemic lupus erythematosus),\(^3\) infections (notably Hepatitis C Virus and HIV)\(^4\) as well as malignancies.\(^5\) Primary ITP is estimated to represent approximately 80% of all adult ITP.\(^4\) Regarding the incidence of ITP, studies from Europe have estimated that to be 2.9-3.9/100,000 annually in adults,\(^6-9\) with an overall incidence slightly higher in females than males. The prevalence is approximately 9.5-23.6/100,000.\(^6,7,10\)

The pathogenesis of ITP involves antibodies recognising membrane glycoprotein (GP) complexes.\(^2,9\) The seminal Harrington-Hollingsworth experiment of self-infusion of ITP plasma led to the discovery of a humoral factor accountable for platelet destruction in ITP.\(^11,12\) Subsequently, Shulman identified that this factor could be adsorbed by platelets and was associated with immunoglobulin G (IgG).\(^13\) Currently, the widely accepted mechanism is that antibody-coated platelets are phagocytosed by splenic and/or hepatic macrophages of the reticuloendothelial system, via their Fc-gamma receptors (Fc\(\gamma\)R), resulting in accelerated platelet clearance.\(^1,9,14\) Equally important in the pathogenesis of ITP is the dysfunction of T cells.\(^15\) Both CD4\(^+\) T regulatory cells reduction\(^16-18\) and CD8\(^+\) T cell-mediated cytotoxicity\(^19-21\) have been reported in ITP.\(^2,9,15\) In addition, CD8\(^+\) T regulatory cells’ immunosuppressive role in ITP has been recognised.\(^22\) This further highlights the significant role of T cells in immune dysregulation and ITP.\(^23\)

Antibody-mediated platelet apoptosis has been suggested in ITP. In 2006, Leytin and colleagues reported in the mouse model that monoclonal anti-GP\(\text{IIb}\) antibody injection induced thrombocytopenia, caspase-3 activation, enhanced phosphatidylycerine (PS) exposure and mitochondrial inner transmembrane potential (\(\Delta\Psi_m\)) depolarisation.\(^24\) In 2012, Winkler et al. demonstrated similar findings in paediatric patients with ITP.\(^25\) More recently, platelet apoptosis was also confirmed in adult ITP patients by Goette and colleagues.\(^26\) However, the relationship between platelet apoptosis and ITP antibody specificity were unclear, as only one patient carried sole anti-GP\(\text{IIb/IX}\) antibodies in this study.
Another ITP pathway that has also been previously\textsuperscript{27} and recently\textsuperscript{28} described is Fc-independent platelet clearance. Li and co-workers reported platelet desialylation through antibody-induced platelet activation by treating donor platelets with monoclonal anti-GPIb/IX antibodies and ITP sera.\textsuperscript{28} Using a murine model of ITP secondary to monoclonal anti-GPIb\(\text{\textalpha}\) antibodies, the group demonstrated evidence of platelet removal via Ashwell-Morell receptors on hepatocytes, and the use of sialidase inhibitors to attenuate thrombocytopenia.\textsuperscript{28} Although the model is not directly relevant to human disease due to the absence of platelet surface FcyRIIA on mouse platelets and the polyclonal nature of primary ITP,\textsuperscript{9,29} these findings still have potential therapeutic implications: patients with anti-GPIb/IX antibodies may respond to sialidase inhibitors while patients with anti-GPIIb/III\(\text{\textalpha}\) antibodies were considered unlikely to have a response with this novel treatment.

Conversely, patients who harbor anti-GPIIb/III\(\text{\textalpha}\) antibodies possibly respond to IVIg therapy better than those with sole anti-GPIIb/IX antibodies, because anti-GPIIb/III\(\text{\textalpha}\) antibodies could drive ITP in an Fc-dependent fashion.\textsuperscript{23,30-32} Similarly, splenectomy may be ineffective in patients with only anti-GPIb/IX autoantibodies as desialylated platelets are removed by the liver.\textsuperscript{28} This differential effect and, therefore, determining antibody specificity, may influence treatment decision and ultimately patient outcomes. Nevertheless, these views have been recently challenged by Cantoni et al., who studied 93 ITP patients and found no predictability of anti-platelet antibody-specificity on the site of platelet clearance.\textsuperscript{33} Thus, ongoing investigations to determine whether antibody specificity predicts therapeutic response is vital.

The true extent to which platelet desialylation and/or platelet apoptosis are involved in ITP pathology remains unclear. Notably, both desialylated and apoptotic platelets were reported to be removed by the liver.\textsuperscript{28,34,35} Yet, platelet apoptosis was observed in a murine ITP model induced by monoclonal anti-GPIIb antibodies,\textsuperscript{24} but desialylation has been demonstrated in ITP patients with anti-GPIb\(\text{\textalpha}\)\textsuperscript{28} and anti-GPIIb/III\(\text{\textalpha}\) antibodies.\textsuperscript{36,37} Whether there is a link between platelet desialylation and platelet intrinsic apoptosis in ITP caused by these two antibodies is still not defined. In addition, the possibility of using neuraminidase inhibitors in the treatment of ITP is also to be investigated.
We recently examined the sera from 61 ITP patients for the presence of antiplatelet antibodies and their specificities. Here, we further scrutinise the sera’s capability to induce platelet desialylation and apoptosis, and studied these two potential thrombocytopenic mechanisms’ relationship with the antibody subtypes. Additionally, we established a mouse model to elucidate the therapeutic effect of neuraminidase inhibitor in preserving human platelet number in the presence of patients’ antibody and provide in vivo data on the feasibility of this agent in the treatment of ITP.

Methods

Patient sample collection
The study was approved by the Human Research and Animal Care Ethics Committees of the University of New South Wales (Sydney, Australia). Sera were obtained from 61 adult ITP patients (aged between 18 to 90 years) and 21 healthy controls with written informed consent. All 61 patients were diagnosed with ITP according to the international working group’s criteria, which was also consistent with the 2019 updated consensus report. Whole blood was centrifuged at 860xg for 10 min. The sera and plasma were stored in aliquots at -80°C until required for analysis. In some experiments, immunoglobulin G fraction (IgG), purified using Protein G affinity chromatography (Sigma-Aldrich, USA), was used. The purity was over 95% as determined by gel densitometry (ImageJ, Version 2.1.0/1.53c).

Antiplatelet antibody (APA) detection and specificity determination
The indirect detection of antiplatelet antibody and the determination of antibody specificities in this cohort of patients have recently been published. Briefly, venous blood was collected into 3.2% trisodium citrate. Platelet pellets were washed with wash buffer (pH 6.0) containing 140mM NaCl, 5mM KCl, 12mM trisodium citrate, 10mM glucose, 12.5mM sucrose, followed by resuspension in buffer (pH 7.4) containing 140mM NaCl, 3mM KCl, 0.5mM MgCl₂, 5mM NaHCO₃, 10mM glucose and 10mM HEPES. Washed platelets (1x10⁶) were incubated with patients’ samples at various dilutions for 30 min at 37°C, washed twice, incubated with Alexa Fluor 488 or 647-labelled anti-human IgG (Invitrogen, 1:100), washed and analysed by flow cytometry (LSRFortessa™ X-20 [BD, USA]).
For antibody specificity determination, monoclonal antibody immobilisation of platelet specific antigens assay (MAIPA) was performed as previously described. Donor platelets were incubated with sera, followed by washing and incubation with antibodies against GPIIb/IIIa complex (AP2; Beckman Coulter, USA), GPIX (FMC25, Millipore, USA) or GPV (G-11, Santa Cruz Biotechnology, Inc. USA). The platelets were washed, solubilised and incubated in goat anti-mouse IgG Fc fragment specific antibody (Jackson ImmunoResearch, USA) pre-coated microtiter plates (ThermoFisher, USA). The reactions were washed and further incubated with goat anti-human IgG Fc fragment specific-horseradish peroxidase-conjugated antibody (Sigma-Aldrich, USA). SureBlue™ TMB-microwell peroxidase substrate (KPL Inc. USA) was then added and stopped at 10 min with 0.18M sulphuric acid. The absorbance was read at dual wavelength (450 nm and 492 nm).

Platelet neuraminidase expression, desialylation and activation
To examine sera-induced platelet neuraminidase expression, 1 x 10^6 washed donor platelets were incubated with patient or control sera (1:25) for 30 min at 37°C followed by washing and incubation with anti-NEU1 mouse monoclonal antibody (1:25, Santa Cruz Biotechnology, Inc. USA) for 20 min at room temperature and washing. Similarly, for platelet desialylation, the washed sera-donor platelet reactions were incubated with FITC-labelled *Ricinus communis* lectin (RCA-1, Vector Laboratories, USA, 0.5 μg/mL). To determine the effect of patients’ IgG, control experiments were performed using purified IgG from healthy donors and ITP patients (50 μg/mL). Platelet activation was assessed by flow cytometry with anti-P-selectin monoclonal antibody APC (1:5, eBioscience™, Germany).

Platelet apoptosis
To evaluate ITP patient sera’s effect on platelet mitochondrial inner membrane depolarization potential, 1 x 10^6 washed donor platelets in phosphate-buffered saline containing 1mM MgCl₂, 5.6mM glucose, 10mM HEPES and 0.1 % BSA were incubated with patients’ or controls’ sera (1:10) for 30 min at 37°C, followed by washing and incubation with 100nM DiOC₆ (Molecular Probes) for 15 min in the dark. To examine the role of Fc-gamma (Fcγ) receptor in platelet apoptosis, platelets were pre-incubated with anti-FcγRIIa antibody (clone IV.3, 2.5 μg/mL) for 10 min at 37°C prior to treatment with patients’ sera. To
determine the effect of patients’ IgG on platelet apoptosis, experiments were performed using 50µg/mL of IgG, followed by washing and incubation with 50nM DiOC₆.

Non-obese diabetic/severe combined immunodeficient (NOD/SCID) mouse model of ITP

Human platelets (450 x 10⁶) were transfused via the tail vein into the NOD/SCID mice and allowed to stabilize for 2.5 h. Forty µg/g of patients’ or controls’ IgG was then administered intravenously. Oseltamivir-treated mice received intraperitoneal (IP) injection of 10µg/g oseltamivir immediately prior to human platelet infusion and again, immediately before IgG injection. Mouse blood was collected at time 0 (prior to IgG injection), 2, 4 and 6 h following IgG injection. Human platelets were identified using anti-human CD41a V450 (BD Biosciences, USA) by flow cytometry. The percentage of human platelets at time 0 was set at 100 percent.

Statistical analysis

Flow cytometry data were processed with FlowJo software (LCC, USA). Data were analysed with GraphPad Prism version 8 (GraphPad Software, USA). The significance level was set at less than 0.05. Kruskal-Wallis test with Dunn’s multiple comparison was carried out for in vitro desialylation and apoptosis experiments. Linear mixed model was used to examine the effect of neuraminidase inhibitor on platelet survival in vivo. Mann Whitney test was applied in other analyses.

Results

Patient characteristics

Of the 61 patients (aged between 18 to 90 years old) included in this study, 61% were female. The majority (84%) had primary ITP. Thirty-five patients (57%) had detectable antiplatelet antibodies in their sera as determined by flow cytometry. MAIPA demonstrated that 9 patients had sole anti-GPⅡb/Ⅲa antibodies, 5 patients had sole anti-GPIb/Ⅸ antibodies while 7 other patients had antibodies against both complexes. The rest (14 patients, 23%) had no detectable anti-GPⅡb/Ⅲa or anti-GPIb/Ⅸ antibodies by MAIPA (Figure 1A). Compared to the direct testing, the lower detection rate of dual antibody positive patients may reflect the lower sensitivity in antibody determination using indirect method, which is consistent with a prior MAIPA report.
Recent work demonstrated that GPV is an important target of antiplatelet antibodies in ITP.\textsuperscript{44, 45} We additionally interrogated patient samples with positive indirect antiplatelet antibodies (sera from 31 patients were available) for the presence of anti-GPV antibodies. Using a cut-off of 3 standard deviations (SD) over the mean of 20 normal controls, 21 patients were positive for antibodies against GPIIb/IIIa, GPIb/IX and/or GPV (Table 1). Seven were found to have anti-GPV antibodies in their sera. However, two patients have co-existing anti-GPIIb/IIIa antibodies, two others have co-existing anti-GPIb/IX antibodies, and one patient had all three antibodies (Table 1, Fig 1B).

**Antiplatelet antibodies predict platelet desialylation**

To determine whether ITP patients’ sera can induce desialylation, we treated donor platelets with ITP or control sera and measured NEU1 expression by flow cytometry. NEU1 expression was significantly increased in ITP sera-treated platelets compared to controls (Figure 2A). Further scrutiny of the patient subgroups, determined by the antibody status, demonstrated that NEU1 translocation was significantly different from controls only in platelets treated with sera from patients with detectable antiplatelet autoantibodies (Figure 2B). Indeed, when we examined the level of platelet desialylation, as assessed by RCA-1 binding, we found that only antibody-positive sera could induce detectable desialylation (Figure 2C). Of note, a significant difference in RCA-1 binding was not detected when comparing the whole ITP group (antibody positive and negative sera) to normal controls (Figure 2D). This is likely due to the weak binding of lectins (such as RCA-1) to their target sugars.\textsuperscript{46}

We further examined whether the IgG fraction was responsible for platelet desialylation. We used purified IgG from two ITP patients containing anti-GPIIb/IIIa antibodies to treat donor’s platelets. Figure 2E shows that the IgG fraction causes significant platelet desialylation. This result confirmed the findings by Marini and colleagues, that ITP patients’ IgG leads to cleavage of platelets’ sialic acid and hence, platelet desialylation.\textsuperscript{36} Together, these data show that ITP autoantibodies induce NEU1 translocation and platelet desialylation.
Given the previous observation of platelet activation by monoclonal antibodies against GPIbα causing platelet desialylation, we examined the ability for ITP patients’ sera to induce P-selectin externalization. Compared to the control group, there was no enhanced anti-CD62P binding to treated platelets (Figure 2F). Unlike NEU1 expression and desialylation, no difference in platelet activation was noted between sera from patients with or without detectable antiplatelet antibodies (Figure 2G). Interestingly, the 3 sera with the highest P-selectin expression were all from secondary ITP patients (SLE, APS and B-cell lymphoma), raising the likelihood of distinct disease biology in secondary ITP. Finally, no difference was seen when patients with GPIIb/IIa antiplatelets antibodies were compared with those with anti-GPIb/IX antibodies (Figure 2H).

**Antiplatelet antibodies predict platelet apoptosis**

To determine the role of ITP autoantibodies in platelet apoptosis, washed platelets were treated with either ITP or control sera. Like NEU1 expression, upon treatment with patients’ sera, platelets showed significantly reduced DiOC6 fluorescence compared to the control (Figure 3A). This denotes the loss of mitochondrial inner membrane potential, ΔΨm, indicating the presence of platelet apoptosis. Consistent with the observations for desialylation, when compared to the controls, antibody positive patients’ sera disrupted mitochondrial ΔΨm more effectively than the antibody negative group (Figure 3B). Importantly, these changes are induced by the IgG fraction of ITP sera (Figure 3C). Therefore, the presence of antiplatelet antibodies in ITP sera induces platelet apoptosis as determined by changes in mitochondrial ΔΨm.

**Anti-GPIIb/IIa antiplatelet antibodies induce platelet apoptosis via Fcγ receptor**

Prior literature demonstrated that the platelet desialylating capacity of anti-GPIbα antibody was Fcγ receptor independent. However, in the case of anti-GPIIb/IIa antibodies induced ITP, we and others have shown that Fcγ receptor is the driver of antibody-mediated platelet desialylation. Also clinically relevant is that, anti-GPIIb/IIa auto-antibodies account for the majority of antibody positive cases in our patient population (Fig 1B), which is consistent with a recent report. Therefore, further examination into the pathogenesis of anti-GPIIb/IIa antibody driven ITP is of clinical significance.
To establish the relationship amongst anti-GPIIb/IIIa antibodies, platelet Fcγ receptors and platelet apoptosis in ITP, we evaluated the impact of FcγRIIA inhibition with the monoclonal antibody IV.3 on platelet apoptosis. Platelets were treated with patient sera with or without the presence of IV.3, and the effect on platelet ΔΨm was examined. In the presence of FcγRIIA inhibitor, antiplatelet antibodies’ ability to induce loss of ΔΨm was impaired (Figure 3D). The rise in ΔΨm in platelets pre-treated with IV.3 was quantified and is shown in Figure 3E. This observation indicates that the anti-GPIIb/IIIa autoantibodies signal via FcγRIIA to initiate platelet apoptosis pathway.

**Antiplatelet antibodies have differential effects on the thrombocytopenic pathway**

We further examined the differential effects of the most common antiplatelet antibodies on platelet desialylation and apoptosis. Although only 9 sera contained anti-GPIIb/IIIa without the presence of anti-GPIb/IX antibodies, 6 of these antibodies induced NEU1 translocation (defined as MFI greater than 2SD of the controls). In contrast, only 1 out of the 5 sera with anti-GPIb/IX antibodies, without anti-GPIIb/IIIa, was able to do so (Figure 4A, table 2). To our surprise, 4 of these 5 anti-GPIb/IX sera led to platelet apoptosis as reflected by significant loss of ΔΨm (defined as MFI lower than 2SD of the controls) while only 3 of the 9 anti-GPIIb/IIIa sera induced this change (Figure 4B, table 2). Together, these novel findings indicate that antiplatelet antibody specificity is an important determinant of platelet fate in ITP and the missing link between the previously unobserved relationship between platelet desialylation and apoptosis.\(^{36}\)

Two patient sera were found to have sole anti-GPV antibodies. Repeated experiments using these 2 sera did not show evidence of desialylation on donor platelets (Figure 5A). However, treatment of donor platelets using one of the 2 patients’ sera produced significant reduction of DiOC6 fluorescence (Figure 5B). The second sample also led to decreased ΔΨm compared to controls but was not statistically significant (P=0.15; Kruskal Wallis with Dunn’s multiple comparison), indicating that anti-GPV antiplatelet antibodies possibly lead to platelet apoptosis. A larger sample size with sole anti-GPV antibody is needed to assess its functional effect on platelets.

**Oseltamivir protects platelets from GPIIb/IIIa antibody mediated destruction in vivo**
Neuraminidase inhibitor oseltamivir has been previously reported to protect platelets from anti-GPIbα monoclonal antibody driven platelet destruction in murine studies. More recently, we demonstrated oseltamivir’s effect on platelet number preservation in the presence of polyclonal human anti-GPIIb/IIIa antibody from a patient with acquired Glanzmann Thrombasthenia. Following our findings that anti-GPIIb/IIIa antibodies induce desialylation (Figure 4A), we extended our in vivo experiments to examine other ITP patients with sole anti-GPIIb/IIIa antibodies. To test whether destruction of human platelets could be prevented in vivo, we treated recipient mice with oseltamivir. As shown in Figure 6, oseltamivir protected human platelets from anti-GPIIb/IIIa antibodies-mediated destruction. Therefore, the protective effect of desialylation inhibitors could be generalized to patients with anti-GPIIb/IIIa antibodies. Oseltamivir reduces platelet destruction in ITP and is potentially an efficacious treatment for a larger proportion of ITP patients as anti-GPIIb/IIIa antibody is a more common antibody than anti-GPIbα antibody.

Discussion

ITP is a heterogenous disease with multiple proposed mechanisms. Potential therapeutic advances require more detailed understanding of the means that lead to platelet destruction. As such, we sought to examine platelet desialylation and apoptosis as contributors to thrombocytopenia in ITP. We studied 61 ITP patient sera and examined the presence of antiplatelet antibodies as a predictor for these two processes. Antibody specificity was interrogated, specifically, anti-GPIb/IX, anti-GPIIb/IIIa and GPV antibodies. GPIa was not examined as isolated anti-GPIa/IIa antibody positivity has not been reported in recent literature. We demonstrated that the presence of antiplatelet antibodies in ITP patients’ sera is associated with platelet desialylation in our patient population. Although desialylation was initially thought to be induced by anti-GPIb/IX antibodies in ITP, here we found that enhanced neuraminidase expression was observed in the majority of our patient cohort with detectable antiplatelet antibodies. This supports recent studies which reported that the loss of sialic acid is a more frequent finding in ITP than previously thought. Furthermore, in a murine model of ITP utilising patients’ anti-GPIIb/IIIa antibodies and human platelets, we found solid and reproducible evidence to support the use of neuraminidase inhibitors as potential new therapeutics for ITP.
The status of whether the patients have detectable ITP antibodies also influences the degree of platelet apoptosis. Sera with ITP antibodies induced significantly greater loss of \( \Delta \Psi_m \) compared to the controls, which was not observed in the antibody negative group. As we and others have found that desialylation depends on Fc receptor activity in anti-GP\( \text{IIb/IIIa} \) antibody-driven ITP,\(^{36,37}\) we further examined the relationship between platelet Fc\( \gamma \) receptors and the downstream signalling of these ITP antibodies in platelet apoptosis. Fc\( \gamma \)RIIa inhibitor IV.3 effectively suppressed antibody induced platelet loss of \( \Delta \Psi_m \), indicating that anti-GP\( \text{IIb/IIIa} \) antibodies may signal through Fc\( \gamma \) receptor to initiate platelet apoptosis.

In addition to the ability to predict desialylation and apoptosis, antibody specificity appears to have an impact on the predominant thrombocytopenic mechanism in ITP. Unlike a prior report,\(^{28}\) we found that anti-GP\( \text{IIb/IIIa} \) antibodies resulted in higher degree of NEU1 translocation. On the other hand, anti-GP\( \text{Ib/IX} \) antibodies appeared to cause more platelet apoptosis. A recent systematic study examined antibody specificity and platelet/megakaryocyte desialylation.\(^{36}\) The report described no association between antibody-induced desialylation and platelet apoptosis in ITP.\(^{36}\) However, this study did not report the relationship between antibody specificity and the induction of either desialylation or apoptosis. In our study, the finding of differential effects with respect to the antibody specificity provides a potential new dimension to the understanding of ITP pathogenesis.

The small number of patients containing sole anti-GP\( \text{V} \) antibodies precludes definite conclusions on its effect on the subsequent platelet events, but it is notable that sera from both patients did not induce desialylation. Interestingly, this result is consistent with the finding by Amini and colleagues, who recently reported the lack of hepatic uptake of Indium-111 labelled platelet in patients with anti-GP\( \text{V} \) antibodies.\(^{49}\) Of note, serum from one anti-GP\( \text{V} \) positive patient induced significant loss of platelet \( \Delta \Psi_m \), indicating anti-GP\( \text{V} \) may drive ITP via platelet apoptosis.

While antiplatelet antibody testing is available in specialised platelet laboratories, routine testing has not been recommended, and ITP remains a disease without any confirmatory
investigation or a “gold standard” test. Given the additional important information provided by a positive result, which is the ability of patients’ antibodies to induce platelet desialylation and apoptosis, we suggest ITP antibody testing to be incorporated into ITP management algorithm. It may provide new insight into ITP pathology and could guide treatment individualisation.

The main weakness of our study is that it was performed on stored patient samples. Prospective evaluation of pathological ITP sera would allow us to correlate the sera’s ability to induce desialylation and apoptosis with patients’ treatment and disease response. Another significant limitation in using stored samples is the lack of direct testing on patient platelets. Direct glycoprotein-specific antiplatelet antibody assays will improve testing sensitivity \(^{43, 50}\) and is the recommended test by the International Society of Thrombosis and Haemostasis (ISTH). \(^{51}\) Nevertheless, indirect examination allowed us to determine the impact of antiplatelet antibodies present in the patient serum on donor platelets and compared such effect of different patient samples as well as with controls.

Another limitation is the small number of patients with single antiplatelet antibody specificity. Larger sample size is desirable to capture the difference between each antibody type and the associated downstream effect on platelet survival more accurately, which may ultimately lead to ITP treatment individualisation. An additional challenge is the difficulties in performing platelet antibody testing outside specialised laboratories. In agreement with the ISTH recommendation, we recommend referral of platelet immunology tests to a centralised laboratory, where staff are adequately trained in specialised methods to minimise laboratory variability. \(^{51}\)

In conclusion, we report the predictive capability of antiplatelet antibody in relation to the potential underlying ITP mechanisms, specifically platelet desialylation and apoptosis. We showed the differential effects of antibody subtypes on these two ITP pathogenesis pathways and the role of Fcγ receptors on anti-GPIIb/IIIa antibodies induced platelet apoptosis. We also demonstrated the therapeutic effect of neuraminidase inhibitor in platelet preservation despite the presence of anti-GPIIb/IIIa antibodies. Hence, such treatment can potentially be applied to most ITP patients since it is likely that both anti-
GPIIb/IIIa antibodies (this study) and anti-GPIb/IX antibodies lead to platelet desialylation. Further collaboration is required to investigate the treatment potential using neuraminidase and/or apoptosis inhibitors in prospective randomized ITP clinical trials.
References

Table 1. Summary of antiplatelet antibodies found in sera as determined by MAIPA

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<th>Patient no.</th>
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Table 2. Relative effects of anti-GPIIb/IIIa and anti-GPIb/IX antibodies on platelet desialylation and apoptosis

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<th>Anti-GPIb/IX sera</th>
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<tr>
<td>Positive NEU1 translocation</td>
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<td>20%</td>
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<td>Significant loss of ΔΨm</td>
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<td>80%</td>
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**Figure legends**

**Figure 1. Antibody pattern by indirect flow cytometry and MAIPA.** (A) Focusing on GPIIb/IIIa and GPIb/IX in 61 ITP patients, 43% had no detectable antibodies by flow cytometry (Ab Negative); of the 35 patients with positive antibody by flow cytometry, 15% had antibodies against GPIIb/IIIa (IIIa); 8% against GPIb/IX (IX); 11% had antibodies against both complexes (IIb/IIIa & Ib/IX). (B) Examination of anti-GPV antibody by MAIPA in 31 patients with available sera, in relation to anti GPIIb/IIIa and GPIb/IX antibodies.

**Figure 2. Effect of ITP patient sera on platelet desialylation and activation.** (A, B) NEU1 surface translocation and (C, D), RCA-1 lectin binding comparing patient subgroups and controls. (E) Effect of purified ITP IgG (50 µg/mL) on RCA-1 lectin binding. (F & G) P-selectin expression on control and patient sera treated platelets. (H) P-Selectin expression on platelets treated with GPIIb/IIIa or GPIb/IX antibodies. CTRL, control. Pt, patient. Neg, antibody negative; Pos, antibody positive. MFI, mean fluorescence intensity. Data shown as mean ± SD. Levels of significance are expressed as p values. ns, non-significant, *p<0.05, **, p<0.01. Mann Whitney and Kruskal-Wallis test with Dunn’s multiple comparison.

**Figure 3. Effect of ITP sera on platelet apoptosis.** Loss of ΔΨm as measured by DiOC₆ in platelets treated by (A) ITP patients’ and controls’ sera, as well as (B) antibody positive and antibody negative patients’ sera. (C) Effect of purified ITP IgG (50 µg/mL) on washed platelets. (D) Histogram representing the effect of anti-FcγRIIa antibody IV.3 on ΔΨm despite the presence of patient sera. (E) Effect of anti-FcγRIIa antibody IV.3 on ΔΨm of 3 patients; data point represents the mean of 3 experiments. CTRL, control. Pt, patient. Neg, antibody negative; Pos, antibody positive. MFI, mean fluorescence intensity. Data shown as mean ± SD. Levels of significance are expressed as p values. ns, non-significant, *p<0.05, ***,p<0.001. Mann Whitney and Kruskal-Wallis test with Dunn’s multiple comparison. Repeated measures ANOVA used in 3E.

**Figure 4. Differential effect of anti-GPIIb/IIIa and anti-GPIb/IX antibodies on NEU1 surface translocation (A) and ΔΨm (B).** CTRL, control. Black horizontal lines represent the means
for each group. Green horizontal lines indicate the cut-off for positive samples (2SD over [for NEU1] and under [for DIOC₆] the average of controls). Two patients in the anti-GPⅡb/Ⅲa antibody subgroup and 2 patients in the anti-GPⅠb/Ⅸ subgroup had concomitant anti-GPⅤ antibodies.

**Figure 5. Effect of anti-GPⅤ antibodies on platelet desialylation (A) and apoptosis (B).** CTRL, control. Pt, patient. Data shown as mean ± SD. ns, non-significant, ***p<0.001. Kruskal-Wallis test with Dunn’s multiple comparison.

**Figure 6. Murine model of ITP with anti-GPⅡb/Ⅲa antibodies.** Effect of 3 ITP patients’ IgG (black dots) and oseltamivir treatment (red dots) in the presence of patient IgG, on human platelet survival in NOD/SCID mice (n=5 for each patient group) measured as human platelet percentage at 2, 4, and 6 hours after IgG injection. Data shown as mean ± s.e.m. Levels of significance are expressed as p values. ns, non-significant, **p<0.01, ****p<0.0001. Linear mixed model.
Figure 5

A

B

DiOC6 MFI

CTRL  Pt1  Pt2

ns

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Figure 6

- Pt IgG
- Pt IgG + Oseltamivir

Human plt (%) vs. Time (h)

ns

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