COMPARISON OF TWO ELISA PROCEDURES FOR DETECTION AND QUANTIFICATION OF PLATELET SPECIFIC ANTIBODIES IN NEONATAL ALLOIMMUNE THROMBOCYTOPENIA: Application of the best method for anti-HPA 5b antibody detection.

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CONTENTS

ABBREVIATIONS

AITP	Autoimmune thrombocytopenia
AU	Arbitrary unit
BSA	Bovine serum albumin
CD	clusters of differentiation system
CS	Caesarean section
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EH	Enzyme inhibitor
ELISA	Enzyme linked immunosorbent assay
GP	Glycoprotein
HGH	Human growth hormone
HLA	Human leukocyte antigen
HPA	Human platelet antigen
ICH	Intracranial haemorrhage
Ig	Immunoglobulin
IVIG	Intravenous immunoglobulin
ISBT	International society of blood transfusion
ITP	Idiopathic thrombocytopenic purpura
IU	International unit
MAIPA	Monoclonal antibody-specific immobilization of platelet antigen assay
MCA	Murine monoclonal antibody
MoAb	Monoclonal antibody
MRI	Magnetic resonance imaging
NAIT	Neonatal alloimmune thrombocytopenia
NIBSC	National institute for biological standards and control
OD	Optical density
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PTP	Post transfusion purpura
PTR	Platelet transfusion refractoriness

RBC	Red blood cell
RT	Room temperature
UNN	University Hospital of North Norway
vWF	von Willebrand Factor

ABSTRACT

Human platelet antigens (HPA) is a group of platelet specific antigens represented with two alleles (a and b) differing by one amino acid in a molecule on the platelet surface. Neonatal alloimmune thrombocytopenia (NAIT) can occur due to foetomaternal mismatch in an HPA. Antibody specificities involved in NAIT varies according to alleles present in the population. In the Caucasians, anti-HPA 1a antibodies are the most frequent cause of NAIT (about 78%). Other antigens involved in NAIT are anti-HPA-5b and anti- HPA-3a antibodies. There is no established screening program for detection and follow up of pregnancies at risk for NAIT. Besides, significant correlation has been shown between the level of anti-HPA 1a antibodies in the mother and the severity of thrombocytopenia in the new born. There is an international demand to standardize the test for antibody detection and quantification. A modern method to detect and quantify platelet specific antibodies is a fairly complicated ELISA test called Monoclonal antibody immobilization of platelet antigen assay (MAIPA). The aims of this study were to standardize and simplify the MAIPA procedure, and to detect the frequency of anti-HPA-5b antibodies by using the MAIPA in samples from Egyptian pregnant women as anti-HPA-5b antibodies have been reported to be a more common cause of NAIT in the Mediterranean countries.

A modified local MAIPA method at the University Hospital of North Norway (UNN) and the National institute for biological standards and control (NIBSC) MAIPA were compared for detection of platelet specific antibodies. For comparison of both the procedure we used fresh and lyophilized platelets, local standard (EA) and NIBSC international standard serum and different monoclonal antibodies (MoAb) to immobilize GP IIb/IIIa.

The local MAIPA has been shown to be more sensitive than the NIBSC MAIPA procedure for detecting anti-HPA 1a antibodies measured as OD values. Also the local MAIPA required less time compared to the NIBSC procedure.

Anti-HPA 5b antibodies were present in 16 of 367 randomly selected samples (4.4%) from the Egyptian population of pregnant women. Surprisingly, anti-HPA 5b antibodies were detected in three women with the platelet genotype HPA 5ab. The explanation for this discrepancy is being studied. In two women both anti-HPA 1a and

anti-HPA 5b antibodies were detected.

The sensitivity of detecting anti-HPA 1a antibody by local MAIPA procedure can be enhanced by using international standard serum, pool of MoAb and fresh platelets.

1. INTRODUCTION

1.1 Human platelets: An overview

Human platelets or thrombocytes are the smallest blood cells, anuclear and discoid in shape, with dimensions of approximately 2.0-4.0 by 0.5 μm [1]. Due to their anuclear nature, they were discovered late in the nineteenth century by Bizzozero [2] and only 'rediscovered' in the 1960s after almost 8 decades of oblivion. Platelets are produced by megakaryocytes in the bone marrow. The life span of a circulating platelet is about 8–10 days. The normal number of platelets in the circulation is between 150–450 × 10^9/L of blood.

Platelets are multifunctional and are involved in many physiological and pathophysiological processes, mainly haemostasis and thrombosis., However platelets are also known to be important participants in inflammation, host defence and tumour biology [3] (Fig.1).

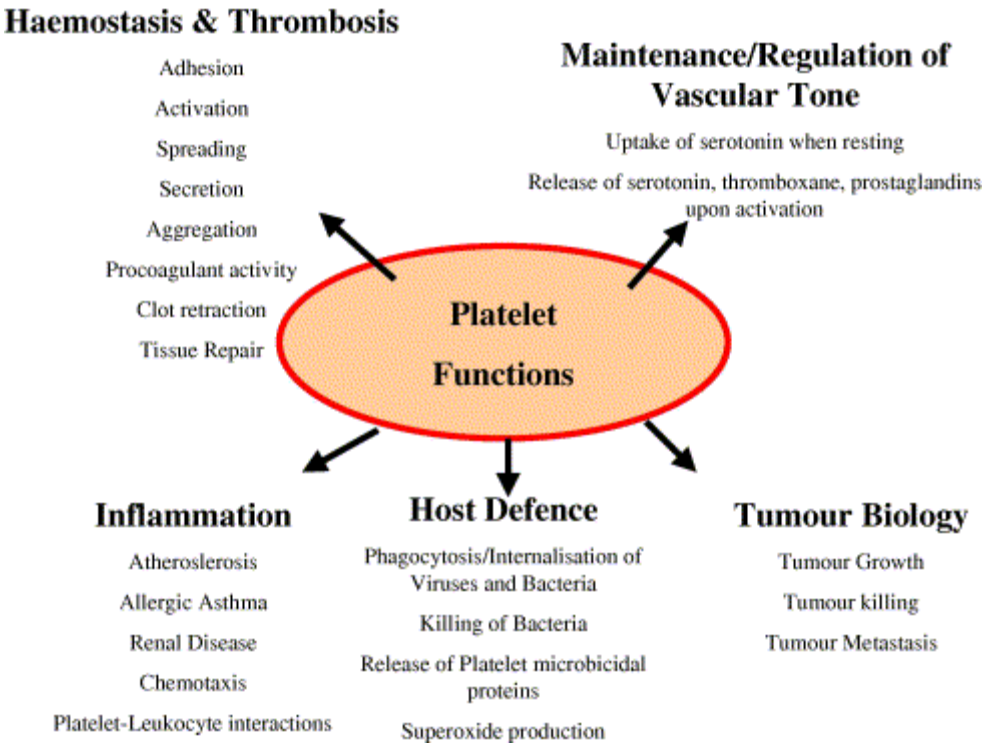

FIGURE 1 The multifunctional platelet [3].

In response to injury of the monolayer of endothelial cells lining the blood vessel wall, alterations of blood flow or chemical stimuli, platelets undergo activation and perform a triad of functional responses: adhesion, activation, secretion and aggregation [3] (Fig.2)

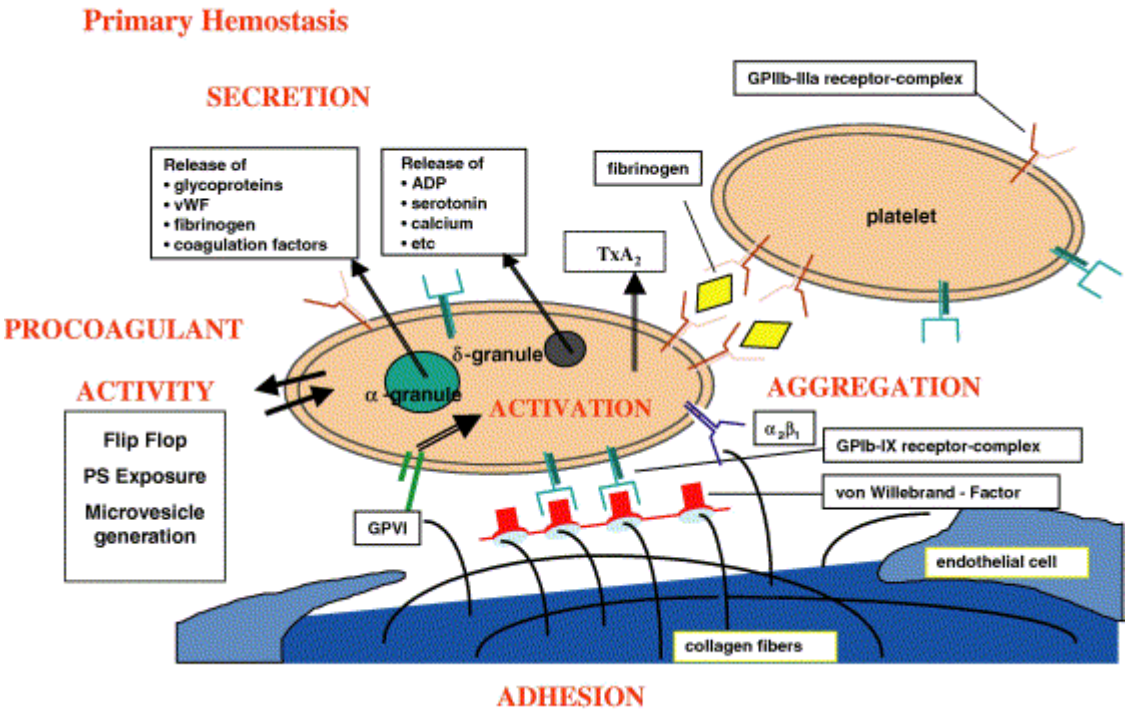

FIGURE 2 The multiple roles of platelets in haemostasis [3].

All of the major platelet responses are coordinated to form a haemostatic plug that occludes the site of damage for preventing blood loss. The functional responses occur via a series of co-ordinated signals that convert the extracellular stimulus into intracellular chemical messengers through the activation of an array of specific membrane receptors, belonging to distinct molecular families, some of which are unique to platelets while others are widely distributed in mammalian tissues. The process of haemostasis can be described in following steps:

1. Exposure of collagen and subendothelial proteins from damaged blood vessel to platelets, with initial platelet adhesion mediated via von Willebrand Factor (vWF) binding to the GP Ib/IX/V complex on the platelet surface.
2. Platelets slow down and transiently adhere or roll along the vessel wall. Collagen binding to GPVI results in cellular activation, inducing firm adhesion and spreading through the activated receptors GP IIb/IIIa and the $\alpha_2\beta_1$ glycoprotein.
3. Adhesion results in intracellular signalling and platelet activation; degranulation including release of ADP, generation of thromboxane, activation of the GP IIb/IIIa complex, exposure of anionic phospholipids and generation of procoagulant microvesicles.
4. Further local recruitment of platelets into the vicinity, resulting in platelet aggregation mediated by fibrinogen and vWF bridging between activated GP IIb/IIIa on adjacent cells.
5. Thrombin generation and fibrin formation resulting in stabilisation of the haemostatic platelet plug.

Defective platelet function or thrombocytopenia can cause abnormal bleeding. Hereditary disorders of the platelet function are rare and they may cause abnormal platelet functions [4]. Bleeding abnormalities that are linked to the hereditary disorders include mucocutaneous bleeding, e.g., menorrhagia, gastrointestinal bleeding, visceral haematomas, haemarthroses and intracerebral haemorrhage in rare cases. Genetically defective platelets inducing severe bleeding symptoms include Glanzmann's thrombasthenia and Bernard-Soulier syndrome (BSS). In Glanzmann's thrombasthenia platelet glycoprotein IIb-IIIa complex is undetectable and bleeding symptoms are sporadic in nature which is explained by the compensation of absent fibrinogen receptor by other platelet receptors [1].

In the Bernard-Soulier syndrome (BSS) GP Ib-IX-V complex may remain absent or depleted or non-functional [5]. BSS is characterized by long bleeding time, thrombocytopenia and morphologically abnormal and abnormally large platelets and

platelets are incapable to interact with vWF [6]. Generally acquired platelet defects are mild, sporadic and ubiquitous [1].

Thrombocytopenia can be caused by autoantibodies, e.g., autoimmune thrombocytopenic purpura, alloantibodies [7], e.g., neonatal alloimmune thrombocytopenia (NAIT), post transfusion purpura (PTP) and platelet transfusion refractoriness (PTR) and by drug-induced antibodies, e.g, GP IIb/IIIa inhibitors.

1.2 Platelet surface glycoproteins

There are more than 45 distinctive platelet membrane structures found on resting platelets, that belong to five different sub-groups of molecules, such as adhesion molecules, e.g., integrins, immune molecules, e.g., MHC class I molecules, glycoprotein receptors, blood group antigens and other molecules, e.g., oligosaccharides [8]. Platelet-membrane glycoprotein receptors are essential for adhesion to subendothelial tissue, and are involved in subsequent aggregation to form the haemostatic plug [6, 9-11]. Platelet glycoprotein nomenclature have been developed based on the integrins (α and β) and clusters of differentiation (CD) system. Structurally, integrins are heterodimeric (α and β) type I transmembrane receptors and each subunit typically contains a large extracellular domain, a transmembrane domain and a short cytoplasmic tail [12]. Upon activation, platelets express several integrins (αIIbβ3, also called glycoprotein IIb/IIIa [GPIIb/IIIa], αVβ3, α2β1, α5β1, α6β1) [13]. All integrins are usually "2-tailed" receptors; one tail is oriented to the extracellular space and the other towards the cell interior. Ligand binding to any of the tails can trigger information transfer, or signalling, across the plasma membrane to "activate" cellular functions [13] (Fig. 3).

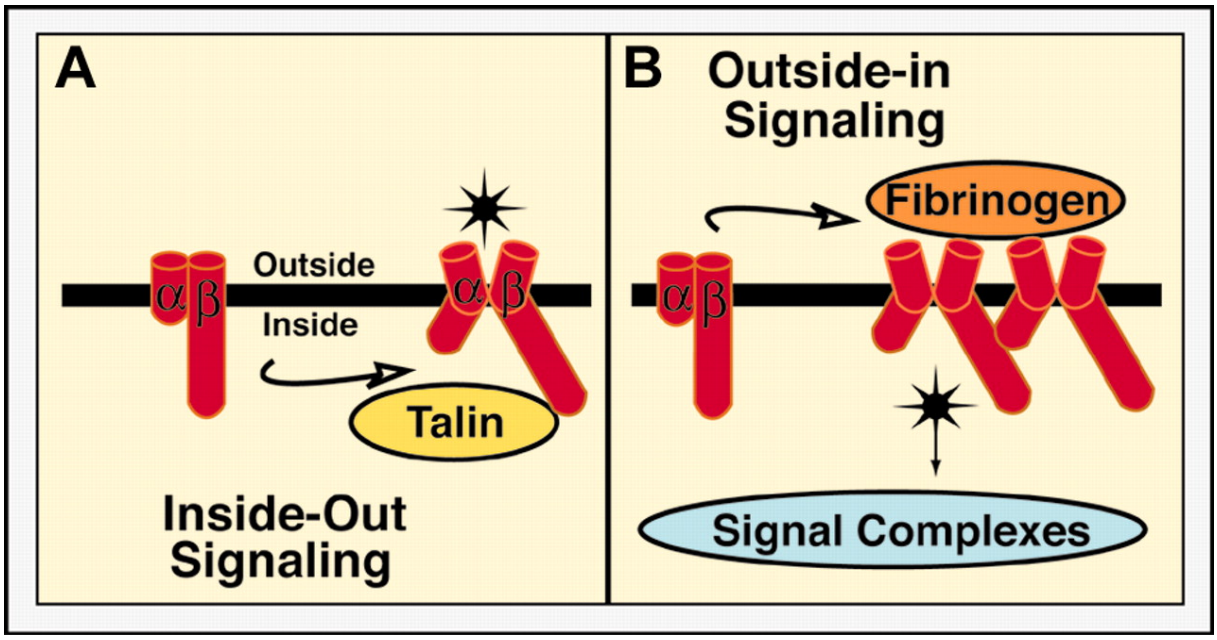

FIGURE 3 Glycoprotein activation is bidirectional and reciprocal. (A) Inside-out signalling; Agonist (Talin)-dependent intracellular signals, and (B) Outside-in signalling; extracellular ligand binding causes integrin clustering and further activation [13].

TABLE 1 Major platelet membrane glycoprotein receptors with associated functions and disease implications [1].

Receptor	Structure	Function	Polymorphisms	Disorder
Glycoprotein IIb/IIIa	Integrin(aIIbβ3), 80,000 molecules per platelet	On platelet activation, becomes a receptor for fibrinogen, vWF, fibronectin and other ligands	L33P, Q62R, I843S, I140T, R143Q, Q489R, A407P, H633R, C636R, M837V, L33Pcreate platelet specific antigens, HPA-1a/1b- which are important antigens in NAIT.	Deficiency causes Glanzmann's thrombasthenia
Glycoprotein Ia/IIa	Integrin ($\alpha_2\beta_1$), 900-2,300 molecules per platelet	Constitutively active receptor for collagen	C807T; silent dimorphism affects $\alpha_2\beta_1$ surface density and collagen receptor activity, associated with coronary thrombosis and stroke	Deficiency may cause mild bleeding
Glycoprotein Ib/V/IX	Complex of four gene products, 25,000 molecules per platelet	Constitutively active receptor for insoluble vWF in the perivascular matrix	No clinically important polymorphisms described	Deficiency results in BSS, and mutations with increased function result in platelet type vWF disease.

1.3 Platelet alloantigens

Blood cells like red blood cell (RBC) and platelets, have a number of alloantigens on their surface, which may cause immunization in cases of blood transfusion, pregnancy and in bone marrow transplantation. In immune-mediated platelet disorders, antibodies

against platelet alloantigens play significant roles [7]. There are two different types of clinically relevant platelet alloantigens. Type I alloantigens are those which are shared with other blood cells and tissues such as glycoconjugates of the blood group ABH system and the highly polymorphic human leukocyte antigen (HLA) class I molecule. Most of the platelet ABH antigens are located on intrinsic platelet membrane glycoproteins, such as GPIb, GPIIa, GPIIb, GPIV, GPV, platelet endothelial cell adhesion molecule, e.g., PECAM-1 and the PI-linked CD109 [14-16].

The expression of HLA antigens on platelets varies substantially. For example, the expression of HLA-A and -B antigens is higher than that of HLA-C antigens [17]. Different studies show that platelets carry HLA class I antigens as an intrinsic component of the platelet membrane [7].

Type II alloantigens are generally specific to platelets and are known as platelet-specific alloantigens. But they can reside on other cells and tissues. For example, platelet alloantigens located on the integrin β_3 (GPIIIa) subunit have been detected on endothelial cells, smooth muscle cells, and fibroblasts [18]. Antigens located on the α_2 integrin (GPIa) subunit are found on activated T lymphocytes and endothelial cells [19, 20], but alloantigens residing on GPIb subunits are specific for platelet lineage [21]. Type II alloantigen-specific antibodies are implicated in the pathophysiology of different immune related disorders. Most of these antigens are defined by one amino acid differences in the sequences of the integrins (Table 2).

A platelet-specific alloantigen is called a human platelet antigen (HPA) when the molecular basis has been defined and the antigen is located on a platelet specific structure. The first human platelet antigen (HPA) system, Zw/Pl^A or HPA-1, was identified in 1959 [22] followed the next system which was characterized 20 years later. The HPA system consists, now of 24 polymorphic epitopes. The previous nomenclature which was based on patients' name, has been replaced by the human platelet alloantigen (HPA) system in accordance with the international System for Human Gene Nomenclature [23, 24] in 1990 by the international society of blood transfusion (ISBT) platelet working party [25]. Antigen systems are numbered chronologically by their date of description where the high-frequency allele is assigned with the letter "a" and its

antithetical low-frequency allele with the letter "b" [24]. A summary of the HPA systems, based on a 1998 update and earlier reports, is presented in Table 2 [7].

TABLE 2 Human platelet antigens [7].

Antigen	Alternative names	Glycoprotein	Nucleotide substitution	Aminoacid substitution
HPA-1a	Zw^a, PI^{A1}	GPIIIa	T_{196}	Leu_{33}
HPA-1b	Zw^b, PI^{A2}		C_{196}	Pro_{33}
HPA-2a	Ko^b	GpIb	C_{524}	Thr_{145}
HPA-2b	Ko^a, Sib^a		T_{524}	Met_{145}
HPA-3a	Bak^a, Lek^a	GPIIb	T_{2622}	Ile_{843}
HPA-3b	Bak^b		G_{2622}	Ser_{843}
HPA-4a	Yuk^b, Pen^a	GPIIIa	G_{526}	Arg_{143}
HPA-4b	Yuk^a, Pen^b		A_{526}	Gln_{143}
HPA-5a	Br^b, Zav^b	GPIa	G_{1648}	Glu_{505}
HPA-5b	Br^a, Zav^a, Hc^a		A_{1648}	Lys_{505}
HPA-6bw	Ca^a, Tu^a	GPIIIa	A_{1564} G_{1564}	Gln_{489} Arg_{489}
HPA-7bw	Mo^a	GPIIIa	$_{G1317}$ C_{1317}	Ala_{407} Pro_{407}
HPA-8bw	Sr^a	GPIIIa	T_{2004} C_{2004}	Cys_{636} Arg_{636}
HPA-9bw	Max^a	GPIIb	A_{2603} G_{2603}	Met_{837} Va_{837}
HPA-10bw	La^a	GPIIIa	A_{281} G_{281}	Gln_{62} Arg_{62}
HPA-11bw	Gro^a	GPIIIa	A_{1996} G_{1996}	His_{633} Arg_{633}
HPA-12bw	Iy^a	GpIbβ	A_{141} G_{141}	Glu_{15} Gly_{15}
HPA-13bw	Sit^a	GPIa	$_{T2531}$ C_{2531}	Met_{799} Thr_{799}
HPA-14bw	Oe^a	GPIIIa	$delAAG_{1929\text{-}1931}$ AAG	$DelLys_{611}$ Lys_{611}
HPA-15a	Gov^b	CD109	C_{2108}	Ser_{703}
HPA-15b	Gov^a		A_{2108}	Tyr_{703}
HPA-16bw	Duv^a	GPIIIa	T_{517} C_{517}	Ile_{140} Thr_{140}

There are 24 platelet-specific alloantigens. Of them, 12 are grouped in six biallelic systems (HPA-1, -2, -3, -4, -5, -15), and in the other 12 , alloantibodies against the thetical but not the antithetical antigens are observed. In 21 of 24 antigens the difference between self and non-self is defined by a single nucleotide polymorphism (SNP) [26]. Six platelet membrane glycoproteins- GPIa, GPIbα, GPIbβ, GPIIb, GPIIIa and GPI-linked CD109- have been characterized as carriers of those platelet-specific alloantigens [7]. Figure 4 shows the GPIIb/IIIa complex which has most HPA epitopes of the platelet glycoproteins. The HPA–1a/1b is localised on GPIIIa (CD61) of the GPIIb/IIIa–complex [21, 27] . The GPIIIa is part of an adhesion molecule (vitronectin receptor) used by invading throphoblasts while replacing maternal endothelial cells in the spiral arteries [28]. At the beginning of the 2nd trimester, GPIIb/IIIa–complex has been shown to appear on foetal platelet membrane where differentiating cytotrophoblasts transform their adhesion receptor phenotype.[28, 29]. The complex is also expressed on the vascular endothelial cells of the umbilical cord [21].

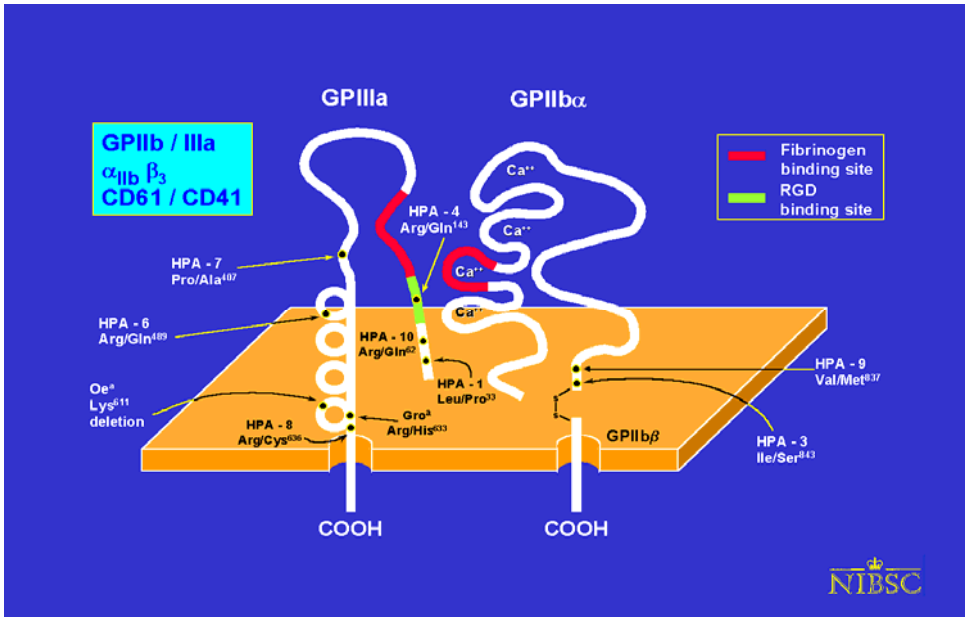

FIGURE 4 GPIIb/IIIa with the HPA epitopes [30].

1.4 Platelet antibodies

Human platelet antigen specific antibodies can be grouped in two categories: autoantibodies and alloantibodies. Autoantibodies are the antibodies that are directed towards epitopes of platelet glycoproteins, among other proteins, of the patients' own platelets and cause autoimmune thrombocytopenia (AITP). AITP may be divided into four broad groups, including idiopathic thrombocytopenic purpura (ITP), secondary immune thrombocytopenia, immune drug-induced thrombocytopenia and viral infection-related thrombocytopenia (e.g., HIV AIDS). Out of these autoimmune disorders, ITP is a significant and major disorder with incidences ranging from 3.2 to 6.6 cases per 100,000 per year [31]. In ITP, platelet specific antibodies can be detected in around 80% of the patients.

Platelet alloantibodies are the antibodies that are directed against alloantigens, such as genetically determined non-self molecular variations of proteins or carbohydrates on the platelet membrane. Alloantibodies are observed in normal individuals from exposure to the alloantigen during pregnancy, blood transfusion, and after bone marrow transplantation. FcR mediated phagocytosis occurs upon binding of antibodies to the target platelet alloantigens [7].

Besides contributing to platelet transfusion refractoriness, alloantibodies are responsible for immunologic mediated thrombocytopenia in foetal and neonatal alloimmune thrombocytopenia (NAIT), post transfusion purpura (PTP), passive alloimmune thrombocytopenia and transplantation-associated thrombocytopenia. Polymorphic platelet glycoprotein structures might also play a key role in the development of platelet related acute coronary and cerebrovascular diseases [32].

1.5 Neonatal alloimmune thrombocytopenia (NAIT)

1.5.1 Pathogenesis

Neonatal alloimmune thrombocytopenia (NAIT) is a disease of foetus and newborn. It is due to foetomaternal mismatch of human platelet alloantigens (HPA). Maternal exposure during pregnancy to HPA antigens of foetal origin, lacking on the mother's own platelets, can stimulate her immune system. IgG antibodies can be produced, cross placenta and enter the foetal circulation (Fig. 5) [33].These antibodies can bind to the platelets and

antibody-coated platelets are removed by the reticuloendothelial system. The baby becomes thrombocytopenic. Severe thrombocytopenia (platelet count $<50\times10^{9}/L$) may result in bleeding such as intracranial haemorrhage (ICH)[34]. Due to ICH the baby may die or have long lasting disability[35].

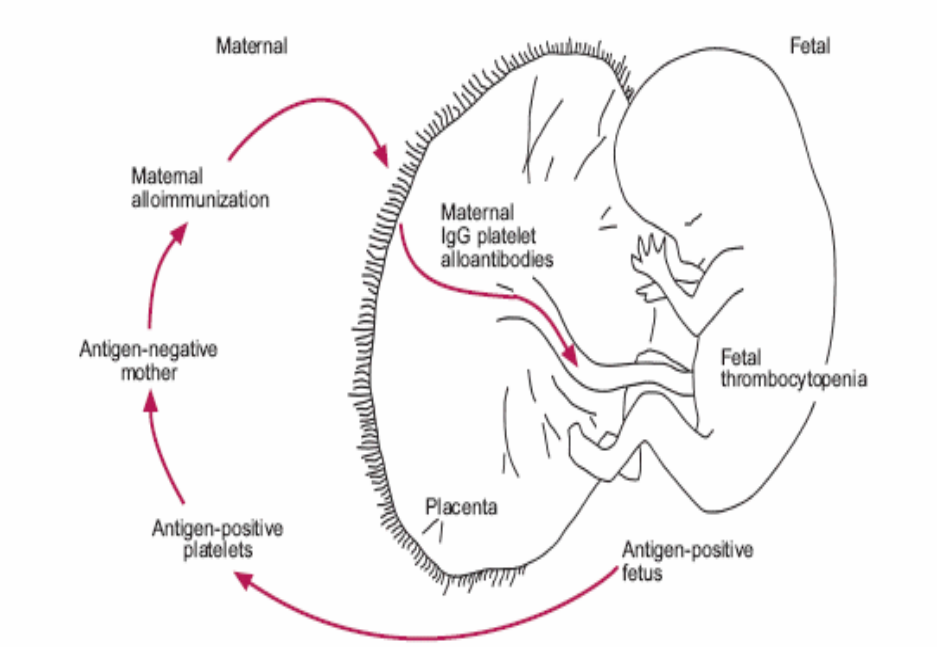

FIGURE 5 Pathogenesis of neonatal alloimmune thrombocytopenia [36].

1.5.2 Incidence

Retrospective studies reported in the late 1950s and early 1960s showed that NAIT as an uncommon disorder [36]. Some recent studies have shown that the incidence of neonatal alloimmune thrombocytopenia ranges from 1.5/1000 [37] to 1/5000 live births [38]. Anti- HPA 1a antibodies (disparity in the biallelic HPA-1 system, with an HPA-1a negative mother and an HPA–1a positive foetus) are the most frequent cause of NAIT (about 78%) [39]. Other antigens involved in NAIT are anti- HPA-5b antibodies (19%) and anti- HPA-3a antibodies [39]. Anti-HPA-4a antibody induced NAIT is often severe, but occurs almost exclusively in the Asian population [40]. Approximately 2-2.5% of the

Caucasian population is HPA –1a negative, however, only 6-12% of HPA-1a negative women develop anti-HPA-1a antibodies, around 90% of these women are HLA-DRw52a (HLA-DRB3*0101) positive[41, 42].

1.5.3 Diagnosis

In newborn babies, NAIT can be diagnosed in the laboratory by analysis of blood samples from the mother and the baby. The characteristics by which NAIT may be diagnosed are: (a) an abnormally low platelet count, such as $<150 \times 10^9$/ L in newborn, (b) foetomaternal incompatibility for a platelet-specific antigen, (c) maternal platelet alloantibodies that are reactive towards the antigen and (d) clinical response to compatible antigen-negative, but not to incompatible antigen-positive platelet transfusions [36].

In clinical term, the diagnosis of NAIT is a diagnosis of exclusion. Although the neonate is born by a healthy mother with no previous history of thrombocytopenia, auto-immune disorders or ingestion of drugs, the baby may present with petechiae or widespread purpura at birth or a few hours after birth. However, the infant may remain unaffected with no clinical signs. Among the severely thrombocytopenic infants approximately 10-30% shows evidence of intracranial haemorrhage leading to death in 50% of those with ICH [39, 43, 44]. Infant platelet count is usually low and anaemia might be seen after bleeding.

Ultrasonography and magnetic resonance imaging (MRI) are the major ways to diagnose ICH. Clinically, infants with alloimmune thrombocytopenia show purpura or haematoma rather than visceral haemorrhages, such as gastrointestinal bleeding or haematuria [45]. Whatever the platelet antigen is involved in NAIT, ICH can arise in severe cases [45]. Antibodies to HPA-3a antigens may induce severe neonatal thrombocytopenia [46]. NAIT that is linked to HPA-5b incompatibility seems to be less severe than HPA-1a related NAIT [47]. Conversely, the infant may be symptomless with thrombocytopenia discovered incidentally, even in case of HPA-1a alloimmunization. Unexpected or unexplained neonatal thrombocytopenia or severe early onset thrombocytopenia in both pre-term and term babies should raise the possibilities of NAIT and guide investigations accordingly.

In NAIT the platelet count in the foetus and newborn is lower than normal (<150x10^9 /L). The platelet immunological investigations of the mother include detection of circulating anti-HPA alloantibodies and identification of homozygocity of the less frequent platelet antigen. The father should carry the platelet antigen that the antibodies are specific for. Flow cytometry, ELISA and the MAIPA techniques can be used to detect antibodies [48] . Thereafter, molecular techniques can be used for genotyping [49-52] . Infant platelet typing, can be performed as confirmatory test when the father is heterozygous or paternity is not certain. Diagnosis of NAIT becomes easy in case of parental incompatibility with a corresponding maternal antibody.
Typically, the infant with NAIT is full-term. Along with the frequent incidence of severe thrombocytopenia, haemorrhage in infants with NAIT may be quite severe.

1.5.4 Management of NAIT

1.5.4.1 Neonatal management

There is a correlation between very low platelet counts and tendency for bleeding. So, management of thrombocytopenia should be carried out on the basis of the clinical observations.

For the infants with haemorrhages and platelet counts below 50-30 $\times 10^9$/L during the first 24 hours of life, the treatment should start by transfusion of compatible platelets which are not destroyed by the antibodies acquired from the mother. Compatible donors or the mother herself may be platelet donors in such a situation. If the mother is used, antibodies must be removed from the product by repetitive washing. Irradiation is performed to prevent graft versus host disease. It is reported that random platelets can be used until compatible platelets are available for transfusion [53] IvIg is used together with transfusion of platelets in some situations [53]. As the effect is delayed for 12-18 hours after injection, in such a situation, intravenous immunoglobulin (IvIg) (1g/kg/day for 2 days) is not considered for the treatment of haemorrhage [54].

For the infants without haemorrhage and a platelet count above 30-50 $\times 10^9$/L, follow up with repetitive platelet counts may be performed. Platelet transfusion is not indicated with platelet counts above 50 $\times 10^9$/L. However, if platelet count drops below 50$\times$ 10^9/L, transfusion of compatible platelets may be considered. At the University Hospital of North Norway, 35 $\times$ 10^9/L is used as a transfusion trigger.

1.5.4.2 Management of subsequent pregnancies

In case of subsequent pregnancies, there are three reports showing a strong correlation between the antibody level in the mother and the severity of thrombocytopenia in the newborn [55]. In some studies foetal blood sampling have been performed to determine the foetal platelet count. The procedure is connected with a high risk for complications [56]. In case of thrombocytopenia, the antenatal therapy is to reverse the foetal thrombocytopenia by platelet transfusions, however intrauterine blood sampling and transfusions are matters of controversy [56, 57]. Alternative therapies include weekly injection of high doses of immunoglobulin (IvIg) +/- steroids to the mother[45] . A consensus protocol cannot be provided as different results are obtained by different research groups in Europe and in USA. However, maternal therapy is suggested as the first line of treatment in a recent study [58]. The University Hospital of North Norway follows a treatment strategy to manage subsequent pregnancies [59] . It includes:
Prenatal interventions are:

- Exclusion of any platelet function inhibitor
- Avoiding vigorous physical activities
- Clinical follow at the university hospital
- Caesarean section 2-4 weeks before term

Postnatal interventions are Compatible transfusion to baby if needed.

1.6 The Norwegian study, design and outcome

A prospective study, including a screening – and intervention program was carried out in collaboration between University Hospital of North Norway, Tromsø and Ullevål University Hospital and the National Hospital, Oslo [59]. A total 100,448 pregnant

women from southern and northern part of Norway were included in this study. Blood samples from pregnant women were HPA 1 typed. Every fourth week samples from all HPA-1a negative women were screened for anti-HPA 1a antibodies. No further follow up was offered to the women who did not develop anti-HPA-1a antibodies. Blood samples from immunized women were obtained every fourth week and quantified for anti-HPA-1a antibodies, and the women were referred for clinical follow up at the nearest University Hospital. The immunized women were examined clinically and underwent ultrasonographic examination twice; between week 28 and 30, and about week 36. Delivery was performed by caesarean section (CS) in 36-38 weeks of gestation. When high level anti-HPA-1 antibodies had been detected, more frequent examinations were carried out. Prior to CS, platelets from HPA-1bb donor were harvested and kept for transfusion to the neonate. Platelets were transfused to the neonates immediately after delivery if platelet count in umbilical cord or capillary blood was less than 35 x 10^9 /L. The outcome of this screening and intervention program was significant reduction of serious complications of NAIT to approximately one fourth [59].

1.7 The Egyptian study, design and outcome

The Egyptian study is a screening and intervention program for antenatal and neonatal management of NAIT and prevention of ICH related morbidity and mortality [60]. In this study 6,774 pregnant Egyptian females were randomly phenotyped for HPA1a. HPA1a negative pregnancies (HPA1bb) were managed as follows:

- HPA1a phenotype of fathers.
- Identification and quantification of anti-HPA1a antibodies in HPA1bb mothers corresponding to HPA1a positive fathers.
- Previous history of pregnancies with NAIT in alloimmunized mothers.
- The HPA 1bb mothers without detectable alloantibodies and with negative obstetric history were followed and allowed for vaginal delivery, unless indicated otherwise. However, a cord blood sample was obtained immediately after delivery to exclude NAIT. If the newborn was found have platelet count $<35\times10^9$/L, they were transfused with compatible HPA1bb platelets.

- The mothers with anti-HPA 1a antibodies were delivered with elective CS at week 36 - 38.

This randomized study showed that Egyptian women have higher (3.6%) frequency of the HPA 1a negative phenotype than the Caucasians (2%). The incidence of alloimmunization was about 9% in HPA 1a negative pregnancies. NAIT has been reported in 1of 1,129 HPA 1a negative women- in most cases it was considered mild to moderate. Unlike the Norwegian study, the study did not show that the intervention reduced NAIT related complications significantly as the number of included women were too low.

1.8 Newer diagnostic and follow-up tools for detecting antibodies

A variety of assays have been developed to detect platelet specific antibodies [61]. Newer diagnostic tools to detect antibodies on platelet surface glycoproteins (GPs) include flow cytometry, enzyme linked immunosorbent assay (ELISA), and monoclonal antibody-specific immobilization of platelet antigen (MAIPA). For genotyping of HPA 1a polymerase chain reaction (PCR) is used [50, 51].

Flow cytometry is a very sensitive method for detection of alloantibodies. This methods is used to detect platelet antibodies that binds to intact platelets [62]. Flow cytometry is also a suitable method for HPA 1a phenotyping [63].

The Enzyme-Linked ImmunoSorbent Assay, or ELISA, is a simple biochemical technique suitable for large scale testing of anti-HPA-1a [64]. In the ELISA two antibodies are used; one is specific to the antigen and the other reacts to antigen-antibody complexes. The second antibody accounts for "enzyme-linked" and can cause a chromogenic or fluorogenic substrate to produce a signal.

MAIPA is used to detect, determine specificity and quantify platelet specific antibodies. It was first described by Kiefel *et al* [48] and modified by Bertrand *et al* [65]. MAIPA assay is the most widely used assay to detect platelet-specific antibodies. It requires the use of monoclonal antibodies (MoAbs) that are used to target antigens, though not competing with the human antibodies. The clinically significant platelet-

reactive antibodies, such as antibodies to epitopes on GPs IIb/IIIa, Ib/IX, Ia/IIa, IV (CD36) and HLA Class I are detected with MAIPA.

MAIPA is a glycoprotein-specific ELISA technique that enables the identification of the antibodies specificity even if plasma contains a mixture of antibodies. In the MAIPA technique, target platelets are sensitized with patient plasma and a MoAbs are supplied to recognize the desired target molecule on the platelet surface [61]. After the initial sensitization has been carried out by incubation with patient plasma, platelets are washed and re-incubated with a murine glycoprotein-specific monoclonal antibody, and solubilised in a non-ionic detergent. Then centrifugation is carried out to remove cytoskeleton fragments. Thereafter an aliquot of the supernatant of the lysate is added to the wells of a microtiter tray containing immobilized goat antibody-specific for mouse IgG. This causes MoAbs to be captured and the platelet surface GP with its bound human antibodies are immobilized. After a subsequent wash step, the human antibody is detected with an enzyme labelled goat anti-human immunoglobulin probe (Fig. 6).

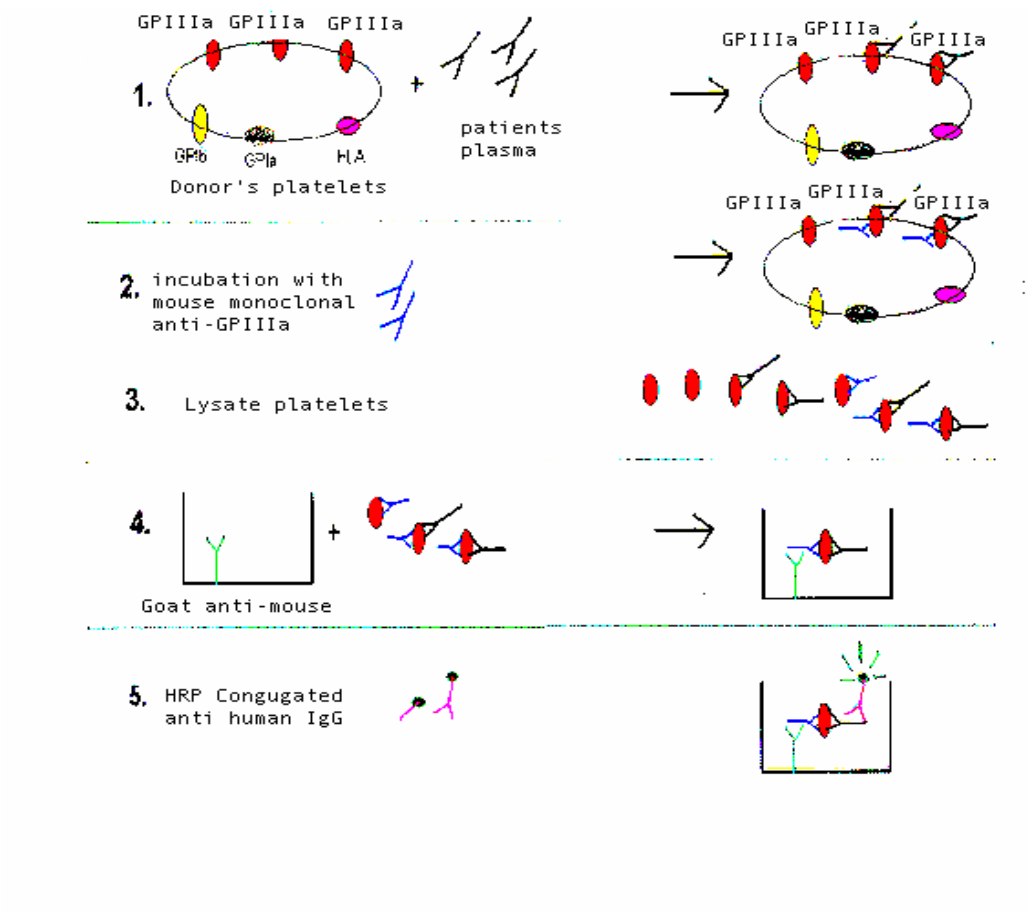

FIGURE 6 Schematic design of MAIPA procedure.

Recently a 5' nuclease assay (5' NA) has been devised for human platelet antigen (HPA) 1a/b allelic discrimination [50]. It is an automated and reliable test for large scale genotyping of HPA. This assay is based on the simultaneous amplification and detection of the two targets in a one-tube system. The procedure is less time-consuming and cheaper than conventional sequence-specific primer PCR (SSP-PCR) [50].

1.9 Lyophilized platelets

The most widely used preparation for treating thrombocytopenic patients are fresh platelet concentrates stored in liquid state at 22 °C. At present, platelets can only be stored for up to 7 days because of possible microbial contamination and partial loss of viability [66]. Storage of conventional 22 °C liquid-stored allogeneic platelet concentrates has achieved a lot of advances in the area of safety and processing of

platelets concentrates for clinical use. The advances includes: a) development of more effective storage containers; b) ability to produce single donor platelets by apheresis; and c) availability of effective prestorage leukoreduction techniques. In research, platelets can be stored frozen or fresh in buffers inhibiting the enzymes responsible for antigen degradation. The need for stable platelet reagents is obvious and new preparations have become available for research use. One such product is lyophilized platelets. Although this new products is promising, there remain numerous questions associated with the use.

Production and research associated with lyophilized platelets were initiated in the 1950s [67] although limited haemostatic efficacy was shown in experimental animals in vivo. The preparation contained washed platelets treated with 1.8% paraformaldehyde, frozen in 5% albumin, and then lyophilized. After rehydration, lyophilized platelets are similar, at least under electron microscopy, to fresh platelets and have been shown to bear most of the platelet glycoproteins [66]. Rehydrated lyophilized platelets have been evaluated clinically in dogs, rats and rabbits and have shown to be haemostatic effective in thrombocytopenic animals.

1.10 Aims of the study

- There is no established screening program for detection and follow up of pregnancies at risk for NAIT. Besides, significant correlation has been shown between the level of anti-HPA 1a antibodies in the mother and the severity of thrombocytopenia in the new born [55]. There is an international demand to standardize the test for antibody detection and quantification. A modern method to detect and quantify platelet specific antibodies is a fairly complicated ELISA test called Monoclonal antibody immobilization of platelet antigen assay (MAIPA). The aim of my study was to standardize and simplify the MAIPA procedure.
- .Different ethnic groups show variable frequencies of HPA alleles. Antibody specificities involved in NAIT varies according to alleles present in the population. In the Caucasian population 2% of the population has the platelet type HPA 1bb and anti-HPA-1a antibodies are responsible for around 80% of NAIT cases. The aim of this part of my study was to detect the frequency of anti-HPA-5b antibodies by using MAIPA in samples from Egyptian pregnant women as anti-HPA-5b antibodies have been reported to be a more common cause of NAIT in the Mediterranean countries (personal communication, Yves Merieux)

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Platelets

Platelets were isolated from healthy blood donors after written consent. Lyophilized platelets were a gift from prof. Dominique Rigal, Lyon, France. The lyophilized platelets were rehydrated in phosphate buffered saline (PBS) containing 0.3% ethylenediaminetetraacetic acid (EDTA), 2% bovine serum albumin (BSA).

2.1.2 Patient plasma/serum

A minimum of 0.5 ml EDTA-plasma or serum was required for testing. Plasma from a woman (EA), known to have a moderate level of anti-HPA 1a antibodies, was assigned 1000 AU/ml and used as local standard. International standard from the National Institute for Biological Standards and Control (NIBSC) (code: 03/152, WHO international laboratory for biological standard, 100 IU/ml) was a mix of plasma from six donors immunised against the HPA-1a antigen. After mixing, the plasma was lyophilized and rehydrated by the customer. The contents of anti-HPA 1a antibodies equal 6 times that of our local standard (EA).

2.1.3 Reagents

2.1.3.1 MAIPA

The reagents included 10% sucrose solution, 1.5M $CaCl_2$, 1M H_2SO_4, PBS containing 3% EDTA and or 2% BSA, coating buffer, solubilisation buffer, TBS washing buffer and substrate solution. Coating buffer was prepared from 15 mM Na_2CO_3, 35 mM $NaHCO_3$ and 3mM NaN_3, pH 9.6. TBS washing buffer (pH 7.4) was prepared from 10 mM Tris Base $C_4H_{11}NO_3$, 0.9% NaCl, 25ml Triton X-100, 2.5ml Tween 20, 2.5ml 1.5M $CaCl_2$. Substrate solution was prepared from 2 mg OPD (1,2 phenylenediamine dihydrochloride)tablets dissolved in 3ml H_2O and 1.25 µl H_2O_2.

<u>Antibodies used in the MAIPA procedure:</u>

- *Coating the microtiter plates:*

Goat anti-mouse IgG: 1.8 mg/ml, diluted 1:300 in coating buffer (Jackson ImmunoResearch, Code 115-005-071, Pennsylvania, USA.)

- *Monoclonal antibodies (MoAbs)*:

Anti-GPIIIa capture antibody: Clone Y2/51, anti-CD61 (Dako, Glostrup, Denmark) 5 μg/ml in PBS 2% BSA, clone P2, anti-CD41 (Immunotech, France) diluted to 0.2mg/ml in PBS 2% BSA and French in-house anti-GPIIIa monoclonal antibodies (kindly provided from C. Kaplan, Paris, France).

Anti-GPIa capture antibody: Anti-CD49b VLA2 (Immunotech, France) 5 μg/ml in PBS 2% BSA.

- *HRP conjugated goat anti-human IgG:* 0.8 mg/ml, dilution 1:4000 in TBS wash buffer. (Jackson ImmunoResearch, Code 109-035-008, Pennsylvania, USA)

2.1.3.2 PCR typing for HPA 5

Blood samples: A minimum of 200 μl of EDTA blood was used to perform the DNA isolation.

Reagents for DNA isolation: QIAamp Spin Blood Kit. It contained Qiagen Protease stock solution, Buffer AL, Buffer AW1, Buffer AW2, ethanol (96-100%).

Reagents for PCR: dNTP (ATP/CTP/GTP/TTP; 100 mM of each), formamide (10% and 15%), ampliTaq DNA polymerase (5 U/μl), 1M KCl , 1M Tris, 1M $MgCl_2$, 1% gelatine. Forward allospecific primers HPA 5a, HPA 5b, common reverse primer HPA 5c 5μM each and Human growth hormone (HGH)1 (forward) and HGH 2 (reverse) primers 1 μM each for detection of house keeping gene (Table 3).

TABLE 3 Primers for HPA 5 typing

Primer Name	Sequence	Producer
HPA 5a	5'-GAG-TCT-ACC-TGT-TTA-CTA-TCA-AAG-3'	Med Probe
HPA 5b	5'- GAG-TCT-ACC-TGT-TTA-CTA-TCA-AA-3'	Med Probe
HPA 5c	5'-GCA-GTA-CAC-TAT-ACA-TTC-AAC-TCT-A-3'	Med Probe
HGH 1	5'-TGC-CTT-CCC-AAC-CAT-TCC-CTT-A-3'	Med Probe
HGH 2	5'-CCA-CTC-ACG-GAT-TTC-TGT-TGT-GTT-TC-3'	Med Probe

Controls: For HPA 5a and HPA 5b typing, positive and negative controls were included (5aa and 5bb) and heterozygote sample (5ab). These were sample that had been typed in previous analysis.

Reagents and solutions for electrophoresis:
TAE buffer: 10 mM Tris base, acetic acid (CH_3COOH) , 0.5 M EDTA and ion-exchanged water.
Loading buffer: 1.8 mM Bromphenol blue, 2.32 mM xylene cyanol, 1.5 M sucrose, 1M Tris to pH 8.0, and ion-exchanged water.
Ethidium bromide (10 mg/ml) solution and 1.6% agarose gel (Ultra pure DNA grade agarose).

2.2 METHODS

2.2.1. Preparation of fresh platelets

a) Anticoagulated whole blood was collected from group O or A_2 donors and centrifuged in order to isolate the buffy coat.
b) The buffy coat was centrifuged at 870 rpm for 15 min and the platelet rich plasma was pipetted off.
c) Platelet rich plasma was centrifuged at 3200 rpm for 5 minutes in order to pellet the platelets. Then the supernatant was sucked off and the pellet was resuspended in fresh enzyme inhibitor (EH)-buffer at a concentration of 1500 x 10^9 / L. EH-buffer contained 21 mM leupeptine, aprotinine (10 mg/ml) and, 54 mM iodacetamide in phosphate buffer saline (PBS) 0.3% EDTA solution.
d) The platelets can be kept in EH-buffer for 2 weeks at 4ºC [68].

2.2.2. Rehydration of lyophilized platelets

To rehydrate lyophilized platelets they were stored in a wet incubator at 42ºC for 1 hour with cap partly open. 1 ml buffer PBS containing 0.3 %EDTA and 2%BSA was added very slowly drop by drop. For better results with MAIPA, the platelets were rehydrated within 24 hours before use.

2.2.3 MAIPA Procedure

Our local MAIPA method (a modified MAIPA method based on procedure described by Kiefel et. al., [48] established at University Hospital of North Norway (UNN), and the National institute for biological standards and control (NIBSC) procedure [69] are thoroughly described in table 4. Negative and positive controls were included in each run. Plasma from a male non-transfused AB-donor served as negative control. A sample containing anti-platelets antibodies with known specificity was run in parallel as a positive control.

TABLE 4 Steps in local MAIPA and NIBSC procedure. Differences in both the procedures are highlighted in blue font color.

Steps	Local MAIPA Procedure	NIBSC Procedure
Preparation of coated microtiter plates	• Coating of Maxisorb microtiterplate (Nunc Inc, 551730) with goat anti-mouse IgG (6 µg/ml), and incubating the plates 2 hours at 37ºC.	• Coating of flat-well microtiterplates (NUNC 44204A) with goat anti-mouse IgG (3 µg/ml), and incubating plates at 2 hours at room temperature (RT; 22ºC).
1st incubation	• Adding 20 ×10^6 of HPA 1aa platelets to each well in an uncoated U-well microtiter plate and spin at 2500 rpm for 3 minutes. The supernatant was discarded, and the platelet pellets were resuspended in 30µl PBS 2% BSA. After that 50 µl of patient plasma was added, mixed and incubated 40 minutes at 37 º C. • After incubation, the plates were washed 3 times and the pellets were mixed by adding 200µl 0.3% EDTA/PBS/ 2% BSA.	• Adding 20 ×10^6 of HPA 1aa platelets to each well in an uncoated U-well microtiter plate and spin at 2500 rpm for 3 minutes. The supernatant was discarded and the platelet pellets were resuspended in 50 µl test serum or plasma and incubated 30 minutes at 37ºC. • After incubation the plates were washed 2 times and the pellets were mixed by adding 200 µl PBS/%?EDTA.
Incubation with MoAb	• The platelet pellets were resuspended by adding 40µl diluted mouse monoclonal anti-GPIIIa (5 µg/ml) to the plates, and then mixed and incubated	• The platelet pellets were resuspended by adding 40µl diluted mouse monoclonal anti-GPIIIa (5 µg/ml) to the plates, and then mixed and

	for 40 minutes at 37ºC. • The samples were washed through 10% sucrose and centrifuged at 2500 rpm for 3 minutes.	incubated for 30 minutes at 37ºC. • The platelets were washed three times with 200 µl PBS/EDTA.
Washing and blocking coating plates	• The coating plate was washed 4 times with TBS washing buffer and blocked with PBS 2%BSA for 30 minutes at RT (22ºC).	• The coating plate was washed 4 times with TBS washing buffer and blocked with PBS 2%BSA for 30 minutes at 4°C.
Lysis	• The platelets were resuspended carefully in 130 µl solubilisation solution and kept for 15 minutes at RT. Then, centrifugation at 2500 rpm for 15 minutes.	• The platelets were resuspended carefully in 130µl solubilisation solution and kept for 30 minutes at 4°C. Then the microtiterplate was centrifuged 3000 rpm for 15 minutes at RT, and 130µl TBS wash buffer was added into U-well microplate. 100 µl solubilised platelets diluted in TBS wash buffer was added.
Attachment to solid phase	• The blocking solution was removed from the coated microtiterplate and 100 µl solubilised platelets was added carefully without disturbing the pellet to the desired well. Then, 100 µl TBS washing solution was added to blank position, and incubated 40 minutes at 37ºC. • After incubation microplate wells were washed 6 times with TBS wash buffer.	• The blocking solution was removed from the coated microtiterplate and 100 µl diluted solubilised platelets was added carefully without disturbing the pellet to the desired well. Then, 100 µl TBS washing solution is added to blank position, and incubated 90 minutes at 4ºC. • After incubation microplate wells were washed 4 times with TBS wash buffer.
Incubation with anti-IgG	• HRP conjugated goat anti-human IgG (0.2 µg/ml) was added to microplate well, and incubated for 40 minutes at 37º C. The microtiter plate was then, washed 6 times with TBS wash buffer.	• HRP conjugated goat anti-human IgG (0.16 µg/ml) was added to microplate well and incubated for 90 minutes at 4°C. The microtiter plate was then, washed 6 times with TBS wash buffer.
Colour	• 100µl substrate solution was added and	• 100µl substrate solution was added and

development	incubated 10-15 minutes in the dark at RT. • 50µl 1M H_2SO_4 was added to stop reaction when suitable colour intensity was developed.	incubated 10-15 minutes in the dark at RT. • 100µl 0.5 M H_2SO_4 was added to stop reaction when suitable colour intensity was developed.
Presentation of results	• Optical density (OD) values were measured at 492 nm. Programmed ELISA-reader automatically redrawed blanks from results and OD values were transferred to an Excel file for automatic calculation of antibody concentration in sample.	• OD values were measured at 492 nm. Programmed ELISA-reader automatically redrawed blanks from results and OD values were transferred to an Excel file for automatic calculation of antibody concentration in sample.

2.2.3.1 Standard curve

A known positive sample with a-HPA1a antibodies (EA) was used as the standard in local MAIPA procedure. Non-diluted EA plasma equals 1000 AU/ml. This standard was set up (4 parallels) in a dilution series from 1:2 to 1:64 (diluted in PBS containing 2% BSA). In order to create a standard curve, NIBSC anti-HPA-1a standard (100 IU/ml) was diluted from 1:1 to 1:1024.

2.2.4 DNA Isolation

Thoroughly mixed 200 µl EDTA blood sample was taken into Eppendrof tube. 20 µl QIAGEN Protease and 200 µl Buffer AL were added to the Eppendrof tube. Then they were mixed immediately in a vortex mixer for 15 seconds and incubated at 56°C for 10 minutes. 200 µl ethanol (96–100%) was added and mixed with the incubated sample. After that the sample was transferred to the QIAamp Spin Column in a 2 ml collection tube. The total mixture was centrifuged at 8,000 rpm for 1 minute. QIAamp Spin Column was placed in a clean 2 ml collection tube, and 500 µl of Buffer AW1 was added and centrifuged at 8,000 rpm for 1 minute. Again, the QIAamp Spin Column was placed in a clean 2 ml collection tube and Buffer AW2 was added followed by centrifugation for 3 minutes. Then the QIAamp Spin Column was placed

in a clean microcentrifuge tube with addition of 200µl distilled H_2O and incubated for 5 minutes and RT (22°C). Subsequently centrifugation was carried out at 8,000 rpm for 1 minute. The prepared DNA sample was stored at 4°C.

2.2.5 PCR analysis

The procedure for PCR analysis using allele-specific primers was performed according to the method described by Skogen B. *et. al*.[51]. The Eppendrof tubes were marked as 5a and 5b for different master-mixes, and pipetted in following order: 3mM $MgCl_2$, dNTP, formamide (15% for 5a and 10% for 5b), diverse primers (HPA 5a primer in 5a marked Eppendrof tube, HPA 5b primer in 5b marked eppendrof tube and the HPA 5c primer in both tubes; Table 2), Taq-polymerase (diluted to1:20 in autoclaved H_2O). 10 µl of autoclaved H_2O were pipetted into each well of a PCR plate with control 5aa/5ab and samples, and 8 µl for control 5bb. Besides, 16 µl of autoclaved H_2O was added for blank. 35µl of respective master mix (5a and 5b) was added to every well. After that 6 µl of DNA (30-100ng) was added to each well for samples and control 5aa/5ab, and 8 µl for 5bb control. The tubes were sealed with "sealing sheet" amplification on Perkin Elmer Thermocycler 9600 was started as quickly as possible. Following amplification, the samples were kept at 4°C. The PCR products were run and visualized by agarose gel electrophoresis.

2.2.6 Electrophoresis of PCR products

After making a tight system around a gel tray, appropriate combs were placed thereon. 1.6% solution of agarose in 100 ml 1× TAE buffer was heated in the microwave oven for 3-4 minutes. The agarose was cooled to 60°C. 5µl ethidium bromide was added to agarose gel and mixed. The agarose was then poured on the gel tray, and kept for 30 minutes for hardening. Then the combs were taken away and gel tray was placed in the electrophoresis tray. 1× TAE buffer was poured over the gel. The samples were prepared in a microtitre well by adding 2 µl of loading buffer and 18 µl of PCR product. 17 µl of prepared samples were pipetted into the wells. The electrophoresis was then carried out at 110V for 30 minutes.

2.2.7 Statistics

Statistical calculations were carried out by Microsoft Excel's (Office 2003) data analysis tool package. Pearson product moment correlation coefficient (r) was calculated when required. Probability (*P*) values were calculated by one-way analysis of variance (ANOVA) method and interpreted as; 'not significant' ($P > 0.05$), 'significant' ($P \leq 0.05$) and 'highly significant' ($P \leq 0.01$).

3. RESULTS

3.1 RESULTS

3.1.2 Comparison of local and NIBSC MAIPA procedures

The local and NIBSC MAIPA procedures using local standard (EA, 1000 AU/ml) were compared (Table 5). The reliability of the MAIPA assay was calculated as the coefficient of variation (CV) in percentage. The inter- and intra -assay variations were < 10% (OD > 0.1). There was a strong correlation between the measurements done with the local procedure compared with the NIBSC procedure (r = 0.999, Fig. 7). However, table 5 show that .for every dilution (1:2 to 1:64), the mean OD values were significantly different (P <0.001) between the two procedures. The NIBSC procedure showed an average of <50% of the sensitivity of the local MAIPA. Similar results were obtained by using international standard serum (100 IU/ml) in both procedures (Table 6).

TABLE 5 Measurement of local standard (EA, 1000 AU/ml). Results obtained by local MAIPA and NIBSC procedure.

	NIBSC MAIPA			Local MAIPA		
Dilution EA	**Mean OD values (n=3)**	**Standard Deviation (SD)**	**Coefficient of variation (%)**	**Mean OD values (n=3)**	**Standard Deviation (SD)**	**Coefficient of variation (%)**
1:2	0.98	0.05	4.6	1.98	0.07	3.29
1:4	0.57	0.01	2.0	1.12	0.06	4.92
1:8	0.31	0.01	3.2	0.61	0.04	5.74
1:16	0.14	0.01	8.4	0.31	0.03	8.12
1:32	0.08	0.01	6.9	0.20	0.01	5.00
1:64	0.03	0.01	17.3	0.11	0.01	9.09

TABLE 6 Measurement of international standard (100 IU/ml). Results obtained by local MAIPA and NIBSC procedure

	NIBSC MAIPA			Local MAIPA		
Dilution EA	**Mean OD values (n=3)**	**Standard Deviation (SD)**	**Coefficient of variation (%)**	**Mean OD values (n=3)**	**Standard Deviation (SD)**	**Coefficient of variation (%)**
1:2	3.69	0.06	1.7	4.45	0.06	1.24
1:4	3.21	0.03	1.0	4.01	0.02	0.50
1:8	2.34	0.06	2.4	3.82	0.06	1.44
1:16	1.15	0.02	1.3	2.77	0.01	0.42
1:32	0.73	0.05	6.3	1.09	0.03	2.30
1:64	0.43	0.02	4.7	1.00	0.02	2.09

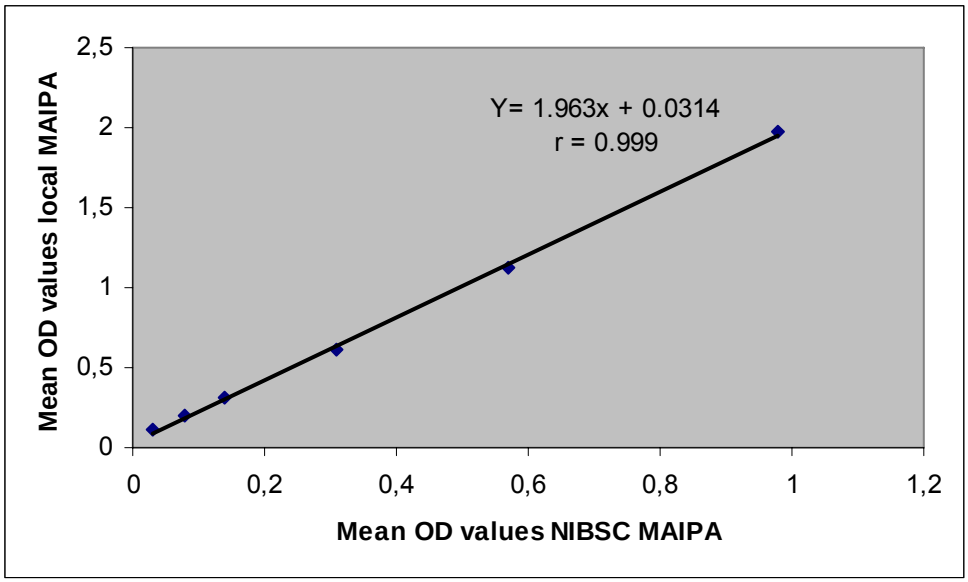

FIGURE 7 Correlation between local MAIPA procedure and NIBSC procedure. Local standard (EA) run in serial two-fold dilution (1:2-1:64). r = Pearson product moment correlation coefficient.

3.1.3 Comparing local (EA, 1000 AU/ml) and international standard (NIBSC, 100 IU/ml).

In this study, both local and NIBSC MAIPA have been run by using local standard (EA) and international standard serum (NIBSC, 100 IU/ml), where 1000 AU/ml of local standard was equivalent to 15 IU/ml of international standard serum. Results have shown that international standard serum had an approximately three times stronger reactivity (OD value) than our local standard (1000 AU/ml) (Figure 8a and 8b). Similar results were obtained when we compared the standards using both the local MAIPA procedure and the NIBSC procedure. .

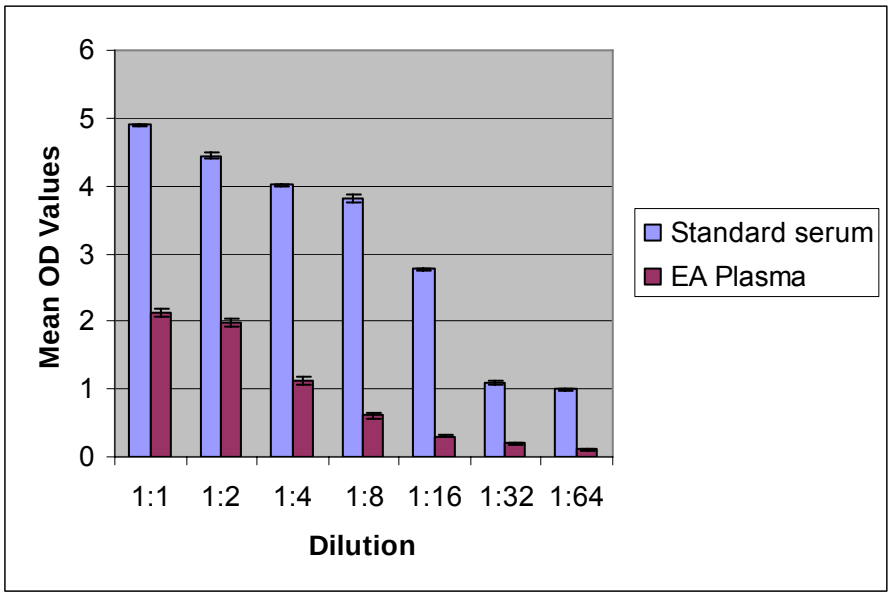

FIGURE 8a. Standard curves obtained using the international standard (100 IU/ml) and the local standard (1000 AU/ml) serum using the local MAIPA procedure.

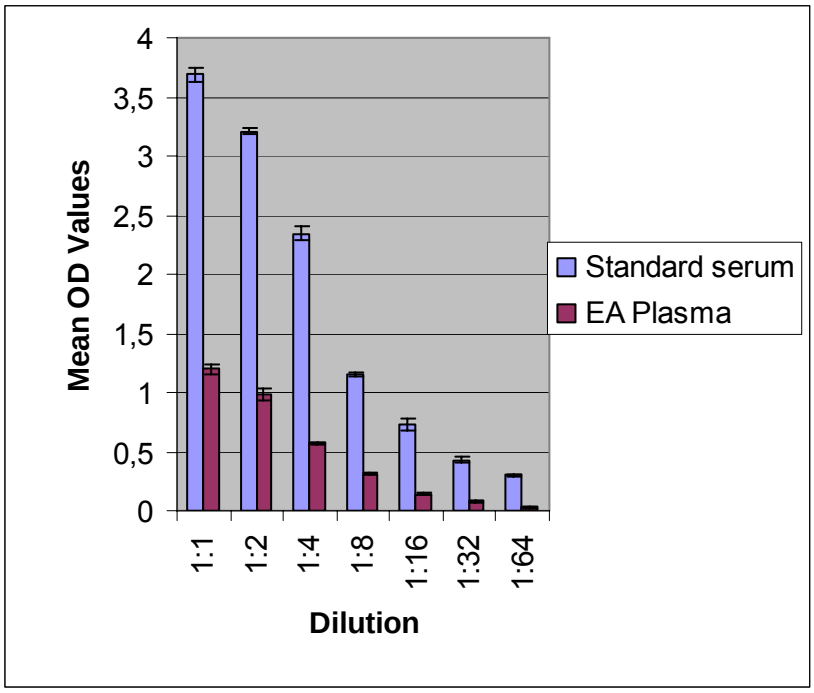

FIGURE 8b Standard curves obtained using the international standard (100 IU/ml) and the local standard (1000 AU/ml) serum in the NIBSC procedure.

3.1.4 Comparison between fresh platelets and lyophilized platelets

We compared local and NIBSC MAIPA procedures by using both fresh and lyophilized platelets. *Local MAIPA procedure:* There was a strong correlation between the measurements done with fresh platelets compared with lyophilized platelets (r = 0.988, Figure 10) by local standard. However, lyophilized platelets showed significantly less sensitivity than fresh platelets (on average 70% less, mean OD values in every dilution, P <0.001) (Table 7, Figure 9).

TABLE 7. Measurement using fresh and lyophilized platelets with local standard and the local MAIPA procedure.

Dilution local standard (1000 AU/ml)	Mean OD values (SD) at 490 nm Fresh platelets (n=3)	Mean OD values (SD) at 490 nm Lyophilized platelets (n=3)
1:2	1.70 (0.09)	0.71 (0.04)
1:4	1.12 (0.06)	0.36 (0.04)
1:8	0.60 (0.05)	0.20 (0.01)
1:16	0.38 (0.03)	0.08 (0.01)
1:32	0.19 (0.02)	0.04 (0.02)
1:64	0.11 (0.02)	0.03 (0.01)

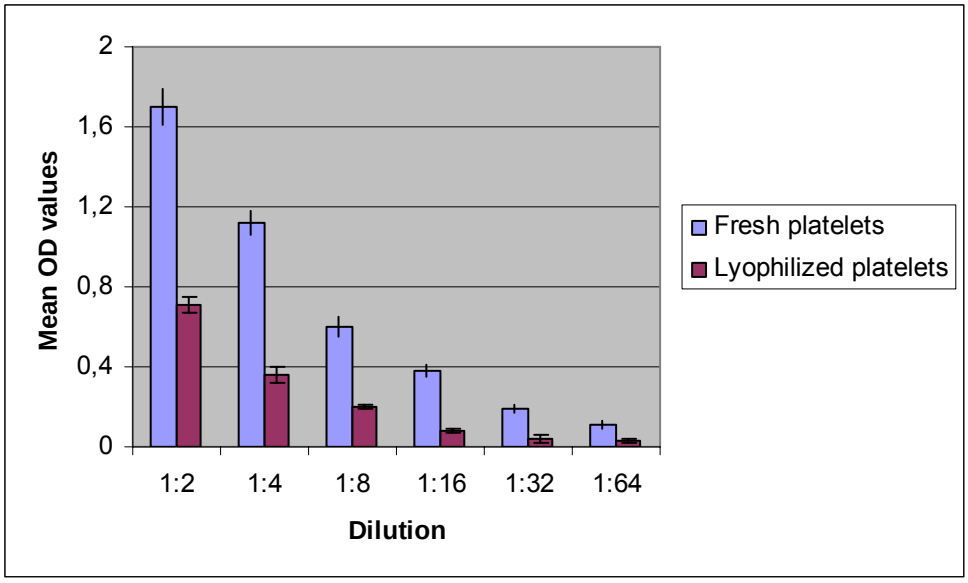

FIGURE 9. Standard curves obtained using the local standard (1000 AU/ml) and hence fresh or lyophilized platelets. Local MAIPA procedure.

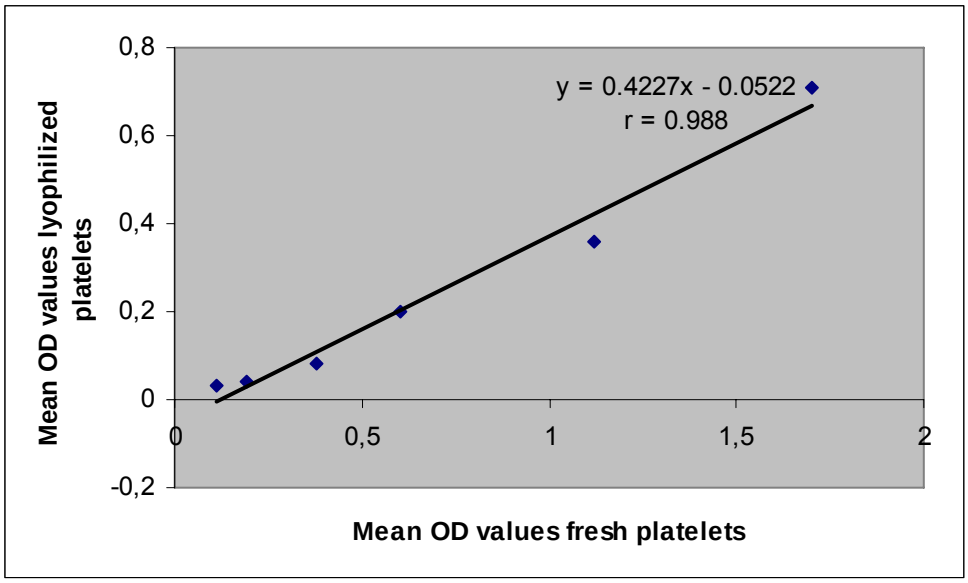

FIGURE 10 Correlation between fresh platelets and lyophilized platelets. Local standard (EA) run in serial two-fold dilution (1:2-1:64). Local MAIPA procedure. r = Pearson product moment correlation coefficient.

NIBSC procedure: We found a strong correlation (Figure 12, r = 0.955) of mean OD values between fresh and lyophilized platelets using international standard in NIBSC MAIPA procedure. Also lyophilized platelets were less reactive than fresh platelets, $p < 0.0001$ (Table 8, Figure 11). The OD value remained negative from dilution 1:64 using lyophilized platelets compared with 1:128 when using fresh platelets.

When local standard was used to compare fresh and lyophilized platelets in NIBSC procedure, OD values had been found to be negative from dilution 1:8 (Table 9).

TABLE 8 Measurement using fresh platelets and lyophilized platelets. International standard and the NIBSC procedure.

Dilution NIBSC standard	Mean OD values (SD) at 490 nm Fresh platelets (n = 3)	Mean OD values (SD) at 490 nm Lyophilized platelets (n = 3)
1:2	3.69 (0.06)	2.75 (0.08)
1:4	3.21 (0.03)	2.19 (0.05)
1:8	2.34 (0.06)	0.78 (0.03)
1:16	1.15 (0.02)	0.34 (0.02)
1:32	0.73 (0.05)	0.18 (0.01)
1:64	0.43 (0.02)	0.09 (0.01)
1:128	0.07 (0.00)	0.08 (0.01)
1:256	0.07 (0.00)	0.04 (0.01)
1:512	0.03 (0.00)	0.02 (0.01)
1:1024	0.02 (0.00)	0.01 (0.01)

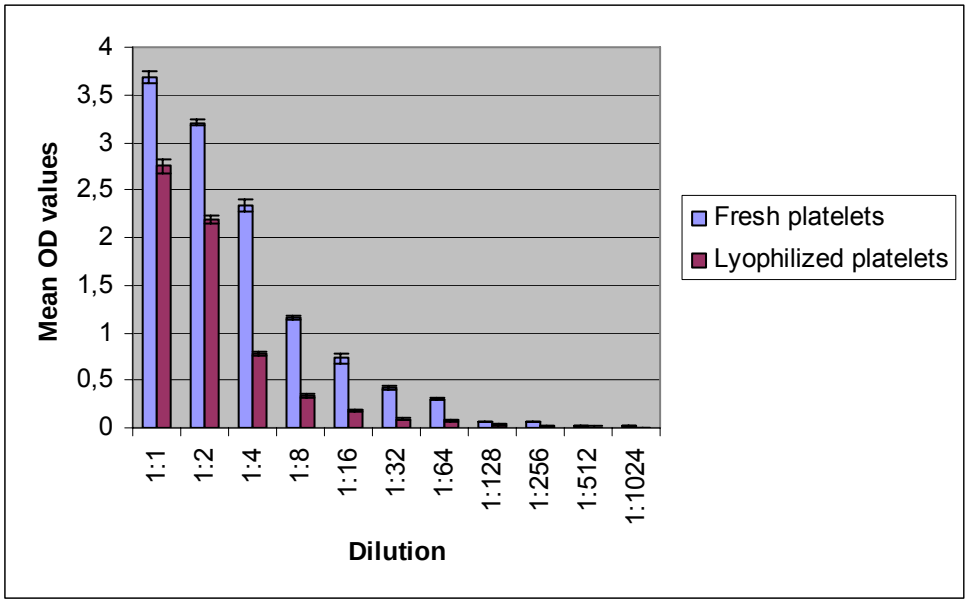

FIGURE 11 Standard curves obtained using the international standard (100 IU/ml) and hence fresh or lyophilized platelets. NIBSC procedure.

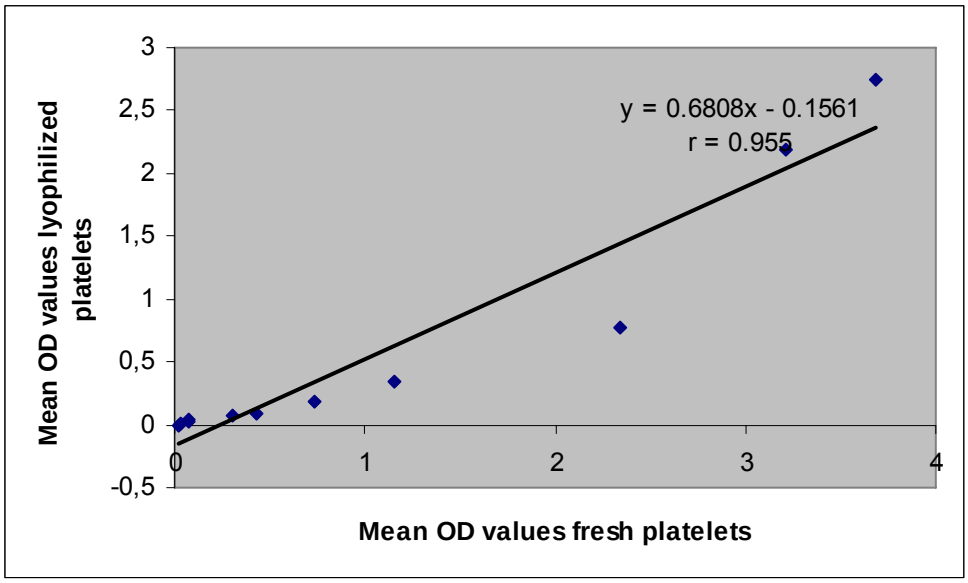

FIGURE 12. Correlation between fresh platelets and lyophilized platelets. International standard run in serial two-fold dilution (1:2-1:1024). NIBSC procedure. r = Pearson product moment correlation coefficient.

TABLE 9 Measurement using fresh platelets and lyophilized platelets. Local standard and the NIBSC procedure.

Dilution EA	Mean OD values (SD) at 490 nm fresh platelets (n=3)	Mean OD values (SD) at 490 nm Lyophilized platelets (n=3)
1:2	0.98 (0.05)	0.20 (0.06)
1:4	0.57 (0.01)	0.11 (0.06)
1:8	0.31 (0.01)	0.02 (0.03)
1:16	0.14 (0.01)	0.00 (0.03)
1:32	0.08 (0.01)	0.00 (0.04)
1:64	0.03 (0.01)	0.00 (0.03)

3.1.5 Different monoclonal antibodies for immobilization of human platelet antigen.

Three different MoAbs (anti-CD41-P2 clone, anti-CD61-clone Y2/51 and a French non-commercial MoAb) have been used in the local MAIPA procedure to identify their similarity

and/or difference. Local standard (1000 AU/ml) was used to compare those MoAbs (Table 10). None of the MoAbs performed better than the other ones in this study, $P > 0.05$. Significant differences ($P < 0.001$) of the mean OD values have been observed only with 1:64 dilutions of the French MoAb and the other two commercially available MoAbs.

TABLE 10 Measurement using three different monoclonal antibodies for immobilisation of human platelet antigen. Local standard and local MAIPA procedure.

Dilution EA	**Mean OD values (SD) at 490 nm French local MoAb (n=5)**	**Mean OD values at (SD) 490 nm Clone P2 (n=5)**	**Mean OD values at 490 nm (SD) Clone Y2/52 (n=5)**
1:2	1.92 (0.07)	1.99 (0.02)	2.06 (0.08)
1:4	1.09 (0.05)	1.14 (0.06)	1.03 (0.04)
1:8	0.85 (0.06)	0.89 (0.03)	0.88 (0.04)
1:16	0.65 (0.03)	0.66 (0.04)	0.60 (0.02)
1:32	0.39 (0.01)	0.41 (0.04)	0.40 (0.02)
1:64*	0.18 (0.01)	0.27 (0.02)	0.28 (0.01)

* indicates $P < 0.001$ of mean OD values between French and other two MoAbs.

Using clone P2 and French MoAb, we further examined 58 selected samples which exhibited positive results with clone Y2/51. Only 16 of the 58 sera containing anti-HPA 1a antibodies were detected by all three different MoAbs (Table 11). Out of these 16 samples, most exhibited similar sensitivity with all three MoAbs; and only two induced high variance in anti-HPA 1a antibody detection levels. When using clone Y2/51 the antibody levels general were 6-8 times higher than clone P2 and French MoAb. In five samples these antibodies could not be identified using clone P2. 39 out of 58 samples did not show positive results by using the French MoAb.

TABLE 11 Quantification of anti-HPA 1a antibodies (AU/ml) using three different monoclonal antibodies for immobilisation of human platelet antigen (CD61). ND = Not detected.

Samples No	Clone Y2/51 (AU/ml)	Clone P2 (AU/ml)	French MoAb (AU/ml)	Samples No	Clone Y2/51 (AU/ml)	Clone P2 (AU/ml)	French MoAb (AU/ml)
1	1263	141	165	30	14335	3564	ND
2	1026	290	117	31	301	120	ND
3	129	76	ND	32	2097	1622	ND
4	1198	543	ND	33	186	178	ND
5	25	<15	ND	34	2099	577	ND
6	8905	5766	6773	35	1191	1088	1124
7	13	<15	ND	36	1075	1972	ND
8	980	765	ND	37	6532	4850	4974
9	1646	1056	967	38	7491	8199	7654
10	13116	1987	ND	39	171	56	ND
11	4387	4119	4229	40	149	243	ND
12	39	30	ND	41	1017	992	1134
13	2884	2619	ND	42	4062	4649	4381
14	3081	2748	2862	43	4644	5582	5065
15	529	220	125	44	178	ND	ND
16	962	543	334	45	10236	17860	ND
17	656	321	ND	46	1627	1151	ND
18	1031	329	ND	47	69	<15	ND
19	800	703	812	48	6622	6810	5983
20	6294	3796	ND	49	3909	2405	ND
21	2613	2093	2354	50	20513	21390	19632
22	106	79	ND	51	575	344	ND
23	1191	1009	ND	52	1724	624	ND
24	3159	3203	3078	53	5019	801	ND
25	2059	2151	ND	54	172	129	ND
26	1956	1234	ND	55	38	<15	ND
27	1678	895	ND	56	2681	1621	ND
28	446	306	ND	57	811	577	ND
29	425	233	ND	58	31	<15	ND

3.1.6 Detection of anti-HPA 5b antibodies in a population of Egyptian pregnant women

Previous results in this study have confirmed that local MAIPA method exhibited better sensitivity over NIBSC procedure for detecting anti-HPA 1a antibodies. So, we employed the local MAIPA to detect anti–HPA 5b antibodies in samples from a population of Egyptian pregnant women, as anti-HPA5b are the second most frequent cause of NAIT. The samples which were found to be anti-HPA 5b positive were genotyped. Anti-HPA-5b antibodies were present in 16 of the 367 randomly selected samples (4·4%, Table 12). Surprisingly, anti-HPA 5b antibodies were also detected in three women with the platelet genotype HPA-5ab (Figure 13). One of the neonates was genotyped HPA-5aa and the other had HPA-5ab genotype, and both the fathers were genotyped HPA-5aa. We did not have genotypic data for neonate and father in the third case of HPA-5ab genotype mother. Two women had both anti-HPA 1a and anti-HPA 5b antibodies (not shown).

TABLE 12. The presence of anti-HPA-5b antibodies samples in 16/367 Egyptian women, and their HPA 5 genotype.

Sample Number	**HPA-5 Genotype**			**Anti-HPA 5b antibody**	**Sample Number**	**HPA-5 Genotype**			**Anti-HPA 5b antibody**
	aa	**bb**	**ab**			**aa**	**bb**	**ab**	
1	aa			Positive	9	aa			Positive
2	aa			Positive	10	aa			Positive
3	aa			Positive	11	aa			Positive
4	aa			Positive	12	aa			Positive
5	aa			Positive	13	aa			Positive
6			ab	Positive	14	aa			Positive
7			ab	Positive	15	aa			Positive
8			ab	Positive	16	aa			Positive

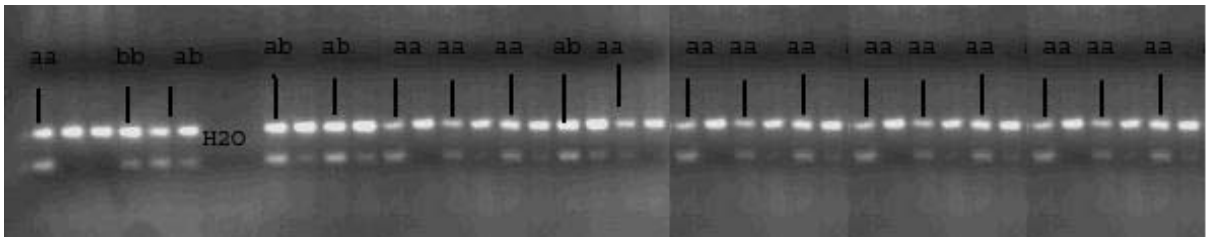

FIGURE 13 Electrophoretic gel picture of HPA 5 genotype in Egyptian anti-HPA 5b positive samples. First four lanes are for controls and the rests are for samples.

4.0 DISCUSSION

The MAIPA procedure is regarded as the reference test of antibodies to epitopes on platelet glycoproteins because the glycoproteins (GP) remain in intact structure in this procedure. This can provide better sensitivity and specificity by MAIPA in comparison with other techniques, such as ELISA and Immunobead assay [70]. Also MAIPA has the advantage of the selective specificity of platelet-specific MoAbs and can achieve higher sensitivity by avoiding direct contact between the plasma/serum and the solid phase. With this procedure the platelets can be sensitized with patient antibodies before platelet lysis, which may stabilize the epitope and retain its conformation and thereby increase the sensitivity [71]. There is an international demand to standardize the test for antibody detection and quantification. Like many laboratories that specialize in detection of platelet antibodies, a modified MAIPA method has been developed in the University hospital of North Norway in order to check specificity and quantify anti-HPA antibodies utilising a more effective procedure than described originally.

This study revealed that the NIBSC procedure required ~5 hours for incubation, which was ~3.5 hours for local MAIPA procedure. This means that the other steps, such as washing, pipetting, mixing, and also preparing platelets, making all necessary reagents and solutions to carry out the NIBSC procedure is hardly possible within a working day. The local MAIPA showed better sensitivity compared with the NIBSC MAIPA. This may not be important if mainly samples from women with high antibody concentration are measured, but if samples from women who are newly immunized are measured the most sensitive assay is preferred. Dilution of the supernatant in NIBSC procedure may have caused less sensitivity than the local MAIPA. During the coating of microtiter plate with goat anti-mouse IgG, the concentration of the antibody and incubating temperature varied between the procedures. These variations may have resulted in less adsorption of antibody to the plate. Unlike NIBSC, PBS 2% BSA was used in

local MIAPA for resuspending the platelets. PBS 2% BSA ensures minimal non-specific interactions of the suspended materials within it- resulting better binding of patient antibodies. Also during incubation with HRP conjugated goat anti-human IgG, the concentration was less in NIBSC that may cause less conjugated antibodies bound to human IgG on the platelet epitopes. All these differences might have contributed to the better sensitivity of local MAIPA over NIBSC.

The better sensitivity of local MAIPA over NIBSC method is clearly shown in its higher OD values by using both local and international standard serum; the NIBSC showed an average of > 50% less sensitivity than the local MAIPA. The strong correlation coefficient value ($r = 0.999$) between the results from both assay indicates that the difference in sensitivity between the methods solely procedural- not due to local reagents, equipment or operator skill. The difference in OD values and amounts of antibodies measured in the two assays can be explained by the dilution of supernatant in NIBSC method.

However, very low level of anti-HPA 1a antibodies can be detected by using international standard serum in both local and NIBSC MAIPA procedures. Because the international standard serum is > 3 times more sensitive than local standard serum with respect to OD values.
One of the major activities of International Society of Blood Transfusion (ISBT) is to identify the extent to which original MAIPA [48] has been modified by different laboratories around the world. The 12th ISBT workshop identified a great variation in reagents and reaction conditions, among other factors, in the performance of MAIPA assay [72]. The next biennial workshop in 2006 at Cape Town aimed at comparing and therefore standardizing different in-house MAIPA methods to a supplied rapid method (NIBSC) of original MAIPA. The 13th ISBT workshop revealed that there was high variability of sensitivity in NIBSC [69] method compared to less variability with in-house methods. Only for detecting anti-HPA 1a (93/710), variation was observed in 2 out of 25 laboratories that used in-house MAIPA method. By using NIBSC MAIPA method, 6 out of 27 laboratories reported variation in sensitivity. The variation in sensitivity with NIBSC procedure was attributed to the different factors, e.g., reagents and laboratory equipments. In this study we followed the exact NIBSC recommended reagents and procedure. Still we observed the variation in sensitivity between local and NIBSC MAIPA. However, a significant difference between the procedures was also hinted in case of larger number of replicates in that workshop.

Although lyophilized platelets have shown to retain a near normal structure and have many of the surface membrane functions of fresh platelets [66], this study with the local MAIPA procedure revealed that lyophilized platelets showed significantly less sensitivity than fresh platelets. This observation is supported by a previous flow cytometry study that has shown that lyophilized platelets with labelled monoclonal antibodies to GPIIIa/IIb demonstrated a fibrinogen receptor binding density of only 42% of that fresh platelets [73]. Similar trend of higher sensitivity of fresh platelets over lyophilized platelets can be observed with NIBSC procedure using international standard. The difference in sensitivity between fresh and lyophilized platelets becomes more distinctive using EA plasma. Antibody levels were not even detected at lower dilutions with lyophilized platelets.

None of the three different MoAbs (clone P2, French and clone Y2/51) has been found to perform better sensitivity than the other ones in local MAIPA procedure using EA plasma. So, use of any of these monoclonal antibodies with EA plasma does not interfere on the local standard curve. However, using 58 different samples selected by the Y2/51 MoAb, different sensitivity with the three MoAbs was shown. P2 and French MoAb showed varying sensitivity in samples selected for positivity with Y2/51. These discrepancies may result from epitopic change in the GPIIb/GPIIIa complex on the platelet surface when immobilized with each of the three MoAbs. For example, if one MoAb binds a conformational epitope in GPIIb/GPIIIa complex, this may be disrupted during solubilisation of platelets that results in decrease in binding of that MoAb. Another discrepancy in sensitivity may arise from the fact that patient and mouse MoAb are required to bind the same epitope on GPIIb/GPIIIa complex. When an epitope for a patient and one of the MoAbs overlap, the anti-HPA 1a antibodies may not be detected [71]. If a patient antibody and a MoAb have same or closely related epitope, they can mutually inhibit their binding. Also the proteins present in the patient serum, such as human anti-mouse antibodies, can inhibit the binding of MoAb to its epitope. In these cases pool of MoAbs directed towards different epitopes on the same molecule could be used to detect antibodies [48].

Considering the results obtained by this study, the local MAIPA procedure is advantageous over NIBSC procedure, especially with respect to better sensitivity as well as less time requirement. In addition, to obtain better results it seems like one should use fresh platelets in stead of lyophilized platelets. International standard serum can be used as a reference serum, as the higher sensitivity (> 3 times) with international standard over local standard demonstrates

the strength of international standard. Also, low level of antibodies can be detected by using international standard serum. This is particularly important to detect alloimmunization in an early stage. From the results in this study, we can recommend to use pool of MoAbs, in stead of a single MoAb, for detecting antibodies.

The local MAIPA procedure was used to study the frequency of anti-HPA-5b antibodies in Egyptian population as anti-HPA-5b antibodies have been reported to be a more common cause of NAIT in the Mediterranean countries. We reported the presence of anti-HPA-5b antibodies in 4.4% randomly selected Egyptian samples [74], in contrast to a recent study from Japan, where a-HPA 5b antibodies were detected in 0·68% of the samples [52]. In both Caucasian and Asian populations the most common pregnancy-induced platelet-specific antibody is anti-HPA-5b [40, 75], but the most frequent cause of NAIT in Caucasians is anti-HPA-1a antibodies(~80%). Anti-HPA-5b is reported to be the cause of NAIT in 8% of the cases, in 2% anti- HPA-5b and an additional alloantibody were responsible for NAIT [61]. Although alloimmunization against HPA-5b causes NAIT in only a few of the affected infants, the bleeding sequelae in the affected infants can become severe [47]. Also, anti-HPA 5b antibodies have been reported to be a more common cause of neonatal alloimmune thrombocytopenia (NAIT) in the Mediterranean countries compared with Northern Europe (personal communication, Yves Merieux). Our study is the first investigation of anti-HPA-5b antibodies in the Egyptian population and the results showed higher frequency than that observed with other population.

The presence of both anti-HPA-1a and anti-HPA-5b antibodies in two of the 16 positive anti-HPA-5b presenting women may be compared with alloimmunization for red cell antigens; making antibodies against one antigen make a patient more susceptible than an non-immunized patient to make alloantibodies to other red cell antigens.

The most significant finding of this study was the detection of ‘anti-HPA 5b’ antibodies in three women with the platelet genotype HPA-5ab; the explanation for this phenomenon is not known. This may arise either from presence of a third allele or autoantibodies against HPA-5b. Nucleotide sequencing can be carried out to find out the underlying reasons, e.g., defect in the intronic area. As studies have shown difference between genotype and phenotype with HPA 1[76], we can also investigate if there were any phenotypic discrepancies in HPA 5. Mueller-Eckhard *et. al.* has reported a strong association between human leukocyte antigen (HLA)-DR6

and immunization against HPA-5b antigen [77]. We, therefore, could look for similar association in the studied population. However, other molecules, such as TAP1 and TAP2 genes and their polymorphism in the MHC class II region, could be also involved in an antigen presentation and processing [78, 79].

5.0. CONCLUSIONS

This study revealed that local MAIPA at the University Hospital of North Norway (UNN) exhibited better sensitivity than NIBSC procedure for anti-HPA 1a antibody detection. However, use of international standard serum, pool of MoAb and fresh platelets can enhance the sensitivity of local MAIPA procedure.

Anti-HPA-1a, anti-HPA-5b antibodies in an Egyptian cohort are more frequent than other population, but the clinical consequences of this antibody specificity are not known.

6.0. REFERENCES

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