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REVIEW ARTICLE

Platelets and platelet alloantigens: Lessons from human patients and animal models of fetal and neonatal alloimmune thrombocytopenia



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Abstract Platelets play critical roles in hemostasis and thrombosis. Emerging evidence indicates that they are versatile cells and also involved in many other physiological processes and disease states. Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a life threatening bleeding disorder caused by fetal platelet destruction by maternal alloantibodies developed during pregnancy. Gene polymorphisms cause platelet surface protein incompatibilities between mother and fetus, and ultimately lead to maternal alloimmunization. FNAIT is the most common cause of intracranial hemorrhage in full-term infants and can also lead to intrauterine growth retardation and miscarriage. Proper diagnosis, prevention and treatment of FNAIT is challenging due to insufficient knowledge of the disease and a lack of routine screening as well as its frequent occurrence in first pregnancies. Given the ethical difficulties in performing basic research on human fetuses and neonates, animal models are essential to improve our understanding of the pathogenesis and treatment of FNAIT. The aim of this review is to provide an

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overview on platelets, hemostasis and thrombocytopenia with a focus on the advancements made in FNAIT by utilizing animal models.

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Platelets are versatile cells – Critical roles in hemostasis and thrombosis

Platelets are small anucleated blood cells, derived from precursor megakaryocytes in bone marrow^{1,2} that play a key role in vascular repair and hemostasis.^{2–4} Their major role is to accumulate at sites of damaged blood vessels and initiate clotting in order to prevent blood loss. Resting platelets in the blood circulation of mammals are smooth and discoid in shape. When the vessel wall becomes injured, collagen and other subendothelial matrix proteins are exposed and interact with receptors on the platelet surface, resulting in a series of intracellular signaling events and platelet activation.^{3–5} The activated platelets then change their shape and adhere to the site of injury. Recruitment of additional platelets to the damaged site leads to platelet aggregation and platelet plug formation (i.e. the first wave of hemostasis).^{4,5}

The second wave of hemostasis is mediated by the coagulation system, which can be activated by either

extrinsic (tissue factor pathway) or intrinsic pathway following vessel damage. The vital product of coagulation cascades is thrombin, which converts fibrinogen to fibrin, leading blood coagulation that further assists the formation of a clot/hemostatic plug. Thrombin is also the most potent platelet agonist that induces platelet activation, granule release and platelet adhesion/aggregation. Thus, the second wave of hemostasis significantly enforces the first wave of hemostasis. On the other hand, following platelet activation, phosphatidylserine exposure occurs, which generates a negatively charged membrane surface that can host coagulation factors and markedly increase thrombin generation.⁶ These intensive interactions between the first and the second waves of hemostasis synergistically contribute to the formation of a hemostatic plug.^{3,7,8}

An interesting and recent discovery describes the deposition of plasma fibronectin^{9,10} into the injured vessel wall and initiating a “protein wave of hemostasis”¹¹ (Fig. 1). This “protein wave” hemostasis occurs even earlier than the classical “first wave of hemostasis”,

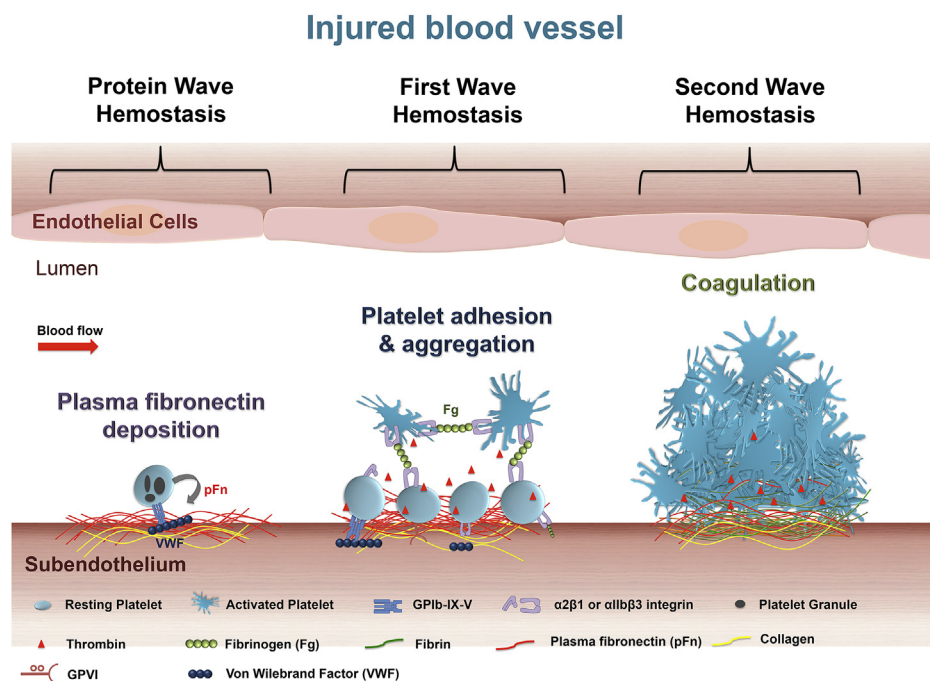


Figure 1 Roles of platelets in thrombosis and hemostasis. After vascular injury, plasma fibronectin quickly deposits onto the injured vessel wall. Platelets may also release their internalized plasma fibronectin from their granules. These plasma and platelet sources of fibronectin likely synergistically contribute to the protein wave of hemostasis. Platelet adhesion and aggregation (i.e. the classical first wave of hemostasis) are then initiated via platelet receptors and their ligands. Activated platelets also provide a negatively charged surface and mediate cell-based thrombin generation, which contributes to blood coagulation that is initiated following tissue damage (i.e. the classical second wave of hemostasis). In a growing hemostatic plug/thrombus, the fibrin and fibronectin matrix is usually formed in the interface between the injured vessel wall and platelet plug.

although platelet recruitment and their locally released granule fibronectin (internalized from plasma fibronectin via α IIb β 3 integrin)^{12–15} may be also involved. However, the importance of this new discovery in diseases including possible plasma fibronectin transfusion in controlling bleeding disorders, particularly those associated with anticoagulant therapies requires further investigation.¹¹ It is notable that platelets contribute to all the aforementioned three waves of hemostasis, which may explain why a decreased platelet count in blood (i.e. thrombocytopenia)¹⁶ usually causes bleeding disorders, such as autoimmune thrombocytopenia (ITP) and fetal and neonatal alloimmune thrombocytopenia (FNAIT).

Despite the vital role in hemostasis, platelet adhesion and aggregation at inappropriate sites may lead to thrombosis and vessel occlusion. In both men and women, heart attack and stroke are the leading cause of morbidity and mortality worldwide^{3,17} and are caused by thrombosis in the coronary or cerebral arteries.^{7,18} Thrombotic events may also occur in the placenta which may lead to miscarriage.¹⁹ In addition to their important roles in thrombosis and hemostasis, platelets have several other functions including acceleration of atherosclerosis,^{20–22} lymphatic vessel development,^{23–25} aiding in tumor growth,²⁶ destruction of microorganisms^{27,28} and infected cells^{29,30} and modulation of inflammation and immune responses.^{31–34} Thus, platelets are versatile cells that play critical roles in multiple human diseases.

Platelet α IIb β 3 integrin and GPIb α : The abundant functional receptors and common targeted antigens in immune mediated thrombocytopenia

There are many receptors expressed on platelets which maintain platelet physiological and pathological functions. The two major platelet surface receptors are glycoprotein (GP) IIb/IIIa (α IIb β 3 integrin) and the GPIb-IX complex, which mediate platelet adhesion and aggregation.^{4,35,36} GPIb α , of the GPIb-IX complex, is a leucine rich repeat family protein, which is essential for platelet tethering and adhesion to the injured vessel wall primarily through binding to immobilized von Willebrand factor (VWF), particularly at high shear stress.^{3,37,38} The interaction between GPIb α and VWF can deliver a signal to platelets, which subsequently causes a conformational change in α IIb β 3 integrin on the platelet surface³⁹ leading to fibrinogen (Fg) binding.⁴⁰ Fg cross-links adjacent platelets, leading to platelet aggregation and platelet plug formation. Although it has been documented for more than a half century that Fg is required for platelet aggregation, interestingly, recent studies demonstrated that the formation of a hemostatic plug and thrombus formation can occur independently of VWF and Fg,^{12,41} but not α IIb β 3 integrin.⁴² These findings suggest that other unidentified ligands of α IIb β 3 integrin exist which can also mediate platelet aggregation, and hemostatic plug/thrombus formation.^{5,7,42} This mechanism and its significance in thrombosis and hemostasis is currently under investigation.^{41–45}

There are approximately 80,000 copies of α IIb β 3 integrin and 25,000 copies of GPIb α on the surface of resting human

platelets.^{46,47} It was previously documented that both GPIb α and the α IIb subunit of the α IIb β 3 integrin are exclusively expressed on platelets and their precursors, while the β 3 subunit of the α IIb β 3 integrin is expressed on many other cell types such as endothelial and endothelial progenitor cells,^{48,49} microglia,⁵⁰ astrocytes,⁵¹ sperm cells⁵² and trophoblast cells of the placenta.⁵³ The expression of GPIb α on endothelial cells (ECs) has been demonstrated under certain conditions,^{54,55} however this remains controversial.⁵⁶ Within the last decade α IIb has been shown to be expressed on early trophoblast cells of the placenta,⁵⁷ mast cells,⁵⁸ embryonic stem cells⁵⁹ and hematopoietic stem cells (HSCs).⁶⁰ Little is known about the role α IIb plays in stem cells, however it is thought to maintain HSC activity during embryonic development,⁶¹ although the exact function of α IIb in stem and mast cells remain unclear.

In addition to their important physiological and pathological roles in hemostasis and thrombosis, platelet α IIb β 3 integrin and GPIb α are the major targeted antigens in both auto- and alloimmune thrombocytopenias (e.g. ITP and FNAIT).^{62,63} Although the immunogenicity and the exact mechanisms of immune response are still not well understood, their abundant expression and significant amounts of polymorphisms likely contribute to the pathogenesis of these immune-mediated thrombocytopenias.

Thrombocytopenia disorders

Thrombocytopenia refers to a decrease in the number of circulating platelets in the blood. In healthy adults, the normal range in circulating platelets is $150\text{--}400 \times 10^9/\text{L}$ and a platelet count below this range may lead to severe bleeding, where in some cases may be life threatening.³¹ The three major categories of thrombocytopenia are immune-mediated, genetic deficiency-associated and malignancy-associated. Immune-mediated thrombocytopenia can be further sub-classified into autoimmune or alloimmune thrombocytopenia.^{33,64}

Autoimmune thrombocytopenia is caused by a loss of self-tolerance resulting in an abnormal immune response, targeting one's own platelets. Autoimmune thrombocytopenia is categorized into primary immune thrombocytopenia (ITP), drug-induced thrombocytopenia and infection associated thrombocytopenia.^{65–67} ITP is the most common with an incidence of 1–2.5 per 10,000 individuals⁶⁸ and the degree of thrombocytopenia can range from mild to severe.⁶² It has been shown that both platelet destruction and impaired platelet production may contribute to low platelet counts, although the exact contributions by each remain unclear.⁶⁹ To date, autoantibodies are considered to be the predominant effector in thrombocytopenia. In adult ITP patients, approximately 70% of detectable platelet autoantibodies are directed against α IIb β 3 integrin, and roughly 20%–40% have specificity for the GPIb α complex, or both.⁷⁰ Interestingly, there is a subpopulation of ITP patients who are thrombocytopenic but have no detectable antibodies. One proposed explanation is that in this group of patients, ITP is mediated by CD8⁺ T cells (i.e. cytotoxic T-lymphocytes).^{71–73} On the other hand, it may be that the current antibody detection systems are not

optimal and may be unable to detect some of the autoantibodies that recognize conformation-dependent epitopes. The conformations of these platelet antigens may be changed during the anticoagulant treatment and sample preparations, subsequently losing their binding sites for some antibodies.⁷⁴ This may be particularly important for α IIb β 3 integrin, since divalent cations play important roles in maintaining integrin structure and function.^{75–79} Divalent cation chelators (e.g. sodium citrate, and EDTA) in the anti-coagulated blood may generate false negative results and decrease the autoantibody detection.

Platelet clearance mediated by anti-platelet autoantibodies typically result in platelet phagocytosis by Fc γ -receptor bearing cells of the reticuloendothelial system (RES) such as macrophages, and the majority of these opsonized platelets are cleared in the spleen. However some ITP patients, particularly those with anti-GPIIb α antibodies who do not respond well to treatments,^{80–83} may mediate platelet clearance through an Fc-independent pathway,^{84,85} leading to alternative sites of platelet clearance.⁸⁶ A recent study found that platelet desialylation⁸⁷ may occur after antibody binding, particularly those platelets opsonized by anti-GPIIb α antibodies.⁸⁸ This mechanism leads to platelet clearance in the liver via Ashwell-Morell receptors on hepatocytes, which is fundamentally different from the classical Fc-Fc γ R-dependent macrophage phagocytosis in spleen.⁸⁸ This discovery may be important not only in basic research, but also for diagnosis and treatment of refractory ITP (Nature Communications *in revision*).^{89,90} It is currently unknown whether this novel Fc-independent platelet clearance pathway also occurs in fetuses and contributes to FNAIT.

ITP in pregnancy and other neonatal thrombocytopenias

Expecting mothers with pre-existing ITP have been less well studied and as such the effect of the disease on neonates is controversial.^{91,92} Neonatal thrombocytopenia in mothers with ITP cannot be predicted by history or platelet count, however an affected older sibling is a reliable risk factor in subsequent pregnancies.⁹³ Despite being rare and less of a clinical concern, some cases of neonates born to mothers with ITP have been associated with extensive hemorrhage and fetal death.^{94,95} Neonatal thrombocytopenias which are not due to anti-platelet antibodies are generally associated with chronic fetal hypoxia, as seen in mothers with pregnancy-induced hypertension, diabetes or intrauterine growth restriction (IUGR).⁹⁶ The mechanism of non-antibody mediated forms of neonatal thrombocytopenia is thought to be reduced megakaryopoiesis⁹⁷ and cases are usually mild since neonate platelet count normally resolves within 10 days.⁹⁸

Alloimmune thrombocytopenia is due to alloantibody-mediated platelet depletion and develops when an immune response is generated following exposure to allogenic platelets. This can occur after transfusion of platelets from allogenic donors, termed post-transfusion purpura (PTP) or during pregnancy following maternal exposure to paternal alloantigens on fetal platelets, termed fetal and neonatal alloimmune thrombocytopenia (FNAIT).⁹⁹ FNAIT is similar to

the more common condition hemolytic disease of the fetus and newborn (HDFN) where maternal alloantibodies target antigens on fetal red blood cells. In contrast to HDFN that occurs following antigen exposure in the previous pregnancy, FNAIT may develop in the first pregnancy in up to 50% of all cases,^{100,101} making diagnosis and treatment more difficult.

Fetal and neonatal alloimmune thrombocytopenia

FNAIT is caused by fetal platelet destruction by maternal alloantibodies developed during pregnancy.^{102,103} Harrington et al were the first to formally describe neonatal thrombocytopenia in 1953, where two infants were born with significantly decreased platelet counts, delivered from mothers without ITP.¹⁰⁴ Although serological techniques were not available at the time, this was the first identification and report describing what we now know as FNAIT. Shulman et al¹⁰⁵ first identified that maternal alloimmunization and antibodies targeting platelet antigens was the reason for platelet destruction in neonates with the disease. Today FNAIT is the most common cause of severe thrombocytopenia in live born neonates¹⁰⁰ and accounts for up to 40% of all neonates admitted into the neonatal intensive care unit.¹⁰⁶ FNAIT is now recognized as a critical complication in pregnancy with severe and adverse outcomes. Due to the life threatening nature of FNAIT, there are ethical concerns in performing basic research on human fetuses and neonates with the disease. The use of animal models in FNAIT research will surely help decode the complexity of this disease and may lead to treatment in humans.

FNAIT is also the most common cause of intracranial hemorrhage (ICH) in full-term infants,¹⁰⁷ which is the most severe clinical complication of FNAIT and may lead to neurological impairments or death.^{95,108–110} ICH occurs at a rate of 10%–20% in neonates born with FNAIT and has fatal consequences in approximately 5% of documented cases.^{102,111} An ICH can also occur in utero as early as week 14 of pregnancy in fetuses affected by FNAIT,¹¹² suggesting that early diagnosis and management are necessary to prevent morbidity and mortality. The recurrence rate of a subsequent pregnancy affected by FNAIT is close to 100% in antigen positive siblings and they usually have a similar or more severe form of thrombocytopenia.^{109,113} Therefore, the only established biomarker for severity of the disease is if a previous sibling presents with ICH.^{113,114} Interestingly, in some cases ICH is independent of the severity of thrombocytopenia,^{113,115} indicating that other mechanisms aside from low platelet count likely contribute to bleeding in the brain as seen in some neonates.

Several large studies have shown an incidence of FNAIT between 1 and 1.5/1000 live births.^{106,116–118} However, these reports do not include the rate of miscarriage associated with the disease, therefore FNAIT may be much more prevalent than previously thought. Although it has been reported by several groups,^{101,119,120} the rate of miscarriage in affected pregnant women has not been adequately studied. One explanation for this is that rare human platelet antigens (HPAs) may induce severe FNAIT

leading to miscarriage, which could confound the severity and frequency of reported cases of FNAIT in humans.

In healthy human fetuses, the platelet count reaches approximately $300 \times 10^9/L$ by 30–35 weeks of gestation¹²¹ and thrombocytopenia in the fetus or neonate has been defined as a platelet count less than $150 \times 10^9/L$, however FNAIT can cause severe thrombocytopenia where platelet counts are often below $20 \times 10^9/L$.¹²⁰ Alloantibodies generated in the mother are transported across the placenta via the neonatal Fc receptor (FcRn) during pregnancy and enter fetal circulation and opsonize platelets and mediate clearance^{122,123} (Fig. 2). It is thought that platelet destruction in FNAIT is similar to that in ITP. Allo-antibody bound platelets interact with Fc receptors on macrophages through the Fc fragment of IgG, resulting in clearance of the antibody-bound platelets by the RES system in the spleen.¹²⁴ However, some ITP patients with antibodies targeting GPIIb α may have Fc-independent platelet clearance,⁸⁶ which may also be occurring in FNAIT patients. It is currently unclear whether there are significant differences between adult and fetal/neonatal platelet clearance mechanisms.

Gene polymorphisms and platelet alloantigens in FNAIT

In 1990, the Platelet Serology Working Party of the International Society of Blood Transfusion (ISTB) established a nomenclature for platelet antigens.¹²⁵ They classified platelet antigens as HPAs, where each platelet antigen is numbered based on date of discovery and the more common alleles are ordered in alphabetical pairs based on frequency – ‘a’ for the common allele and ‘b’ for the rare allele.¹²⁶ All HPAs result in a single amino acid substitution except for HPA-14w where the antigen is a result of a one amino acid deletion.¹²⁷ The database of all immunizing

HPAs that have caused at least one case of FNAIT can be found at <http://www.ebi.ac.uk/ipd/hpa/table2.html>. To date there are 36 HPAs on six platelet surface proteins that have been discovered and may lead to FNAIT.¹²⁸ Of these, 18 are located on integrin $\beta 3$ subunit (including HPAs 1a, 1b, 1c, 4a, 4b, 6bw, 7b, 7c, 8bw, 10bw, 11bw, 14bw, 16bw, 17bw, 19bw, 21bw, 23bw and 26bw), 8 are located on the αIIb subunit (HPAs 3a, 3b, 9bw, 20bw, 22bw, 24bw, 27bw and 28bw), 5 on integrin $\alpha 2$ (HPAs 5a, 5b, 13bw, 18bw and 25bw), 2 on GPIIb α (HPAs 2a and 2b), 1 on GPIIb β (HPA 12bw) and 2 on CD109 (HPAs 15a and 15b). 26 HPAs are caused by bi-allelic polymorphisms and two HPAs are due to tri-allelic polymorphisms.¹²⁹

Despite the existence of multiple platelet antigens, only few are responsible for the majority of reported cases of FNAIT. Incompatibility of HPA-1 (a leucine–proline difference at residue 33 of the $\beta 3$ integrin¹³⁰) accounts for approximately 75%–85% of cases in Caucasians.¹³¹ Although FNAIT due to HPA-1a incompatibility is very rare in African and Asian populations,^{132,133} irrespective of race, HPAs in the $\beta 3$ integrin subunit account for the vast majority of reported cases.^{134–136} It is interesting that certain polymorphisms are found much more abundantly in certain populations than others. The HPA-4 polymorphism, also located on the subunit $\beta 3$ integrin, resides on residue 143 and is more prevalent in the Asian population, as well as HPA-21bw.¹³⁷ In addition, HPA-6bw is a more common polymorphism to the Finnish population that causes FNAIT.¹³⁸ HPA-5 is located on integrin $\alpha 2$ subunit of the collagen receptor and is the second most common antigen causing FNAIT accounting for 10%–15% of all cases.¹³⁹ The reported incidence of FNAIT due to HPA-2 on GPIIb α is rare.^{140,141} However, it is currently unknown whether pro-thrombotic events are induced by anti-GPIIb α antibodies in the placenta,¹⁹ which may cause miscarriage and mask the reported incidence in humans. FNAIT caused by HPA-2 may also be rare due to the lower immunogenicity of this

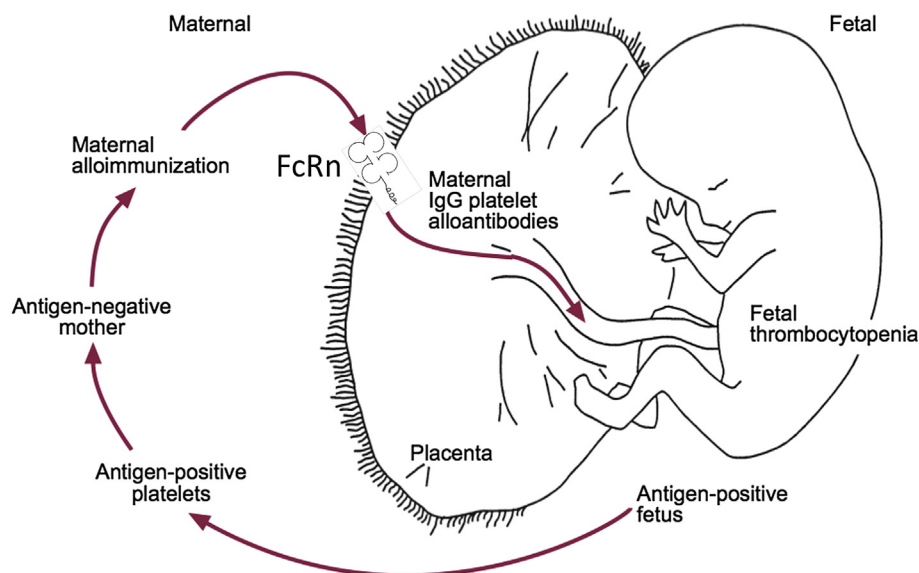


Figure 2 Pathogenesis of fetal and neonatal alloimmune thrombocytopenia (FNAIT). This figure is adapted from Blanchette VS, Johnson J and Rand M, *Baillieres Best Pract. Res Clin Haematol*, 2000, 13(3): 365–90 and Chen P, Li C, Lang S, Zhu G, Reheman A, Spring CM, Freedman J and Ni H, *Blood*, 2010, 116 (18), 3660–3668.

leucine repeat family protein that may stimulate a weaker immune response in women.⁸⁶

Interestingly, 5 out of 8 HPAs on the α IIb subunit are located in very close proximity within the calf-2 domain, including the most common α IIb antigens HPA-3a and HPA-9bw. These HPAs only account for 2%–5% of all cases of FNAIT,^{106,131} however they are typically involved in more severe cases of FNAIT.^{142,143} HPA-3b and HPA-9b are in a linkage disequilibrium, explained by the fact that they are located only 19 base pairs apart.¹²⁹ HPA-22bw resides on the β -propeller domain, close to the ligand-binding site, which may interfere with fibrinogen and other ligand binding and cause severe bleeding in affected neonates.¹⁴⁴ Both polymorphisms themselves and alloantibody binding may affect hemostasis/thrombosis. HPA-13w on the α 2 integrin and both HPA-1b and HPA-21bw on the β 3 integrin subunit have also been identified as HPAs that interfere with receptor function.^{145,146} However the extent to which various HPAs contribute to hemorrhagic or thrombotic risk remains to be determined.¹⁴⁷

Glycoprotein IV (CD36) deficiency occurs in 3%–5% of individuals of African or Asian ancestry. It is a member of the class B scavenger receptor family of proteins expressed on platelets, red blood cells, endothelial cells, monocytes and macrophages.¹⁴⁸ Mothers who lack CD36 are at risk of becoming immunized if exposed to the protein by transfusion or pregnancy.¹⁴⁹ It is interesting that affected neonates have a severely decreased platelet count without solid evidence of other tissue damage despite CD36 being expressed on endothelial cells and other blood cells.¹⁴⁸ Immunization against CD36 is termed 'Nak' isoantibody generation. Since CD36 is expressed on multiple cells, it does not have HPA nomenclature.¹⁵⁰

Mechanisms of alloantibody generation in FNAIT

FNAIT is thought to be initiated by fetal platelet alloantigens that are inherited from the father but absent in the mother. The mechanism of exposure of the mother to fetal alloantigens is not well understood. Similar to HDFN, immunization may occur as a result of fetomaternal hemorrhage during parturition.^{116,151} Since FNAIT often occurs during the first pregnancy, fetal platelets may "leak" into the maternal circulation and initiate an immune response. The smaller size and greater mobility of platelets compared to red blood cells may attribute to FNAIT development in the first pregnancy as compared to HDFN. It has been demonstrated that β 3 integrin is expressed on human trophoblast cells of the placenta,^{57,152} suggesting that an immune response may be generated independently of platelets but via a maternal immune response against β 3 integrins on trophoblast cells. In addition, β 3 integrin is also expressed on spermatozoa,^{52,153} therefore suggesting that women may become immunized with preconceptional exposure.

When platelet antigens are exposed to the maternal circulation, they are processed by antigen presenting cells and subsequently presented to T helper lymphocytes similar to other foreign antigens.^{99,153–157} T cells may then stimulate B cells to become activated and differentiate into

plasma cells and secrete antigen-specific IgG antibodies. Macrophages themselves may also directly stimulate the differentiation of B cells to plasma cells.¹⁵⁸ Interestingly, not all mothers with incompatible fetuses will develop FNAIT; prospective studies indicate only approximately 10% of women with an HPA-1a incompatible fetus will become immunized during pregnancy.^{116,159,160} The most plausible explanation for this may be the strong association between the human leucocyte antigen (HLA) class II DRB3*0101 and DRB4*0101 alleles and HPA-1a alloantibody generation.¹⁵⁵ The DRB3 allele has also been demonstrated to have binding affinity for HPA-1a but not HPA-1b.¹⁵⁴ This implies that specific maternal antigen presentation pathway and a specific genetic background may be involved in fetal antigen alloimmunization. In addition, co-existing viral or bacterial infections may also enhance the maternal immune response against fetal platelet antigens.¹⁵⁶

Once antibodies against fetal platelet alloantigens are generated in the mother, they can enter fetal circulation via FcRn¹²² and cause platelet opsonization and clearance.¹²³ It is thought that platelet destruction in FNAIT is similar to that in ITP. Alloantibody-bound platelets interact with Fc receptors on macrophages through the Fc fragment of IgG, resulting in clearance of the antibody-bound platelets by the RES system in the spleen.¹²⁴ However, some ITP patients with antibodies targeting GPIIb/IIIa may have Fc-independent platelet clearance,⁸⁶ which may also be occurring in FNAIT patients. It is currently unclear whether there are significant differences between adult and fetal/neonatal platelet clearance mechanisms.

Therapies and management of FNAIT

Although there have been significant advancements in the diagnosis and management of FNAIT within the past decade, existing therapies are currently limited and prophylactic treatments are lacking. FNAIT is a life-threatening disease that may occur during the first pregnancy and antenatal management is challenging but necessary. Since there is currently no screening procedure in practice, diagnosis occurs after the delivery of a symptomatic child.¹⁶¹ Several treatments including intravenous immunoglobulin G (IVIG), corticosteroids and fetal and neonatal platelet transfusions have been used to manage FNAIT,¹⁶² however antenatal treatment has not been standardized.¹⁶³ Although in utero platelet transfusions may be effective in some FNAIT patients, most centers have abandoned this technique due to its invasiveness and due to the high risk of fetal loss.^{161,164} It is notable that recent studies in murine models suggested that neither platelets nor fibrin clots are essential for hemostasis in fetuses,^{165,166} therefore in utero fetal platelet transfusion may be less important. However, platelet transfusion after birth has been demonstrated to be very beneficial for neonates.^{120,167} IVIG has been used to treat FNAIT albeit with varying results.^{118,168} IVIG is pooled from the plasma of healthy donors; it is expensive and in limited supply. There are also risks of transmitting blood born pathogens with administration of IVIG and it is often not well tolerated in pregnant women.¹⁶⁹ Recent clinical studies suggest a combination of IVIG and corticosteroids is the most

effective treatment, specifically when corticosteroids are administered during the last trimester.¹⁷⁰

Postnatal therapeutic options depend on the severity of thrombocytopenia, since severe bleeding and ICH often occur in neonates with platelet counts less than $20 \times 10^9/L$.¹⁷⁰ Platelet transfusions are the first line of therapy in neonates with low platelet counts. Fortunately, random donor platelets, even when incompatible, are frequently effective either as sole treatment of FNAIT^{167,171} or when administered together with IVIG. This is of significance since matched platelets may not be available and obtaining washed maternal platelets may not be possible in certain cases. The challenge for effective therapy and management is not only due to FNAIT occurring in the first pregnancy, rather it is due to a shortage of knowledge, which requires research and animal models of this disease.

Animal models of FNAIT

Given the ethical difficulties in performing basic research on human fetuses and neonates, animal models are essential to improve our understanding of the pathogenesis and treatment of FNAIT. Previously we established the first animal model of FNAIT, while other animal models have been generated in order to study immune thrombocytopenia.^{73,172–177} These include mouse models such as (NZW $\times$ BXSB) F1 mice¹⁷³ that develop lupus nephritis, myocardial infarction, and thrombocytopenia; and an immunodeficient SCID mouse/human chimera model, in which human cord blood cells or splenocytes from ITP patients were implanted into SCID mice.¹⁷⁴ Other animal models such as murine,^{178,179} rat,¹⁷⁵ dog,¹⁷⁶ and baboon¹⁷⁷ have been studied, which were generated by injection of anti- $\beta 3$ integrin antibodies. In models of alloimmune thrombocytopenia, genetically or chemically modified anti-HPA-1a competitive antibodies (B2G1 and SZ21) have been demonstrated to be able to block anti-HPA-1a antibodies binding to HPA-1a-positive platelets in murine models, suggesting their therapeutic potential.^{180,181} These models provide important information for gaining a better understanding of the antibody-mediated macrophage phagocytosis and the role of IVIG, which shed light on the mechanisms of platelet destruction in FNAIT. However, they provide little information to our understanding of the maternal immune responses causing FNAIT or how pathogenic antibodies affect the fetus throughout development.

Previous experiments from our laboratory have demonstrated $\beta 3$ integrin deficient ($\beta 3^{-/-}$) mice generate antibodies when transfused with wild type (WT) platelets. After immunized $\beta 3^{-/-}$ females were bred with WT males, thrombocytopenia and ICH were observed in the heterozygote pups. We also examined the role of FcRn in FNAIT via combined deficiencies of $\beta 3^{-/-}$ and FcRn $^{-/-}$ mice.¹²² Furthermore, we established a model of FNAIT mediated by anti-GPIIb α antibodies.¹⁹

Lessons learned from animal models of FNAIT

Prior to establishing a murine model of FNAIT, the process of the maternal immune response to fetal platelet antigens

was largely unknown. Although in clinical cases, mothers usually generate antibodies against one specific site on the integrin; our animal models have the advantage in that they generate anti-platelet antibodies which are capable of targeting multiple epitopes on platelet receptors as seen in some cases.¹²⁸ In addition, there are 18 alloantigens (approximately half of all HPAs) located throughout the $\beta 3$ integrin subunit (from the N-terminus to the C-terminus of the extracellular domain), which are capable of eliciting an immune response. Therefore the study of the immune response against the entire $\beta 3$ integrin subunit is of importance to the understanding of FNAIT that are mediated by different anti-HPA antibodies.

Our first model was established by transfusing female $\beta 3$ deficient mice with WT platelets.¹⁵³ These immunized female mice were then bred with WT male mice, to generate a heterozygous fetus. Antibodies from the maternal circulation cross the placenta via the FcRn and bind to fetal platelets, leading to bleeding symptoms.¹²² Some mothers underwent miscarriage and did not deliver pups, while other pups were delivered stillborn. The severity of fetal symptoms is correlated with the antibody titers in the maternal circulation. Both IgG1 and IgG2a antibodies were detected in the maternal serum, indicating that both T-helper 1 (Th1) and Th2-like immune responses exist. Therapeutic administration of IVIG to immunized mothers decreased antibody titers in both mothers and neonates, however, no anti-idiotypic activity of IVIG was found in this model.

Alloantibodies may have other roles in the pathogenesis of FNAIT besides the destruction of fetal platelets. ICH has been observed in some patients whose platelet counts are within the normal range.¹¹⁵ Our lab demonstrated that anti- $\beta 3$ antibodies generated in our model of FNAIT can cross react with $\alpha V\beta 3$ integrin on angiogenic endothelial cells *in vivo*, leading to impaired angiogenesis.¹⁸² Neonates born to our model of anti- $\beta 3$ mediated FNAIT present with ICH and decreased blood vessel density in both their brains and retinas. However neonates from the anti-GPIIb α model of FNAIT did not show any evidence of bleeding in the brain or decreased vascular density. *In vitro* studies with anti-HPA-1a antibodies demonstrate inhibited proliferation and network formation of human umbilical vein endothelial cells (HUVECs). Interestingly, anti- $\beta 3$ antibodies injected into $\alpha IIb^{-/-}$ neonates induce ICH, suggesting that ICH may develop independently of thrombocytopenia. Therefore, ICH likely results from interference of $\alpha V\beta 3$ integrin on ECs by antibodies in FNAIT, leading to impairments in blood vessel development in the brains of fetuses and neonates.^{182,183}

Miscarriage is a devastating outcome of FNAIT, however the incidence and mechanism of miscarriage has not been adequately studied. Therefore, an animal model of FNAIT is particularly useful in examining the pathophysiological mechanism of miscarriage. In contrast to the 20%–40% prevalence of anti-GPIIb α antibodies in patients with immune thrombocytopenia,⁶⁷ there are few reported cases of anti-GPIIb α FNAIT.¹⁴⁰ Our laboratory has demonstrated in an FNAIT animal model that anti-GPIIb α antibodies cause spontaneous miscarriage,¹⁹ which may explain the low frequency of anti-GPIIb α mediated FNAIT in humans. Miscarriage may also account for the low frequency of other

rare antigens reported in FNAIT such as anti- α IIb mediated FNAIT, however more studies are needed to confirm this phenomenon.

Antenatal treatments such as IVIG and steroids have been used to treat FNAIT, however the mechanisms of these treatments are still not well understood and they are often accompanied by side effects. Therefore, a safer and more effective therapy remains to be developed. The FcRn has been shown to be important in transplacental transfer of IgG antibodies from mother to the fetus during pregnancy.^{122,184} However, it has been speculated that other IgG associated proteins may also contribute for the transport of IgG across the placenta, specifically in inflammatory conditions. We have shown that in an animal model of FNAIT, fetuses lacking the FcRn prevented the transfer of pathogenic antibodies from the mothers.¹²² In addition, targeting the FcRn with anti-FcRn antibody or IVIG ameliorates FNAIT. Prior to our discovery, it was previously unknown whether FcRn independent IgG placental transport plays a significant role in FNAIT, and whether this pathway can be upregulated during inflammation or other pathologies that occur in the placenta during FNAIT. However, in our animal models, we clearly demonstrated that fetal but not maternal FcRn is required for all IgG placental transport and required for induction of FNAIT.¹²²

While infection in the pathogenesis of ITP has been investigated,⁶⁶ infection in FNAIT has not. In our mouse model of FNAIT, injections of lipopolysaccharide (LPS) (mimicking bacterial infection) or Poly I:C (mimicking viral infection) was performed together with immunization of WT platelet in GPIIb α ^{-/-} and β 3^{-/-} mice. We found enhanced antibody generation, more severe bleeding and increased rates of miscarriage in these mice.¹⁵⁶ This suggests that bacterial or viral infection may enhance the severity of FNAIT and also other alloimmune thrombocytopenias such as post-transfusion purpura (PTP). Control of infection may be an important strategy during pregnancy to attenuate FNAIT and PTP, and after pregnancy to prevent miscarriage in subsequent pregnancies.

Future treatments of FNAIT

Treating and preventing FNAIT is especially difficult because of the severity of bleeding symptoms and also because it may occur during the first pregnancy. However results from a retrospective study indicate that FNAIT occurring in the first pregnancy may be less common than expected¹⁸⁵ and that FNAIT may be treated prophylactically similar to HDFN. Antibody-mediated immune suppression (AMIS) has successfully been used for prevention of RhD immunization for the past 4 decades.¹⁸⁶ Results from a proof of concept study using our mouse model of FNAIT suggest that the same principle may be applied for the prevention of FNAIT. In our murine model of FNAIT, AMIS led to a 90% reduction in anti-platelet antibodies in maternal circulation, significantly elevated neonatal platelet counts and vastly improved pregnancy outcomes.¹⁸⁷ The idea of a prophylactic approach in FNAIT is supported and deserves further studies in clinical trials.

Therapeutics targeting the FcRn may be considered as a useful therapy alternative to current treatments. The dose of

anti-FcRn required to mediate therapeutic effects are much lower than that of IVIG (at least 200-fold less), suggesting that treatment with anti-FcRn antibody may be a more efficient therapy.¹²² Anti-FcRn therapy would also be advantageous, as it is not prepared from pooled human plasma (as is the case for IVIG), thereby decreasing the chance of patient exposure to blood born micropathogens as well as being a more cost effective therapy. Therefore, the use of anti-FcRn monoclonal antibody is promising as a future treatment for this disease and deserves further clinical consideration. In conclusion, further clinical studies on treatments are warranted for FNAIT. It is also of significance to investigate the possible differences of platelet function, the coagulation system, and RES system between fetus and adult.

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