Platelet physiology and immunology: pathogenesis and treatment of classical and non-classical fetal and neonatal alloimmune thrombocytopenia

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Abstract: Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a devastating disorder affecting approximately 0.5–1.5/1,000 live neonates. It occurs due to maternal immune responses against paternally inherited human platelet antigens (HPAs), resulting in low platelet counts, severe bleeding (e.g., intracranial hemorrhage; ICH), intrauterine growth restriction (IUGR), and miscarriage. There are currently 37 known HPAs; and 15 HPAs are located on the integrin β3 subunit. Fetomaternal incompatibilities in this protein have been most frequently reported, in which HPA-1 system accounts for more than 75% of FNAIT cases. The data from our animal models and from human anti-HPA-1a demonstrate that maternal anti-β3 antibodies have an anti-angiogenic effect, and that anti-angiogenesis, not thrombocytopenia, may be the key cause of ICH, suggesting that fetal platelet transfusion may have limited clinical benefits. Anti-β3 antibodies may also damage antigen positive trophoblasts via natural killer (NK) cells, causing placental dysfunction and miscarriage. Notably, anti-GPIbα alloantibody (e.g., anti-HPA-2) may induce platelet cell-based thrombin generation and thrombosis in placenta, leading to miscarriage. The consequences of anti-angiogenesis and pathology in placenta have not been adequately explored but may cause symptoms beyond thrombocytopenia and bleeding disorders, termed non-classical FNAIT. Meanwhile, maternal intravenous IgG (IVIG) transfusion is likely able to block pathogenic antibody transport across placenta, and ameliorate thrombocytopenia in fetal reticuloendothelial system (RES), although it is currently unclear whether IVIG has equal efficacy for all anti-HPAs or other platelet antigens such as CD36. Further research is required to define standard treatment protocols and explore new treatment options, such as anti-HPA-1a prophylaxis, anti-neonatal Fc receptor (FcRn) and anti-NK therapies. In this review, we summarize the current state of literature comprising platelet versatilities and hemostasis, and integrate new discoveries related to FNAIT etiological factors in order to develop better diagnostic and therapeutic strategies against this life-threatening disease.

Keywords: Alloimmune thrombocytopenia; platelets; integrin; antibodies; hemostasis

Introduction

Platelets, thrombocytes, and thrombocytopenia

Platelets are anucleate blood cells derived from megakaryocyte cells in the bone marrow of mammals (1,2). Interestingly, recent studies have demonstrated that platelets could also be produced in lungs (3), however the portion of these lung-derived platelets in blood circulation and whether they possess different and/or additional functions are still under debate. Platelets have been known for more than a century for their critical functions in hemostasis and thrombosis (4,5). Even so, continuing research in the last few decades, particularly last few years, reveals platelets to be versatile and significantly contribute to many other physiological and pathological processes such as atherosclerosis (6,7), angiogenesis (8,9), tumor metastasis (10), lymphatic vessel development (11,12) and liver regeneration (13,14). Importantly, platelets are considered as an important part of the immune system and actively engage in both innate and adaptive immune responses via their surface adhesion molecules and intracellular components (15-17). For instance, platelets demonstrate "innate immune cell-like" roles by possessing rudimentary phagocytic and antimicrobial activity (15,16), and releasing pro-inflammatory cytokines such as interleukin-1 (IL-1) during infections (15,16,18). Platelets may also have anti-inflammatory and immune regulatory functions through the release of immunosuppressive cytokines such as transforming growth factor beta (TGF-β) and IL-10, although more evidence is required to establish their dual roles in immune responses (15).

In lower (non-mammalian) vertebrates, thrombocytes are nucleated blood cells which play similar hemostatic and immune protective roles, suggesting that platelet functions in mammals are important and evolutionarily conserved (1,15,16,19).

Thrombocytopenia, defined as a low number of thrombocytes or platelets in the body, is a hematologic disorder which can be inherited or acquired (4). When not properly treated, this may result in severe bleeding diathesis and become life-threatening. The typical acquired thrombocytopenia result from the immune system targeting platelets for destruction, which can occur in either autoimmune or alloimmune disorders such as immune thrombocytopenia (ITP) and fetal and neonatal alloimmune thrombocytopenia (FNAIT), respectively (20-22).

FNAIT, the most common cause of severe thrombocytopenia in both fetuses and neonates (22), will be the main focus of this review, particularly elaborating on non-classical FNAIT such as early miscarriage and intrauterine growth restriction (IUGR), as well as intracranial hemorrhage (ICH). In order to better understand the complex pathogenesis of FNAIT, especially for the non-classical FNAIT, and develop better treatments, it is important to take into account that platelets are versatile and play the roles of both passive targets and active immune regulators beyond their well-recognized function in arrest of bleeding. Following, we will discuss the roles of platelets in hemostasis as well as mutual interactions between platelets and the immune system.

Platelets play central roles in hemostasis: old topic with new discoveries

Platelets are small cells or "fragments" of megakaryocytes. After being released into the blood, the physical force of larger red blood cells "pushes" platelets aside, maintaining a majority of them in close proximity to the vessel wall (1,2,5). At the site of vascular injury, subendothelial matrix proteins such as collagen are exposed to the blood flow, which anchors von Willebrand factor (VWF), and initiates platelet glycoprotein (GP) GPIbα-VWF interaction. This advances platelet tethering/adhesion, particularly at high shear conditions such as the area of arterial stenosis (23-25). GPIbα-VWF transient interactions mediate platelet rolling and translocation onto the injured vessel wall, slowing them down, and provide the opportunity for subsequent GPVI-collagen interactions, an important step for further activation of platelets (26,27). In addition to GPIbα-VWF interaction, some soluble factors released from platelets or generated from blood coagulation, such as ADP and thrombin, can also deliver signals for platelet activation (24,28-30). These activated platelets then express active conformations of integrins such as αIIbβ3 integrin (GPIIbIIIa) (31-33). Through interactions with their ligands on the vessel wall such as VWF, fibrinogen/fibrin, collagen, fibronectin, laminins, and thrombospondin-1, these integrins (αIIbβ3, likely also αVβ3 and α2β1, α5β1 α6β1) can mediate firm platelet adhesion onto the injured vessel wall (31,34-36). Under low shear conditions such as in veins, these integrin-ligand interactions may be sufficient to initiate platelet adhesion (23,31).

Following the first layer of platelet adhesion, the aggregation between adjacent platelets can occur through the binding of plasma fibrinogen to activated αIIbβ3 integrins (37-39). Interestingly, although the theory that fibrinogen is required for platelet aggregation has been established for

more than 50 years, platelet aggregation persists in mice lacking fibrinogen, fibrinogen and VWF, and even triple deficient fibrinogen/VWF/plasma fibronectin (40,41), but does not occur in β3 deficient mice (42). These results can be further demonstrated *in vitro* in aggregometry using non-anticoagulated (but not anticoagulated) blood (41-43). Thus, anti-coagulant agents have masked the fibrinogen-independent platelet aggregation for the last half-century (39,42). These findings shift the paradigm in the field and provide insight into the mechanisms which support the survival of some patients with afibrinogenemia (44,45). Since αIIbβ3 is required and both plasma and platelet proteins contribute to this platelet aggregation (42), it is worthwhile to further identify these αIIbβ3 ligands and characterize their roles in hemostasis (45-50).

Platelet accumulation (adhesion and aggregation) at the site of vascular injury has been considered as the first wave of hemostasis. The second wave is mediated by blood coagulation, which generates thrombin via extrinsic and/or intrinsic pathways (20,23,29,30). Thrombin converts the soluble fibrinogen to insoluble fibrin, leading to blood coagulation and hemostasis (51,52). Notably, there are many interactions between the first wave and the second wave of hemostasis. For example, thrombin generated from blood coagulation (the second wave) is the most potent platelet agonist triggering platelet activation, enhancing platelet adhesion/aggregation (the first wave). On the other hand, activated platelets may generate phosphatidylserine (PS) on their surfaces, harboring the coagulation factors that markedly enhance cell-based thrombin generation (23,53,54). Understanding this process is useful for us to explore the possible mechanisms of fibrin and thrombus formation in placenta induced by maternal anti-fetal platelet antibodies in FNAIT (55).

Interestingly, we recently observed a "protein wave of hemostasis", which occurs even prior to platelet accumulation (the first wave) (23,43). We found plasma fibronectin plays previously unreported hemostatic roles. Although it by itself does not support, but rather, inhibits platelet aggregation (41,43), plasma fibronectin can covalently link to fibrin and subsequently support platelet aggregation (43,45,56). Most importantly, plasma fibronectin can quickly deposit onto the injured vessel wall, likely via interaction with collagen or other proteins, and form a hemostatic matrix (the protein wave) (43). Since there is a 3-5 fold increase of plasma fibronectin content in platelets from fibrinogen deficient/γ chain mutant mice and afibrinogenemic patients (40,44,45,57), these platelets may release fibronectin from their α granules and deliver them into the site of injury to compensate for the bleeding disorder in afibrinogenemic conditions, although more evidence is required to establish this compensatory pathway. Thus, platelets contribute to both first and the second waves as well as the newly termed "protein wave" of hemostasis (20,23,43).

It is notable that the levels and function of plasma fibrinogen and other coagulation factors as well as platelets may have considerable differences in fetuses compared to adults (58,59). Understanding these differences should be very useful for apprehending the bleeding disorders and other symptoms of FNAIT.

Thrombocytopenia: autoimmune and alloimmune bleeding disorders

Multiple genetic and environmental factors, including those that are malignancy-associated and immune-mediated, may contribute to the impairment of platelet production and clearance, leading to thrombocytopenia (4,15,60,61). Inherited thrombocytopenias include genetic defects leading to abnormal platelet size and function (62,63), and/or impaired platelet production (i.e., micro-, normo-, macrothrombocytopenia) (63). Malignancy-associated thrombocytopenias arise due to underlying conditions (e.g., chronic lymphocytic leukemia and lymphomas, breast, and ovarian cancers, etc.) (64,65). Immune-mediated thrombocytopenias, which can be further classified as autoimmune or alloimmune thrombocytopenia, result from an abnormal immune response against either one's own platelets or other sources of platelet alloantigens (21,66,67).

Autoimmune thrombocytopenia includes primary thrombocytopenia (ITP) and secondary thrombocytopenia, which may be induced after infection (e.g., HIV, HCV) or drugs (e.g., heparin) (60). While most thrombocytopenic patients are asymptomatic, some can experience a wide range of bleeding conditions such as petechiae, mucosal bleeding, epistaxes, and/or menorrhagia (4). Most concerning is the risk of ICH which is estimated to occur in 1.5–1.8% of adult ITP patients and cause fatality in 25% of these cases (4,68). While it was previously known that autoantibodies against platelet antigens opsonized the platelet to be engulfed by Fc-receptor (FcR) expressing phagocytes (e.g., macrophages) and cleared in the spleen (69,70), recent research revealed an antibody Fc-independent pathway (71-73), as well as the involvement of CD8^{+} cytotoxic T lymphocytes (CTL) in the disease process (74-76). Notably, CD8^{+} T regulatory cells have also been

identified and may play some unique roles (77), synergizing with CD4$^+$ T regulatory cells during immune-mediated thrombocytopenia (77,78).

Whether an Fc-dependent or independent immune response in ITP is dependent on antigen specificities is a hot topic in the field (71-73,79-82). GPIbα complex and GPIIbIIIa (αIIbβ3 integrin), the two key platelet receptors for VWF and fibrinogen, are the major autoantigens in ITP. These two receptors belong to distinctive protein families, which have their own unique signal pathways affecting platelet activities and likely eliciting different immune responses (83,84). Earlier studies found that anti-GPIbα monoclonal antibodies can induce thrombocytopenia without their Fc portions or Fc receptor (FcR) (i.e., FcγR) in mice (71,72). Subsequent studies demonstrated that anti-GPIbα-antibody-mediated thrombocytopenia is not sensitive to intravenous IgG (IVIG) therapies in both mice and human ITP patients (72,85,86), which is consistent with the prevailing mechanism of FcR blockage by IVIG (87-89). Interestingly, it is also not sensitive to steroid therapies (90). Thus, anti-GPIbα-mediated ITP seems to have 2–3 times higher likelihood of being refractory to the first line therapies (i.e., steroid and IVIG) compared with anti-αIIbβ3-antibody-mediated ITP (86,90). Recent studies demonstrated that some anti-GPIbα antibodies can induce conformational changes in GPIbα mechanosensory domain, which delivers signals for platelet activation (81), causes platelet desialylation (79,80,91) and apoptosis (55,79,92), leading to platelet clearance likely by Kupffer cells in the liver (80,93). Notably, some anti-αIIbβ3 antibodies can also induce platelet apoptosis (89) and desialylation, particularly in humans, since the FcR on human platelets can crosslink with these autoantibody Fc portions and provide additional signals for platelets (80). Whether this type of anti-αIIbβ3-mediated ITP is also refractory for the first line therapies and/or splenectomy remains to be further investigated. The available data suggests that platelet desialylation is inversely correlated with efficacy of the first-line therapies (94) and sialidase inhibitors may be useful to ameliorate thrombocytopenia (95-98).

There is no doubt that there are significant similarities for platelet opsonization and clearance between autoimmune- and alloimmune thrombocytopenias (e.g., ITP and FNAIT). Platelets, including platelet released cytokines, likely affect the immune responses and the disease processes in both ITP and FNAIT (15,99). However, immune response/reactions in pregnant women to alloantigens on fetal platelets, particularly in the context of maternal immune tolerance, may have considerable differences, and little information is available today for the reticuloendothelial system (RES) in fetuses. In addition, it has not been addressed whether different autoantibodies (Fc-dependent versus Fc-independent) in pregnant women with ITP may differently affect their fetuses and fetal platelet clearance. These interesting questions should be further studied in the near future.

FNAIT

In alloimmune thrombocytopenia, antibody-mediated platelet destruction may occur following transfusions of platelets derived from genetically different donors (termed post-transfusion purpura; PTP), or during pregnancy against paternally-derived platelet antigens (FNAIT) (66). Unlike hemolytic disease of the fetus and newborn (HDFN), a condition where antigens on fetal red blood cells are targeted by maternal alloantibodies, FNAIT may develop in the first pregnancy in 50% of cases, and close to 100% of subsequent pregnancies that have siblings who are similarly platelet-antigen positive (100-102). It is likely that the antigen targeted, the accessibility of location, and length of exposure are all critical factors that influence the pathogenesis of disease. In comparison to ITP patients, fetuses and neonates can face 10-100 fold increased risk of severe bleeding conditions (103). Life-threatening complications include ICH, IUGR, and neurological sequelae (66,100,104). Murine models developed by our laboratory has revealed new insights into the pathogenesis of FNAIT (55,88,102,105-107), however the mechanisms leading to miscarriage are still largely unknown (108-110). Here, we will discuss the "classical" FNAIT that manifest with thrombocytopenia and fetal/neonatal bleeding, and the "non-classical" FNAIT that do not present with typical bleeding symptoms and have not been well described or recognized in the field.

Alloantigens in FNAIT

There are 37 human platelet antigens (HPAs) that have been defined by Platelet Nomenclature Committee to be targeted in FNAIT (*Figure 1*), that are spread across six platelet surface proteins: αIIb (GPIIb), β3 (GPIIIa), and α2 (GPIa), GPIbα, GPIbβ, and cluster of differentiation CD109 (101,111-113). When referring to HPAs, "a" following the number indicates high frequency forms, and "b" for low frequency forms (114). Amongst the HPAs targeted in FNAIT, more than 80% of FNAIT cases in Caucasians are targeting the extracellular domain of β3
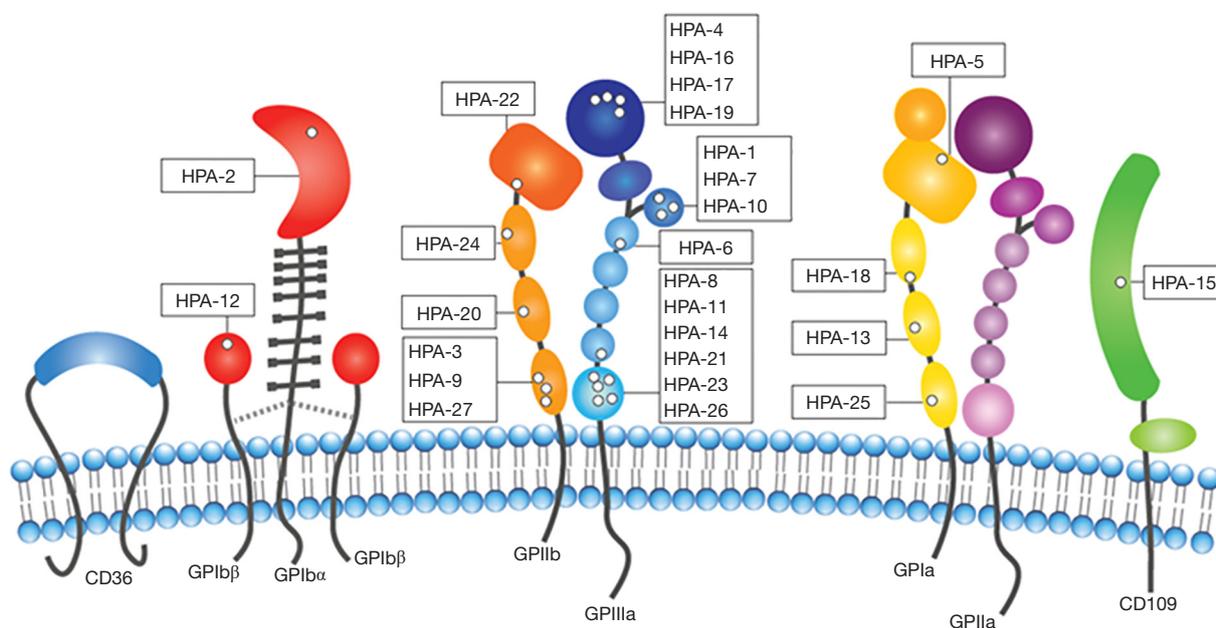
Figure 1 Platelet glycoprotein and HPA known to have induced alloimmunization. Diagram of human platelet antigen (HPA) and surface proteins known to have induced alloimmunization during pregnancy, which can lead to FNAIT. Platelet glycoproteins can possess specific polymorphisms that have been characterised and named through the HPA system. Presently, 37 HPAs have been discovered. Maternal CD36 (CD109) deficiency can also result in FNAIT however this condition is not associated with a polymorphism and therefore CD36 has not been designated an HPA.

subunit (115,116), and less than 20% of the cases are targeting other platelet surface proteins. Interestingly, although integrin subunit αIIb, which pairs exclusively with β3, has a similar number of polymorphisms as β3 (117), it is not understood why there is a much lower reported incidence of anti-αIIb mediated FNAIT. Moreover, there are <1% of reports on anti-GPIbα (e.g., HPA-2) mediated FNAIT, in contrast to 20–40% prevalence of anti-GPIbα complex antibodies reported for ITP patients (102,118). Ongoing studies from animal models suggest that the rarity of anti-αIIb and anti-GPIbα-mediated FNAIT may be attributed to life-threatening phenotypes, resulting in underreported miscarriages (55,119-121).

Certain single nucleotide polymorphisms (SNPs) on the same integrin may elicit different severities of immune responses. While HPA-1a-mediated alloimmune response (Leu to Pro substitution at residue 33 of β3) is attributed to >75% of the FNAIT cases in people with mixed European descent (66,101,122), this polymorphism is relatively rare in both African and Asian populations (102). In comparison, HPA-4b mediated FNAIT (Gln to Arg substitution at residue 143 of β3) is more prevalent in Japan (123). Likewise, HPA-2a (Thr to Met substitution on

reside 145 on GPIbα) and HPA-3a (Ile to Ser substitution on residue 843 on αIIb) are not frequently reported as targets of FNAIT in Caucasian populations (113,118,119). Structural analysis into the SNPs which are responsible for HPAs reveals that many of them are exposed on the surface of the protein, far away from the ligand binding site (124). However, some anti-HPAs associated with impairment of ligand binding sites are found (101,102). It is speculated that single amino acid substitutions may lead to changes in charges and electrostatic potential of the receptor, and contribute to changes in its antigenicity (124). Although allele frequencies within certain ethnic groups may allow us to predict whether an incompatibility might arise, incompatibility does not necessarily correspond with the incidence of disease.

While not formally designated as an HPA in FNAIT, anti-CD36 mediated FNAIT is common in Chinese, Japanese, and other Asian populations, but have not been reported in Caucasian populations (125,126). One explanation could be a lack of CD36 on the surface of platelets and/or monocytes which is prevalent in 2–5% of Asian populations compared to less than 0.3% of Caucasians (126). While CD36 deficiency has not been definitely linked to a

disease, the roles of CD36 in diseases have been reported (127-130). In the case of FNAIT, CD36 deficiency may lead to the risk to develop isoantibodies against the entire CD36 protein (125,131,132). Future studies assessing the frequency of anti-CD36 in different ethnic groups requires improved international collaboration, and aids in better understanding the genetic and environmental influences of anti-CD36 mediated FNAIT and other anti-CD36 mediated alloimmune disorders (101,126,133).

Alloantigen presentation and antibody generation

There may be multiple opportunities before, during, and after pregnancy for an alloimmune response to occur. For instance, some women are pre-exposed to HPAs prior to pregnancy (i.e., through semen, blood transfusions) which could lead to the development of alloantibodies and predispose the fetus to develop FNAIT (66,88). Whether alloantibodies against platelets cross-react with semen, or vice versa, and lead to difficulties in conception and FNAIT is currently unknown. In the event of pregnancy, it is unclear how fetal platelet antigens trigger the maternal immune system, considering that the placenta is an immunoprivileged site and the mother is in a "physiological immune tolerance" condition. However, fetal microparticles have been detected in maternal serum as early as 7 weeks (134-136), thereby becoming a potential source of foreign antigens for the maternal immune system to generate antibodies against. Notably, the trophoblast cells of the placenta, which express fetal polymorphic platelet surface proteins, may be another accessible source of alloantigens (106,137-139).

Fetal alloantigens can be processed by maternal antigen presenting cells (APCs) such as dendritic cells and macrophages, and subsequently presented to naïve CD4 T helper (Th) cells. These T cells become activated, proliferate, and release cytokines which can further induce B cells to differentiate and produce anti-fetal platelet antigen antibodies (66). The ability of an antigen to be presented to a naïve T cell depends on the affinity of the major histocompatibility complex (MHC) allele of the APC to the antigen. For instance, women carrying the MHC allele DRB3*01:01 allele are 25 times more likely to develop alloantibodies against HPA-1a compared to women who lack this allele. In one study, 90% of immunized HPA-1a negative women that were DRB3*01:01 positive had significantly increased antibody titers compared to DRB3*01:01 negative women (140,141). In a retrospective

study, male neonates who were found with anti-HPA-1a antibodies experienced on average significantly lower than average birth weights. A similar, although non-significant negative correlation existed for FNAIT-affected females (142). While these data support the notion that male sex is an independent risk factor for adverse pregnancy outcomes (143), more research is required to better understand how offspring sex is involved in FNAIT (142). It is important to note, however, that given its frequency in the Caucasian population, HPA-1a-mediated FNAIT has been studied more in depth than any other HPAs. It is currently unknown whether other HPA antigens are processed and presented less efficiently to antigen-specific T helper cells, and whether this is linked to differences in certain anti-HPA antibody titers (66,101).

Antibody cross-placental transport via neonatal FcRn

Maternal alloantibodies against paternally-derived platelet antigens can be detected in fetal circulation at approximately 13 weeks gestation in humans and embryonic day (E) 10.5 in mice (143-145). Using β3 integrin and neonatal FcRn deficient murine models, we demonstrated that FcRn expressed by the syncytiotrophoblast cells of the outermost layer chorionic villi of the placenta, not maternal FcRn, is required for the transfer of maternal IgG from mother to fetus (105). This leads to several interesting questions which remain unanswered such as: (I) where and how IgG-FcRn interaction occurs in the placenta; (II) whether human FcRn polymorphisms may result in different affinities of maternal IgG transport across the placenta and (III) if this mitigates the likelihood or severity of FNAIT development during pregnancy. Our preliminary results also show that FNAIT symptoms can be ameliorated through the use of fetal FcRn blockade, thus arising the potential to develop into a novel FNAIT therapeutic intervention (105).

Antibody-mediated fetal thrombocytopenia

Research efforts in the past few decades have elucidated several distinct antibody-mediated mechanisms that contribute to the pathogenesis of thrombocytopenias. Generally, the mechanisms of platelet destruction and clearance in FNAIT is assumed to be similar as ITP: the Fc portion of the alloantibody that is bound to fetal platelet antigens interacts with the FcRs (e.g., FcγRIIa, FcγIIIa) expressed by macrophages, initiating phagocytosis and clearance of platelets in the RES of the spleen. Recently, it

was demonstrated in mice that fetal macrophage populations are heterogeneous, for instance, CD11b^{HI} macrophage from fetal liver released pro-inflammatory cytokines in response to antigens such lipopolysaccharide (LPS) while low CD11b^{HI} macrophages derived from yolk sac were non-responsive (146). These findings provide new insight into fetal immunity especially within an immunosuppressive environment as the placenta. Indeed, how these pro-inflammatory macrophages respond to maternal antibodies, especially in the context of FNAIT, is unknown. In addition, as previously mentioned, in ITP murine models, we have shown an Fc-independent mechanism where anti-GPIbα antibodies cause platelet activation and desialylation, subsequently leading to clearance in the liver (80,147). However it is not well understood whether fetuses also clear platelets via the same pathways.

Other immune-cell involvement of FNAIT pathogenesis

While other immune cell involvement has not been extensively studied on the fetal-maternal interface in the context of FNAIT, it is clear that maternal immune competent (e.g., APCs, T and B) cells are involved in the immune response against fetal platelet antigens. Research on ITP found that CD4+ helper T cells secrete immune regulating cytokines (e.g., IL-2) and can proliferate *in vitro* when stimulated with autologous platelets (75,76) while CD8+ CTLs may mediate platelet lysis (148). Whether these cells also contribute to damage antigen positive trophoblasts in FNAIT and impairment of placenta development remains to be further studied. In addition, how maternal CD4+ and CD8+ T regulatory cells impact pro-inflammatory immune responses during pregnancy and affect placenta development requires further investigation (66). The immune reactions in the fetal RES system have not been adequately explored; the roles of fetal spleen/liver macrophages, as well as other immune competent cells in FNAIT are of interest in future studies.

Non-classical FNAIT: mechanisms of miscarriage

The "classical" FNAIT is characterized as fetal and neonatal thrombocytopenia along with bleeding disorders. However, since platelets are versatile and platelet alloantigens may expressed on other cells, anti-platelet responses may therefore lead to different pathogenesis from the classical FNAIT. These "non-classical" FNAIT symptoms have been observed in our animal models, which do not present with typical bleeding and have not been well described or recognized in the field. We will introduce some of them here.

Anti-GPIbα-mediated thrombosis in the placenta

GPIbα is one of the major antibody targets in ITP, and approximately 20–40% serum positive patients have anti-GPIbα antibodies. Interestingly, only a few cases of FNAIT have been linked with anti-GPIbα antibodies. The rarity of anti-GPIbα-mediated FNAIT cannot be explained by the frequency of HPA-2 polymorphism on GPIbα (101,149). Utilizing both immunized GPIbα and β3 deficient mice as active murine models of FNAIT, we discovered that miscarriage occurred in >83% of pregnant mice with anti-GPIbα antibodies, which is far more frequent than those pregnant mice with anti-β3 antibodies (55). Interestingly, these anti-GPIbα-mediated miscarriages were not observed with bleeding symptoms. Instead, anti-GPIbα caused a previously unidentified, non-classical FNAIT where anti-GPIbα antibodies activated platelets (i.e., platelet calcium mobilization, enhanced PS expression, enhanced cell-based thrombin generation), leading to fibrin deposition in the placenta, blockage of fetal blood supply and apoptosis/necrosis in the placenta (55). These data are consistent with recent observations in ITP studies (79-81). Collectively, our results demonstrated that differences in FNAIT pathophysiology may depend on the platelet antigen targeted.

Whether increased miscarriages caused by anti-GPIbα antibodies accounts for the rarity of reported human anti-GPIbα-mediated FNAIT remains unknown (66). Notably, similar to anti-β3-mediated FNAIT, in our murine studies, IVIG therapy or anti-FcRn antibodies were able to efficiently prevent anti-GPIbα-mediated FNAIT by blocking FcRn and preventing pathogenic maternal antibodies to cross the placenta (55). Screening GPIbα polymorphisms for women who experience frequent miscarriages and detecting anti-GPIbα antibodies may be useful strategies to identify and assess the risks of miscarriage (150-152). Recall this is unlike anti-GPIbα-mediated ITP murine models where IVIG was not as effective in ameliorating thrombocytopenia due to an Fc-independent pathway (78,80). However, it is still unknown whether downstream platelet clearance by anti-GPIbα in FNAIT is also mediated by an Fc-independent pathway in the fetal RES system. The efficacy of IVIG has not yet been translated to human patients with anti-GPIb-mediated FNAIT due to its rarity.

Impairment of angiogenesis by targeting β3 integrin

One of the most serious complications of FNAIT is ICH (66). On average, 10–20% of neonates born with FNAIT experience ICH, and approximately 5% of these ICH cases are fatal (66,102). In fetuses, ICH can be detected before 20 weeks of age, and about 10% of these fetuses may develop permanent neurological impairment (66,101,153).

While it is generally thought that fetal thrombocytopenia caused by maternal alloantibodies is the main cause of ICH seen in FNAIT, emerging research suggests there are other factors mediating ICH. Genetically engineered mice lacking circulating platelets (deficient for the hematopoietic subunit of the heterodimeric erythroid transcription factor NF-E2) do not experience ICH when delivered by caesarian section nor significant fetal death (154). Additionally, a lack in the ability to form fibrin clots in fetuses and neonates was not the major cause of observed hemorrhages, as shown by fibrinogen deficient mice (41,42,155). Even more strikingly, the fetuses with double deficient of NF-E2 and fibrinogen exhibited normal embryonic development, were morphologically indistinguishable from their wild-type controls, and experienced no obvious bleeding (58).Taken together, current data stimulate another "non-classical" approach describing mechanisms of bleeding in FNAIT beyond thrombocytopenia and/or blood coagulation.

These findings prompted further research into the mechanisms behind ICH. Integrin β3 (GPIIIa) subunit, targeted in many FNAIT cases, can be expressed with αIIb in the GPIIbIIIa complex, or with αV as a part of vitronectin receptors widely prevalent on endothelial cells and several other cell types (156,157). Our group found anti-β3 antibodies may also target endothelial cells and impair angiogenesis in a murine model of FNAIT (158), contributing to haemorrhage during embryogenesis and fetal growth (107,159). Impaired development of angiogenic signaling and other abnormalities in angiogenesis are linked to severe hemorrhage in the brain, and has been demonstrated in both zebra fish and rabbit models, respectively (160,161). Indeed, the brain, one of the most active angiogenic organs during the fetal growth, may be significantly impacted as a consequence of anti-β3 antibodies, leading to the development of ICH (162-164). Our laboratory demonstrated that fetuses and neonates of immunized β3$^{-/-}$ murine models experienced ICH and impaired retinal angiogenesis, and the polyclonal human anti-HPA-1a IgG has also showed inhibitory effects on vessel tube formation using human umbilical vein endothelial cells (HUVECs) (107). Importantly, this observation was further supported by elegant work done in human FNAIT patients, which demonstrated that anti-endothelial αVβ3 antibodies are associated with ICH in human patients (165). These findings may give rise to new clinical interventions such as better detection of anti-αVβ3 antibodies in expectant mothers with higher risks of developing ICH in their fetuses.

It seems that there are significant mechanistic differences in hemostasis between fetuses and adults since combined deficiencies of both platelets and coagulation do not lead to bleeding in fetuses (58). If this is true for humans, platelet transfusion to fetus may therefore be a less efficient therapy to control fetal bleeding. This question should be addressed in the near future.

Disruption of placental structure and function

Placental pathology and its role in miscarriage have not been as extensively studied as bleeding diatheses in FNAIT (106). Healthy placenta development involves blastocyst implantation followed by spiral artery development (166,167). These crucial processes involve trophoblast cell migration which is tightly controlled by a subset of NK cells (i.e., decidual and uterine NK cells; d/μNK cells) (168,169). Maternal alloantibodies in FNAIT targeting integrin β3, expressed with αV or αIIb on the surface of trophoblasts, may significantly impair trophoblast function, and lead to life-threatening complications as IUGR or miscarriage (106,167,170,171).

Recent discoveries, however, demonstrated that IUGR was perpetuated by the recruitment of pro-inflammatory μNK cells (i.e., upregulation of activating markers NKp46, FcγRIIIa, perforin release) in mid-gestation (106,172). While it is previously known that perturbation of μNK function results from a variety of self and non-self signals (e.g., IL-17 produced from Th17 cells, LPS), how NK cells are involved in antibody-mediated pathologies such as FNAIT has not been previously well studied. Our findings in the β3 model showed activation of NK cells from a quiescent to cytotoxic state (106).

We observed that pro-inflammatory μNK cells recruited to the placenta in mid-gestation induced apoptosis in trophoblasts that were bound by anti-β3 integrin complexes. NK cells expressing FcγRIIIa receptors interact with the Fc portion of antibodies, become activated and release perforin and other cytotoxic molecules, in a process known

as antibody-dependent cell-mediated cytotoxicity (ADCC). Conversely, this process may be inhibited by NK depletion (anti-asialo-GM-1) or inhibition of activating receptors (e.g., NKp46) (106). FNAIT was abrogated through administration of IVIG or anti-FcRn antibodies (55,88,106). These findings have demonstrated yet another non-classical, antibody-mediated mechanism resulting in the pathogenesis of FNAIT (i.e., apoptosis of trophoblasts, impairment of vascular development in placenta, decrease of blood supply to the fetuses, leading to IUGR and miscarriage).

Diagnosis, treatment, and prophylaxis

Diagnosis and prophylaxis screening for FNAIT

The diagnosis of FNAIT is usually made after detecting severe bleeding or low platelet count ($<150 \times 10^9$/L) in fetuses or neonates (66). FNAIT is only diagnosed if no other etiology can be identified or if there is a previous history of FNAIT. The relatively more common prevalence of HPA-1a mediated FNAIT has prompted investigation into the benefits of screening women for HPA-1a genotype (141). Both the expectant mother and her partner can be screened to determine an HPA mismatch, however, the results may be inconclusive if the father is heterozygous (i.e., has shared and distinct HPAs from his partner). The HPA status of the fetus can be determined from fetal DNA present in maternal plasma, however this is not a routine laboratory test yet. The mother can also be screened for the presence of the human leukocyte antigen DRB3*0101 allele (173), which may be an indicator for developing alloantibodies against HPA-1a (174,175).

Another method of screening is to detect anti-HPA alloantibodies in the maternal serum at approximately 20 weeks of gestation (4,113,176). The gold standard method to perform this task is the monoclonal antibody immobilization of platelet antigens (MAIPA) assay (177-180). Even with the sensitivity of MAIPA, antibodies may not be detectable at birth in some rare cases of FNAIT (181). For instance, HPA-3 (αIIb/GPIIb) and HPA-15 (CD109) are challenging to detect as their antigen expression varies with the method of platelet storage and preparations (182-184). Furthermore, divalent cation-chelating anticoagulants used during maternal blood collection can potentially attenuate MAIPA recognition of certain alloantibodies, possibly reducing detection of anti-αIIb-mediated FNAIT (67,185-187). Therefore, MAIPA may not be sensitive enough to detect all pathological

maternal antibodies in FNAIT. In such cases it is possible to detect antibodies with a different assay such as surface plasmon resonance (188), however this is not commonly practiced. Most recently, the new technique of self-assembling monolayer coupled to platelet receptors has emerged with great potential to increase the sensitivity and specificity for anti-platelet antibody detection (189). While some studies suggest that detection of maternal anti-HPA alloantibodies is predictive of developing FNAIT (179), other studies don't find a correlation (104,190). Currently, there is no basis for screening as the cost-benefit analysis is unfavorable and there may be an increased risk of overdiagnosis and unnecessary intervention.

Prophylaxis: antibody mediated immune suppression (AMIS)

FNAIT can be considered the platelet analogue of haemolytic disease of the fetus and newborn (HDFN), another antibody mediated pregnancy complication. HDFN is treated using anti-D prophylaxis, a type of AMIS, which works by preventing a maternal immune response via antibody administration. Due to the prevalence of HPA-1a mediated FNAIT, the potential clinical benefit of anti-HPA-1a prophylaxis was investigated using the β3$^{-/-}$ mouse model (191). The study of this preventative strategy was a proof-of-concept that AMIS could also be an effective prophylactic treatment in a β3-mediated model of FNAIT (191). These studies have led to an initiative to collect plasma to manufacture an anti-HPA-1a IgG product for the purpose of testing the efficacy of HPA-1a prophylaxis (191). The PROFNAIT project (http://www.profnait.eu/profnait-project/project-funding/) was a European union funded project from 2012–2018 to develop anti-HPA-1a immunoglobulin for prophylaxis, which has orphan status in Europe and the USA and clinical studies are in progress.

Interestingly, it seems that the AMIS is not antigen but platelet specific since anti-β3 antibody can also decrease the immune response against platelet GPIbα (191). This broadens its application for other alloantigens in FNAIT. It is currently unknown whether a monoclonal antibody or mixed monoclonal antibodies can be used to replace the anti-HPA-1a immunoglobulin collected from pregnant women with FNAIT (187,192). If so, the limited resource of anti-HPA-1a antisera can be overcome.

Antenatal treatment

In multigravida with a history of FNAIT, or if anti-platelet

alloantibodies are detected, antenatal treatment of FNAIT becomes possible. Several studies have shown a fetal benefit to administering IVIG to the mother during gestation (193,194), however efficacy is still controversial (194-199). Cases mediated by different platelet antigens may be more or less responsive to IVIG therapy, therefore, further study is required to determine responders versus non-responders, and standardize treatment (115). Steroid treatments, such as prednisone may also be administered during pregnancy however the efficacy and potentially harmful side effects must be carefully considered. In a study comparing the usage of single and combination IVIG and prednisone drug therapies, a favorable response was observed in those treated with IVIG alone (196) whereas combination therapy of IVIG and prednisone was reported to have more unfavorable side effects (200). It is now generally accepted to stratify patients based on relative risk of developing FNAIT as judged by maternal history, and propose treatments accordingly (201).

Postnatal treatment

If FNAIT is diagnosed in the neonate, treatment must begin promptly. Some guidelines call for the use of intravenous immunoglobulin (IVIG) therapy, a blood product made from pooled IgG from >1,000 donors. If the infant is severely thrombocytopenic, platelet transfusion should be considered. Despite a lack of standardized trials, a lower threshold of 30×10^9/L is normally used to indicate treatment for neonates (i.e., immediate transfusion with random donor platelets, if antigen negative platelets are not available) (66,113,202-204). Thrombopoietin (TPO), a primarily liver-generated hormone that stimulates the production of platelets (205,206), and TPO mimetics are useful treatments in ITP, however it is unknown whether these drugs may be of some value in FNAIT to increase neonatal platelet production.

The novel and potential new therapies for FNAIT

Recombinant anti-HPA-1a

A newly developed human recombinant anti-HPA-1a antibody (B2G1) (207), competes with the maternal alloantibodies for binding to fetal HPA-1a positive platelets and may be a potential future therapy. The antibody Fc region of B2G1 is modified to prevent binding to Fcγ receptors on macrophages and subsequent phagocytosis. Promising preclinical studies show that in the presence of

maternal and B2G1 antibodies the platelets lasted 3 times longer in circulation. Another anti-HPA-1a antibody, known as 26.4, was developed with a higher affinity for HPA-1a⁺ platelets than B2GI (208) and works to opsonize HPA-1a⁺ platelets for destruction. In addition, several monoclonal antibodies developed by our group have also been demonstrated to be able to block pathogenic anti-HPA-1a binding to platelets (187,192). Future clinical trials should be performed to confirm their therapeutic potential.

Anti-FcRn therapy

FcRn is the Fc receptor responsible for extending the life of circulating IgG, transporting maternal IgG from milk across neonatal gut epithelium, and placental transport of maternal IgG from maternal circulation to fetal circulation (209). IgG naturally binds FcRn at low pH and is released at physiological pH (210). The efficacy of anti-FcRn monoclonal antibodies in ameliorating FNAIT symptoms (e.g., low platelet count) have been applied in several mouse models with promising results (55,105-107). As previously mentioned, anti-FcRn is thought to block fetal FcRn and prevent the transport of maternal IgG into fetal circulation. Although monoclonal antibodies are frequently used as therapeutics, there is still a lot more work that needs to be done to assess the safety and efficacy of anti-FcRn as a therapeutic before its use in a clinical setting.

Anti-NK cell therapy

Our group recently demonstrated that uterine resident NK cells in anti-β3-mediated FNAIT can switch from a quiescent non-cytotoxic phenotype to an activated cytotoxic phenotype (106). Monoclonal antibodies that were used to deplete NK cells or block activating receptors (NKp46 or FcγRIIIa), proved to be an effective in utero treatment shown in our FNAIT mouse models, ameliorating much of the miscarriage and bleeding associated with anti-β3-mediated FNAIT. Although still in its early stages, these observations open the door to other therapeutic targets on NK cells and potentially other placental resident immune cells (59). Again, the efficacy and safety should be evaluated in future clinical trials.

Conclusions and future perspectives

This article summarizes the progress that has been made in FNAIT pathogenesis and treatment. We have identified several "non-classical" immune-mediated and antigen specific mechanisms resulting in potential miscarriage

in FNAIT. For instance, alloantibodies against platelet antigens (i.e., β3) can bind a wide range of cells (e.g., endothelial) that further contribute to pathogenesis of FNAIT like ICH (107), than previously known. Integrin β3⁺ trophoblasts may also be targeted to impair placenta development and contribute to IUGR and miscarriage (106). We have also shown that uterine resident NK cells may switch to a cytotoxic phenotype and impair placental development in FNAIT (106). However, the role of other regulatory cells in the vicinity is unknown. Another non-classical FNAIT pathology includes anti-GPIbα alloantibodies leading to platelet activation, thrombosis and placental dysfunction (55). Collectively, these mechanisms cause distinct pathophysiology leading to miscarriage compared thrombocytopenia alone, and lead to new approaches in modulating treatment. Broadening our understanding of the pathophysiology of FNAIT has fueled the exploration of novel treatments including anti-HPA-1a recombinant antibodies for prophylaxis, anti-FcRn and anti-NK cell therapies. Overall, since there is no standardized protocol for the treatment of FNAIT so far, therein lies great opportunity for potential development of new therapeutics and prophylaxis targeting this life-threatening disease.

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Footnote

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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