AUTOANTIBODIES IN IMMUNE THROMBOCYTOPENIA

BIOLAYER INTERFEROMETRY AS A NOVEL METHOD FOR DETECTING AUTOANTIBODIES IN PATIENTS WITH IMMUNE THROMBOCYTOPENIA

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A Thesis

Submitted to the School of Graduate Studies in

Partial Fulfillment of the Requirements for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (2021) McMaster University

(Biochemistry and Biomedical Science) Hamilton, Ontario

TITLE: Biolayer interferometry as a novel method for detecting autoantibodies in patients with immune thrombocytopenia

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NUMBER OF PAGES: xx, 97

LAY ABSTRACT:

Platelets are blood cells involved in clotting at sites of injury. Immune thrombocytopenia (ITP) is a disease defined by a low platelet count that can lead to bleeding. ITP is a rare disease that affects 3 in 100 000 adults every year. ITP is thought to be caused by proteins known as antibodies that bind self-platelets and lead to their destruction. These antibodies are directly found on approximately 50% of patients' platelets, and only 18% of patients have antibodies in circulation. It is possible in many patients, antibodies are present at a low concentration, or are too weak to be detected in antibody tests. In this study, a new technology known as biolayer interferometry was employed to find antibodies in a higher percentage of patients. Results showed only 6% of ITP patients had detectable antibodies in their circulation. This research will improve our understanding of antibodies in ITP.

ABSTRACT:

Immune thrombocytopenia (ITP) is an autoimmune hematologic disorder characterized by a low platelet count due to increased platelet destruction or decreased production. In primary ITP, the patient can have a low platelet count ($<100x10^9$ cells/L) for clinically unknown reasons. ITP is a rare disease that affects approximately 3/100 000 adults each year and some patients may experience bleeding symptoms. Autoantibody-mediated autoimmunity plays a role in the destruction of platelets by targeting platelet glycoproteins (GPs). Autoantibodies against platelet membrane GPIIbIIIa and GPIbIX are observed in about 50% of patients through direct antigen-capture assays, and 18% in patients through indirect antigen-capture assays. It is possible that some antibodies may not be detectable due to affinity or titre, or there may be other factors involved in platelet destruction. Currently, there is no definitive diagnostic test available for ITP, as a result of low assay sensitivity and different mechanisms involved in disease pathogenesis. The objective of this study was to use a novel approach to increase autoantibody detection unique to ITP patients. Total IgG was purified from patient and control plasma samples. A streptavidin-based antigen-capture assay was optimized to test the effect of biotinylation on the detection of anti-GPIIbIIIa and anti-GPIbIX autoantibodies in primary ITP patients (n=14), secondary ITP patients (n=3), non-immune thrombocytopenic controls (n=2) and healthy controls (n=16). Streptavidin-coated biosensors were used in an optimized biolayer interferometry (BLI) assay to study autoantibodies binding to biotinylated GPIIbIIIa and GPIbIX. Detection of anti-GPIIbIIIa autoantibodies in the streptavidin antigen-capture assay had a sensitivity of 24% and anti-

GPIbIX autoantibodies had a sensitivity of 25%. BLI showed binding of autoantibodies in approximately 5% of ITP samples for both GPIIbIIIa and GPIbIX. The samples that had detectable autoantibodies in the antigen-capture assay did not have detectable antibodies in the BLI assay. BLI was not able to confirm antibody detection found in enzyme immunoassays.

ACKNOWLEDGEMENTS:

I have greatly enjoyed this experience in science research and owe my successes to many people for their support during this degree. First and foremost, I would like to thank my supervisor Dr. Ishac Nazy, for his guidance in this project. I have learned so much from you and always appreciated your lab email signatures “happy science”, which constantly reminded me of how much I enjoy research. I would also like to thank my co-supervisor Dr. Donald Arnold, as well as my committee member Dr. Matthew Miller. I appreciate all of the feedback you have both provided me with in the last two years, which have helped me better my lab skills, writing skills, presentation skills and people skills.

I could not have made it without the help of my lab mates: Dr. Nikola Ivetic, Dr. Angela Huynh, John Vrbensky, Taylor Elliott, Mercy Daka, Anna Bissola, Julie Briscoe. The learning process was always so fun when we could learn from each other in the lab (and through Zoom). Thank you for all of the laughs we had during late lab hours when we were all so tired or frustrated with our experiments.

Thank you to Rumi Clare for all of your last-minute editing and help in discovering the capabilities in the wonderful technology we know as BLI. I am so grateful that I was able to work alongside you in this lab and appreciate your knowledge and humor more than I can ever express. Thank you Jim Smith, Jane Moore and Hina Bhakta for always bleeding donors for me even if you were busy with your own work.

Finally, thank you to my sister Barbora Hucik, and my parents, for listening to me talk about research, helping me become a better person during this experience, and for doing my laundry and cooking when I was too busy with my school work. I could not have completed this project without your love and support, and I am so grateful that you encouraged me.

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LIST OF ABBREVIATIONS:

ACD- acid citrate dextrose

AID- autoimmune disease

ALPS- autoimmune lymphoproliferative syndrome

AMIDA- antibody-mediated identification of autoantigens

APS- antiphospholipid syndrome

ASH- American Society of Hematology

BCA- bicinchoninic acid

BLI- biolayer interferometry

BSA- bovine serum albumin

CD- cluster of differentiation

CLL- chronic lymphocytic leukemia

conA- concanavalin A

Da- dalton

DEA- Diethylalamine

dsDNA- double-stranded deoxyribonucleic acid

EIA- enzyme immunoassay

ELISA- enzyme-linked immunsorbent assay

EDTA- Ethylenediamineteraacetic acid

Fab- fragment-antigen binding

Fc- fragment crystallizable

GP- glycoprotein

GT- Glanzmann thrombasthenia

HIREB- Hamilton integrated research ethics board

HIV- human immunodeficiency virus

HPA- human platelet antigen

ITC- isothermal titration calorimetry

IFN- interferon

Ig- immunoglobulin

IL- interleukin

ITP- Immune thrombocytopenia

IVIg- intravenous immunoglobulin

IWG- International ITP Working Group

k_a- association rate

k_d- dissociation rate1

K_D- equilibrium constant

kDa- kilodalton

LC- long chain

mAb- monoclonal antibody

MACE- modified antigen-capture assay

MAIPA- monoclonal antibody-specific immobilization of platelet antigen

mM- millimolar

NEM- N-ethylmaleimide

NHS- N-hydroxysuccinimide

NPP- nitrophenyl phosphate

OD- optical density

pAb- polyclonal antibody

PAGE- polyacrylamide gel electrophoresis

PBS- phosphate buffered saline

PDH- pyruvate dehydrogenase

pH- potential of hydrogen

PRP- platelet rich plasma

RA- rheumatoid arthritis

RBC- red blood cell

RNA- ribonucleic acid

SD- standard deviation

SDS- sodium dodecyl-sulfate

SLE- systemic lupus erythematosus

SPR- surface plasmon resonance

Tc- cytotoxic T cell

Th- helper T cell

TPO- thrombopoeitin

Treg- regulatory T cell

vWF- von Willebrand factor

WGA- wheat germ agglutinin

DECLARATION OF ACADEMIC ACHIEVEMENT:

All the experiments described in this thesis were performed by Andrea Hucik, under the supervision of Dr. Ishac Nazy and Dr. Donald M. Arnold, excluding the following:

- Dr. Peter Horsewood and Sabrina Shrestha purified glycoprotein IbIX
- Rumi Clare assisted in the purification of glycoprotein IIbIIIa
- James Smith conducted the indirect antigen-capture assays for autoantibody detection using platelet lysate

1.0 INTRODUCTION

1.1 Platelets

Platelets, or thrombocytes, are small, 1-2μm in diameter blood cells that are involved in many physiologic processes (Schultze, 1865).They are primarily involved in hemostasis but are also involved in inflammation and innate immunity (Ali, Wuescher, & Worth, 2015; Morrell, Aggrey, Chapman, & Modjeski, 2014). Platelets are derived from megakaryocytes in the bone marrow and are released into circulation, where they remain until they are recruited to respond to bleeding upon blood vessel damage (Wright, 1906). The process of hemostasis is a multi-step process that arrests bleeding when a blood vessel has been damaged. In the initial step of hemostasis, the blood vessel compresses and becomes narrower (vasoconstriction) (Gremmel, Frelinger, & Michelson, 2016; Broos, Feys, De Meyer, Vanhoorelbeke, & Deckmyn, 2011). Second, platelets interact with extracellular matrix proteins and are immobilized to the vessel wall at the site of injury (adhesion). Then, biochemical factors involved in the clotting process bind platelet receptors or receptors cross-link to induce a cascade of signal responses (activation), leading to platelet-platelet interactions occurring (aggregation) to form a hemostatic plug. Finally, through a process known as coagulation, fibrinogen is converted to fibrin and a meshwork of cross-linked fibrin forms around the platelet plug to stabilize it resulting in a blood clot. Platelets are also involved in responding to blood-borne infections and bacterial clearance, and can quickly respond to local infections because of their close proximity to the blood vessel walls while in circulation (McDonald & Dunbar, 2019).

Platelets express integrins and lectins that regulate their interaction with viruses, and they have several receptors which respond to invading bacteria by binding their surface proteins (C. Li et al., 2012; C. Li, Li, & Ni, 2020). Platelet production has recently been shown to be regulated by inflammatory mediators, which supports their need during a rapid infection response (C. Li et al., 2012; C. Li et al., 2020).A healthy adult has a platelet count between $150x10^9$ cells/L and $450x10^9$ cells/L for regular function (Gauer & Braun, 2012; Smock & Perkins, 2014). A person may have a lower platelet count caused by a decrease in bone marrow platelet production and increased platelet destruction or splenic sequestration, leading to the diagnosis of thrombocytopenia (Gauer & Braun, 2012; Smock & Perkins, 2014).

1.2 Thrombocytopenia

Thrombocytopenia is a condition in which a patient presents with a platelet count less than $150x10^9$ cells/L. It is typically discovered when a complete blood count is taken by a physician, and the cause is not always obvious. Signs or symptoms of a systemic disease may be present and patients typically require further clinical investigation. Thrombocytopenia is considered severe if the platelet count is less than $20x10^9$ cells/L (Rodeghiero et al., 2009). Thrombocytopenia may be a result of decreased platelet production, increased platelet apoptosis or sequestration (Smock & Perkins, 2014). There are several etiologies of thrombocytopenia including chronic liver disease, congenital thrombocytopenia, drug-induced thrombocytopenia and immune thrombocytopenia (ITP) (Gauer & Braun, 2012).

1.3 Immune thrombocytopenia (ITP)

ITP is an autoimmune hematologic disorder characterized by a decrease in platelet count (less than $100x10^9$cells/L) due to accelerated platelet destruction or abnormal platelet production (Kistangari & McCrae, 2013). ITP is classified as primary ITP, which is thrombocytopenia in the absence of infection, drugs or other autoimmune disorders (AIDs). Primary ITP has no known cause and is diagnosed by exclusion (McCrae, 2011). Secondary ITP is any form other than primary, including thrombocytopenia secondary to drugs (e.g. quinine), viral infection such as Hepatitis C or bacterial infection such as *Helicobacter pylori*, autoimmune disorders such as systemic lupus erythematosus (SLE), antiphospholipid syndrome (APS), or other disorders (Kistangari & McCrae, 2013). Although initially known as idiopathic thrombocytopenic purpura, it has been designated *immune* thrombocytopenia to acknowledge the immune pathogenesis and variability in symptoms (Rodeghiero et al., 2009). A patient suspected of having ITP should typically undergo several evaluations including a personal history as well as family history to rule out possible causes of thrombocytopenia including medical conditions and inherited disorders. ITP disease stages are classified as acute, or newly diagnosed (within 3 months), persistent (3-12 months), chronic (>12 months) or remission. ITP is a rare disease; 3.3/100 000 adults are diagnosed each year, with a higher incidence of diagnosis in the female population (Lambert & Gernsheimer, 2017). Patients over the age of 60 are also more commonly affected. ITP is easier to manage in children with about 80% undergoing spontaneous recovery(McCrae, 2011). Adult management is more complex

and they have an increased risk of bleeding, infection and mortality as the disease usually progresses to become chronic (Stasi et al., 1995).

1.4 ITP treatment

ITP can be severe with bleeding mandating immediate treatment. Patients with ITP may exhibit the following bleeding symptoms: bleeding in the skin (petechiae), mucosal bleeding in the oral cavity (e.g. blood blisters), or gastrointestinal tract, dry purpura (bruising), and in very severe cases, intracranial haemorrhage (Provan et al., 2010; Nomura, 2016).Corticosteroids such as prednisone and dexamethasone are used as a first line of therapy with initial response to treatment being about 85% within 7-10 days; however, most of these patients relapse over the following 6-12 months (McCrae, 2011). Intravenous immunoglobulin (IVIg) or anti-Rho(D) are typically used in conjunction with other treatment options; IVIg increases platelet count by decreasing platelet clearance in the spleen through mechanisms such as modified Fcγ receptor activity and is usually used as an emergency therapy (Leontyev, Katsman, & Branch, 2012).Anti-Rho(D) has also been used to treat ITP and this binds the Rh(D) antigen on erythrocytes to block clearance of antibody-coated platelets (Cooper, 2009). Common second line therapies are the immunosuppressant rituximab, which is an anti-CD20 antibody that normalizes T cell type and decreases B cells typically for up to a year, or thrombopoietin (TPO) receptor agonists, which bind and activate TPO receptors to stimulate megakaryocyte maturation and ultimately increase platelet production (Patel, 2006; Stasi et al., 2010). Open or laparoscopic splenectomy is also an option for patients who fail steroid therapy and this

treatment typically has a high success rate: approximately 80% of patients respond to the treatment within a week and 70% of patients respond to this treatment over a multi-year period; splenectomy has a low relapse rate (approximately 15%) (Schwartz et al., 2003; Kojouri, Vesely, Terrell, & George, 2004; Mikhael et al., 2009).

1.5 ITP pathogenesis

ITP pathogenesis is complex and involves a loss of tolerance to platelet and megakaryocyte glycoproteins (GPs), leading to destruction by autoantibodies or impaired production (Kistangari & McCrae, 2013; Cines, Bussel, Liebman, & Luning Prak, 2009). This tolerance defect is categorized into 3 groups: peripheral tolerance, differentiation blocks and central tolerance (Kistangari & McCrae, 2013). Secondary ITP related to a peripheral tolerance loss includes exposure to human immunodeficiency virus (HIV), where megakaryocyte infection may result in decreased platelet production (Sakaguchi, Sato, & Groopman, 1991; Sundell & Koka, 2006). Blocks in cellular differentiation may result in ITP, such as in the case of chronic lymphocytic leukemia (CLL) where B cell skewing leads to a low memory B cell subset (Kistangari & McCrae, 2013; Liebman, 2009). This is important in autoimmunity as memory B cells have an enhanced ability to respond to specific antigens upon re-exposure (Dorner, Jacobi, & Lipsky, 2009). Central tolerance defects associated with ITP include autoimmune lymphoproliferative syndrome (ALPS), where abnormal B and T cell death is associated with gene mutations (Kistangari & McCrae, 2013; Teachey, 2012). Patients with primary ITP typically display an increased $CD4^+$ T helper (Th) 1 cytokine profile, with increased type II interferon (IFN-γ) and interleukin 2 (IL-2) which play roles in adaptive and innate immunity as well as

inflammation response, and decreased regulatory T cells (Tregs) and Th2 cells which are important to infection response (Toltl, Nazi, Jafari, & Arnold, 2011; Panitsas et al., 2004; Sakakura et al., 2007). ITP may be caused by different pathologic mechanisms including an abnormal immune response by T cells, which leads to an increase in B cell clonal expansion and somatic hypermutation (Audia, Mahevas, Samson, Godeau, & Bonnotte, 2017; Perera & Garrido, 2017). Autoantibodies against platelet GPs develop as a result of the T-cell dependent clonal expansion (Hou & Peng, 2004). Platelet destruction can be triggered by recognition of anti-platelet antibodies on platelets which induces platelet opsonisation and phagocytosis by splenic macrophages through Fcγ-receptor ligation (Kuwana, Okazaki, & Ikeda, 2009). Cytotoxic T cells ($CD8^+$ Tc) are also thought to contribute to ITP onset by platelet lysis through apoptotic factors including Apo1/Fas, perforin and granzymes A and B (F. Zhang et al., 2006; Olsson et al., 2003).

1.6 Autoantibodies in ITP

ITP is thought to be primarily antibody-mediated, where immunoglobulin G (IgG)-type autoantibodies are involved in the destruction of the patient's own platelets by recognizing membrane GPs (van Leeuwen, van der Ven, Engelfriet, & von dem Borne, 1982; Swinkels et al., 2018). This was first presented in the studies of Harrington *et al.* in 1951 where a disease-related circulating plasma-derived component was identified following a blood transfusion from a patient with chronic ITP (Harrington, Minnich, Hollingsworth, & Moore, 1951). In 1965, Shulman *et al.* identified this factor to be an IgG antibody (Shulman, Marder, & Weinrach, 1965). Subsequent studies determined much of the IgG bound to platelets non-specifically (Dixon & Rosse, 1975). IgG is the

main class found in the human body and has the longest half-life in serum at concentrations between 10 and 16mg/mL (Schroeder & Cavacini, 2010). Four subclasses have been identified: IgG1, IgG2, IgG3, IgG4, with IgG1 being most prevalent in healthy individuals and IgG1 and IgG3 most typically induced in a protein antigen response (Vidarsson, Dekkers, & Rispens, 2014). In ITP, IgG1 autoantibodies are most typically found in patients, although IgG2, IgG3 and IgG4 can be found as well (C. Li et al., 2012). Typically, autoantibodies found in ITP patients are of the IgG isotype, however, IgM and IgA can be less frequently detected (Swinkels et al., 2018; Cines, Wilson, Tomaski, & Schreiber, 1985). IgA antibodies are commonly found at mucosal surfaces and serum antibodies are present at low levels, however they are usually higher than IgM (Schroeder & Cavacini, 2010). IgM is the first Ig expressed in B cell development and in a pentameric state the molecule can reach high avidity through multiple interactions as the multimer has ten antigen binding sites. IgM has been shown to stimulate complement fixing on platelets, which can initiate platelet clearance by complement activation (Cines et al., 1985).

Similar to platelets, megakaryocytes express GPs which may be targeted by autoantibodies in ITP patients (Rabellino, Levene, Leung, & Nachman, 1981). A 2003 study of plasma from paediatric ITP patients with anti-GP antibodies determined that in the presence of TPO, they inhibited maturation of mononuclear cells into megakaryocytes (Chang et al., 2003). A subsequent study showed a decrease in megakaryocyte production from $CD34^+$ cells using ITP patient plasma and found this to be mediated by anti-GP IgG (McMillan, Wang, Tomer, Nichol, & Pistillo, 2004). A recent study looking at anti-TPO

antibodies or antibodies against the TPO receptor cMpl found that 44% of ITP patients had antibodies against either TPO or cMpl; this proportion was similar in patients with other thrombocytopenic disorders such as splenomegaly and myelodysplastic syndrome (Nazy et al., 2018) Another study aimed to quantify megakaryocytes with bound IgG in bone marrow samples from primary ITP patients; from a study of 17 patients, it was determined that 12/17 (71%) had over 50% bone marrow-derived megakaryocytes with bound IgG, compared to 3/13 (23%) healthy controls (Arnold et al., 2015). Additional work looking at platelet-autoantibodies in the bone marrow used a modified platelet-GP antigen-capture assay to study bound and circulating antibodies in ITP patients, as well as non-immune thrombocytopenia and healthy controls, finding 56% of patients to have autoantibodies present in the bone marrow (Shrestha, Nazy, Smith, Kelton, & Arnold, 2020).

Antibodies that react with self-molecules in healthy individuals are known as autoantibodies and are specific to mechanisms of some AIDs where they target various intracellular or extracellular molecules (Casali & Schettino, 1996). They are typically IgM class and have a moderate affinity, providing defense against infection and contributing to immune system homeostasis (Suurmond & Diamond, 2015). High affinity somatically-mutated IgG autoantibodies occur in pathologic conditions where pathways and normal cell functions are disrupted (Elkon & Casali, 2008). There are three main mechanisms of antibody-mediated autoimmunity. First, it has been found that in the most common systemic AID, such as SLE, the target antigens are intracellular molecules (Elkon & Casali, 2008). Since they are most commonly located in the nucleus and not

readily accessible to antibody binding, nuclear components such as histones, dsDNA and RNA are only released and bound by antibodies following cell death, where they can be then found in the extracellular environment (Suurmond & Diamond, 2015). Secondly, autoantibodies found in patients with rheumatoid arthritis (RA) function differently and can target mutated autoantigens that are typically extracellular and located on long-lived proteins (Suurmond & Diamond, 2015; Elkon & Casali, 2008). The modifications are suggested to be induced prior to the development of autoantibodies and the eventual autoimmune state (Suurmond & Diamond, 2015). Finally, autoantibodies may be produced in response to foreign antigens such as during Epstein-Barr virus or pneumococcal bacterial infection; these cross-react with self-antigens, leading to autoreactive B cell clonal expansion (Suurmond & Diamond, 2015). This is different in ITP, where the target antigens are located on the platelet surface, the anti-platelet autoantibodies have a shorter lifespan and post-translational modifications of the antigens are induced in the presence of autoantibodies (Swinkels et al., 2018; Elkon & Casali, 2008). When B cells are presented with platelet antigens, they can secrete these autoantibodies by differentiating into plasma cells (Figure 1). These are found in the bone marrow and peripheral blood and they produce autoantibodies which increase platelet clearance by dendritic cells and splenic macrophages, cell apoptosis, or by inhibiting production of platelets from megakaryocytes (Dorner et al., 2009).

1.7 Autoantibody targets in ITP

Anti-platelet autoantibodies in ITP patients are mainly against platelet surface GPIIbIIIa(~220 kDa) and GPIbIX(~190 kDa) and are observed in approximately 50% of

patients (van Leeuwen et al., 1982; Horsewood, Smith, & Kelton, 1993; Tomiyama & Kosugi, 2005). These GPs function as receptors for coagulation factors involved in platelet adhesion and wound repair such as fibrinogen and von Willebrand Factor (vWF) (Broos et al., 2011). GPIIbIIIa is the main mediator in platelet adhesion and platelet-platelet interactions, and the heterodimer complex is the most abundant of surface GPs, with approximately 80 000 copies per cell (Audia et al., 2017; Horsewood et al., 1993). It has been shown that autoantibodies bind epitopes in both the extracellular and cytoplasmic domain of this complex, and additional studies show a favoured binding to the GPIIb subunit (Horsewood et al., 1993; Sela-Culang, Kunik, & Ofran, 2013). GPIIbIIIa is the primary target of anti-platelet autoantibodies (Audia et al., 2017).GPIbIX is the next common complex, with approximately 25 000 copies per cell (Horsewood et al., 1993). It is important in early hemostasis, as GPIb is the receptor for preliminary platelet adhesion at sites of injury (Broos et al., 2011). The target epitope on this complex is not well-studied, although typically autoantibodies are directed to bind the GPIb subunit and they are detected in approximately 25% of cases (Swinkels et al., 2018; Sela-Culang et al., 2013). There are other known platelet GPs, however they are less prevalent on the platelet surface. ITP studies show autoantibodies binding to GPIaIIa (approximately 2000 molecules per platelet), and GPIV (over 12 000 copies per cell) (Broos, De Meyer, Feys, Vanhoorelbeke, & Deckmyn, 2012); both proteins are less frequently identified as antigens in this disorder (~5% of cases) (Swinkels et al., 2018). Recently it was shown that GPV, with approximately 10 000 copies per platelet, is an autoantibody target in ITP; it is already well characterized as an antigen in Varicella-

associated thrombocytopenia and from the 1140 patients included in this new study almost 20% had platelet-bound anti-GPV autoantibodies: of the 343 platelet-bound antibody-positive samples, 10 (2.9%) had anti-GPV only, 10 (2.9%) were anti-GPIIbIIIa plus GPV, 61 (17.8%) were anti-GPIbIX plus GPV, and 141 (41.1%) were positive for GPIIbIIIa, GPIbIX and GPV (Vollenberg et al., 2019).

A study to determine novel antigens in ITP used antibody-mediated identification of autoantigens (AMIDA) through mass spectrometry (Kamhieh-Milz et al., 2017). These tests were conducted with antibodies isolated from pooled sera from patients with both detectable and undetectable autoantibodies. GPIIbIIIa, which was used as a control, was detected in ITP antibody+/- groups, as well as healthy controls and there was no quantitative difference between the groups (Kamhieh-Milz et al., 2017). The antigens which were detected in patients were validated through dot-blot analysis and included intracellular antigens involved in cell motility and cellular interactions such as myosin 9 heavy chain, coagulation factor 8, hexokinase 1, E1 pyruvate dehydrogenase (E1-PDH) and filamin A (Kamhieh-Milz et al., 2017). Interestingly, it has previously been demonstrated that oxidative stress is present in ITP patients and the enzymes involved in glycolysis such as E1-PDH and pyruvate kinase, can be affected by oxidative damage and potentially become autoantigenic (Kamhieh-Milz et al., 2012; Jin et al., 2013; Kamhieh-Milz & Salama, 2014; Ohno, Kanoh, & Uchino, 1984).

Some patients with chronic ITP have autoantibodies to multiple platelet GPs (Beardsley & Ertem, 1998). It is thought that ITP begins with autoantibodies against a single antigen like GPIIbIIIa, and the antibody-coated platelets are destroyed in the spleen

(McMillan, Longmire, Tavassoli, Armstrong, & Yelenosky, 1974). However, through this degradation process of platelets, antigenic peptides are released from the lysed platelets. It is possible that specific T cells may be recruited and activated as a result to stimulate B cells for novel autoantibody production against the peptides from the lysed cells (Vanderlugt & Miller, 2002). This phenomenon is known as epitope spreading and may explain why many chronic ITP patients have autoantibodies against multiple targets.

1.8 Standard immunoassays for autoantibody detection

Detection of anti-platelet autoantibodies is typically achieved through indirect tests (in circulation in plasma or serum) or direct tests (bound to platelet surface GPs/antigens). Assays for the detection of anti-platelet autoantibodies include an enzyme immunoassay (EIA), monoclonal antibody-specific immobilization of platelet antigen (MAIPA) and modified antigen capture enzyme-linked immunosorbent assay (ELISA) (MACE) (Figure 2) (Kiefel, Santoso, Weisheit, & Mueller-Eckhardt, 1987; Johnen et al., 2007; Murakami et al., 1985; Kosugi et al., 1996). These EIA-based antigen capture assays allow for analysis of multiple antigens including proteins and peptides (Kelton, Vrbensky, & Arnold, 2018). Since the antigen is immobilized on a microtitre plate, it can react with specific antibodies which are detected via enzyme-labelled secondary antibodies. The direct antigen capture assay involves the following steps: coating of a microtitre plate with an anti-mouse antibody, binding of a GP-specific monoclonal antibody to capture platelet GPs from patient whole cell lysate, and antibody detection using an enzyme-tagged anti-human IgG antibody (Lochowicz, 2011). The indirect assays

uses purified platelet GPs or donor platelet lysate coated in microtitre plates to bind autoantibodies in patient plasma and serum, which are detected using an enzyme-tagged anti-human IgG antibody (Lochowicz, 2011). Generally, immunoassays are simple to perform and typically have high sensitivity (ability to identify and rule in true positives) and specificity (ability to rule out true negatives) (Sakamoto et al., 2018). A recent systematic review and meta-analysis was completed to understand the performance of diagnostic testing in ITP; 18 eligible studies were included in this review with data from 1170 patients (Vrbensky et al., 2019). It was found that the sensitivity for GPIIbIIIa or GPIbIX anti-platelet autoantibody testing is 49% for direct tests, and 18% for indirect tests (Vrbensky et al., 2019).However, the occurrence of false positives is low, as depicted in the study with a 93% specificity in direct testing, and a 96% specificity in indirect testing (Vrbensky et al., 2019).

1.9 Limitations of standard immunoassays in autoantibody detection

The indirect and direct antigen capture assays have many limitations in their use and application. ITP patients have a low frequency of platelets as a result of decreased platelet production and increased platelet destruction, so the discovery of autoantibodies bound to platelets depends on the availability of circulating platelets (McMillan, 2003). Furthermore, antigen-capture assays such as the MAIPA and EIA rely on immobilization of platelet GPs or lysate bound by a mAb on a 96-well plate for autoantibody detection; having the antigens of interest bound to a surface or another antibody may result in steric hindrance and inaccessible epitopes, which may lead to a lower detection rate of ITP-related autoantibodies (Arnold, Santoso, Greinacher, & Platelet Immunology Scientific Subcommittee of the, 2012). This may

be a major factor in the low sensitivity of currently-employed immunoassays, and it will be important to determine whether the detection method is limited, or whether only half of ITP patients truly have anti-platelet autoantibodies.

2.0 RESEARCH OUTLINE

2.1. Overall Objective

The main objective of this study was to design an assay to detect the presence of anti-GPIIbIIIa and anti-GPIbIX autoantibodies in ITP patient samples using biolayer interferometry (BLI) and compare the sensitivity and specificity of the assay to standard immunoassays such as the EIA.

2.2. Rationale

It is important to address the ongoing issue of defining a reliable diagnostic marker and test for ITP. Although ITP is thought to be mainly autoantibody-mediated, disease pathogenesis is not fully understood and as a result there is currently no cure (Lambert & Gernsheimer, 2017). The International ITP Working Group (IWG) and American Society of Hematology (ASH) both presented guidelines which do not indicate anti-platelet antibodies as an appropriate test for disease diagnosis (Lambert & Gernsheimer, 2017). This is due to the low diagnostic sensitivity of autoantibody detection assays, the different possible mechanisms involved in disease onset, as well as the lack of knowledge in antibody targets. This suggests that there may be other antigens being targeted by anti-

platelet antibodies that are not yet known to be ITP-related, or that other mechanisms play a greater role in ITP onset and progression. More importantly, it proposes that current test methods are limited in their ability to detect antibodies in most patients if the antibodies are not high affinity and fall off after wash steps, or if they are present at a low titre.

2.3 Hypothesis

Only 18% of ITP patients test positive for anti-GPIIbIIIa or -GPIbIX autoantibodies in the indirect antigen-capture assay because they have undetectable low affinity or low titre autoantibodies.

2.4 Specific objectives

1. Establish a streptavidin-capture EIA and test feasibility of BLI using monoclonal antibodies against GPIIbIIIa and GPIbIX.

2. Test patient samples, establish cut-offs and determine assay sensitivity for the detection of autoantibodies in ITP using the streptavidin-based EIA.

3. Test patient samples, establish cut-offs and determine instrument sensitivity for detection of autoantibodies in ITP patients using BLI then compare to streptavidin-based EIA and previous indirect antigen-capture assay.

These objectives were completed using a modified antigen-capture assay using biotin-labeled GPs, as well as using BLI as the novel method for autoantibody detection. Biotin is a vitamin that binds with high affinity (10^{-14}mol/L) and high specificity to streptavidin and avidin proteins, unaffected by extreme pH or denaturing agents; because

biotin is a relatively small molecule (244 Da), it can be conjugated to many proteins without altering the biological activity of the protein (Green, Konieczny, Toms, & Valentine, 1971). For these reasons, this capture method was used in both the immunoassay as well as the BLI assay. BLI is a label-free technology that monitors binding interactions in real-time between a ligand, or antibody, immobilized on a sensor and a receptor, or antigen, in solution (Shah & Duncan, 2014; Kumaraswamy & Tobias, 2015). BLI measures interference patterns of white light causing a wavelength shift as a result of molecules binding to a probe (Kumaraswamy & Tobias, 2015). Only molecules binding to or dissociating from the biosensor can shift the interference pattern and generate a response profile so BLI is not susceptible to changes in fluid dynamic or refractive index (Shah & Duncan, 2014; Peterson, 2017). Using this technology, antibody-antigen interactions have been monitored previously by measuring the interference pattern generated through association and dissociation steps (Shah & Duncan, 2014). This data is used to analyze molecular interaction information such as kinetic rates and binding affinities of target autoantibodies (Shah & Duncan, 2014; Kumaraswamy & Tobias, 2015). BLI is a sensitive technology with a detection limit of about 0.1x K_D (Tobias, N.D.). Previous studies have shown that BLI is an appropriate tool to measure antibodies of various affinities; a kinetics screen of 2000 hybridomas against a single antigen was able to identify clones containing high affinity antibodies (KD: 10^{-12}M) to lower affinity antibodies (KD: 10^{-7}M) (Lad et al., 2015). A recent study of the interaction between a methyltransferase and a bacterial chemoreceptor and its derivatives showed an ability to detect low binding affinities (K_D:10^{-4}– 10^{-6}M) (M. Li &

Hazelbauer, 2020), therefore showing the feasibility of this technology in detecting low-affinity antibody interactions. In addition, a previous study successfully implemented BLI in the detection of viral-specific antibodies in patient sera diluted up to 1:10000, therefore, the use of BLI may be successful in further optimizing diagnostic strategies in ITP patients by testing very dilute samples (Auer, 2015).

3.0 MATERIALS AND METHODS

3.1 Patient samples and controls

The different cohorts being tested in this study are as follows: healthy controls (n=16), primary ITP patients (n=14), secondary ITP patients (n=3), non-immune thrombocytopenic controls (n=2) (Supplementary table 1, supplementary figure 1). Several controls were also used in this study, such as samples with antibodies against the human platelet antigen (HPA) -1 and 5 system, particularly the antigens HPA-1a and HPA-5a. These platelet antigen polymorphisms induce alloantibody production after a patient has received a platelet transfusion from a donor with a different HPA (Tomiyama & Kosugi, 2005; Smith, Hayward, Warkentin, Horsewood, & Kelton, 1993). Those with the HPA-1a antigen produce antibodies against GPIIIa, and those with the HPA-5a antigen produce antibodies against GPIa (Smith et al., 1993). Antibodies against HPA-1a are approximately 80% of those detected in alloimmunized patient sera (Smith et al., 1993). Neonatal alloimmune thrombocytopenia (NAIT) patients (n=5) were used as a

positive control, as they commonly have alloantibodies against HPA-1a. Plasma from a patient with Glanzmann's thrombasthenia (n=1) was used as a control, as patients with this disorder have very low amounts or no GPIIbIIIa on their platelets, or the GP has qualitative deficiencies from mutations and produces strong antibodies against this ITP-related GP (Nurden, Fiore, Nurden, & Pillois, 2011). Monoclonal antibodies (mAb) Raj-1 and TW-1 were used as controls to optimize each assay, as they target the most common platelet GPs (Supplementary table 2). Other mAbs against GPIIbIIIa (HIP2, HIP8, M148, CRC64, 96.2C1), and GPIbIX (SZ2, AK2, HIP1) were used as controls for optimizing, as it is important to test the efficiency of different antibodies binding to different epitopes. Isotype control mouse IgG1 kappa mAb, as well as anti-platelet factor 4 (PF4) mAbs 5B9 and KKO were used as negative controls. Consent was obtained from all donors and this study was approved by the Hamilton Integrated Research Ethics Board (HIREB).

3.2 Purification of GPIIbIIIa from platelet lysate

Platelet-rich plasma (PRP) was centrifuged at 2400 x g for 45 minutes to pellet platelets. Platelets were resuspended in 10 mL of PBS with acid citrate dextrose (ACD) pH 6.2 and topped up to 30 mL and centrifuged for 20 minutes at 2400 x g at room temperature. Platelets were washed twice with wash buffer (20 mM Tris HCl, 150 mM NaCl pH 7.5) in 25 mL buffer per 2 mL platelets at 2400 x g for 15 minutes at room temperature. The platelet pellet was resuspended in lysing buffer (20 mM Tris HCl, 150 mM NaCl, 1 mM $CaCl_2$, 0.02% NaN_3, 1% Triton X-100 and Ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor tablet (1 tablet per 50mL buffer) pH 7.5) with 15 mL buffer per 2 mL pellet. Platelets were lysed at room temperature for 10 minutes,

resuspended, and left overnight at 4 degrees Celsius on a shaker. Lysate was stored at -20 degrees Celsius until use. Platelet lysate was thawed and left at room temperature for 30 minutes and centrifuged at 45,000 x g for 30 minutes at 4 degrees Celsius in an ultracentrifuge. Supernatant was removed and stored at 4 degrees Celsius until use. The platelet pellet was further lysed with lysing buffer at 5 mL per 2 mL pellet and ultracentrifuged. Ten mL concanavalin A-sepharose (ConA) beads were washed with buffer A (20 mM Tris HCl, 500 mM NaCl, 0.1% Triton X-100 pH 7.5) three times for 15 minutes at 500 x g. ConA beads were mixed with solubilized platelet lysate on a shaker for 2 hours at room temperature or overnight at 4 degrees Celsius. The beads were poured into a 15 mL column and washed with 20 mL aliquots of wash buffer B (20 mM Tris HCl, 500 mM NaCl, 0.1% Triton X-100, 100 mM dextrose pH 7.5). Flow through was collected and the OD was measured. Beads were washed with buffer A until the OD reached 0. GPIIbIIIa was eluted off the beads with elution buffer (20mM Tris HCl, 500 mM NaCl, 0.1% Triton X-100, 200 mM methyl α-D mannopyranoside pH 7.5). Three mL fractions were collected and OD was measured. GPIIbIIIa presence and purity was verified through SDS-PAGE gel electrophoresis (Supplementary Figure 2).

3.3 Purification of GPIbIX from platelet lysate

PRP was pooled in 50 mL falcon tubes and centrifuged at 350 x g for 15 minutes at room temperature to pellet red blood cells (RBCs). PRP was centrifuged at 2400 x g for 45 minutes at room temperature to pellet platelets. Platelets were washed three times with wash buffer (120 mM NaCl, 30 mM D-glucose, 30 mM sodium citrate, 10 mM EDTA pH 6.5) at 2400 x g for 15 minutes at room temperature. Platelets were lysed in lysing buffer

(150 mM NaCl, 2 mM EDTA, 20 mM Tris, 2 mM N-ethylmaleimide (NEM), 1% Triton X-100, EDTA-free protease inhibitor tablet (in 50 mL lysing buffer) pH 7.4) and mixed on rotator for 30 minutes at room temperature. Platelet lysate was collected over time and stored at -20 degrees Celsius until use. Platelet lysate was ultracentrifuged for 2 hours at 7 degrees Celsius at 35,000 rpm. Supernatant was collected and the cell pellet was collected separately and frozen at -20 degrees Celsius. GPIbIX was purified using 10 mL wheat germ lectin (WGA) beads regenerated with a 30 minute incubation and one wash using regeneration buffer (500 mM NaCl, 1 mM Mg, 1 mM Mn, 1 mM Ca, 1 mM Zn). Beads were washed using washing buffer (150 mM NaCl, 2 mM EDTA, 20 mM Tris, 2 mM NEM, 0.1% Triton X-100 pH 7.4). 10 mL WGA beads were incubated with 100 mL of supernatant on a shaker at room temperature for 2 hours. A 500 mL flask was set up with a black column holder and a 60 mL coarse column, and the supernatant mixture was poured into the column. Flow-through was collected and stored at -20 degrees Celsius. The beads were washed with 25 mL wash buffer 5 times and washed 6 times in a 15 mL column. GPIbIX was eluted from the beads using elution buffer (150 mM NaCl, 2 mM EDTA, 20 mM Tris, 2 mM NEM, 0.01% Triton X-100, 2.5% N-acetyl-D-glucosamine pH 7.4). OD was recorded with each 3 mL fraction. Beads were stored in at 4 degrees Celsius in regeneration buffer with NaN_3. GPIbIX presence and purity was verified through SDS-PAGE gel electrophoresis (Supplementary Figure 3).

3.4 Biotinylation of platelet GPIIbIIIa and GPIbIX

One mL of 1 mg/mL or 1.1 mL of 307 μg/mL of purified GPIIbIIIa was incubated for one hour at room temperature with EZ-Link NHS-LC-Biotin, with a 1:1 ratio of biotin to protein and 3:1 ratio of biotin to protein, respectively, for a final molar ratio of 1:1. One mL of 300 μg/mL purified GPIbIX was incubated for one hour at room temperature with EZ-Link NHS-LC-Biotin, with a 3:1 ratio of biotin to protein, for a final molar ratio of 1:1. Excess biotin was quenched with 5 mL Tris-glycine buffer (20 mM Tris-HCl pH 7.4, 5 mM glycine, 10 mM EDTA, 140 mM NaCl) for 10 minutes and proteins were washed with Tris-glycine buffer once at 1900 x g and three times with phosphate-buffered saline (PBS) pH 7.4 using Amicon-Ultra 4 100 kDa cut-off centrifugal tube filter units before being re-suspended in 1 mL PBS. Proteins were stored at 4 degrees Celsius short term and -80 degrees Celsius long term.

3.5 Antigen-capture immunoassay

A 96-well Maxisorp plate was coated with 20 μg/mL of GPIIbIIIa or GPIbIX in bicarbonate buffer pH 9.6 ($NaHCO_3$, Na_2CO_3) overnight at 4 degrees Celsius. Wells were washed with PBS pH 7.4 + 0.05% Tween 20 twice and PBS pH 7.4 three times before being blocked by PBS pH 7.4 + 3% BSA overnight at 4 degrees Celsius. Wells were washed with PBS pH 7.4 + 0.05% Tween 20 and PBS pH 7.4 before a 1 hour incubation with purified human IgG using 100 μL of 200 μg/mL IgG with subsequent dilutions in 1% bovine serum albumin (BSA), 100 μL of 4 μg/mL monoclonal antibodies with subsequent dilutions in 1% BSA, or 100 μL of 1/50 human plasma with subsequent dilutions in 1% BSA for 1 hour at room temperature, washed, and incubated 100 μL of

1/3000 Alkaline Phosphatase-conjugated AffiniPure F(ab')$_2$ Fragment Goat Anti-Human IgG, Fcγ Fragment Specific in 1% BSA or 1/2000 Alkaline Phosphatase-conjugated AffiniPure Goat Anti-Mouse IgG, Fcγ Fragment Specific in 1% BSA for 1 hour at room temperature. The plate was washed six times before the addition of 100 μL of 0.5 mg/mL P-nitrophenyl phosphate (PNPP) tablets dissolved in Diethylalamine (DEA) buffer for IgG detection. The plate was read for 58 minutes at OD 405 nm, every 2 minutes with a reference OD 492 nm, shaking for 3 seconds between each read.

3.6 Modified streptavidin-capture immunoassay using GPIIbIIIa and GPIbIX

A 96-well Maxisorp plate was coated with 10 μg/mL Streptavidin in bicarbonate buffer pH 9.6 ($NaHCO_3$, Na_2CO_3) overnight at 4 degrees C. Wells were washed with PBS pH 7.4 + 0.05% Tween 20 twice and PBS pH 7.4 three times before being blocked by PBS pH 7.4 + 3% BSA overnight at 4 degrees C. Wells were washed with PBS pH 7.4 + 0.05% Tween 20 and PBS pH 7.4 before a 1 hour incubation with 100 μL of 20 μg/mL biotinylated GPIIbIIIa or GPIbIX diluted in 1% BSA. After following previous wash steps, the protein was incubated with purified human IgG with 100 μL of 200 μg/mL IgG with subsequent dilutions in 1% BSA, 100 μL of 4 μg/mL monoclonal antibodies with subsequent dilutions in 1% BSA, or 100 μL of 1/50 human plasma with subsequent dilutions in 1% BSA for 1 hour at room temperature, washed, and incubated 100 μL of 1/3000 Alkaline Phosphatase-conjugated AffiniPure F(ab')$_2$ Fragment Goat Anti-Human IgG, Fcγ Fragment Specific in 1% BSA or 1/2000 Alkaline Phosphatase-conjugated AffiniPure Goat Anti-Mouse IgG, Fcγ Fragment Specific in 1% BSA for 1 hour at room

temperature. The plate was washed six times before the addition of 100 μL of 0.5 mg/mL PNPP tablets dissolved in DEA buffer for IgG detection. The plate was read for 58 minutes at OD 405 nm, every 2 minutes with a reference OD 492 nm, shaking for 3 seconds between each read.

3.7 Total IgG purification from plasma samples

Fifty μL of Protein G beads were washed in Eppendorf tubes three times using PBS pH 7.4 for 5 seconds at 2000 x g. One hundred μL of patient and control plasma was diluted 1/10 in PBS pH 7.4 and incubated with the beads for 1 hour at room temperature, rotating. The beads were washed 3 times with PBS pH 7.4 before IgG was eluted in 0.1 M glycine pH 2.8, three times with 250 μL per elution at room temperature for 15 minutes, rotating. The eluate was collected and neutralized with 75 μL 1.5 M Tris pH 8.8 (1:1). A BCA assay kit or spectrophotometer was used to determine sample concentrations. Amicon Ultra-15 30 kDa cutoff centrifugal filter units were washed with PBS pH 7.4 prior to washing the IgG three times for 15 minutes at 2400 x g. The protein was re-suspended in 1mL PBS pH 7.4 and quantified. IgG was stored at 4 degrees Celsius short term and -80 degrees Celsius long term.

3.8 Streptavidin BLI assay for kinetics analysis

GPs were biotinylated as described. Streptavidin-coated sensors were used to determine antibody affinity using the ForteBio Octet RED96e (Supplementary figure 4). The initial assays were performed through ForteBio Data Acquisition 9 and were defined

as following: 10 minutes pre-soaking of the streptavidin probes, 60 seconds to establish a baseline, 1200 seconds for loading of GPIIbIIIa or GPIbIX onto the probes, 1800 seconds to re-establish the baseline, 800 seconds for antibody association, 3000 seconds for antibody dissociation as per standard protocol parameters. Baseline and dissociation steps were completed using PBS pH 7.4 or PBS with varying concentrations of BSA for blocking. Blocking of free streptavidin sites using 100 μM of biocytin was tested in preliminary assays through a 300 second quench step following loading of biotinylated ligand. Each well contained 200 μL of buffer, ligand or analyte. The experiments were completed at 30 degrees Celsius. Assay parameters were adjusted following initial experiments for optimal antibody binding conditions: 10 minutes pre-soaking of the Streptavidin sensors, 60 seconds to establish a baseline, 1200 seconds for loading of biotinylated GPIIbIIIa or GPIbIX, 1000 seconds to re-establish the baseline, 800 seconds for antibody association and 3000 seconds for antibody dissociation. Association and dissociation rates and the affinity constant were determined through ForteBio Data Acquisition 9.0 for data extraction.

3.9 Statistical Analysis

GraphPad Prism version 8.4.3 was used to produce graphs for the comparison of patient sample groups, as well as for statistical analysis. Cut-offs for all assays were calculated as a mean + 3 standard deviations (SD). Statistical significance was determined with a p value<0.05. A two-tailed unpaired t-test was used to compare distinct sample groups.

3.10 BLI analysis

ForteBio Data Analysis 9.0 was used for kinetics analysis. Sample concentrations were converted from μg/mL to nM for rate calculations. Binding responses were calculated as an average over 795 seconds. MAb studies were fit to a 1:1 homogeneous binding model, and pAb studies were fit to a 2:1 heterogeneous binding model.

4.0 RESULTS

4.1 Objective 1: Establish a streptavidin-capture EIA and test feasibility of BLI using monoclonal antibodies against GPIbIX and GPIIbIIIa.

4.1.1 Determining cut-off ODs using negative control mAb in EIA and streptavidin-capture EIA

In order to develop an antigen-capture assay that could be compared to the BLI assay to determine assay sensitivities, a streptavidin-capture assay was designed using purified GPs which were biotinylated using Sulfo-NHS LC biotin at a final concentration of 1 mol biotin to 1 mol protein. The assays were performed using an optimized concentration of 20 μg/mL of purified GPIbIX or GPIIbIIIa, both biotin-labeled and unlabelled. These conditions were tested to determine whether biotin would interfere with the binding of mAb and pAb from patient samples, as any significant changes in the

binding capabilities would render the capture method unfit. The biotinylated proteins were immobilized on streptavidin-coated plates and incubated for 1 hour with dilutions of mAb against GPIIb and GPIb starting at 2 μg/mL. Negative controls (mouse IgG1 kappa, 5B9, KKO) at 2 μg/mL were used to establish the background and cut-off values of the assays prior to studying mAb binding. These were calculated as the mean and 3SD. The cut-off for GPIbIX was OD: 0.087, calculated using the negative control mAb ODs as follows: mouse IgG1: 0.062; KKO: 0.036; PF4: 0.046. The cut-off for GPIIbIIIa was OD: 0.125, based on the following negative control mAb ODs: mouse IgG1: 0.087; KKO: 0.052; PF4: 0.076.

4.1.2 Measuring binding of mAb to GPIbIX and GPIIbIIIa using the EIA and streptavidin-EIA

To determine the levels of mAb binding to GPIbIX, four anti-GPIb mAb were tested. All four antibodies were subclass 1, IgG mAb. The comparison of biotinylated versus non-biotinylated GPIbIX showed a decrease in binding of the mAb to biotinylated antigen, although binding to non-biotinylated GPIbIX was still detectable at a low concentration (Figure 3). At a concentration of 2 μg/mL, TW-1 had an OD of 3.31 in the assay using purified protein, however, the OD was only 1.027 in the assay using biotinylated protein. AK2, which had a similar level of binding, dropped from OD: 3.23 to OD: 1.71 in the streptavidin-capture assay. HIP1 also exhibited strong binding in the EIA (OD: 3.19), with a decrease in binding in the streptavidin EIA (OD: 1.05). SZ2, an

IgG1 mAb, exhibited the weakest binding of all anti-GPIb mAbs, (OD: 2.35 in the EIA, OD: 0.73 in the streptavidin EIA), possibly as a result of epitope accessibility.

To determine the levels of mAb binding to GPIIbIIIa, four anti-GPIIb antibodies were tested in the EIA and streptavidin-capture EIA. The comparison of biotinylated versus non-biotinylated GPIIbIIIa also showed a decrease in binding of the mAb to biotinylated antigen based on the ODs (Figure 4), a phenomenon similar to the tests using mAb against GPIb; however, binding to non-biotinylated GPIbIX was also still detectable at a low concentration. With the biotin-labeled protein, Raj-1 exhibited a decrease in binding weaker (OD: 3.48 to OD: 1.27). Likewise, binding of CRC64 and 96.2C1 decreased with the streptavidin-capture method (OD: 3.50 to OD: 1.26 and OD: 3.49 to OD: 1.14, respectively). HIP8 mAb decreased from OD: 3.54 to OD: 2.12. Raj-1 and 96.2C1 has the largest decreases in ODs compared to the other mAb, and it is possible that this is due to the biotin having a greater impact on epitope accessibility for these antibodies. However, antibody binding was detectable after several dilutions; the antigen-capture assays showed feasibility of using a streptavidin-based protein immobilization, and this system was applied to BLI.

4.1.3 Measuring binding of mAbs to GPIbIX and GPIIbIIIa using BLI

In order to determine the feasibility of BLI assays for immobilizing GPIIbIIIa and GPIbIX and measuring antibody binding interactions, mAbs were used in the optimization assays. The BLI assay was designed using biotinylated GPs labelled with a final ratio of 1 mol biotin to 1 mol protein, which were optimized to use at a

concentration of 20 μg/mL. MAbs were used at 2 μg/mL and diluted in 0.01% BSA for the GPIIbIIIa assay, and PBS for the GPIbIX assay; the binding response, equilibrium constant and on and off rates were measured. Cut-offs were established using negative control mAbs (KKO, mouse IgG1, anti-GPIIb antibodies and anti-GPIb antibodies) at 2 μg/mL. Responses were recorded as the average of the 800s association period. For GPIbIX cut-offs, the negative controls as well as anti-GPIIbIIIa mAb were tested in the BLI assay. The mean (-0.0054 nm) and 3SD (0.02787 nm) resulted in a cut-off value of 0.02247nm based on the following binding response values: KKO: -0.0082; mouse IgG1: -0.0092; Raj-1: -0.0148; HIP2: -0.015; HIP8: -0.0041; CRC64: 0.003; 96.2C1: 0.0102. For the GPIIbIIIa BLI cut-off, the negative controls as well as anti-GPIbIX mAb were tested. The mean (-0.001 nm) and 3SD (0.01388 nm) resulted in a cut-off value of 0.01288nm based on the following binding response values: KKO: 0.0004; mouse IgG1: 0.0054; AK2: -0.0002; SZ2: -0.0037; HIP1: -0.0069.

Following the establishment of background mAb binding signal, streptavidin biosensors with 20μg/mL of biotinylated GPIbIX and GPIIbIIIa were incubated with 2μg/mL of monoclonal antibodies. Binding affinities of various mAbs against GPIb were tested using the protein bound to streptavidin sensors and results were analyzed using a 1:1 homogeneous ligand binding model (Supplementary Figure 5). This model was chosen as it had the most accurate fit with the binding pattern of the various mAb, and it demonstrated the association of one Fab portion of each antibody binding one epitope on its cognate protein. Kinetics analysis shows HIP1, an IgG1 antibody, had the lowest binding response to GPIbIX as well as the lowest affinity (10^{-7} M), compared to AK2

which had a quick initial binding response, with an on-rate of 10^6 /Ms and high affinity (10^{-10} M) (Table 1). TW-1 and SZ2 had similar association rates (10^3/Ms). Although TW-1 had a lower overall binding response compared to SZ2 (0.0247 nm versus 0.0426 nm), TW-1 was a slightly stronger antibody, with an equilibrium constant of 9.09×10^{-9} M compared to 1.34×10^{-8} M for SZ2. The negative controls 5B9 and mouse IgG1 both had a negative binding response (-0.0212 nm and -0.0273 nm, respectively) and the equilibrium constant could not be calculated due to the lack of antibody association, as expected.

A similar experiment was performed using GPIIbIIIa and was also analyzed using a 1:1 homogeneous ligand binding model as it showed the most accurate fit to the binding pattern exhibited by the mAb (Supplementary Figure 6). Kinetics analysis shows HIP2, an IgG3 antibody, exhibits the least binding to the protein (0.0097 nm) and this may be due to epitope proximity to biotin binding sites (Table 2). It was concluded through optimization of the antigen-capture assay that the mAb did not bind when the protein was both biotinylated and non-biotinylated; this may be a result of protein density on the plate, and in this assay, on the sensor, inhibiting its binding. Antibodies HIP8 and CRC64 showed the largest binding responses (0.1087 nm and 0.1011 nm, respectively), with both antibodies having the fastest on rates (10^7 /Ms). Both antibodies has the strongest binding affinities of the five tested, with HIP8 at 10^{-12} M and CRC64 at 10^{-11} M. Raj-1 had a similar K_D to 96.2C1, also at 10^{-10} M, however, the average binding response was much weaker at 0.0658 nm. The negative control mouse IgG1 exhibited a small binding response, at -0.0002 nm. These results show that BLI is an optimal tool for studying antibody binding to platelet GPs.

4.1.4 Measuring dilution limits of mAb in BLI assay

Following the optimization of the mAb assays, it was important to determine the limits of detection in terms of mAb concentration. The strongest mAb to each GP were not chosen for this experiment, for the reason that this assay would be later used to study autoantibodies in ITP. Following the hypothesis that current antigen-capture assays are not sensitive enough to detect weak antibodies in ITP patients, it was important to pick mAb that had a moderate affinity from their group in order to determine the concentration limits in these scenarios, since it would be expected that the highest affinity antibodies could be diluted further than medium-affinity antibodies. SZ2 was picked as an anti-GPIb mAb, and 96.2C1 was picked as the anti-GPIIb mAb and they were diluted from 2μg/ml (13.3 nM) to 0.06 μg/mL (0.42 nM). These values were compared alongside the OD at the same concentration (Figure 5, Table 3, Figure 6, Table 4).

Kinetics analysis shows a concentration dilution limit in BLI a few dilutions before the dilution limit in the streptavidin-based antigen capture assays. In BLI, binding of anti-GPIb mAb SZ2 is not measurable at 0.25 μg/mL, while in the streptavidin-EIA it is considered positive to a dilution of 0.125 μg/mL (OD: 0.208 with background OD: 0.068 subtracted, cut-off OD: 0.087). Binding of anti GPIIb mAb 96.2C1 is not measurable at 0.25 μg/mL, and in the streptavidin-EIA is considered positive to a dilution of 0.06 μg/mL (OD: 0.429 with background OD: 0.265 subtracted, cut-off OD: 0.125). This may be due to the protein density and conformation when immobilized on a streptavidin-coated plate versus immobilization on a streptavidin-coated sensor. It is

possible that there is a discrepancy due to the availability of biotin-binding sites on the matrix.

4.2 Objective 2: Test patient samples, establish cut-offs and determine assay sensitivity for the detection of autoantibodies in ITP using the streptavidin-based EIA

4.2.1 Determining cut-off ODs using healthy controls in streptavidin-capture EIA

Following the EIAs design and optimization using mAb, antigen-capture assays and streptavidin-capture EIAs were performed as previously described, using purified GPIbIX and GPIIbIIIa as well as GPIbIX and GPIIbIIIa labeled with Sulfo-NHS LC biotin, at a final concentration of 1 mol biotin to 1 mol protein. Patients who previously tested both positive and negative for autoantibodies in the indirect antigen-capture assay using platelet lysate were studied, including: primary ITP patients (n=14), secondary ITP patients (n=3), non-immune thrombocytopenic controls (n=2) and healthy controls (n=16). For these experiments, there was no true positive control for anti-GPIbIX antibodies: patients with Bernard-Soulier syndrome have a deficiency in GPIb expression (Clemetson, McGregor, James, Dechavanne, & Luscher, 1982), however due to the inability to obtain an antibody-positive sample, this control was not used. A Glanzmann's

thrombasthenia (GT) patient was used as a strong positive control for GPIIbIIIa, as well as NAIT samples, which are known to have antibodies against GPIIb.

Based on preliminary experiments using BLI, it was determined that healthy control plasma produced a high rate of background binding, likely due to the presence of plasma proteins such as albumin. For this reason, total IgG was purified using Protein G beads for all samples and only the binding of IgG to the target antigens was measured. Healthy donors were used as negative controls and cut-offs were established for both biotinylated and non-biotinylated GPs. Although previous cut-offs were established for direct and indirect antigen-capture assays (OD: 0.21) using the mean and 2SD, the cut-offs were re-calculated using the mean and 3 SD, when the positive control had an OD of at least 1.0 for GPIIbIIIa. For GPIbIX, the same samples were tested for consistency; however, it is important to note that GT and NAIT patients were not antibody-positive for antibodies against GPIb and are additional negative controls in this circumstance, although they were not used to calculate the cut-off. Additionally, the background binding was considered for all final conclusions; any background binding, possibly from residual IgG bound to the purified protein originally was subtracted. The cut-offs using healthy controls for the GPIbIX streptavidin-capture assay (n=15) and antigen-capture assay (n=9) were OD: 0.40 and OD: 0.34, respectively, with backgrounds of OD: 0.084 and OD: 0.032, respectively. Following background subtraction, the cut-off ODs were 0.319 and 0.313 for the streptavidin capture and the antigen capture assay, respectively. These were taken at 10 minutes of a kinetics read measured at 405 nm. The cut-offs using healthy controls for the GPIIbIIIa streptavidin-capture assay (n=16) and antigen-capture

assay (n=16) were OD: 0.35 and OD: 0.40, respectively, with backgrounds of OD: 0.108 and OD: 0.048, respectively. Following background subtraction, the cut-offs were OD: 0.244 for the streptavidin-capture assay and OD: 0.356 for the antigen-capture assay. These were recorded at a 10 minute time point for the streptavidin-capture, and a 12 minute time point for the antigen-capture during a kinetics read measured at 405 nm.

4.2.2 Detecting anti-GPIbIX and anti-GPIIbIIIa autoantibodies in purified IgG from ITP patients

After determining the cut-off values, 18 samples were tested in the GPIbIX assays and 19 samples were tested in the GPIIbIIIa assays to determine which of the ITP patient samples were autoantibody-positive. Several samples that had previously been tested in an indirect antigen-capture assay using a 1/50 dilution of plasma with platelet lysate, were used in these experiments. It was important to compare the ability of the streptavidin-capture test to the non-streptavidin based test, as well as the sensitivity of the assay when using IgG instead of plasma. The experiments were conducted using GPIbIX (Table 5, Figure 7) and GPIIbIIIa (Table 6, Figure 8) and represented with the ODs for the antibody positive samples, as well as using a dot plot. Any ITP samples that were autoantibody-negative based on the calculated cut-offs were not recorded. Furthermore, background binding levels were considered and subtracted in order to determine which samples were autoantibody-positive. The ODs were subtracted as follows: for the GPIbIX streptavidin-capture, OD: 0.035; for the GPIbIX antigen-capture, OD: 0.031; for the GPIIbIIIa streptavidin-capture, OD: 0.163; for the GPIIbIIIa antigen-capture, OD: 0.086.

Based on the results, it was determined that biotinylation of GPIbIX did not affect the detection of antibody-positive ITP patient samples; in fact, it increased the binding of antibodies to the GP. The samples which were positive in the non-biotinylated assay (samples 4, 1, 15), were also positive in the streptavidin-capture assay using biotinylated GP. Two additional samples were positive in the streptavidin-capture assay (sample 8, 17), with an OD of 0.414 and 0.368, respectively. Since the cut-off is 0.319, this suggests that the samples are weak positives. The three other positive samples were all strong positives (OD>0.64). Next, this experiment was performed using GPIIbIIIa.

Based on the initial results, several samples were positive in the streptavidin-capture assay, with an OD>0.35, however, after subtracting the background IgG on the protein, only five ITP samples were positive for autoantibodies: samples 20, 15, 8, 1, 9. The results of the non-biotinylated assay shows the positive control antibodies to bind strongly to the immobilized GPIIbIIIa in the wells, however, no ITP patient IgG was above the cut-off, therefore no samples were determined to be antibody-positive. To further analyze the results of the antigen-capture assays, the ODs were compared to those from previous indirect antigen-capture assays using a plasma dilution and binding free autoantibodies to their cognate GP in platelet lysate (Table 7).

Based on the final comparison, only two samples (1, 15) were positive in all of the assays (platelet lysate, GPIIbIIIa, GPIbIX). Sample 15, which was not positive in the original indirect assay using TW-1 to capture GPIbIX from platelet lysate, was antibody positive in both GPIbIX capture assays using the purified and biotinylated protein. Interestingly, sample 20, which was not positive for anti-GPIIbIIIa antibodies but was

positive for anti-GPIbIX antibodies had the opposite results in the new assays: it was anti-GPIIbIIIa antibody positive in the streptavidin-capture, however, anti-GPIbIX antibody negative. It is important to consider the affect of biotin in these EIAs; since biotin binds streptavidin with a strong affinity, it may ensure that the protein remains bound to the plate in between washes, where in the non-biotin capture assays some of it may be washed off. This may be true for samples 15 and 20, which were antibody positive in the streptavidin capture assays using biotinylated GPIIbIIIa but not in the non-labeled protein capture. Overall, the percentage of samples that were antibody-positive in either of the streptavidin-capture assays was approximately 25%.

4.3 Objective 3: Establish cut-offs for detection of autoantibodies in ITP patients using BLI and determine instrument sensitivity compared to streptavidin-based EIA and previous indirect antigen-capture assay.

4.3.1 Determining BLI cut-offs using healthy controls

In order to test the sensitivity of BLI in ITP sample autoantibody detection, it was important to determine what the background levels of total IgG binding were to establish a cut-off based on healthy control IgG. BLI was performed as previously described, using biotinylated purified GPIIbIIIa and GPIbIX. To determine which samples were

autoantibody-positive in this assay, cut-offs were established using 14 healthy controls. Total IgG was purified from the 14 healthy donors, and antibody binding was measured using 200 μg/mL of total IgG diluted in buffer. The response was recorded (in nm), to determine the background amount of IgG binding from human plasma. The cut-offs were established using total purified IgG diluted in 0.01% BSA (Tables 9 and 11). BSA allowed for the strongest and ultimately the truest binding to be captured and analyzed. A dilution of 0.01% BSA was chosen based on optimization experiments, where 1%, 0.5%, 0.1% and 0.01% BSA concentrations were used for the dilution buffer. Although IgG binding from anti-GPIIbIIIa positive control GT was detected at all concentrations of BSA, preliminary experiments showed that IgG binding from ITP patients was completely inhibited at higher BSA concentrations. Based on these results, it was determined that the most dilute buffer should be used in future studies to ensure that no potentially important binding signals are blocked.

Cut-offs were initially established for both GPIbIX and GPIIbIIIa in PBS as well as 0.01% BSA, however, it was determined that dilutions in PBS resulted in high background binding of healthy donor IgG. When BSA is introduced into the buffer, it has a significant effect on the ability of antibodies to bind to the sensor, even at a very low concentration. For this reason, only the BLI experiments using ITP samples diluted in 0.01% BSA are included in this analysis. For GPIbIX diluted in 0.01% BSA, the cut-off was calculated with the mean (0.0089 nm) and 3SD (0.01222) and determined to be 0.04556nm (Table 8). For GPIIbIIIa diluted in 0.01% BSA the cut-off was calculated with the mean (0.04556nm) and 3SD (0.01055) and determined to be 0.05282nm (Table

9). The association and dissociation of antibodies to GPIbIX was recorded over time (800 s association and 3000 s dissociation) and kinetics data was produced through ForteBio data analysis. For each protein and condition, a healthy control was chosen that had a similar value to the cut-off, either as a single or doubled binding response measurement (Supplementary tables 3, 4). The binding response of each sample was calculated as a ratio over the binding response of the healthy controls (1x or 2x if a doubled measurement was necessary to match the cut-off for each condition). Any value over 1.0 was considered an anti-GPIb or anti-GPIIb antibody-positive sample (Figure 9, 10).

4.3.2 Detecting anti-GPIbIX and anti-GPIIbIIIa autoantibodies in purified IgG from ITP patients

ITP samples were diluted in BSA to ensure that only true signals were detected. In the assay, one sample (ITP 21) was positive for antibodies specific to GPIbIX (binding ratio of 2.551). It was not possible to calculate an accurate K_D as the BSA causes a small interference with the signal which results in large K_D error values. The experiments were performed using GPIIbIIIa with dilutions in BSA. Following data analysis, it was determined that one sample, ITP 21, was positive for anti-GPIIbIIIa antibodies as well. The ratio to the cut-off was significantly higher in the GPIIbIIIa assay (3.4) compared to the GPIbIX assay for this sample (2.5). Based on these results it can be concluded that the assays performed using BSA detect both anti-GPIbIX and –GPIIbIIIa antibodies, as the conditions allowed for detection of a true antibody binding signal from positive control GT.

5.0 DISCUSSION

Primary ITP is an autoimmune bleeding disorder of platelets, which contain coagulation factors that function to arrest bleeding. Primary ITP is caused by a decrease in platelet count due to platelet destruction or abnormal platelet production in the absence of infection, drugs or other autoimmune disorders. Although different mechanisms such as an abnormal T cell response have been suggested, the disease is thought to be primarily antibody-mediated, where anti-GPIIbIIIa and GPIbIX IgG autoantibodies are involved in the destruction of the patient's own platelets. Antigen capture assays are able to identify disease in 53% of patients through direct tests (bound autoantibodies on platelets) and the detection rate is even lower in indirect studies to quantify circulating antibodies in serum (18%) (Vrbensky et al., 2019). Since anti-platelet autoantibodies are not detectable in about half of ITP patients, the following hypothesis was tested: only 18% of ITP patients test positive for anti-GPIIbIIIa or -GPIbIX autoantibodies in the indirect antigen-capture assays because they have low affinity or low titre autoantibodies that are undetectable in current assay.

This project aimed to approach this hypothesis with three main objectives:

1. Establish streptavidin-capture EIA and test feasibility of BLI using monoclonal antibodies against GPIIbIIIa and GPIbIX.

2. Establish cut-offs for detection of autoantibodies in ITP patients in a streptavidin-based EIA and determine assay sensitivity.

3. Establish cut-offs for detection of autoantibodies in ITP patients in BLI and determine instrument sensitivity compared to streptavidin-based EIA and previous indirect antigen-capture assay.

5.1 Objective 1

In objective 1, a modified antigen-capture assays using a streptavidin coat for immobilization was optimized to show that the biotin-streptavidin capture method did not inhibit mAbs binding to their cognate proteins. Modified ELISAs using biotin-streptavidin have been used in previous studies in order to increase assay sensitivity. These include the biotin-avidin immobilization of platelet glycoproteins for detection of anti-platelet antibodies, where in one assay, test sera were added to the microtitre wells directly for incubation with biotinylated platelet GPs (Cordiano et al., 1995). Antigen-capture using biotinylated and non-biotinylated GPIbIX was performed in order to determine the concentrations of antibody which will still produce a high OD in the assay. Dilutions of up to approximately 0.125 μg/mL showed positive binding in both the streptavidin capture, as well as the non-biotinylated antigen-capture assay, although in the non-biotinylated assay the antibodies could be diluted slightly further. It is possible that the biotin molecules are blocking some antibody binding epitopes or causing conformation changes when the proteins are immobilized to the plate. Since the biotin

used for labeling has a medium length spacer arm, it should provide enough length to minimize steric hindrance that may be caused by biotin binding. However, considering these are mAb, it is important to note that these antibodies would have less epitopes on their target protein than a polyclonal antibody; any reduction or inhibition of these binding sites or steric hindrance from the streptavidin-biotin interaction will have a greater effect on mAb than it would only a polyclonal sample. Based on these results, it was concluded that biotinylation of GPIbIX still produces a strong binding OD, although the antibodies do dilute out quicker because of the decrease in binding compared to non-biotinylated protein. Furthermore, it is important to consider the possibility biotinylating the protein and capturing it to the wells using streptavidin results in less-non specific binding, since there is a decrease in background binding.

Antigen-capture using biotinylated and non-biotinylated GPIIbIIIa was also performed in order to determine the concentrations of antibody which still produced a high OD in the assay. Dilutions of up to 0.06 μg/mL still produce a positive signal, although mAbs could be diluted further in the non-biotinylated assay which may be a result of the biotin blocking antibody binding sites, or the protein density or conformation in the wells due to the high affinity streptavidin-biotin interaction. However, the results of the antigen-capture assay confirmed the ability to detect anti-GP antibodies using labelled proteins and biotinylation was favourable for detecting antibody binding in the ITP samples. Subsequent mAb studies using BLI confirmed the feasibility of using this bioanalytical tool to study antibodies in ITP, since strong but varied binding affinities were calculated for all mAbs against their cognate GP. This was important to establish, as

it was necessary to detect binding of antibodies with different affinities to the GPs of interest.

BLI was performed using purified biotinylated GPs and monoclonal antibodies to determine the feasibility of this technology. It was important that various antibodies of different affinities with different binding epitopes were detected using this technology, as it is possible that the antibodies bind at different epitopes and protein conformation may affect accessibility to these sites. In the GPIbIX studies, the AK2 or TW-1 epitopes may be more accessible in comparison to the other antibody epitopes as they had the strongest binding response. In the GPIIbIIIa studies, CRC64 and HIP8, both IgG1 mAbs, exhibited the strongest and most quantifiable binding signals. It is important to note that this may also be due to their epitope accessibility. A comparison of the mAb dilutions in the BLI assay versus the antigen-capture assays shows that the modified streptavidin EIA is able to detect antibody binding from a more dilute sample than BLI. These discrepancies show that the EIA is more sensitive in detecting the binding of mAb. Interestingly, a previous study found that assay sensitivity of mAb was dependent on their binding affinities, with the EIA being superior to BLI in detecting high affinity mAb binding (J. Li, Schantz, Schwegler, & Shankar, 2011). However, BLI was more sensitive in detecting low-affinity mAbs than the EIA (J. Li et al., 2011). Other studies comparing ELISA to BLI found the ELISA to be more sensitive. In a study to optimize BLI for quantifying antibodies in monkey plasma, it was found that the ELISA is more sensitive than BLI, although BLI displayed a greater dynamic range in dilution, being 0.4-50 μg/mL for BLI and 0.1-10 μg/mL for ELISA (Dysinger & King, 2012). Additionally, screening of a metabolite of

two fungi species through assays including ELISA and BLI showed BLI to detect the metabolite conjugate in a picomolar range 20 times lower than in the ELISA; further investigation showed higher affinity antibodies to the metabolite conjugate had a lower sensitivity in BLI, although it was not proven that BLI is less sensitive than the ELISA (Sanders et al., 2016).

The modified antigen-capture assays using a streptavidin coat for immobilization showed that the biotin-streptavidin capture method did not inhibit mAbs binding to their cognate proteins in a well so this capture method was chosen for the biosensor studies. There was a decrease in the ODs of some of the positive control samples, which may be a result of the biotin blocking antibody binding sites, or the protein density or conformation in the wells due to the high affinity streptavidin-biotin interaction. However, the results of the antigen-capture assay confirmed the ability to detect anti-GP antibodies using labelled proteins and biotinylation was favourable for detecting antibody binding in the ITP samples. Subsequent mAb studies using BLI confirmed the feasibility of using this bioanalytical tool to study antibodies in ITP, as strong but varied binding affinities were calculated for all mAb against their cognate GP. This was important to establish, as it was necessary to detect binding of antibodies with different affinities to the GPs of interest Based on these results, it was concluded that using biotinylated GPs and a streptavidin-capture system was still an appropriate tool for conducting the future studies to test the sensitivity of BLI in detecting autoantibodies against GPIIbIIIa and GPIbIX in ITP patients.

5.2 Objective 2

Antigen-capture assays were performed using purified GPIbIX and GPIIbIIIa, along with the method of protein biotinylation in a streptavidin-capture assay to determine if protein labelling has an effect on anti-GP autoantibody detection. Since previous indirect antigen-capture assays were performed using plasma antibodies diluted and bound to platelet lysate prior to immobilization, a comparison was performed with these new assays using total IgG purified from plasma, to determine if there was any non-specific signal or if the binding was increased or decreased using biotinylated GP for capture. It is possible that the biotin can block some binding sites as was previously seen with the ELISA optimization experiments using mAbs, and since GPIbIX is present on the cell surface at a lower concentration than GPIIbIIIa, the effect of biotinylating may be more significant. Furthermore, this modified assay allowed for the study of anti-GP antibodies directly rather than using mAb to capture GPs of interest from cell lysate. In the GPIbIX assays there was an increase in the binding OD in the biotin-streptavidin capture method compared to the non-biotinylated assay, as well as one additional patient sample was determined to be antibody positive compared to the GPIIbIIIa study. This suggests that the protein remains immobilized on the plate for antibody-binding detection and this bond is strengthened when the GP is labelled with biotin. In addition, when a protein is conjugated with several biotin molecules, four of which can bind one molecule of avidin, it may greatly increase the sensitivity of many assays (Livnah, Bayer, Wilchek, & Sussman, 1993). Interestingly, a previous study of an ELISA coupled with a streptavidin-biotin complex lead to increased sensitivity in viral antigen detection, supporting the use of this enhanced ELISA (Davidson, Malkinson, Strenger, & Becker,

1986). This phenomenon was also seen in the GPIIbIIIa assays, as the comparison of the streptavidin-capture versus no streptavidin showed an increase in ODs using the biotinylated protein. The only samples with a high OD that were antibody-positive were only positive in the streptavidin-capture assay, showing that the biotin may be useful in capturing enough protein to the wells for binding detection and allowing them to stay strongly bound during wash steps.

These assays show the validity of using a biotin-streptavidin capture method, as the biotinylation does not interfere with the binding of antibodies that were strongly-positive in previous indirect antigen-capture assay. Additionally, these assays detected anti-GPIIbIIIa and anti-GPIbIX antibodies in two previously antibody-negative samples, as well anti-GPIbIX antibodies in a previously weak-positive sample (OD: 0.274 with cut-off 0.21). Although there is a limitation in the detection of antibodies in a few ITP samples using the purified GP capture method, it is possible that this is a result of the previously positive samples being weak positives and not true antibody positive samples. Interestingly, a previously strong anti-GPIIbIIIa and anti-GPIbIX antibody positive sample (OD: 0.78 and 0.9, respectively) was only positive in the GPIbIX studies. The autoantibody-positive samples had the following diagnoses: non-immune thrombocytopenia (n=1), primary ITP (n=1) and secondary ITP (n=2). The original indirect EIA used platelet lysate and therefore, the concentration of GPIIbIIIa and GPIbIX in the lysate will differ from the concentration of purified GPIIbIIIa and GPIbIX. It is important to consider protein density and conformation as well; purified protein may be more densely packed which may make some binding sites inaccessible to the

antibodies and can result in the samples being antibody-negative in the new assays although this is unlikely as biotinylation positively influenced the results of the assays. It is still important to consider, as epitope accessibility may vary between the samples, as well as the epitopes themselves. Furthermore, purified IgG was used rather than patient plasma. A high concentration of total IgG (200 μg/mL) was used with the estimation that if the average adult has 10 mg/mL of circulating IgG, 200 μg/mL was equivalent to the antibodies present in a 1/50 plasma dilution. Previous indirect antigen-capture assay cut-offs were calculated using the mean and 2 SD; but here 3 SD were used instead. It was considered that this may be the reason for the limitation in the detection of antibodies in some ITP samples, however, when the cut-offs were recalculated using the mean and 2 SD, the previously antibody-positive samples were still not antibody-positive in these new assays. In addition, since the original assays were performed several years prior, it is possible that an updated plate reader could have contributed to the different absorbance readings. Finally, background levels of antibody binding were considered. Since platelets have antibodies bound to them non-specifically, it is possible that these antibodies are bound to the GPs of interest that were purified for these assays. Therefore, any antibodies originally bound to the platelet on these proteins may contribute to a higher OD reading that would be confounding. For this reason, a separate control well with protein only (no antibody sample) was added and the background level was taken at the same time point of the other readings and subtracted to ensure that the ODs were accurate. Since this was not done previously in the indirect antigen captures using platelet lysate, it is possible that many of the antibody-positive samples would now be antibody-negative with the

background binding level subtraction. Lastly, it is important to consider the ITP samples and patients themselves. Many of the samples used are dated several years after the clinical diagnosis; in particular, the patients who were antibody-positive, received their ITP diagnosis ranging from 8-39 years prior to sample collection. Sample viability should be considered, as should the possibility that some patients may not have many antibodies in circulation many years later, instead antibodies may be present in the spleen or bone marrow. BLI could determine whether the positive-to-negative signals are a result protein density and conformation, or if the weak-positive samples are truly negative.

This raises the question of whether there truly are low affinity/low titre antibodies that are undetectable, or if the ELISA-based antigen capture assays have a high rate of false positives due to background from not properly blocking the antigen, cross-reactivity or simply from nonspecific, or non-pathogenic antibody detection. Since platelets play a role in the immune system and are coated in antibodies, it is possible that the slightly elevated levels of IgG detected are a result of infection and the inflammatory response (C. Li et al., 2012). It is important to consider that platelets are being destroyed during an infection response. Given their involvement in the first line of immune defence, it is possible that ITP patients were exposed to a pathogen that may cause an increase in IgG bound non-specifically to their platelets, which may lead to inaccurate quantification of IgG during antigen-capture studies. A recent study assessed the prevalence of various autoimmune markers in adult ITP patients, and it was determined that they have a high rate of autoimmune markers; these did not correlate with autoantibody levels suggesting that there is a dysregulation in their immune system that goes beyond anti-GPIIbIIIa and

GPIbIX autoantibodies (Hollenhorst, Al-Samkari, & Kuter, 2019). It is possible that some ITP patients have an unknown secondary cause, particularly an AID, which is causing increased thrombocytopenia and increased antibodies. There may also be an undetectable number of antibodies in circulation, if any, that are specific to GPIIbIIIa and GPIbIX. The nature of autoantibodies in AID show that they are typically IgM class and have moderate affinities, or, following somatic mutations or clonal selection, high affinity IgG class antibodies; however, the antibody characteristics in ITP are undetermined. Although low titre antibodies should be considered, it may be an unlikely phenomenon to see in patient samples that are taken several years after diagnosis due to affinity maturation. During this process, B cells will produce antibodies with an increased affinity over the course of a (repeated) immune response (Rajewsky, 1996). If low affinity antibodies are out-competed, then they might only be detected early in disease onset.

In terms of assay sensitivity and specificity, it was determined that the modified streptavidin-based assays are of a lower sensitivity compared to the original assays based on how many samples are actually positive for autoantibodies. The EIA using purified GPIbIX had a sensitivity of 14% (3/21) and specificity of 100% as there were no non-diseased individuals classified as antibody-positive; the assay using purified GPIIbIIIa had a sensitivity of 0%. The streptavidin capture had a sensitivity of 35% (7/20) for detection of anti-GPIBIX or GPIIbIIIa autoantibodies and a specificity of 100%; individually, the biotinylated GPIbIX assay had a sensitivity of 24% and the biotinylated GPIIbIIIa assay had a sensitivity of 25%. However, it is critical to test more ITP patients to determine an accurate percentage for sensitivity and specificity. It is unknown if these

antibodies are more specific to their target antigens or if they are only able to bind purified GPs if they are of a higher titer or affinity; binding affinities were later studied using BLI. The sensitivity and specificity of platelet autoantibody testing in ITP is 18% and 96%, respectively, for indirect assays (Vrbensky et al., 2019). The results of these assays show slightly increased assay sensitivity when using a streptavidin-capture method versus using unlabeled purified protein. However, since the number of patients tested was low, it is not yet appropriate to state that this assay is more sensitive. Thus, more ITP patient samples would need to be analyzed to quantify the antibody presence.

5.3 Objective 3

BLI has been used as a tool in many antibody binding and vaccine studies, due to the high assay sensitivity of the technology. Since it was previously shown to detect a positive signal in plasma dilutions up to 1:100 000, it was hypothesized that it could be a useful tool to examine the binding of ITP autoantibodies from patient plasma to purified protein (Auer, 2015). To test antibody binding to GPIbIX and GPIIbIIIa, IgG was purified from samples that had previously tested positive in the indirect antigen-capture assay, as well as samples that were previously antibody-negative, healthy controls, and positive control GT. Since purified IgG binding is a cleaner interaction than using antibodies in plasma, it was expected that the four patients that who were autoantibody-positive in the antigen-capture assays would also exhibit antibody binding in the BLI assay, as both methods used biotinylated GPs immobilized by streptavidin.

Objective 3 aimed to understand antibody binding kinetics in ITP patients, as well as determine the sensitivity of the BLI assay compared to the modified antigen-capture assays. However, after analyses using a low BSA concentration to block non-specific signals, only one ITP patient had detectable anti-platelet GP autoantibodies, against both GPIIbIIIa and GPIbIX. This particular patient was negative for autoantibodies in the original indirect EIAs as well as the streptavidin-based assays. It was determined through the McMaster ITP registry that this patient was diagnosed with secondary ITP, specifically common variable immunodeficiency disorder; however, it is unclear why the patient was negative for autoantibodies in the antigen-capture assay yet positive in the BLI assay. It is possible that BLI is able to detect true antibody-binding signals, based on the success of this technology in many applications. The ITP patient samples that were positive for anti-GPIIbIIIa antibodies in the streptavidin-capture assay did not exhibit any antibody binding in the BLI assay. This result was unexpected as the IgG was able to bind the biotinylated GPs in the streptavidin-capture assays, and it was concluded that the biotin did not have a negative impact on the binding ability. There was no difference in the other patient samples that had previously been described antibody-negative, as they were still negative in both BLI assays. The positive control GT was positive and very strongly positive. The samples were diluted in PBS to ensure that any signal present could be detected, and compared to BSA dilutions that would block any non-specific binding. Here, the BLI assay was unable to detect antibodies in about 50% of patients as per a typical antigen-capture assay; instead, only 1/19 (6%) and 1/18 (5%) patients had an antibody-binding response in the GPIbIX and GPIIbIIIa assays, respectively. The

conformation of immobilized GPs on the sensory may be inhibiting binding of antibodies even though the conformation of GPs immobilized in the wells in EIAs does not seem to have an effect. Since BLI is generally used to measure and confirm antibody binding, it may not incorrect to conclude that the instrument is unsuitable for ITP studies. It is possible that there is a limited understanding of the antibody mechanism in ITP instead. It is important to consider that BLI may be detecting a real IgG binding signal against the specific GPs based on a physiological change occurring following antibody binding, and not just a signal of antibodies binding the proteins. This may be verified in the future using PFA to fix the proteins prior to immobilizing them on the sensors.

Alternatively, future studies should include designing an assay using protein G sensors to immobilize the purified patient IgG rather than the protein, as this would avoid the biotinylation step and would not interfere with any binding sites. This may also allow for better binding conformation. This assay design may confirm whether or not biotin interferes with binding of low affinity or low titer antibodies in ITP, although biotinylation does not affect detection of mAb binding or of the positive control, even with higher concentrations of BSA. It is also important to consider that some of the previously positive samples had low affinity or low titer antibodies which were blocked by BSA. Protein G sensors could also be used to quantify antibody concentrations in plasma samples to determine if low titer is the cause of the limits in detection. It is important to consider the possibility that there may be anti-platelet antibodies of a different class such as IgM, and future studies should be conducted to determine these statistics in ITP patients compared to IgG antibodies. Antibody binding epitopes may also

have an effect on autoantibody detection, and future proteomic studies can compare the binding sites of different antigens so that BLI assays can be designed to optimize these aspects and potentially enhance the binding signals. A study of influenza vaccine potency using mAb concluded that BLI was less sensitive than the ELISA, unless an additional amplification step was performed (Vasudevan et al., 2018). It may be beneficial to explore different conjugates that may increase BLI signal in future assay design.

5.4 Limitations and Future Directions

There are several limitations of streptavidin-capture assays, the first being the biotinylation of the proteins. The indirect EIA is typically performed with a dilution of ITP patient plasma incubated with donor platelet lysate; this complex is then immobilized to a microtitre plate using a mAb specific to the target GPs. This allows any circulating antibodies to bind their cognate protein as long as the epitopes are accessible in the lysate solution. With the addition of biotin molecules, it is important to consider the possibility of a negative effect, in case biotin blocks some of the binding sites. Biotin is a small water-soluble vitamin with a molecular mass of only 244 Da that does not change the structure of the protein and does not interfere with biologic function(Green et al., 1971). The strong affinity biotin has for streptavidin (approximately 10^{-15} M) may result in blocked epitopes that would have been accessible without the streptavidin binding step. However, the assays in this study were optimized to use the lowest ratio of biotin to protein to minimize this effect and used Sulfo-NHS LC biotin with a medium-length spacer arm, such that the total length added to the target is only 22.4 angstroms for minimal steric hindrance. Future studies should also include designing an assay using

anti-human IgG Fc capture sensors to immobilize the purified patient IgG rather than the protein, as this would avoid the biotinylation labeling step and would not interfere with any binding sites. This may also allow for better binding conformation, since only the Fc portion of the antibodies would bind the sensors, leaving the Fab region free. This assay design may confirm whether or not biotin interferes with binding of low affinity or low titre antibodies in ITP, although biotinylation does not affect detection of mAb binding or of the detection of positive control sera.

Previous studies have discussed the importance of immobilizing the smaller of the molecules used in the interaction analysis (Shah & Duncan, 2014). BLI is sensitive to the mass of the protein being immobilized on the sensors; to effectively monitor association and dissociation, it is important to produce a strong binding signal and a larger binding signal will result from the immobilization of the smaller molecule (Kumaraswamy & Tobias, 2015). It could be beneficial to reverse the immobilization for BLI and bind the purified patient IgG to the sensors instead of the purified protein to determine if binding sensitivity increases, since IgG (150 kDa) is slightly smaller in comparison to GPIbIX (190 kDa) and GPIIbIIIa (220 kDa). Availability of antibody binding epitopes may also have an effect on autoantibody detection, and future proteomic studies can compare the binding sites of different antigens so that BLI assays can be designed to optimize these aspects based on conformation and structure analysis, and potentially enhance the binding signals. A study of influenza vaccine potency using mAb concluded that BLI was less sensitive than the ELISA (Vasudevan et al., 2018). It may be beneficial to explore different conjugates that may increase BLI signal in future assay design.

The modified EIAs were performed with multiple wash steps. This is an important consideration, since the goal of this study was to determine if there are low affinity or low titre antibodies present in ITP plasma. It is possible that multiple wash steps can result in a loss of weak antibodies. It may be important to optimize an assay with reduced wash steps, but with increased BSA to block non-specific binding, in order to properly quantify how many ITP patients are antibody-positive in the indirect EIAs.

The ITP sample collection is another critical consideration. Future studies should include samples which were taken at a time point closer to the date of diagnosis, since in this study's patient samples ITP diagnosis were made 8-39 years prior to the sample collection date. The low assay sensitivities may be a result of the lack of antibodies in circulation from patient samples taken many years after the clinical diagnosis. It is unknown how long the anti-platelet antibodies remain in circulation in ITP, in particular any potential low-affinity antibodies. It is also important to consider the possibility that there may be anti-platelet antibodies of a different class such as IgM, and future studies should be conducted to determine their presence in ITP patients compared to IgG antibodies. Samples should be tested for IgM titers during active ITP episodes of low platelet counts, as they are the first antibody type produced by B cells in response to antigen exposure, and followed with IgG antibody testing approximately two weeks later. Following an appropriate timeline will allow for optimal design and detection capabilities of the anti-platelet antibody tests. ITP is difficult to diagnose clinically, due to the lack of a common marker to test for. Since antibodies are present on only about half of patient's platelets and in a fifth of patient's plasma, it is possible that these limitations resulted in

misdiagnoses (Arnold et al., 2017). Sample size increases are necessary in order to determine the true sensitivity of BLI as well, since it is difficult to conclude the usefulness of this technology in ITP studies based on 18 patient samples.

Additionally, there is a limitation in pAb studies using BLI, particularly when determining kinetics binding rates and affinities. The use of a block such as BSA creates some fluctuations in the binding curves from noise, even at low concentrations, and unlike in the analysis of samples diluted in PBS, the equilibrium constant calculations are susceptible to large error values. This is due to the increased variations in the binding curves, which restricts the use of a very accurate fitting model in the calculations. Furthermore, BSA has a greater effect on binding in the GPIbIX studies, possibly due to incompatibility of the block with the chemical structure of the protein complex. It may be necessary to design an assay using a different blocking solution that is more suitable for this application. Furthermore, there are multiple binding options possible in antibody interaction studies which must be considered. They are as follows: monovalent, with one IgG paratope binding one single epitope; homogeneous bivalent, with two paratopes from IgG binding two separate identical epitopes; heterogeneous bivalent, with two paratopes from IgG binding separate distinct epitopes; multiple antibodies binding, with two IgGs binding one single epitope; cross-linking, with IgG binding two separate epitopes on two antigens(Kumaraswamy & Tobias, 2015; Zhang, Williams, Zborowski, & Chalmers, 2006). Since there are several possibilities in the binding interaction, it may be incorrect to conclude that the monovalent 1:1 fitting model chosen is the appropriate models for ITP studies, even if it displays the best fit. This model is based on a suspension in

homogeneous solution; however, with plasma IgG interaction studies this is not guaranteed. Although BLI is a well-established bioanalytical tool, it is still novel in comparison to similar technologies such as surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC), and has a sensitivity 100x lower than SPR(Sanders et al., 2016). There are several limitations with these technologies, such as that SPR is very expensive and is prone to interference due to changes in the refractive index of solution flowing over the sensor, and ITC has a limited range in determining binding affinities while also requiring a large sample volume (Renaud et al., 2016). For these reasons, BLI may be a more appropriate tool for continuing ITP autoantibody studies. The novelty of BLI may contribute to a decrease in sensitivity in comparison to the EIA and further studies and assay optimizations may improve antibody detection in ITP samples.

Finally, there is a possible lack of understanding of the antibody-mediated mechanism of ITP. It must be considered that although BLI has a slightly lower sensitivity in comparison to indirect EIAs, including the modified streptavidin-capture assays, it may be distinguishing between true disease samples and false positives. It is unknown whether ITP patients have pathogenic autoantibodies or if these antibodies have a more important role in the pathogenesis. We were able to determine assay sensitivities ranging from 5%-33% using a streptavidin-capture method, in comparison to the 18% overall sensitivity determined from all other studies. This low assay sensitivity contributes to the decision that these tests are inappropriate for clinical diagnosis. Further

studies looking at the role of autoantibodies in ITP may answer how important antibodies truly are in diagnosing this disease, and for patient recovery.

6.0 CONCLUSION

In conclusion, the hypothesis that only 18% of ITP patients test positive for antibodies in circulation and the ability to increase detection of low affinity or low titre anti-GPIIbIIIa or GPIbIX antibodies using BLI was not proven to be true. Known anti-GP monoclonal antibodies and their binding affinities were quantified using the improved streptavidin-capture assays and novel BLI assays, respectively; however, improving the detection of polyclonal antibodies in ITP patient sera may be more challenging. These studies were performed using patient samples that had tested positive for autoantibodies in indirect antigen-capture assays, which in the literature has a sensitivity of 18%. The antigen-capture assays using purified GPIbIX and GPIIbIIIa had sensitivities of 14% and 0%, respectively. The novel streptavidin-capture assays showed a slightly increased sensitivity of approximately 25% in either the GPIbIX or GPIIbIIIa capture, although it is important to test many more patients prior to making this claim. The BLI assay had a sensitivity of 6% (1/18), which is much lower compared to the EIAs. All assays were able to detect mAb binding against GPIbIX and GPIIbIIIa as well as GT patient sera as a positive control. These novel assays set the stage for further investigation into the role of antibodies in ITP. For instance, subsequent studies of the binding of IgG directly to its

cognate proteins will allow for better understanding of the function of both the antibodies and the GP antigens in ITP, and will hopefully provide further insight on the role of autoantibodies in ITP.

FIGURES

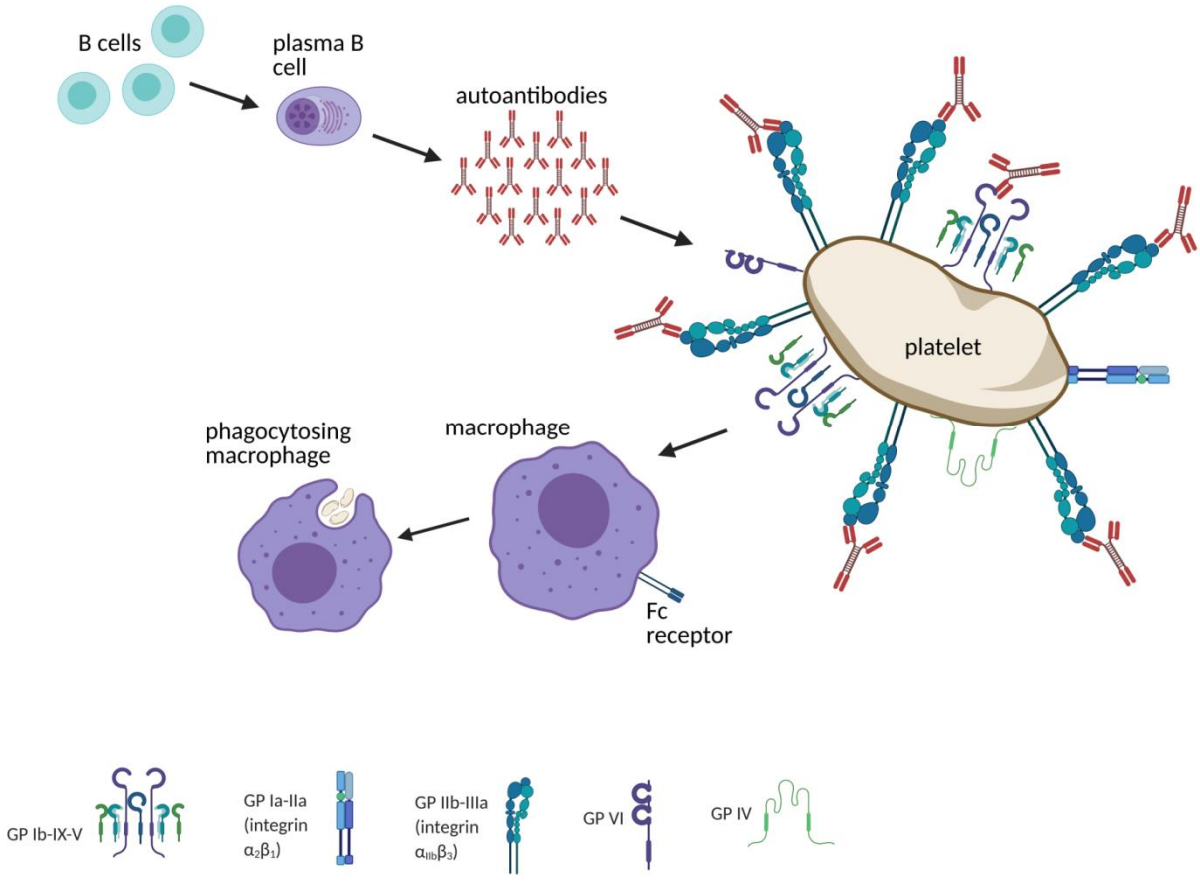


Figure 1. Proposed mechanism of autoantibody production by plasma B cells and subsequent autoantibody-mediated platelet destruction in ITP. First, a platelet antigen binds B cells and stimulates clonal expansion into antibody-producing plasma cells. Then, autoantibodies are produced against platelet antigens and bind their target epitope on membrane glycoproteins. Finally, the Fc receptor interacts with macrophage Fcγ receptors, leading to phagocytosis of the opsonized platelets. Created with biorender.com.

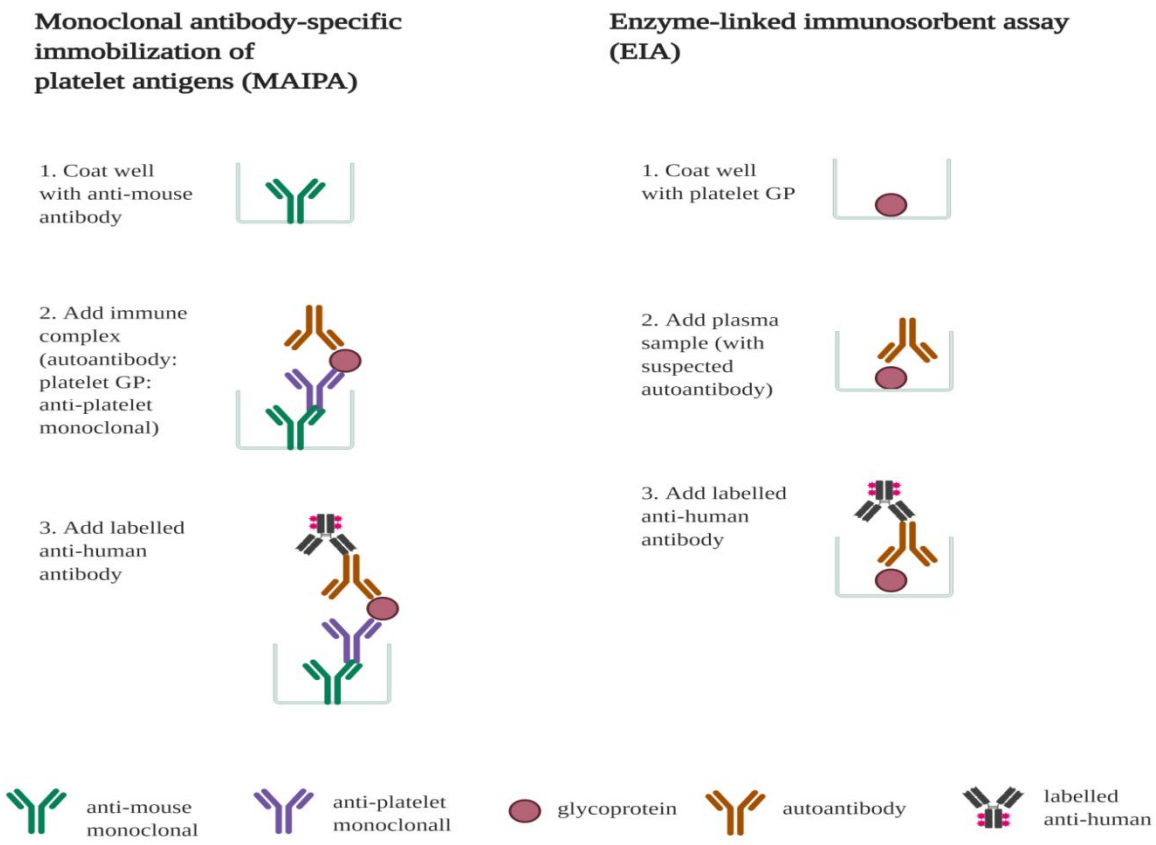
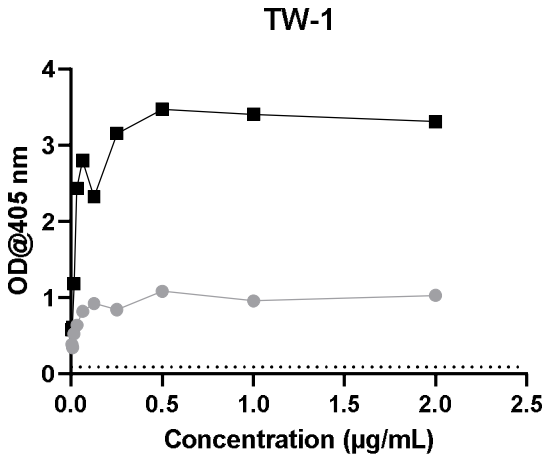
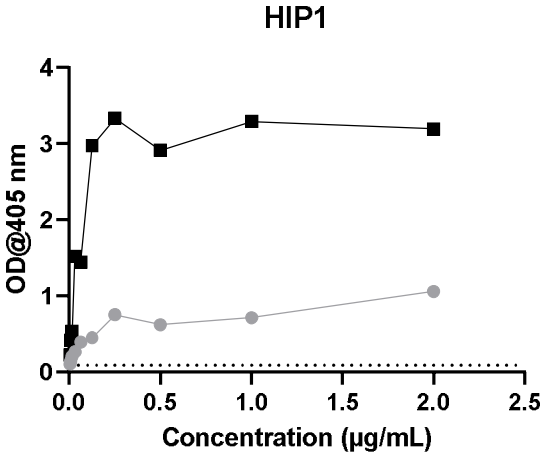


Figure 2. Antigen-capture tests for direct (on platelets; left side) and indirect (in circulation; right side) platelet autoantibody quantification. Testing for autoantibodies involves the use of purified platelet GPs or platelet GPs in cell lysate as the antigen for binding detection. Created with biorender.com.


TW-1
OD@405 nm
0
1
2
3
4
0.0
0.5
1.0
1.5
2.0
2.5
Concentration (µg/mL)

A


HIP1
OD@405 nm
0
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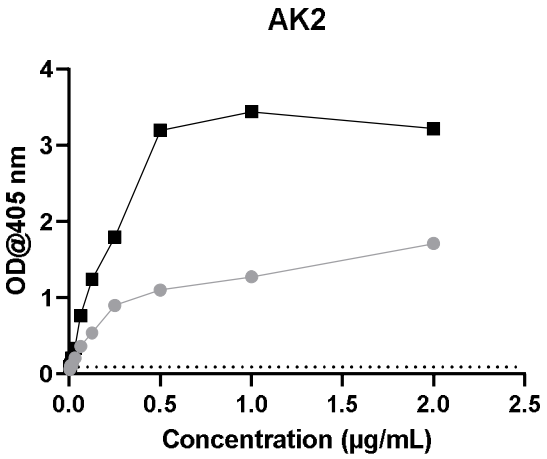
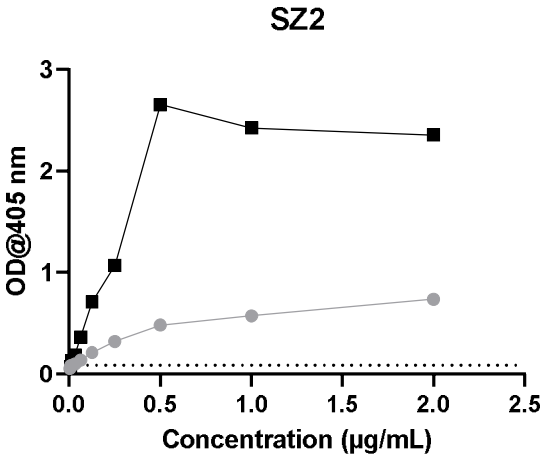
2
3
4
0.0
0.5
1.0
1.5
2.0
2.5
Concentration (µg/mL)

B



C

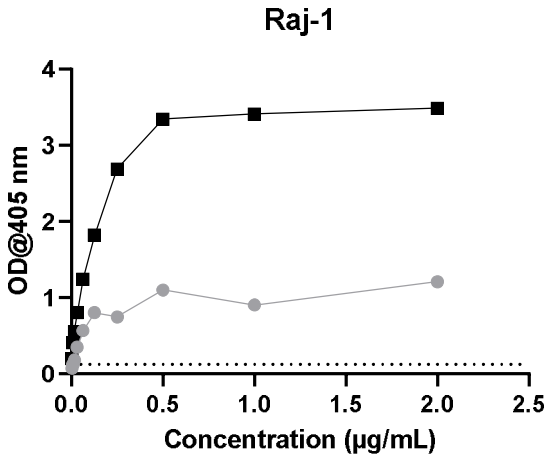


D

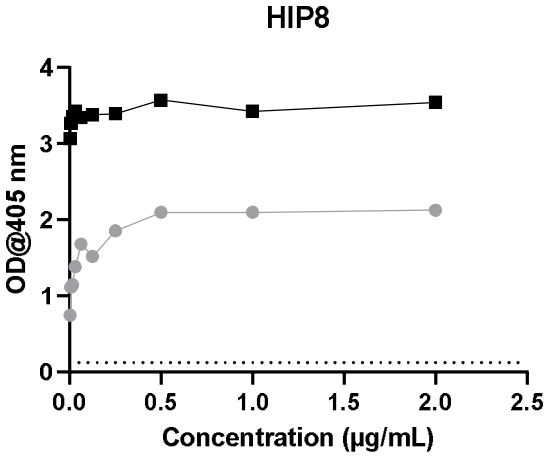
Biotinylated GPIbIX

GPIbIX

Figure 3. Antigen-capture assays showing binding of anti-GPIb monoclonal antibodies to biotinylated (black) versus non-biotinylated (grey) purified GPIbIX. Dotted line indicates cut-off OD 0.087.


Raj-1
OD@405 nm
Concentration (µg/mL)
0
1
2
3
4
0.0
0.5
1.0
1.5
2.0
2.5

A


HIP8
OD@405 nm
Concentration (µg/mL)
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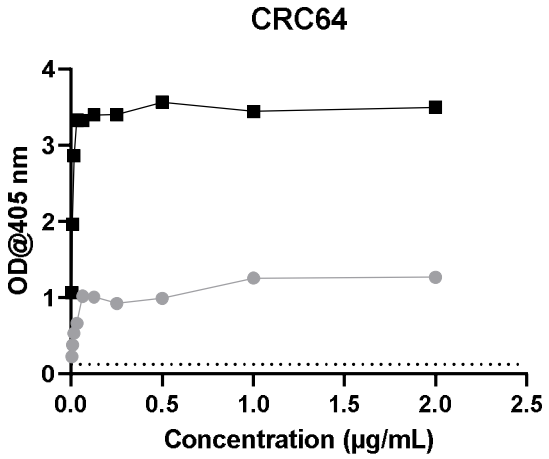
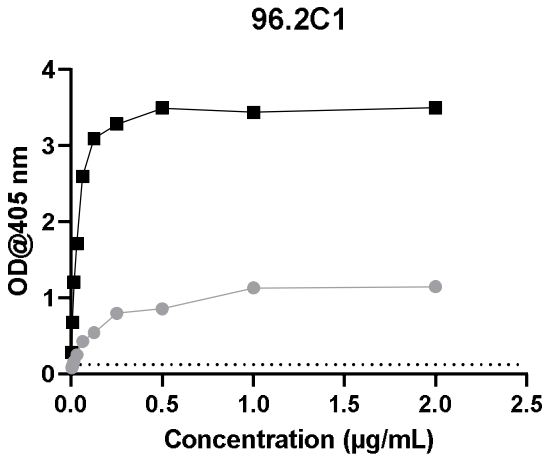
2
3
4
0.0
0.5
1.0
1.5
2.0
2.5

B



C



D

Biotinylated GPIIbIIIa
GPIIbIIIa

Figure 4. Antigen-capture assays showing binding of anti-GPIIb monoclonal antibodies to biotinylated (black) versus non-biotinylated (grey) purified GPIIbIIIa. Dotted line indicates cut-off OD 0.125.

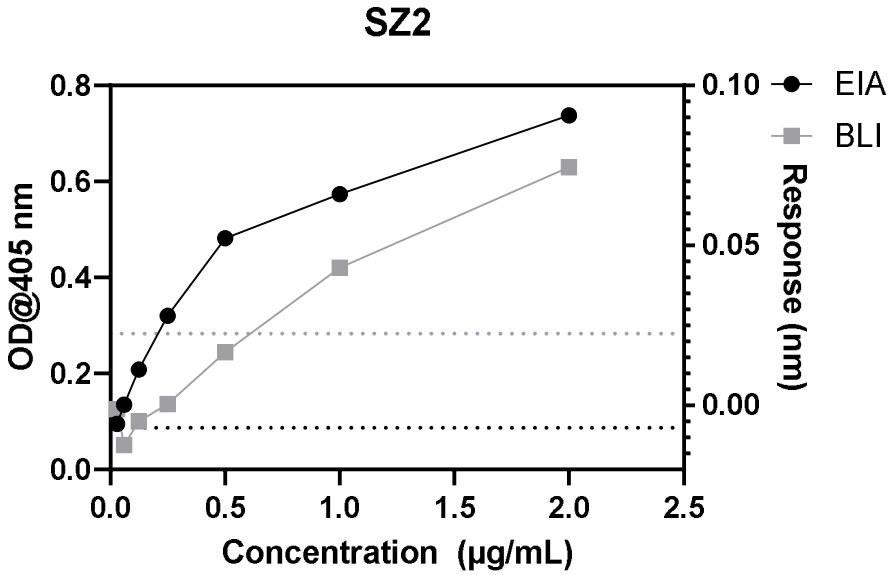


Figure 5. Comparison of EIA and BLI binding limits based on SZ2 anti-GPIb monoclonal antibody dilution. Binding limit (based on cut-offs) is marked by dotted lines: cut-off for EIA is an OD of 0.087; cut-off for BLI is a response of 0.02247 nm.

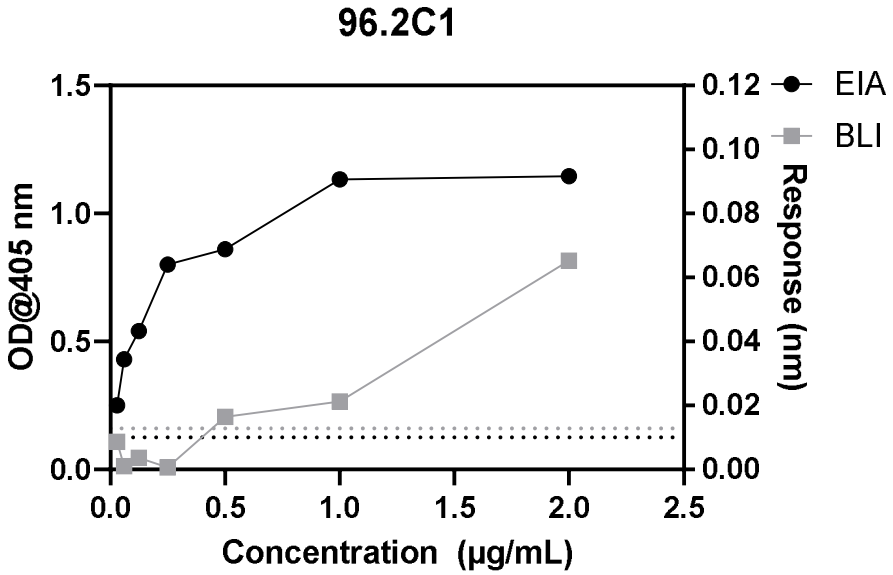


Figure 6. Comparison of EIA and BLI binding limits based on 96.2C1 anti-GPIIb monoclonal antibody dilution. Binding limit (based on cut-offs) is marked by dotted lines: cut-off for EIA is and OD of 0.125; cut-off for BLI is a response of 0.01288 nm.

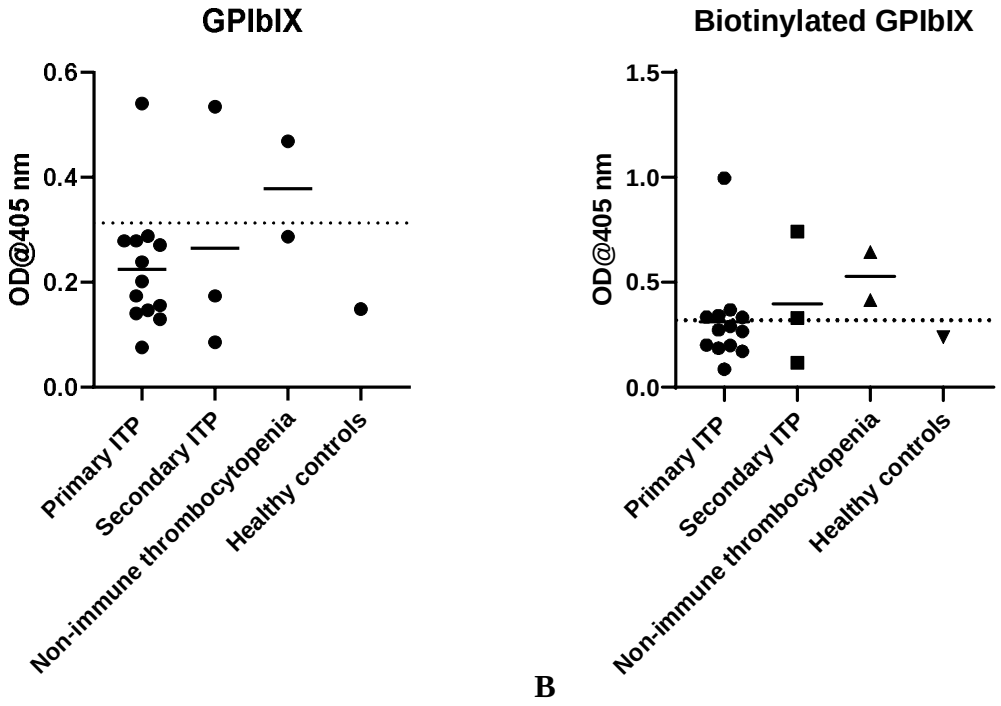


Figure 7. Comparison of antibody-positive ITP samples in EIA using purified GPIbIX versus biotinylated GPIbIX. A) Autoantibody detection measured in an antigen-capture assay. The dotted line indicates the assay cut-off (OD: 0.313). B) Autoantibody detection measured in a streptavidin-capture assay. The dotted line indicates the assay cut-off (OD: 0.319). Solid line indicates mean of sample group. No statistical significance was reported between any of the test groups, $p<0.05$.

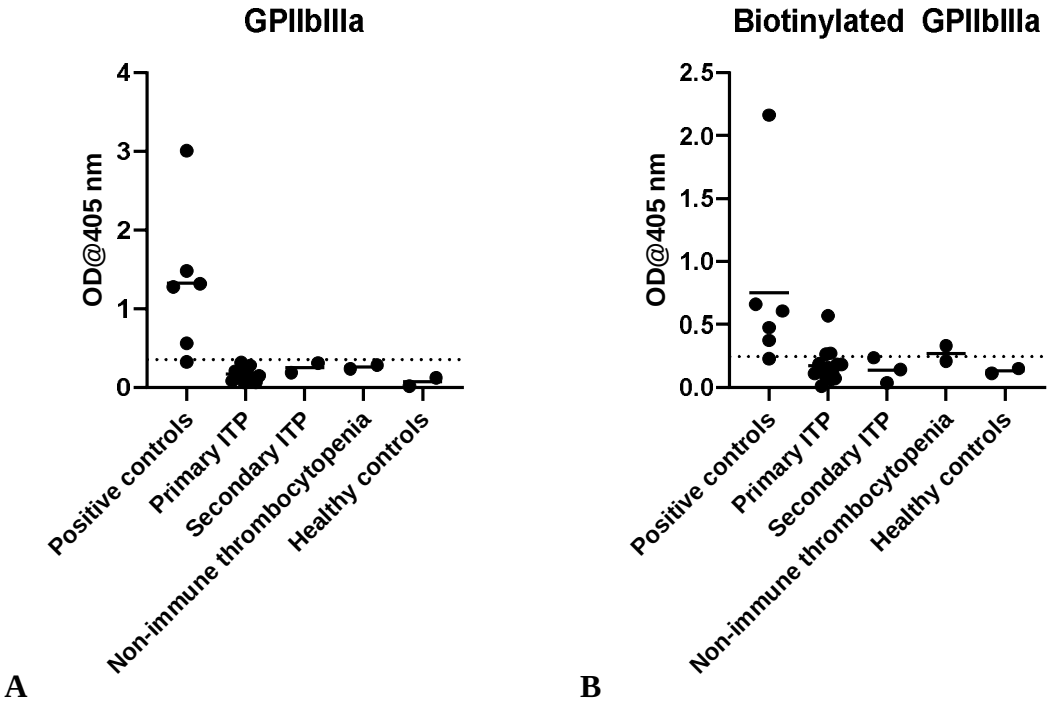


Figure 8. Comparison of antibody-positive ITP samples in EIA using purified GPIIbIIIa versus biotinylated GPIIbIIIa. A) Autoantibody detection is measured in an antigen-capture assay. The dotted line indicates the assay cut-off (OD: 0.356). B) Autoantibody detection is measured in a streptavidin-capture assay. The dotted line indicates the assay cut-off (OD: 0.244). Solid line indicates mean of sample group. No statistical significance was reported between any of the test groups, $p>0.05$. Statistical significance reported following two-tailed t test comparing positive control set to patient sets, $p<0.05$.

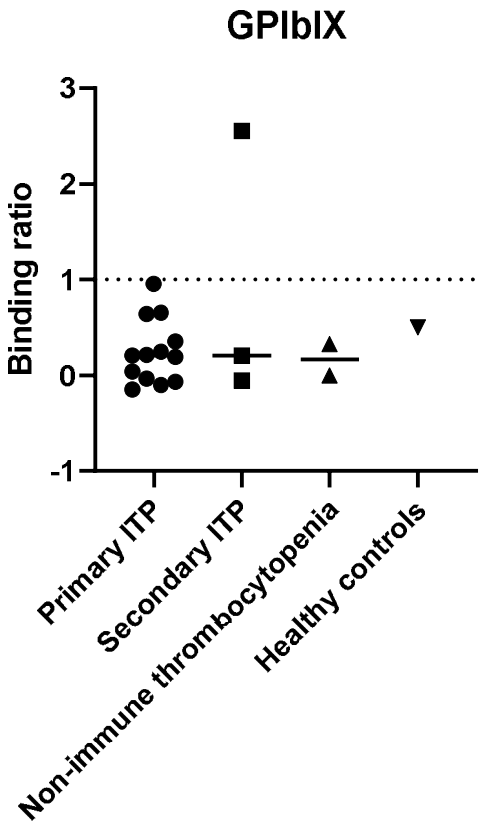


Figure 9. Binding responses of ITP samples in BLI using biotinylated GPIbIX. 18 samples were evaluated for anti-GPIbIX autoantibodies from total purified IgG. Healthy control (n=1) was repeated for consistency and reproducibility.

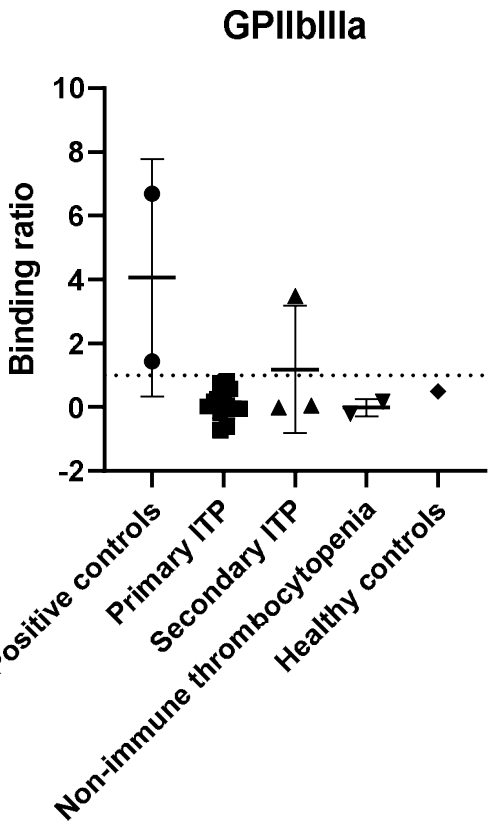


Figure 10. Binding responses of ITP samples in BLI using biotinylated GPIIbIIIa. 18 samples were evaluated for anti-GPIIbIIIa autoantibodies from total purified IgG, and the positive controls GT (n=1) and NAIT (n=1). Healthy control (n=1) and positive control GT were repeated for consistency and reproducibility.

TABLES

Table 1. Kinetics analysis of monoclonal antibody binding GPIbIX in BLI. Four monoclonal antibodies (13.3 nM) against GPIb were tested in BLI to determine their binding affinities. Mouse IgG1 and 5B9 were used as negative controls. Analysis was completed using ForteBio Data Analysis 9.

Sample	Response (nm)	K_D (M)	K_{on}(1/Ms)	K_{off}(1/s)
TW-1	0.0247	1.69E-08	2.71E+03	4.58E-05
HIP1	0.0006	1.33E-07	1.03E+03	1.37E-04
AK2	0.0558	1.52E-10	1.67E+06	2.53E-04
SZ2	0.0426	1.19E-08	6.07E+03	7.19E-05
5B9	-0.0212	<1.0E-12	2.80E-3	<1.0E-7
Mouse IgG1	-0.0273	<1.0E-12	2.80E-03	<1.0E-7

Table 2. Kinetics analysis of monoclonal antibody binding GPIIbIIIa in BLI. Five monoclonal antibodies (13.3 nM) against GPIIb were tested in BLI to determine their binding affinities. Mouse IgG1 was used as a negative control. Analysis was completed using ForteBio Data Analysis 9.

Sample	**Response (nm)**	**K_D (M)**	**K_{on}(1/Ms)**	**K_{off}(1/s)**
Raj-1	0.0658	5.28E-10	2.82E+05	1.49E-04
HIP2	0.0097	<1.0E-12	3.75E+86	1.85E-03
HIP8	0.1087	4.26E-12	6.24E+06	2.66E-05
CRC64	0.1011	4.50E-11	2.73E+06	1.23E-04
96.2C1	0.0771	2.62E-10	3.89E+05	1.02E-04
Mouse IgG1	-0.0002	6.59E-07	7.59E+03	5.00E-03

Table 3. SZ2 dilutions to detect concentration limit in binding GPIbIX. Samples were diluted in PBS and analyzed using ForteBio Data Analysis 9. Bold values indicate concentrations of mAb that are considered anti-GPIb antibody positive following subtraction of cut-off and background signal. K_D was compared to OD to determine which streptavidin-capture assay was more sensitive.

Concentration (µg/mL)	**Response (nm)**	**K_D (M)**	**OD (@405nm)**
2	**0.0743**	**1.58E-8**	**0.737**
1	**0.043**	**4.52E-9**	**0.573**
0.5	**0.0165**	**1.51E-8**	**0.482**
0.25	0.0004	1.65E-8	**0.32**
0.125	-0.0051	<1.0E-12	**0.208**
0.06	-0.0125	<1.0E-12	0.135
0.03	-0.0012	<1.0E-12	0.095

Table 4. 96.2C1 dilutions to detect concentration limit in binding GPIIbIIIa. Samples were diluted in PBS and analyzed using ForteBio Data Analysis 9. Bold values indicate concentrations of mAb that are considered anti-GPIIb antibody positive following subtraction of cut-off and background signal. K_D was compared to OD to determine which streptavidin-capture assay was more sensitive.

Concentration (µg/mL)	**Response (nm)**	**K_D (M)**	**OD (@405nm)**
2	**0.0651**	**1.46E-9**	**1.145**
1	**0.0212**	**2.98E-9**	**1.133**
0.5	**0.0164**	**8.89E-11**	**0.859**
0.25	0.0005	<1.0E-12	**0.799**
0.125	0.0035	<1.0E-12	**0.54**
0.06	0.001	<1.0E-12	**0.429**
0.03	0.0086	<1.0E-12	0.25

Table 5. Autoantibody-positive samples determined from antigen-capture and streptavidin-capture EIA from total purified IgG. 200µg/mL of each sample was incubated with 20µg/mL purified GPIbIX (left columns) or purified biotinylated GPIbIX (right columns). OD was read at 405nm for 1 hour with kinetic reads every 2 minutes. For the non-streptavidin EIA a background OD of 0.031 was subtracted, as well as a background of OD: 0.035 for the streptavidin EIA prior to highlighting positive ITP samples.

Sample	Non-biotinylated GPIbIX	Antibody positive (based on cut-off OD: 0.313 and background subtraction)	Biotinylated GPIbIX	Antibody positive (based on cut-off OD: 0.319 and background subtraction)
4	0.469	**Yes**	0.643	**Yes**
1	0.541	**Yes**	0.995	**Yes**
15	0.535	**Yes**	0.740	**Yes**
8	0.287	No	0.414	**Yes**
17	0.130	No	0.368	**Yes**

Table 6. Autoantibody-positive samples determined from antigen-capture and streptavidin-capture EIA from total purified IgG. 200μg/mL of each sample was incubated with 20μg/mL purified GPIIbIIIa (left columns) or purified biotinylated GPIIbIIIa (right columns). OD was read at 405nm for 1 hour with kinetic reads every 2 minutes. The non-streptavidin EIA had a background OD: 0.086 subtracted, and the streptavidin EIA background of OD: 0.163 was subtracted as well prior to highlighting any antibody positive samples. Positive control GT and NAITs 1-5 are recorded for reference.

Sample	**Non-biotinylated GPIIbIIIa**	**Antibody positive (based on cut-off OD: 0.356 and background subtraction)**	**Biotinylated GPIIbIIIa**	**Antibody positive (based on cut-off OD: 0.244 and background subtraction)**
GT	3.010	Yes	2.163	Yes
NAIT1	1.317	Yes	0.475	Yes
NAIT2	0.561	Yes	0.374	Yes
NAIT3	1.482	Yes	0.662	Yes
NAIT4	0.325	No	0.229	No
NAIT5	1.277	Yes	0.607	Yes
20	0.242	No	0.569	**Yes**
15	0.313	No	0.362	**Yes**
8	0.286	No	0.333	**Yes**
1	0.315	No	0.263	**Yes**
9	0.193	No	0.272	**Yes**

Table 7. Comparison of EIAs using platelet lysate, purified GPIIbIIIa or GPIbIX, and biotinylated GPIIbIIIa or GPIbIX. Samples are listed as antibody positive based on cut-offs determined or original cut-off for indirect assay (OD: 0.21) for platelet lysate capture. Antigen-capture results are summarized based on percentage of samples positive for autoantibodies against cognate protein.

	GPIIbIIIa using platelet lysate	**Purified GPIIbIIIa**	**Biotinylated GPIIbIIIa**	**GPIbIX using platelet lysate**	**Purified GPIbIX**	**Biotinylated GPIbIX**
Percent antibody positive	6/20=30%	0%	5/20=25%	7/20=35%	3/21=14%	5/21=24%

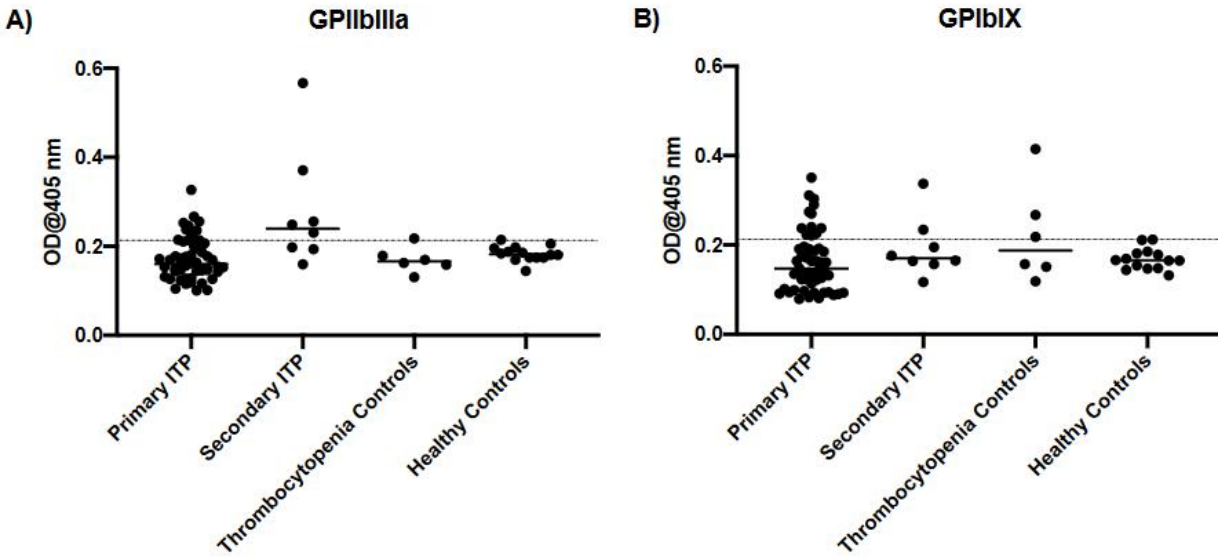
Table 8. Binding response cut-offs for GPIbIX in PBS with 0.01% BSA. 200 μg/mL of purified IgG from healthy controls was diluted in BSA to determine the cut-off for background IgG binding.

Sample	Response (nm)
HC1	0.0241
HC2	0.0107
HC3	0.0121
HC4	0.0017
HC5	0.0052
HC6	0.0013
HC7	0.0153
HC8	-0.0034
HC9	0.0291
HC10	0.0027
HC11	0.0307
HC12	0.0073
HC13	-0.0079
HC14	-0.0043

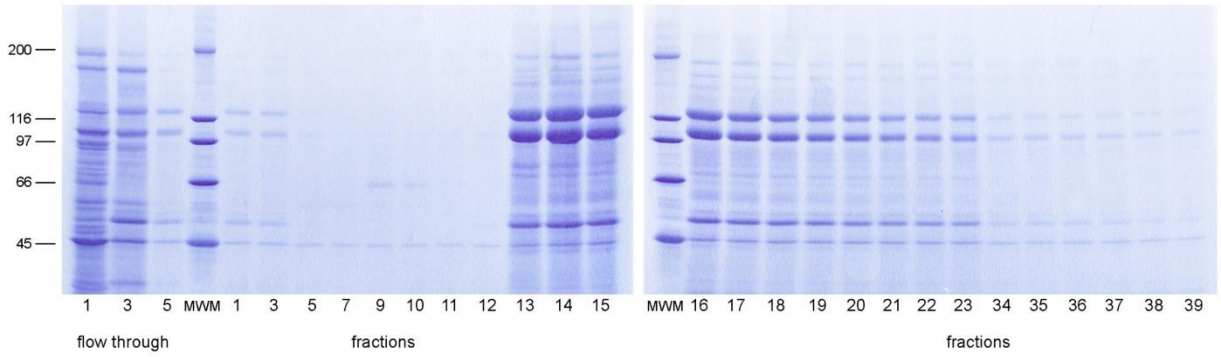
Table 9. Binding response cut-offs for GPIIbIIIa in PBS with 0.01% BSA. 200 μg/mL of purified IgG from healthy controls was diluted in BSA to determine the cut-off for background IgG binding.

Sample	Response (nm)
HC1	0.0258
HC2	0.0121
HC3	0.0152
HC4	-0.0051
HC5	0.01
HC6	0.0031
HC7	0.0071
HC8	-0.0179
HC9	0.0308
HC10	0.0133
HC11	0.0344
HC12	0.0156
HC13	-0.0024
HC14	0.0057

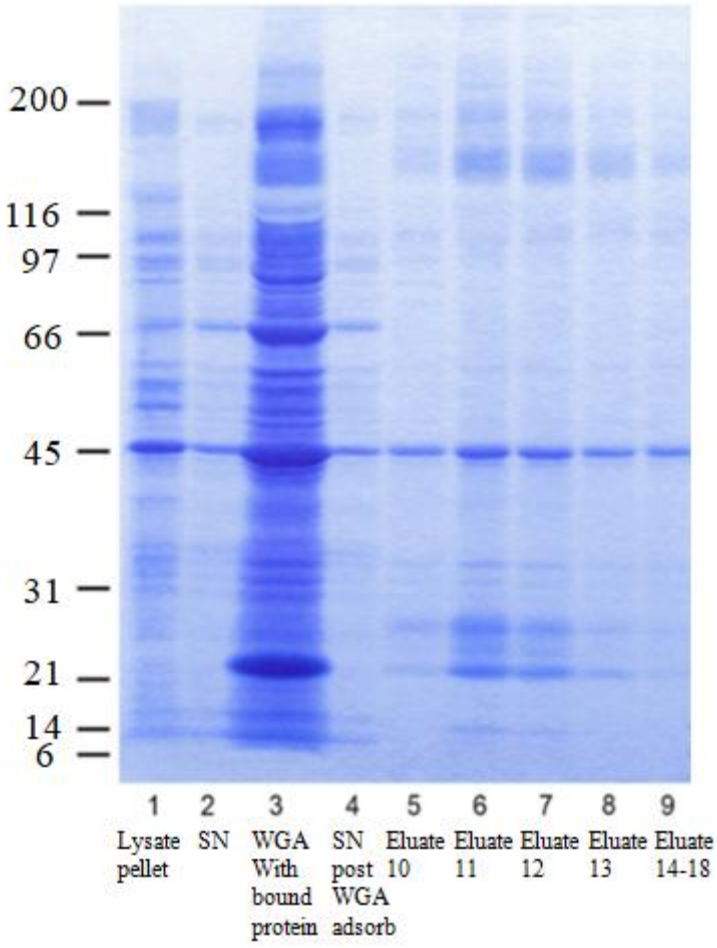
SUPPLEMENTARY FIGURES



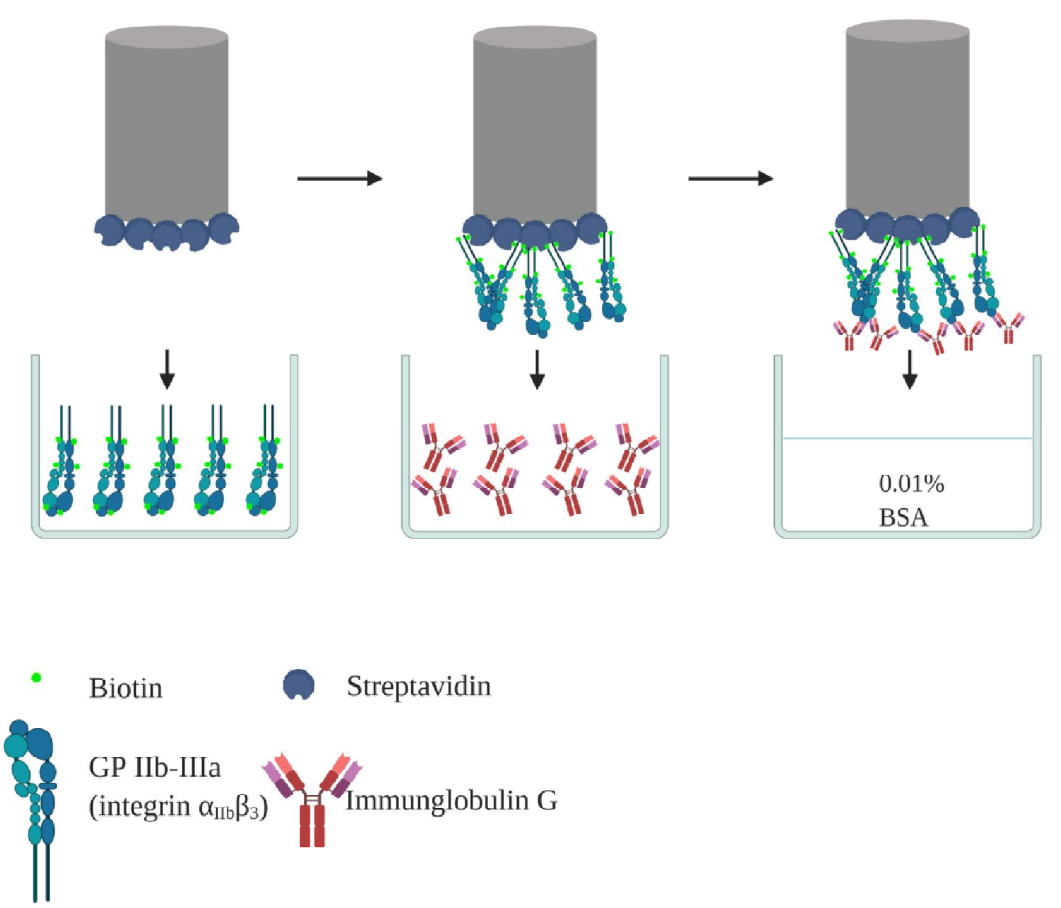
Supplementary figure 1. Current laboratory statistics of indirect antigen-capture assays. A) Indirect tests for anti GPIIbIIIa autoantibodies in patient plasma. B) Indirect tests for anti GPIbIX autoantibodies in patient plasma. Primary ITP n=52; secondary ITP n=8; thrombocytopenic controls n=6 (3 TTP, 2 hereditary TCP, 1 essential thrombocythemia); healthy controls n=14.



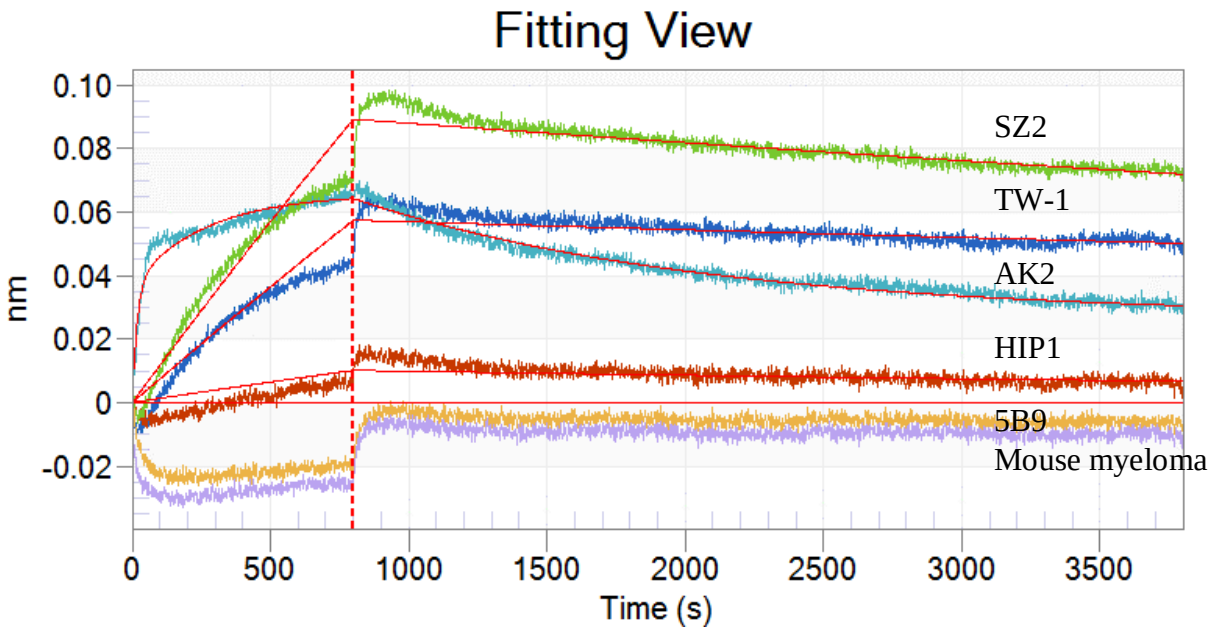
Supplementary figure 2. GPIIbIIIa purification. Purity of GPIIbIIIa was determined using gel electrophoresis. 39 fractions were analyzed and fractions 13-17 were pooled for use in future assays as these contained the highest concentrations of GPIIb (110 kDa) and GPIIIa (125 kDa).



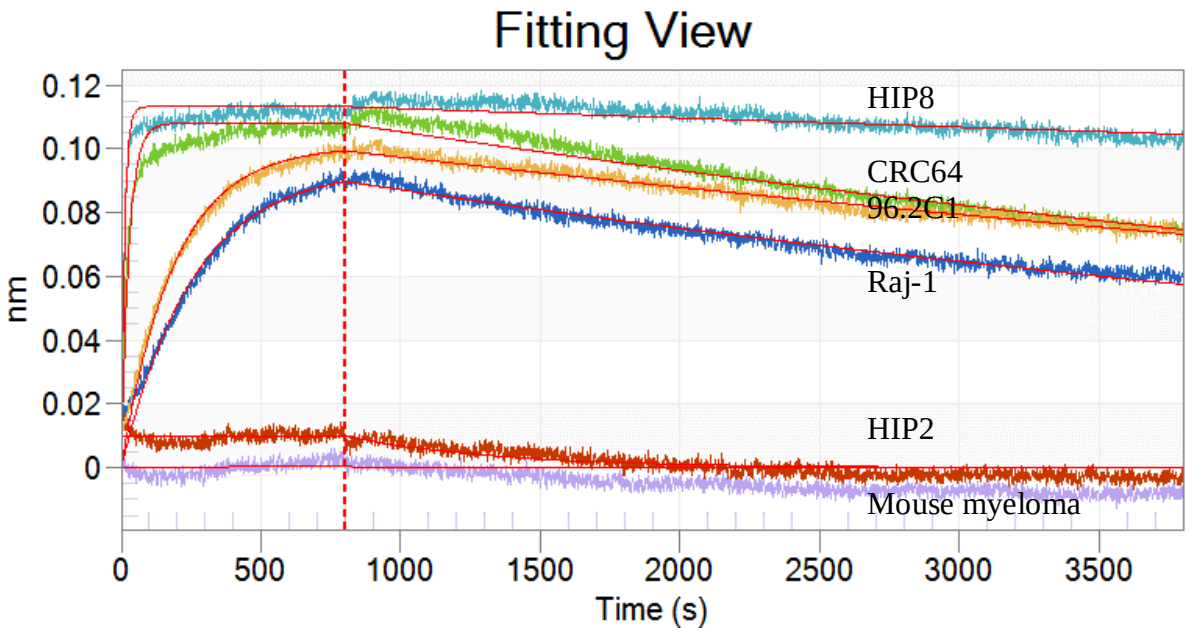
Supplementary figure 3. Purification of GPIbIX. The GP of interest was purified by another lab member and used for future assays. SDS-PAGE was used to verify purity and elution fractions 11-13 were used for experiments, as these contained GPIbα (135 kDa), GPIbβ (26 kDa) and GPIX (20kDa).



Supplementary figure 4. BLI assay design using streptavidin sensors. Biotinylated GPs were immobilized onto streptavidin biosensors to measure binding of cognate antibodies from total IgG samples. Created with biorender.com.



Supplementary figure 5. BLI assay spectrogram showing binding of monoclonal antibodies to GPIbIX. Monoclonal antibodies are as follows: dark blue: TW-1, red: HIP1, light blue: AK2, green: SZ2, yellow: 5B9 (negative control), purple: mouse IgG1 (negative control). Samples were tested at 13.3 nM to determine kinetics data. Analysis was performed using ForteBio Data Analysis 9.



Supplementary figure 6. BLI assay spectrogram showing binding of monoclonal antibodies to GPIIbIIIa. Monoclonal antibodies are as follows: dark blue: Raj-1, red: HIP2, light blue: HIP8, green: CRC64, yellow: 96.2C1, purple: mouse IgG1 (negative control. Samples were tested at 13.3 nM to determine kinetics data. Analysis was performed using ForteBio Data Analysis 9.

SUPPLEMENTARY TABLES

Supplementary table 1. Thrombocytopenia patient demographics. Patients used in the studies are described by gender, age at sample collection, platelet count, diagnosis, year of diagnosis, indirect antigen capture assay results from platelet lysate studies previously done in the lab of GPIIbIIIa and GPIbIX.

ID	Diagnosis	Sex	Age	Sample date	Indirect IIbIIIa	Indirect IbIX	Platelet count	Treatment	Date of ITP diagnosis
1	Primary ITP	F	69	Jan 30, 2017	0.246	0.274	5	N/A	Jan 1, 1978
2	Primary ITP	M	74	Feb 6, 2017	0.151	0.132	42	N/A	Dec 1, 2013
3	Primary ITP	M	74	Feb 6, 2018	0.187	0.126	N/A	N/A	Jan 1, 2006
5	Primary ITP	F	39	Jan 19, 2009	0.256	0.303	116	Cellcept, cyclosporine	Jan 1, 1990
6	Primary ITP	M	66	Feb 28, 2018	N/A	N/A	N/A	Nplate, vincristine	Apr 1, 2001
7	Primary ITP	F	29	Jan 24, 2020	N/A	N/A	1	Nplate	Jan 1, 2003
9	Primary ITP	M	60	Mar 6, 2017	0.172	0.114	5	None	Aug 5, 2015
10	Primary ITP	M	67	Apr 3, 2017	0.162	0.123	6	Prednisone	Jan 1, 1958
13	Primary ITP	M	67	May 8, 2017	0.129	0.079	1	N/A	Jan 1, 1958
14	Primary ITP	M	90	June 5, 2017	0.153	0.135	15	Prednisone, omeprazole, cellcept, azathiprine	Jan 1, 2005
16	Primary ITP	F	34	Sept 25, 2017	0.178	0.123	145	None	Jan 1, 2009
17	Primary ITP	F	33	Oct 18, 2017	0.178	0.133	48	N/A	unknown

18	Primary ITP	F	78	Oct 23, 2017	0.159	0.122	252	Nplate, prednisone	Aug 18, 2016
19	Primary ITP	M	29	Jan 22, 2018	0.147	0.097	2	N/A	Aug 14, 2010
20	Primary ITP	F	57	Feb 23, 2018	0.17	0.351	1	N/A	Jan 1, 1991
22	Primary ITP	F	63	Nov 19, 2018	0.441	0.345	201	N/A	unknown
21	Secondary ITP-common variable immune deficiency	M	31	Jan 18, 2018	N/A	N/A	143	Cellcept	Oct 8, 2014
12	Secondary ITP-lymphoma-associated ITP	F	71	May 1, 2017	0.16	0.164	N/A	N/A	July 24, 2006
15	Secondary ITP-systemic lupus erythematosus	F	38	June 12, 2017	0.567	0.195	109	N/A	Jan 1, 2009
4	Non-immune thrombocytopenia-pancytopenia due to hypersplenism	M	26	Oct 1, 2019	0.783	0.9035	32	N/A	NOT ITP (Aug 29, 2007)
8	Non-immune thrombocytopenia-essential thrombocythemia	F	81	Dec 1, 2008	0.17	0.415	798	N/A	NOT ITP (unknown)

Supplementary table 2. Monoclonal antibodies used in EIA and BLI assays.

Monoclonal Antibody	Cognate antigen	Subclass
Raj-1	CD41	IgG1
HIP2	CD41	IgG3
HIP8	CD41	IgG1
M148	CD41	IgG1
96.2C1	CD41	IgG1
CRC64	CD41	IgG1
TW-1	CD42b	IgG1
HIP1	CD42b	IgG1
AK2	CD42b	IgG1
SZ2	CD42b	IgG1
KKO	PF4	IgG2
5B9	PF4	IgG1
Mouse IgG1 kappa	Isotype control	IgG1

Supplementary table 3. Purified IgG from ITP patients binding to GPIbIX in 0.01% BSA. IgG was diluted to 200μg/mL in BSA and the antibody binding association was measured over time. The kinetics data was created through ForteBio Data Acquisition and analyzed using ForteBio Data Analysis 9. Association and dissociation were fitted to a 2:1 heterogenous ligand binding model and the binding response was calculated as an average over 800 seconds. Samples were tested in groups of 6 with a healthy control.

Sample	Response (nm)	/2xHC1
1	-0.0048	-0.1026
4	0.0001	0.0021
10	0.0098	0.2094
12	-0.0025	-0.0534
13	0.03	0.641
18	0.002	0.0427
Healthy control	0.0234	-
7	-0.0095	-0.1471
5	-0.0041	-0.0635
21	**0.1648**	**2.5511**
3	0.0139	0.2152
2	0.0124	0.1919
20	-0.0022	-0.0341
Healthy control	0.0323	-
6	0.0118	0.2489
22	0.0454	0.9578
8	0.0156	0.3291
15	0.0098	0.2067
19	0.0169	0.3565
16	0.031	0.654
Healthy control	0.0237	-

Supplementary table 4. Purified IgG from ITP patients binding to GPIIbIIIa in 0.01% BSA. IgG was diluted to 200μg/mL in BSA and the antibody binding association was measured over time. The kinetics data was created through ForteBio Data Acquisition and analyzed using ForteBio Data Analysis 9. Association and dissociation were fitted to a 2:1 heterogenous ligand binding model and the binding response was calculated as an average over 800 seconds. Samples were tested in groups of 5 and 6 with a healthy control.

Sample	Response (nm)	/2xHC1
Positive control GT	0.3085	7.3803
1	0.0027	0.0646
4	-0.0088	-0.2105
10	0.0242	0.5789
12	-0.0002	-0.0049
13	0.032	0.7655
Healthy control	0.0209	-
Positive control GT	0.2363	4.9852
7	-0.0345	-0.7278
5	-0.0293	-0.6181
21	**0.1653**	**3.4873**
3	-0.009	-0.1899
2	-0.0019	-0.0401
Healthy control	0.0237	-
Positive control GT	0.3013	8.6086
6	0.0009	0.0257
22	0.0284	0.8114
Positive control NAIT1	0.0502	1.4343
19	0.013	0.3714
16	0.0061	0.1743
Healthy control	0.0175	-
Positive control GT	0.3248	5.7793
8	0.0099	0.1761
15	0.0032	0.0569
17	0.0143	0.2544
18	-0.0027	-0.048
Healthy control	0.0281	-

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