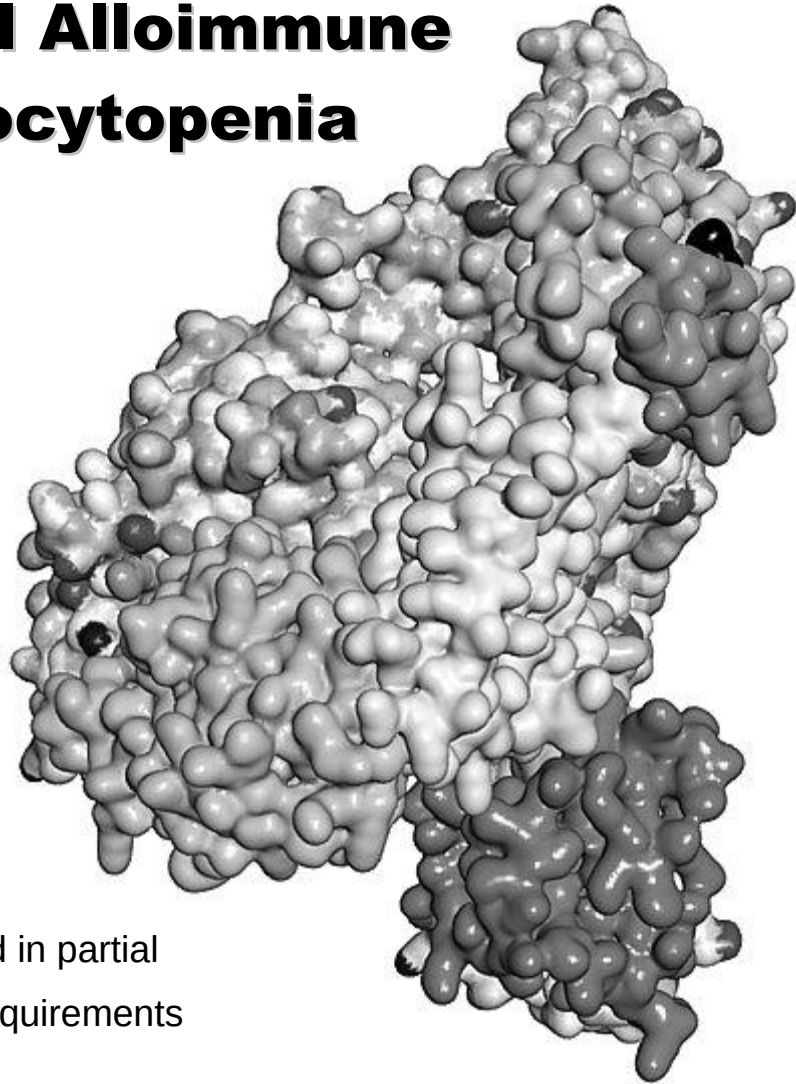


A Study of the Human Platelet Antigen 1a (HPA-1a) Antibody Response in Neonatal Alloimmune Thrombocytopenia (NAIT)

By

David L Allen



A thesis submitted in partial
fulfilment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

at the University of Oxford

St Catherine's College, Trinity Term 2012.

(Do HPA-1a antibodies preferentially recognise $\beta 3$ in association with...)

**“Ib, or not Ib, that is the
question”**

Adapted from Hamlet, Act III, Scene I

To Teresa, Frances and Claire

To my father

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List of publications arising from this study

- I. The incidence and outcomes of fetomaternal alloimmune thrombocytopenia: A UK national study using three data sources. Knight,M.; Pierce,M.; **Allen,D.**; Kurinczuk,J.J.; Spark,P.; Roberts,D.J.; Murphy,M.F. (2011), *B.J. Haem.* **152**, 460-468.
- II. Human Platelet Antigen 1a epitopes are dependant on the cation-regulated conformation of integrin GPIIb/IIIa (alpha-IIb beta-III, CD41/61). **Allen D.L.**; Abrahamsson S.V.; Murphy M.F.; Roberts D.J. *J.Imm.Methods* (2012), **375**, 166-75.
- III. Sensitivity of assays for the detection of HPA-1a antibodies: results of an international workshop demonstrating the impact of cation chelation from integrin $\alpha\text{IIb}\beta 3$ on three widely used assays. **Allen,D.L.**; Metcalfe,P.; Kaplan,C.; Kekomaki,R.; de Haas,M.; Yusuf,R.; Ouwehand,W.H. *Vox Sanguinis* (2013), doi: 10.1111/vox.12043.

Other publications

- I. An international reference reagent (minimum sensitivity) for the detection of anti-human platelet antigen 1a. Metcalfe,P.; **Allen,D.**; Kekomaki,R.; Kaplan,C.; De,Haas M.; Ouwehand,W.H. *Vox Sang* (2009), 96, 146-152.

Awards arising from this study

Early Stage Investigator Prize, British Society for Haematology 51st Annual Scientific Meeting, Brighton, 2011. Measured levels of maternal HPA-1a antibody vary depending on the conformation and density of integrin $\beta 3$. Allen DL and Roberts DJ.

Presentations arising from this study

Feto-maternal alloimmune thrombocytopenia (FMAIT) in the UK: A prospective national study of incidence and outcomes using obstetric, paediatric and laboratory reporting systems. Allen D, Knight M, Pierce M, Spark P, Roberts DJ, Murphy MF. (2010) *Br J Haem.*, **149** (Supple 1), 11. *Free Communication: Presidential Selection - 50th Annual Scientific Meeting of the British Society for Haematology, 19–21 April 2010, Edinburgh, UK.*

Measured levels of maternal HPA-1a antibody vary depending on the conformation and density of integrin $\beta 3$. Allen D and Roberts DJ (2011). *Br J Haem*, **153** (Supple 1), 6. *Free Communication: Presidential Session – 51st Annual Scientific Meeting of the British Society for Haematology 4–6 April 2011, Brighton, UK.*

Neonatal alloimmune thrombocytopenia (NAIT): unusual presentations and regional variations in referral. Allen DL, Knight M, Pierce M, Murphy M, Green A, Lucas G, Roberts DJ (2011). *Br J Haem*, **153** (Supple 1), 10. *Free Communication: Transfusion and Nursing Session – 51st Annual Scientific Meeting of the British Society for Haematology 4–6 April 2011, Brighton, UK.*

Poster presentations arising from this study

Effect of conformation of platelet glycoprotein IIb/IIIa on the expression of the Human Platelet Antigen 1a. Allen D, Abrahamsson S, Murphy M, and Roberts D (2009). *Academic Department of Haematology Annual Meeting, Oxford.*

Factors affecting measurement of anti-HPA-1a levels. Allen D and Roberts D. (2010). *NHS Blood & Transplant Annual R&D conference, Oxford.*

Prediction of Severity of Neonatal Alloimmune Thrombocytopenia. Allen D and Roberts D (2010). *Nuffield Department of Clinical Laboratory Sciences Graduate Studies Open Day, Oxford.*

Abstract

A Study of the Human Platelet Antigen 1a (HPA-1a) Antibody Response in Neonatal Alloimmune Thrombocytopenia (NAIT)

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Thesis submitted in partial fulfilment for the degree of Doctor of Philosophy, Trinity 2012

Neonatal alloimmune thrombocytopenia (NAIT) is caused by maternal alloantibodies against fetal platelet antigens inherited from the father and which are absent from maternal platelets. In Caucasians, antibodies against the Leu33 (HPA-1a) polymorphism of integrin $\beta 3$ (part of the platelet $\alpha \text{IIb}\beta 3$ complex) account for >70% of cases. Antenatal screening for these antibodies does not currently take place in the UK, partly because of the absence of sensitive, predictive tests.

We hypothesized that the poor sensitivity and predictive abilities of current assays are due to the use of $\beta 3$ in an inappropriate conformation, resulting in sub-optimal binding of HPA-1a antibodies. We hypothesized firstly that *in vitro* induced changes to $\alpha \text{IIb}\beta 3$ might alter accessibility of the HPA-1a epitopes to alloantibodies, thus reducing assay sensitivity. Secondly, we hypothesized that HPA-1a antibodies are stimulated by, and preferentially recognise, $\beta 3$ in association with αv , a molecule present on placental syncytiotrophoblasts, and that reactivity against platelet $\alpha \text{IIb}\beta 3$ reflects only cross-reactivity with $\alpha \text{v}\beta 3$.

Our first hypothesis was proven by demonstrating that use of the cation chelating compound EDTA, used by many diagnostic laboratories as a component of assay reagents or present in blood samples as anticoagulant, resulted in significantly reduced assay sensitivity. These findings were confirmed in an international workshop. Support for our second hypothesis was provided by demonstrating enhanced reactivity of a small panel of examples of anti-HPA-1a against $\alpha \text{v}\beta 3$ compared to $\alpha \text{IIb}\beta 3$ and by molecular modelling data.

We also showed that HPA-1a antibodies can inhibit platelet function by using a novel application of the ROTEM® *delta* thromboelastograph and an immunofluorescence assay in which we demonstrated blocking of platelet function using a monoclonal antibody, PAC-1, that binds only to activated $\alpha \text{IIb}\beta 3$.

These studies provide possible explanations for the poor sensitivity and predictive abilities of current assays and suggest further areas for research.

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The undertaking of this thesis would not have been possible without the immense support provided by my supervisor, Professor David Roberts and it is to him that I am greatly indebted. His guidance and constant words of encouragement have ensured that this manuscript reached completion.

It also would not have been possible without the encouragement from my wife, Teresa, and daughters Frances and Claire, with whom I spent less time than I should have done during the writing of this thesis – thank you for being so patient.

There are so many people that have helped me over the years to gain the knowledge that I have, that a further chapter would be required for this already extensive document so I will mention only a few. To Tony Hunt, who sparked my interest in research many years ago, I will be eternally grateful. My interest in the field of NAIT developed as a result of working with many colleagues and experts in the field but I would particularly like to mention and thank Professors Mike Murphy and Willem Ouwehand and Drs Paul Metcalfe and Geoff Lucas, all of whom made it such a stimulating field in which to work.

Thanks are also due to all of the staff within the Blood Research Group at Oxford for their constructive comments and support during my research but I would specifically like to thank Dr Robert Danby, my fellow DPhil student who ensured that I 'stayed the course'.

Of course, this work would not have been possible without access to the samples from blood donors and mothers of children affected by NAIT and I therefore would like to record my gratitude to all of those individuals that either donated or collected samples for me, particularly to the staff of the Component Donation Unit at NHSBT Oxford, Professor Mike Murphy and Dr Nicola Curry.

The work in **Chapter 5** was made possible (and easier) by the generosity of Prof. Felding-Habermann at the Scripps Research Institute, La Jolla, California who supplied the M21 and M21-LIIb cell lines - I am very grateful for these gifts.

Finally, I would like to thank the manager, organiser and participants of the NIBSC international platelet immunology quality assurance workshops for allowing me to use the scheme to validate my findings and for allowing me to use their data.

Declaration of Authorship

I declare that this thesis entitled “A Study of the Human Platelet Antigen 1a (HPA-1a) Antibody Response in Neonatal Alloimmune Thrombocytopenia (NAIT)” and the work presented herein is my own and is the result of my own original research. Where the thesis is based on work done jointly with others the contribution of others is made clear below. I affirm that I have taken reasonable care to ensure that the work is original and has not been copied from elsewhere. Where data has been used from elsewhere, the source and authors have been duly cited and acknowledged in the text.

Chapter 3. The project in context: a study of the epidemiology of NAIT in England and North Wales.

The data in this chapter were obtained as part of a larger collaborative study with the National Perinatal Epidemiology Unit under the auspices of Professor Marian Knight. Data from contributing clinicians regarding the incidence and outcomes of NAIT were collated by NPEU; these data were used in **Tables 3.2** and **3.3**, and **Figure 3.1**.

Data on laboratory referrals was collated and entered on to databases by staff of the Platelet Immunology Reference Laboratory at NHSBT Bristol.

Chapter 4. Integrin $\alpha\text{IIb}\beta 3$ conformation I: effect of integrin activation and dissociation on HPA-1a epitope expression.

Dr Sofia Abrahamsson performed the initial experiments shown in **Figure 4.1**.

Chapter 5. Integrin $\alpha\text{IIb}\beta 3$ conformation I: effect of α -chains on HPA-1a epitope expression.

Dr Winnie Chong performed the Luminex assays and Mr Rizwan Yusuf performed HPA-1 genotyping on the cell lines.

Chapter 1.

Introduction and Aims of the Study

1.1 Platelets

Platelets are the smallest cells in the blood (approximately 2 - 4 μm). The normal platelet concentration is between 150 - 400 $\times 10^3/\mu\text{L}$ (Giles, 1981) and their lifespan is approximately 10 days (Harker & Finch, 1969). Each day about 10^{10} to 10^{11} are removed from the circulation and are replaced by new cells produced from megakaryocytes in the bone marrow (Drouin & Cramer, 2002; Hartwig & Italiano, Jr., 2003).

Platelets were first observed over 170 years ago by A Donné (Donné M, 1842). Although he correctly observed their ability to change shape and aggregate, he ascribed them to being 'chyle globules' that were absorbed from the gut into the circulation and did not realise that they were true blood cells. That they were in fact blood cells was first reported by Addison (Addison, 1842) and Gulliver (Gulliver, 1842), both of whom published illustrations of platelets although it was only Addison who realised the association between platelets and fibrin.

The name 'platelet' and the role of these cells in haemostasis is generally credited to Bizzozero (Bizzozero, 1882) although there is evidence to suggest that he ignored previously published findings of others (Robb-Smith, 1967; Cooper, 2005).

1.2 Platelet membrane receptors and their role in primary haemostasis

Platelets play a key role in the haemostatic response to tissue injury, forming the initial plug at the site of tissue damage by interaction with the damaged endothelium via a number of receptors, including integrins, on the platelet surface that are able to recognise various components of the extracellular matrix (ECM) (Rozman, 2002; Kasirer-Friede *et al*, 2007; Rivera *et al*, 2009) (**Table 1.1**).

TABLE 1.1. Platelet surface membrane receptors for extracellular matrix ligands.

Integrin	Alternative names	CD	Copy number per platelet	Receptor for:
α IIb β 3	GPIIb/IIIa	CD41/61	25 - 80,000	Fibrinogen, fibronectin, von Willebrand factor (vWF), vitronectin
α v β 3		CD51/61	50 - 100	Vitronectin
α 2 β 1	GPIa/IIa	CD49b/CD29	2,000	Collagen
α 5 β 1		CD49e/CD29	1,000	Fibronectin
α 6 β 1		CD49f/CD29	1,000	Laminin
	GPIb/IX/V	CD42a/b/c	25,000	vWF
	GPIV	CD36	25,000	Collagen
	GPVI		1 - 3,000	Collagen

The first event following tissue damage depends on the site of injury and whether the flowing platelets are subject to low shear stresses (e.g. in veins or larger arteries) or high shear stresses (e.g. in the microvasculature) (Kulkarni *et al*, 2000). In conditions of low shear stress, the platelets adhere to collagen, fibronectin and laminin whilst under conditions of high shear, von Willebrand Factor (vWF) is the critical ligand and GPIb/IX/V the critical platelet receptor.

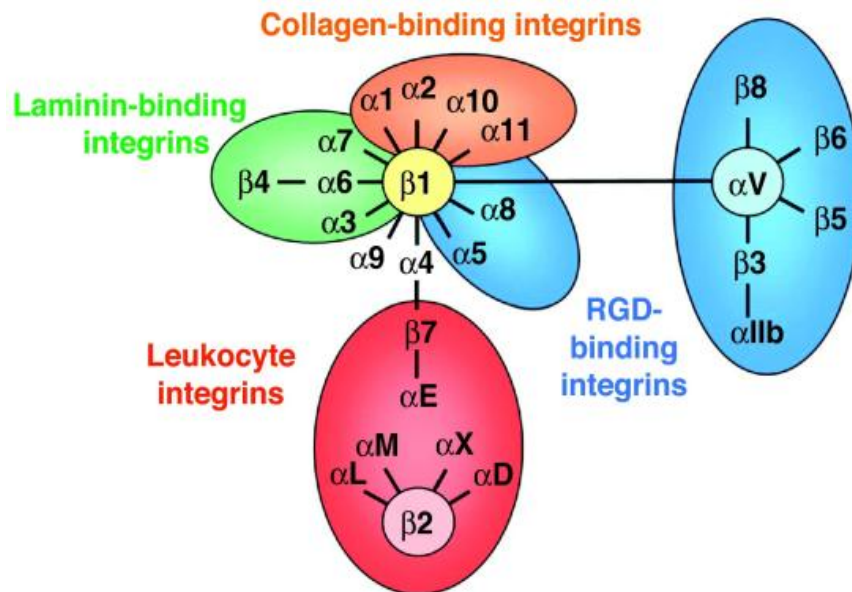
Contact of platelets with the ECM results in tethering of platelets at the site of injury, after which a range of interactions and processes occur (Clemetson, 2012). The platelets start to spread across the site of injury and release the contents of their internal granules e.g. vWF, fibrinogen and fibronectin. Further platelets are recruited to the platelet layer via GPIb/IX and vWF interactions; these interactions are relatively weak and require additional ligand/receptor interactions to stabilise the plug (Gibbins, 2004). Integrin α 2 β 1 and GPVI interact with collagen within the ECM, and binding of ligand to GPVI triggers signalling through to the platelet cytoplasm by a mechanism of 'outside-in' signalling; this then results in activation of platelet integrins α 2 β 1 and α IIb β 3 by 'inside-out' signals transmitted from the cytoplasmic tails to the

extracellular domains. Activation of $\alpha\text{IIb}\beta 3$ is a key part of the primary haemostatic process as it exposes the binding site on $\alpha\text{IIb}\beta 3$ which is the receptor for fibrinogen thus allowing binding of this ligand (and its conversion to fibrin), and binding of vWF, both of which are important for cross-linking of platelets and formation of stable platelet aggregates. Further platelet activatory signals are generated by the presence of thrombin (a potent platelet activator) at the site of injury, and activation of platelets results in release of additional prothrombotic factors such as ADP, serotonin and thromboxane A₂ (Gibbins, 2004; Denis & Wagner, 2007). Thus, a complex series of events occur that ultimately result in activated platelets forming a plug held together by an adhesive network.

In addition to contributing to this primary haemostatic plug, platelets play an important role in secondary haemostasis. Activation of platelets results in generation of procoagulant microparticles and exposure of the highly-charged molecule phosphatidylserine on both platelet and microparticle membranes. This molecule is able to initiate the classic coagulation cascade and the platelet membrane provides a surface on which the prothrombinase complex may be assembled (Heemskerk *et al*, 2002; Gibbins, 2004).

1.3 Integrin $\alpha\text{IIb}\beta 3$

This glycoprotein is the most abundant and one of the most important glycoproteins on the platelet surface, not only being involved in haemostasis, but also in auto- and alloimmunity and as a receptor for viruses. It is a member of the integrin family, glycoproteins that consist of an α -chain non-covalently linked to a β -chain. There are 18 known α -chains and 8 known β -chains which can associate to form 24 different permutations (**Figure 1.1**) (Margadant *et al*, 2011).

FIGURE 1.1 Integrin families (from Margadant *et al*, 2011).

1.3.1 Genetics and biogenesis

The genes encoding αIIb (*ITGA2B*) and $\beta 3$ (*ITGB3*) are located on chromosome 17 within a 260-kilobase segment of each other at 17q21-32 (Bray *et al*, 1987; Rosa *et al*, 1988; Bray *et al*, 1988; Thornton *et al*, 1999). *ITGA2B* spans approximately 17.2 Kb and consists of 30 exons and *ITGB3*, 90Kb over 15 exons (http://www.nextprot.org/db/entry/NX_P08514/exons; accessed 19th April 2012).

Both αIIb and $\beta 3$ can be detected as early as day 5 during culture of megakaryocytes (Vinci *et al*, 1984). The $\beta 3$ chain is synthesised as a single chain (molecular mass 100 kD/90 kD under non-reducing or reducing conditions respectively). The αIIb chain is synthesised as a precursor single chain molecule ('pro- αIIb ', molecular mass 130-140 kD or 120 kD under non-reducing/reducing conditions) (Duperray *et al*, 1987; Nurden, 1997).

After entry to the endoplasmic reticulum, pro- αIIb undergoes folding to form the bent conformation observed in the mature protein and the $\beta 3$ chain is then non-covalently

attached; this also assumes the bent configuration as a result of its attachment to the bent α -chain (Rosa & McEver, 1989; Mitchell *et al*, 2007). The molecules also undergo glycosylation, intra-chain disulphide bond formation and removal of the signal peptide (Nurden *et al*, 2011). After transportation to the Golgi apparatus they undergo the final stages of oligosaccharide processing and pro- α IIb is cleaved into the heavy and light chains of mature α IIb. Finally, the mature complex is transported to the α -granule membrane from where it is exported to the platelet surface membrane (Shen *et al*, 2009).

The mature α IIb and β 3 proteins are comprised of 1008 and 762 amino acids (aa's) respectively (Ma *et al*, 2007). Both chains have a short, single pass transmembrane domain and a short cytoplasmic tail.

1.3.2 Surface expression

The platelet surface membrane expresses 25,000 to 80,000 copies of α IIb β 3 with the large differences in reported copy number related to platelet size and use of whole antibodies or Fab fragments in immunoassays; the molecules therefore are extremely densely packed with an inter-molecule separation of <20 nm (McEver *et al*, 1980; Wagner *et al*, 1996; Kunicki *et al*, 2012). Pools of α IIb β 3 are also located within the platelet, in the surface connected canicular system (SCCS) and in α -granules; upon platelet activation these may be translocated to the platelet surface thus increasing the number of surface molecules (Wencel-Drake *et al*, 1986c; Cramer *et al*, 1990; Heilmann *et al*, 1991). Conversely, the number of surface molecules may be reduced as a result of internalisation into the SCCS and ultimately into the α -granules as part of the normal mechanism to regulate levels of surface expressed glycoproteins (Wencel-Drake, 1990; Morgenstern *et al*, 1992; Wencel-Drake *et al*, 1993; Nurden *et al*, 1999).

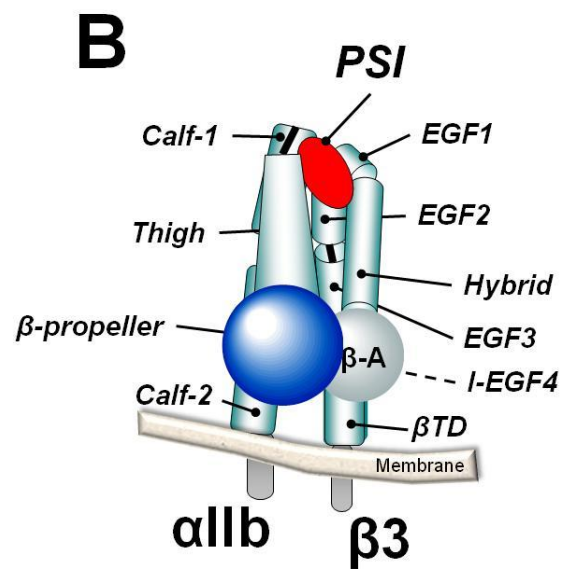
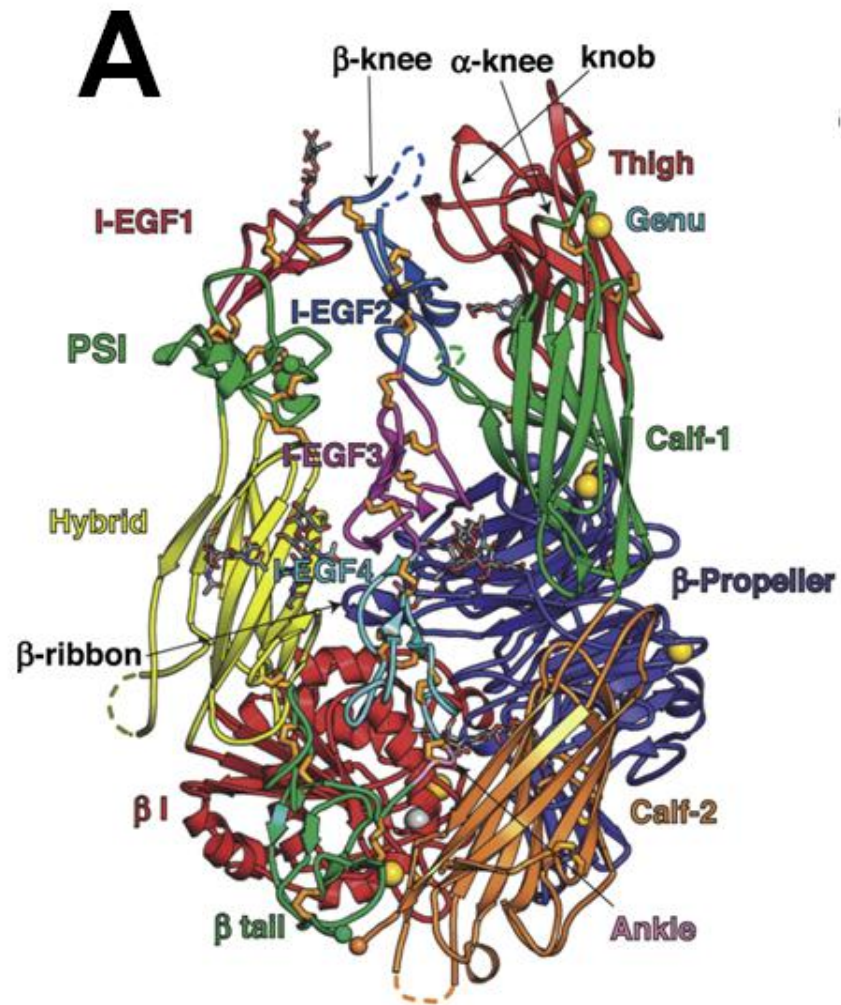
1.3.3 Structure and conformation

Increasingly detailed images of $\alpha\text{IIb}\beta 3$ and the similar molecule $\alpha\text{v}\beta 3$ were obtained using purified $\alpha\text{IIb}\beta 3$ obtained from detergent-solubilised platelet membranes and, more recently, using intact platelets using a range of electron microscopy techniques including scanning, transmission and atomic force (Carrell *et al*, 1985; Weisel *et al*, 1992; Erlandsen *et al*, 2001; Adair & Yeager, 2002; Hussain & Siedlecki, 2004; Adair *et al*, 2005). These images showed what appeared to be a globular head-like structure approximately 8 nm in diameter, from which extended a tail-like structure approximately 18 nm in length; in some images the molecules appeared to be folded over whilst in others they appeared extended.

The most important development in understanding these images was when the crystal structure of the extracellular segments of $\alpha\text{v}\beta 3$ was published (Xiong *et al*, 2001; Xiong *et al*, 2002b). This clearly showed head and tail pieces, and organisation of the molecule into 12 domains. Unfortunately the organisation of the PSI domain, of most relevance to this project, was not clear.

The crystal structure of $\alpha\text{IIb}\beta 3$ published by Zhu (**Figure 1.2**) clearly showed the close similarities between this molecule and $\alpha\text{v}\beta 3$, and more importantly confirmed and extended the findings of Xiao *et al* showing the organisation of the molecule in the region of the PSI domain which was shown to be situated in the fold between head and tail pieces adjacent to the hybrid and EGF-1 (also known as I-EGF-1) domains (Xiao *et al*, 2004; Zhu *et al*, 2008). The hybrid domain was shown to be inserted into the PSI domain and the EGF-1 domain extended from the same end of the hybrid domain as the PSI domain.

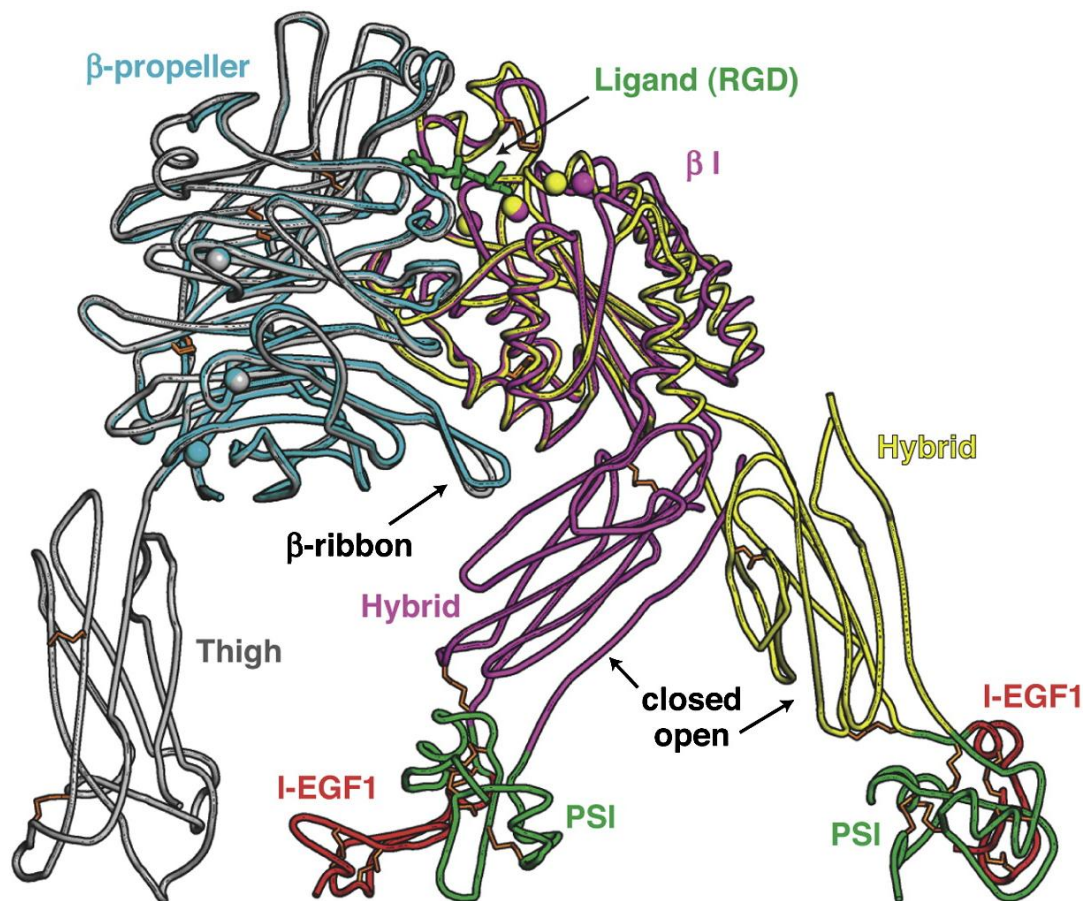
FIGURE 1.2. Domain organisation of $\alpha\text{IIb}\beta 3$. (A) is taken from Zhu (Zhu *et al*, 2008). Cations are shown as gold (Ca^{2+}) or silver (Mg^{2+}) spheres. (B) A simplified version to show more clearly the organisation of the domains; the molecule is horizontally rotated 180° compared to (A).



Domains not to scale

The PSI to EGF-1, and EGF-1 to EGF-2 junctions are highly flexible and permit the molecule to undergo significant rotation and change from the folded structure observed in some electron micrographs, where the molecule is folded at approximately 45°, to the extended 180° form which was also observed. Upon extension, the hybrid domain swings out several times its own diameter (**Figure 1.3**).

FIGURE 1.3. Re-organisation of α IIb β 3 following activation of molecule. Taken from Zhu (Zhu et al, 2008). The hybrid, PSI and EGF-1 domains undergo a massive swing-out and rotation during the transition of the molecule from 'closed' to 'open' conformation.



Importantly, the interface between the PSI domain and the hybrid domain is rigid and key to this rigidity is Arg-93 within the hybrid domain with the hydrogen bonds in the side chain of Arg-93 making contact with one hybrid, and three PSI domain residues although disulphide bonds also play a role (Xiao *et al*, 2004). Thus, movement of the hybrid domain also entails movement of the PSI domain (**Figure 1.3**) and is of relevance to **Chapter 6** (Takagi *et al*, 2003).

1.3.4 Cation dependency

Embedded within, and at the interface of, the α - and β -chains of the $\alpha\text{IIb}\beta 3$ complex are divalent cations; four within the α -chain (within β -propeller) and three within the β -chain (β -A domain) (**Figure 1.2**) (Xiong *et al*, 2002a; Gidwitz *et al*, 2004; Xiao *et al*, 2004). They play a critical role in maintaining the integrity, function and conformation of the $\alpha\text{IIb}\beta 3$ complex.

Naturally-occurring and *in vitro* mutations of $\alpha\text{IIb}\beta 3$ that affect the calcium-binding abilities (as well as other mutations) have been shown to result in incorrect folding of the molecule and impair trafficking of the complex, or expression of functionally-impaired complex on the platelet surface (Nurden *et al*, 1987; Bajt & Loftus, 1994; Wilcox *et al*, 1994; Gidwitz *et al*, 2004; Murcia *et al*, 2008; Mansour *et al*, 2011; Raborn *et al*, 2011).

More importantly, from the point of view of this project, chelation of calcium by chelating agents such as ethylene diamine tetraacetic acid (EDTA) or ethylene glycol tetraacetic acid (EGTA) drastically alters the integrity of the complex (and thus platelet function) in a temperature, time, and pH-dependent manner.

Zucker showed that when platelets were incubated for as little as 5 - 7 minutes in the presence of 5 mM EDTA or EGTA at pH 7.8 they lost their ability to aggregate in response to agonists such as ADP (Zucker & Grant, 1978). As the pH decreased to 7.2 the degree of function loss decreased and as the temperature was reduced

towards 23°C the function loss also decreased.

Fitzgerald showed that the loss of function was due to the effects of EDTA on the $\alpha\text{IIb}\beta 3$ complex (Fitzgerald & Phillips, 1985). They showed, using Western blotting, that the complex existed as a cation-dependent dimer under normal physiological concentrations of calcium (1 – 2 mM) and that concentrations as low as 1 - 10 μM were sufficient to maintain the complex. Exposure of the complex to 0.5 mM EDTA for <10 mins was shown to dissociate the complex but the complex could re-associate in the presence of excess Ca^{2+} or Mn^{2+} . However, exposure to EDTA for ≥ 30 mins resulted in irreversible dissociation. They also were able to show that at temperatures below 25°C little, or no dissociation took place.

Advances in understanding the involvement of cations in regulation of the $\alpha\text{IIb}\beta 3$ complex were made by Ginsberg (Ginsberg *et al*, 1986). They showed that normal platelets did not express the epitope recognised by the monoclonal antibody (mAb) PMI-1; however, exposure of platelets to 5 mM EDTA (even at 22°C) resulted in binding of this antibody. Thus, they concluded, EDTA was able to induce structural changes in $\alpha\text{IIb}\beta 3$ that were recognised by this antibody. Conversely, Pidard showed that the binding of another mAb, AP-2, was decreased after platelets were treated with EDTA at 37°C, supporting the hypothesis that EDTA induced structural changes in the integrin (Pidard *et al*, 1986). Similar results were obtained with a human isoantibody against $\alpha\text{IIb}\beta 3$ (Rosa *et al*, 1984).

A possible explanation for the irreversible nature of the dissociation observed at 37°C was provided by Rivas who observed that, following dissociation at 37°C, the α -chains formed disulphide bonded homopolymers, whilst the β -chains formed non-covalent homopolymers thus preventing heterotypic re-association (Rivas *et al*, 1991).

1.3.5 Homology with viral proteins and target for viruses

Autoantibodies against $\alpha\text{IIb}\beta 3$ can be detected in the serum of patients with autoimmune thrombocytopenia (AITP) (Woods, Jr. *et al*, 1984; McMillan *et al*, 2001). There is some evidence to suggest that these may develop as a result of exposure to glycoproteins present on viruses (Boughton *et al*, 1995). In support of this, the PSI domain of $\beta 3$ was observed to contain sequences similar to those found on hepatitis C virus, and antibodies found in individuals with hepatitis C infection have been shown to react with both virus and platelets (Zhang *et al*, 2009).

Integrins $\alpha\text{IIb}\beta 3$ and $\alpha\text{v}\beta 3$ are receptors used by a number of viruses e.g. adenovirus, echovirus and rotaviruses to gain entry into cells (Switala-Jelen *et al*, 2004). Interestingly, the pathogenic Hantaviruses e.g. Hantaan, Sin Nombre, and Seoul viruses that are able to increase vascular permeability and impair platelet function target $\beta 3$, whilst non-pathogenic Hantaviruses e.g. Prospect Hill, fail to recognise $\alpha\text{IIb}\beta 3$ but bind instead to $\alpha 5\beta 1$ (Gavrilovskaya *et al*, 1998; Gavrilovskaya *et al*, 1999), suggesting that binding of virus affects signalling through $\alpha\text{IIb}\beta 3$. Work by Raymond showed that the pathogenic strains bound to the $\beta 3$ PSI domain which, as described above, undergoes significant movement during platelet activation (Raymond *et al*, 2005). Thus, the observation that binding of virus affected platelet function supports the hypothesis that impairment of PSI domain movement affects platelet activation. A further observation of note is that the epitope recognised by Andes virus is the Pro-33 (HPA-1b) form of $\beta 3$ and the virus is unable to bind to the Leu-33 (HPA-1a) form (Matthys *et al*, 2010). Thus the PSI domain of $\beta 3$ is a key domain in a number of pathologies.

1.4 Polymorphisms of platelet integrins

A large number of mutations of integrin genes have been described. These may result in platelet membrane abnormalities, or in the production of alloantibodies

against alternate forms of the protein following exposure to that protein via pregnancy or blood transfusion.

1.4.1 Non-alloantigenic, pathologic polymorphisms

The inheritance of two mutant genes i.e. recessive Medelian inheritance, coding for proteins within the $\alpha\text{IIb}\beta 3$ or GPIb/IX/V complexes may result in functionally-defective or reduced expression of complexes at the platelet surface and result in the clinical conditions known as Glanzmanns' thrombasthenia and Bernard-Soulier syndrome respectively.

186 mutations within the αIIb gene and 123 within the $\beta 3$ gene have been described (<http://sinaicentral.mssm.edu/intranet/research/glanzmann/menu>; accessed 29th August 2012). Amongst these are mis-sense, nonsense, single nucleotide insertions and deletions and multiple nucleotide deletions. Expression of the mutant integrins on the platelet surface ranges from almost zero to normal depending on the mutation, although the apparent expression level may depend of the specificity of the mAb used to assess expression level (D'Andrea *et al*, 2002; Nurden *et al*, 2011).

Individuals heterozygous for Glanzmanns' mutations have normal platelet function and do not have bleeding symptoms (George *et al*, 1990; Watkins *et al*, 2002a; Nurden *et al*, 2011). Studies of individuals homozygous or compound heterozygous for mutations show that there is no correlation between the subtype (Type I, <5% $\alpha\text{IIb}\beta 3$ expression, Type II, 5-20% or variant-type, >20% expression but with a qualitative defect of $\alpha\text{IIb}\beta 3$) of Glanzmanns' and the clinical bleeding tendency (George *et al*, 1990; Nurden *et al*, 2011).

1.4.2 Alloantigenic polymorphisms

Importantly, a number of mutations have been described, in otherwise normal individuals, that have been shown to be immunogenic. Exposure to these mutations,

or alloantigens, through pregnancy or allogeneic transfusion may result in the production of alloantibodies directed against the mutation. Alloantigens present on platelet integrins are known as Human Platelet Antigens (HPAs) following an internationally agreed protocol (von dem Borne & Decary, 1990)

The majority of the HPAs are located on $\alpha\text{IIb}\beta 3$ (**Table 1.2**); others have been found on integrins GPIb/IX, GPIa/IIa and CD109 (**Table 1.3**). A number of other mutations have been reported that have not yet formally been accepted as HPAs (**Table 1.4**).

Almost all of the HPAs result from a single nucleotide change that results in a single amino acid substitution in the mature protein (**Table 1.2**).

Most HPAs were discovered as a result of immunisation of the mother during pregnancy.

TABLE 1.2. Human platelet antigens on integrin $\alpha\text{IIb}\beta 3$.

HPA	Antigen	Alternative names	Phenotype frequency (%) [*]	Integrin	SNP	Amino acid change	Domain
1	1a	Zw ^a , Pl ^{A1}	97.9	$\beta 3$	T ¹⁹⁶	Leu ³³	PSI
	1b	Zw ^b , Pl ^{A2}	28.8		C ¹⁹⁶	Pro ³³	
	1c		<0.1		G ¹⁹⁵	Val ³³	
3	3a	Bak ^a , Lek ^a	80.95	αIIb	T ²⁶²²	Iso ⁸⁴³	Calf-2
	3b	Bak ^b			G ²⁶²²	Ser ⁸⁴³	
4	4a	Yuk ^b , Pen ^a	>99.9	$\beta 3$	G ⁵²⁶	Arg ¹⁴³	βA
	4b	Yuk ^a , Pen ^b	<0.1		A ⁵²⁶	Glu ¹⁴³	
6				$\beta 3$	G ¹⁵⁶⁴	Arg ⁴⁸⁹	EGF1-2
	6bw	Ca ^a , Tu ^a	0.7		A ¹⁵⁶⁴	Glu ⁴⁸⁹	
7				$\beta 3$	C ¹²⁶⁷	Pro ⁴⁰⁷	Hybrid
	7bw	Mo ^a	0.2		G ¹²⁶⁷	Ala ⁴⁰⁷	
		Hit ^a	Unknown		T ¹²⁶⁷	Ser ⁴⁰⁷	
8				$\beta 3$	T ²⁰⁰⁴	Arg ⁶³⁶	$\beta\text{-TD}$
	8bw	Sr ^a	<0.01		C ²⁰⁰⁴	Cys ⁶³⁶	
9				αIIb	G ²⁶⁰³	Val ⁸³⁷	Calf-2
	9bw	Max ^a	0.6		A ²⁶⁰³	Met ⁸³⁷	
10				$\beta 3$	G ²⁸¹	Arg ⁶²	Hybrid
	10bw	La ^a	<1.6		A ²⁸¹	Glu ⁶²	
11				$\beta 3$	G ¹⁹⁹⁶	Arg ⁶³³	$\beta\text{-TD}$
	11bw	Gro ^a	<0.25		A ¹⁹⁹⁶	His ⁶³³	
14				$\beta 3$			$\beta\text{-TD}$
	14bw	Oe ^a	<0.17		$\Delta\text{AAG}^{1929-1931}$	ΔLys^{611}	
16				$\beta 3$	C ⁵¹⁷	Thr ¹⁴⁰	βA
	16bw	Duv ^a	<1		T ⁵¹⁷	Iso ¹⁴⁰	
17				$\beta 3$	C ⁶²²	Thr ¹⁹⁵	βA
	17bw	Va ^a	<0.4		T ⁶²²	Met ¹⁹⁵	
19				$\beta 3$	A ⁴⁸⁷	Lys ¹³⁷	βA
	19bw	Sta	<1		C ⁴⁸⁷	Glu ¹³⁷	
20				αIIb	C ¹⁹⁴⁹	Thr ⁶¹⁹	Calf-1
	20bw	Kno	<1		T ¹⁹⁴⁹	Met ⁶¹⁹	
21				$\beta 3$	G ¹⁹⁶⁰	Glut ⁶²⁸	$\beta\text{-TD}$
	21bw	Nos	<1		A ¹⁹⁶⁰	Lys ⁶²⁸	
22				αIIb	A ⁵⁸⁴	Lys ¹⁶⁴	$\beta\text{-propeller}$
	22bw	Sey	<1		C ⁵⁸⁴	Thr ¹⁶⁴	
23				$\beta 3$	C ¹⁹⁴²	Arg ⁶²²	$\beta\text{-TD}$
	23bw	Hug	<1		T ¹⁹⁴²	Try ⁶²²	
24				αIIb	G ¹⁵⁰⁸	Ser ⁴⁷²	Thigh
	24bw	Cab ^{2a}	<1		A ¹⁵⁰⁸	Asp ⁴⁷²	
26				$\beta 3$	G ¹⁸¹⁸	Lys ⁵⁸⁰	EGF-4
	26bw	Sec ^a	<1		>T ¹⁸¹⁸	Asp ⁵⁸⁰	
27				αIIb	C ²⁶¹⁴	Leu ⁸⁴¹	Thigh
	27bw	Cab ^{3a}	<1		A ²⁶¹⁴	Met ⁸⁴¹	

The location of these HPAs in relation to the domains of $\alpha\text{IIb}\beta 3$ is shown in **Figure**

1.4.

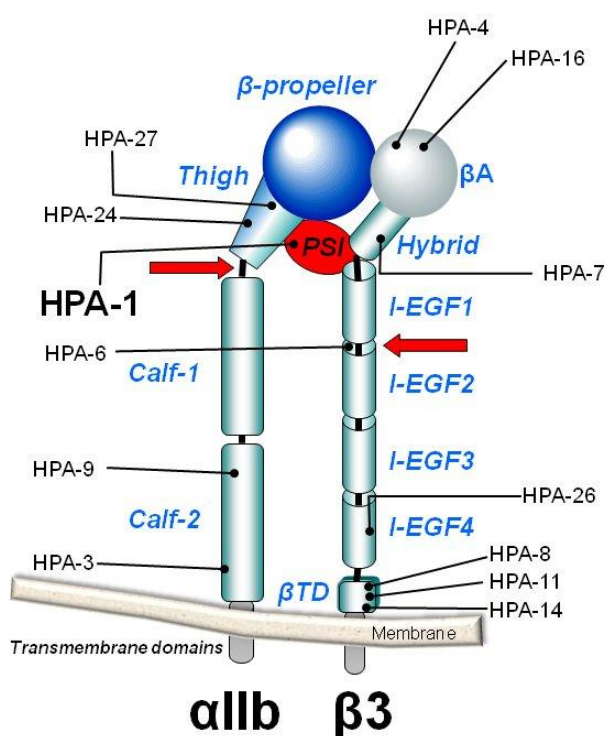
TABLE 1.3. HPAs on other integrins.

HPA	Antigen	Alternative names	Phenotype frequency* (%)	Integrin	SNP	Amino acid change
2	2a	Ko ^b	>99.9	Ib α	C ⁵²⁴	Thr ¹⁴⁵
	2b	Ko ^a , Sib ^a	13.2		T ⁵²⁴	Met ¹⁴⁵
12				Ib β	G ¹⁴¹	Gly ¹⁵
	12bw	ly ^a	0.4		A ¹⁴¹	Glut ¹⁵
5	5a	Br ^b , Zav ^b	99.0	α 2	G ¹⁶⁴⁸	Glut ⁵⁰⁵
	5b	Br ^a , Zav ^a , Hc ^a	19.7		A ¹⁶⁴⁸	Lys ⁵⁰⁵
	5c	Br ^c	Unknown		T ¹⁶⁴⁹	Val ⁵⁰⁵
13				α 2	C ²⁵³¹	Thr ⁷⁹⁹
	13bw	Sit ^a	0.25		T ²⁵³¹	Met ⁷⁹⁹
18				α 2	G ²²³⁵	Glu ⁷¹⁶
	18bw	Cab ^a	<1		T ²²³⁵	His ⁷¹⁶
15	15a	Gov ^b	74	CD109	C ²¹⁰⁸	Ser ⁷⁰³
	15b	Gov ^a	81		A ²¹⁰⁸	Tyr ⁷⁰³
25				α 2	C ³³⁴⁷	Thr ¹⁰⁸⁷
	25bw	Swi ^a	<1		T ³³⁴⁷	Met ¹⁰⁸⁷

TABLE 1.4. Antigenic polymorphisms not yet designated as HPAs.

Antigen	Phenotype frequency* (%)	Integrin	SNP	Amino acid change	Domain
Tib ^a	Unknown	β 3	Unknown	Unknown	Unknown
Mat ^a	<1	β 3	A ⁵²⁰ > G ⁵²⁰	Glu > Arg ¹⁴¹	β A

FIGURE 1.4. Cartoon of α IIb β 3 showing location of HPAs in relation to domains. The molecule is shown in extended, open (activated) configuration for clarity; arrows indicate the location of the genu. Note the location of the HPA-1 polymorphism.



1.4.3 HPA-1a epitopes

The most important HPA is HPA-1a. This was the first antigen to be localised to a specific platelet integrin. In 1978, Kunicki and Aster noted that five patients with Glanzmanns' thrombasthenia had reduced expression of HPA-1a and that the reduction correlated with the degree of expression of $\alpha\text{IIb}\beta\text{3}$ complex (Kunicki & Aster, 1978). From these findings they inferred that the HPA-1a antigen was located on either αIIb or β3 ; subsequent work showed it to be located on β3 (Kunicki & Aster, 1979). Additional findings of this later study were that the antigen was undetectable after heat (100°C) treatment of platelet membranes or after treatment with trypsin or reducing agents such as 2-mercaptoethanol, all of which suggested that the antigen was dependent on a particular conformation of the $\alpha\text{IIb}\beta\text{3}$ complex that was maintained by intact disulphide bonds.

Although Kunicki and Aster had shown that simple sugars such as D-glucose and D-galactose were unable to inhibit the binding of HPA-1a antibodies, the possibility that the epitope was dependent on more complex carbohydrate structures remained (Kunicki & Aster, 1979). Newman used an alternative approach to investigate possible carbohydrate contributions (Newman *et al*, 1985). They used three endoglycosidase enzymes to selectively remove different carbohydrate moieties, and trifluoromethane sulfonic acid to deglycosylate $\alpha\text{IIb}\beta\text{3}$ extracted from platelet membranes. This treatment had no effect on reactivity of HPA-1a antibodies, confirming the findings of Kunicki and Aster. Importantly, they were further able to narrow the location of the epitope to a 17 kD fragment of β3 .

The precise location of the mutation responsible for the HPA-1 polymorphism was determined by Newman following the publication of the nucleotide sequence for β3 cDNA (Fitzgerald *et al*, 1987b; Newman *et al*, 1989). Newman showed that a C to T mutation at position 196 of *ITGB3* resulted in a mutation of Leucine (HPA-1a) to Proline (HPA-1b) in the amino-terminal of mature β3 . The authors suggested that

this simple mutation could result in the HPA-1a antigen by either being directly part of the epitope recognised by antibodies and/or the mutation influenced the secondary structure of $\beta 3$ and the HPA-1a epitope was actually elsewhere in the molecule.

Evidence that conformation of $\beta 3$ (and of the HPA-1a epitope) was important for recognition by HPA-1a antibodies was provided by Flug (Flug *et al*, 1991). Using two 13-mer peptides that straddled and theoretically expressed the HPA-1a and HPA-1b epitopes (SDEALP**L**GSPRCD and SDEALP**P**GSPRCD respectively), they were able to show that the HPA-1a peptide was not recognised by HPA-1a alloantibodies and did not block the binding of HPA-1a antibodies to platelet membranes. These data therefore showed that the amino acid sequence itself was not sufficient to confer HPA-1a specificity.

Flug also reported that the smallest fragment of $\beta 3$ that remained reactive with one particular example of anti-HPA-1a was 55 kD; this differed from the findings of Newman who observed reactivity with a 17 kD fragment (Newman *et al*, 1985). One possible explanation for these differences is that the HPA-1a antibodies used in the two studies were of Type I and Type II, recognising either simple or complex epitopes respectively (see below).

Since synthetic peptides had failed to recreate the HPA-1a epitope, Goldberger attempted to express the epitope in a mammalian (COS-1 cells) expression system using vectors containing cDNA coding for HPA-1a or HPA-1b (Goldberger *et al*, 1991). They successfully showed that the protein expressed by these cells was reactive with examples of anti-HPA-1a or HPA-1b respectively. This provided further support for the premise that conformation of the protein was important since recombinant protein is expressed in a correctly-folded form whereas synthetic peptides, even those containing multiple cysteines, are not (Bodary *et al*, 1989; O'Toole *et al*, 1989).

Bowditch used a prokaryotic expression vector system that was able to express medium-sized (100 - 200 amino acid) fragments of $\beta 3$ to identify a number of fragments that expressed HPA-1a (Bowditch *et al*, 1992). The smallest of these consisted of the 26 aa leader sequence and the first 66 aa's of the mature protein i.e. predominantly PSI domain; this was reactive with four of four examples of anti-HPA-1a. The reactivity of this 66 amino acid fragment with further examples of anti-HPA-1a, and of anti-HPA-1b with a Pro-33 form was confirmed by Barron-Casella (Barron-Casella *et al*, 1994).

Work to date therefore suggested that all examples of anti-HPA-1a recognised similar epitopes. However, Valentin showed quite clearly that this was not the case (Valentin *et al*, 1995). Using site-directed mutagenesis to mutate a number of cysteine residues near to, or remote from, the amino terminus of $\beta 3$ to alanine they made a number of important observations.

Firstly, mutation of Cys-435 (between hybrid and EGF-1 domains) to alanine, a mutation predicted from the above data not to impact on reactivity of HPA-1a antibodies, resulted in complete loss of reactivity of two of eight examples of anti-HPA-1a. Two other examples showed no difference in reactivity compared to native $\beta 3$ and the remaining four exhibited some loss of reactivity.

Secondly, they showed that the different effects were not related to the immunising event i.e. pregnancy or blood transfusion.

Thirdly, they showed that the examples exhibiting the two extreme effects (i.e. non-reactivity or normal reactivity with Ala-435) were both non-reactive with $\beta 3$ with Cys→Ala mutations near the amino terminus (cysteines 5 [later shown to be 13], 23, 26, and 38) thus confirming earlier work regarding the requirement for correct conformation of this terminus. The authors suggested that there were two categories of HPA-1a antibodies, *Type I*, that recognised an epitope at, or near, Leu-33, whilst

Type II antibodies also required the cysteine-rich core (amino acids 433 - 692 [hybrid, EGF1-4 and β -TD domains]) to be orientated closely to Leu-33. Finally they showed that *Type I* and *Type II* antibodies were able to cross-block each other suggesting that the epitopes were extremely close. This was therefore a landmark paper in establishing that HPA-1a antibodies were variable in their epitope requirements and in demonstrating their requirement for conformationally-correct integrin.

Our knowledge of conformational requirements for the binding of HPA-1a antibodies was extended by Honda (Honda *et al*, 1995a) and Kunicki (Kunicki *et al*, 1995). Honda used chimeric human/Xenopus $\beta 3$ expressed in an insect vector system to demonstrate that the Cys-26 to Cys-38 loop containing the HPA-1a polymorphism required distant sequences (within aa's 288 - 490, β -A, hybrid and EGF domains) of $\beta 3$ to be in proximity to the loop in order for it to be appropriately constrained; they suggested that the putative Cys-5 to Cys-435 disulphide bond played a key role in this. Interestingly they observed that the mAb SZ21, which recognises an epitope spanning aa's 28 - 35 and binds preferentially to HPA-1a positive platelets, was able to bind to the Cys-26 to Cys-38 loop regardless of other $\beta 3$ sequences suggesting that this antibody recognised a very simple epitope. Further chimeric $\beta 3$ constructs, consisting of segments of human, avian and Xenopus sequences (all of which differ in sequence to a varying extent), were used by Kunicki to show that the distal sequences that played a role in orienting the Cys-26 to Cys-38 loop were amino acids 435 - 490 (EGF-1 and EGF-2 domains) (Kunicki *et al*, 1995). Kunicki also identified that a more proximal sequence (aa's 53 - 133, within hybrid and β -A domains) was required for correct orientation of the loop. An unexpected finding from this latter study was that one example of PTP-derived anti-HPA-1a was identified that contained a sub-population of antibodies that, whilst still HPA-1a specific, reacted only with chemically-denatured (SDS-treated) $\alpha IIb\beta 3$ and not with whole platelets or purified $\alpha IIb\beta 3$; the clinical importance of this type of antibody was unknown but it

clearly demonstrated the heterogeneity of HPA-1a antibody specificities.

Further support for the hypothesis of Valentin regarding the existence of two types of HPA-1a epitopes was provided by Liu (Valentin *et al*, 1995; Liu *et al*, 1996). This group had previously produced a mAb (LK-4) that was able to differentiate lysates prepared from HPA-1a1a platelets from those prepared from HPA-1b1b platelets, although in flow cytometric assays it bound equally well to both. They showed that this antibody was able to block the binding of nine of nine examples of anti-HPA-1a to both recombinant $\beta 3$ (aa's 1 - 66) and to platelets. The IC_{50} values for blocking of binding to the recombinant protein were in the range 8 - 160 $\mu\text{g/mL}$. However, when using platelets, six of the nine examples had values of 1 - 10 $\mu\text{g/mL}$ and three had values ten to 100-fold higher (380 - 1,013 $\mu\text{g/mL}$). The different platelet IC_{50} data therefore suggested that there were two types of HPA-1a antibody and the difference in reactivity of the HPA-1a antibodies against native $\alpha\text{IIb}\beta 3$ versus recombinant $\beta 3$ supported this theory.

Barron-Casella, using murine $\beta 3$ in which amino acids were substituted one at a time to the human $\beta 3$ sequence, showed that within the 66 amino acid fragment that had previously shown to be the smallest fragment reactive with HPA-1a antibodies there seemed to be a core target structure comprised of amino acids 9 - 50 (Barron-Casella *et al*, 1999). Within this smaller fragment there were key amino acids (30, 32, 33, and 39), the deletion of which significantly affected binding of HPA-1a Type I antibodies. However, they also showed that amino acid sequences at the beginning and end of this smaller fragment had some importance to the HPA-1a epitope since removal of these sequences reduced antibody binding by 30%.

Recently, Stafford used recombinant soluble $\beta 3$ proteins in which various domains were deleted to determine which domains were critical for recognition by a large panel of human polyclonal HPA-1a antibodies and, in conjunction with studies of

canine platelets, to determine precise epitopes of three human monoclonal HPA-1a antibodies (Stafford *et al*, 2008). This study provided a large amount of informative data. Not only did it again confirm the existence of Type I and Type II antibodies, but it showed that, unlike the study of Valentin, Type I antibodies were present in approximately 75% cases of HPA-1a mediated NAIT (Valentin *et al*, 1995). An interesting finding was that deletion of the whole β -A domain rather than part of the domain **increased** sensitivity by 6%, suggesting that perhaps access to HPA-1a epitopes was partially masked by the β -A domain. This β -A domain-deleted peptide (' $\Delta\beta$ -A Leu-33') was selected as having the most sensitivity (96%) and specificity (95%) for the detection of HPA-1a antibodies and was used as the candidate protein in further studies using the high-throughput Luminex assay (see below and **Chapter 5**). Lastly, this study was able to show that the three human anti-HPA-1a mAbs used in this project have different fine specificities. CamTran007 (also known as 'B2') was shown to be a Type II antibody, requiring correct orientation of multiple β 3 domains. Two other clones, '19-7' and '23-15' were shown to be Type I and required only the presence of the PSI domain. However, the epitopes recognised by these latter two antibodies were not identical, as shown by their reactivity with canine platelets.

Since Type II HPA-1a antibodies are dependent on amino acid contributions other than Leu-33 for correct orientation of the HPA-1a epitope, the possibility of identifying mutant α IIb β 3 molecules when using such reagents as phenotyping reagents was a possibility. In 2002, The Oxford Platelet Immunology Laboratory, during routine use of a semi-quantitative HPA-1a typing ELISA assay which used the Type II anti-HPA-1a 'B2,' identified a blood donor who had a HPA-1a1b genotype but whose platelets expressed HPA-1a at a much lower level than 'normal' HPA-1a1b controls (Watkins *et al*, 2002a) despite having normal levels of α IIb β 3. Subsequent analysis showed an Arg-93 $\rightarrow$ Gln-93 mutation of β 3. This novel finding showed the critical importance of the hybrid domain for expression of HPA-1a epitopes.

A similar case was reported by Jallu who phenotyped the mother in a NAIT case as HPA-1b1b but molecular typing showed her to be HPA-1a1b (Jallu *et al*, 2005). Sequencing of *ITGB3* showed a T→C mutation at position 1447, resulting in a Cys-457 → Arg-457 mutation in the mature protein. The authors showed that the mothers' platelets expressed only 50% of the normal amount of $\alpha\text{IIb}\beta_3$; however, they did not state how they arrived at this figure and it is possible that use of other mAb clones may have shown different results.

Furthermore, Santoso identified an individual with a Leu-33 → Val-33 mutation (Santoso *et al*, 2006). Testing of 40 samples containing anti-HPA-1a showed that whilst 29 of these failed to react with heterologously expressed β_3 containing this mutation, 11 samples were reactive (six equally strongly and five less reactive than with Leu-33). This study clearly showed that a HPA-1a-like epitope was formed by Val-33. and that this mutation occurred in approximately 1 in 3,000 individuals.

An interesting case of phenotype/genotype discrepancy was reported by Smith (Smith *et al*, 2007). In this particular case, the mother of an infant diagnosed with NAIT did not express HPA-1a on her platelets despite having an *HPA-1a* gene and had also made anti-HPA-1a. Sequencing studies showed that she had a single nucleotide insertion in exon 10 of *ITGB3*, leading to a frame-shift and premature termination at amino acid 471 (within EGF-1 domain). The authors concluded that this stop codon resulted in loss of the transmembrane domain and/or production of an unstable complex that did not express HPA-1a.

All of these data highlight that the HPA-1a epitope is far more complex than originally thought.

1.5 Techniques for the detection of alloantibodies against platelet polymorphisms

Numerous techniques for the detection of platelet antibodies have been described.

These can conveniently be divided into techniques that are intended to detect antibodies and which utilise intact platelets or whole solubilised membranes, and those that are utilised to both detect and identify such antibodies.

The correct choice of techniques is fundamental to the sensitive detection of antibodies and use of inappropriate or poorly performed assays may lead to erroneous conclusions regarding antibody incidence and potency.

1.5.1 Techniques I: methods using whole, intact platelets

The earliest reports of the detection of platelet antibodies were by Ackroyd who described the detection of drug-dependent antibodies by direct platelet agglutination and complement-fixation/platelet lysis techniques in a series of reports (Ackroyd, 1949a; Ackroyd, 1949b; Ackroyd, 1949c; Ackroyd, 1951).

The first use of the platelet agglutination test to investigate platelet alloantibodies was by Harrington who, using samples from 110 multi-transfused patients, described the existence of eight platelet groups (Harrington *et al*, 1953). Similar results using the agglutination assay were reported by Stefanini who concluded that there were six platelet groups (Stefanini *et al*, 1953). Current knowledge would suggest that the platelet groups were probably HLA class I antigens that can also be detected on platelets. The use of agglutination by van Loghem led to the discovery of the first truly platelet-specific alloantigen, named Zw^a by the authors and now known as HPA-1a (Van Loghem *et al*, 1959). This assay was also used to identify the antithetical antigen, Zw^b (HPA-1b) (van der Weerdt *et al*, 1963).

However, both agglutination and complement fixation assays suffered from the disadvantages that not all platelet-specific antibodies were able to fix complement or were directly agglutinating. To overcome these problems, indirect assays using a secondary, anti-human immunoglobulin reagent were developed e.g. the Platelet Radioactive Antiglobulin Test and the Indirect Platelet Suspension

Immunofluorescence Test (PSIFT) (van Boxtel *et al*, 1973; van Boxtel & Engelfriet, 1973; Soulier *et al*, 1975; von dem Borne *et al*, 1978; Mueller-Eckhardt *et al*, 1978a; Mueller-Eckhardt *et al*, 1978b). Comparison of the sensitivity of these assays showed that the PSIFT was the most sensitive (Helmerhorst *et al*, 1980; von dem Borne *et al*, 1981).

This assay is now the most widely used 'whole platelet' method in platelet immunology reference laboratories across Europe (Allen *et al*, 2007) although in Japan, for reasons that are not apparent, the mixed passive haemagglutination assay is the predominant method (Shibata *et al*, 1981; Fujiwara *et al*, 2009).

However, all of the above methods that utilise whole platelets are subject to false-positive results arising from the presence of blood group A and B, and HLA class I antigens on the platelet surface that can mask reactivity due to HPA alloantibodies.

The problem of false reactivity due to the presence of A and B antigens may be simply overcome by the use of blood group O platelets as screening cells, but cross-matching of paternal platelets against maternal serum/plasma samples that may be ABO-incompatible may require pre-absorption of the maternal sample with washed, platelet-free red cells. The presence of HLA class I antibodies is more problematic since such antibodies are present in up to approximately 40% of pregnant women (Skacel *et al*, 1989; Taaning & Skibsted, 1990; Regan *et al*, 1991; Dankers *et al*, 2003) and may complicate the investigation of suspected NAIT in such cases. A number of methods to overcome this problem have been described.

Blumberg (Blumberg *et al*, 1984) and Nordhagen and Flaathen (Nordhagen & Flaathen, 1985) used chloroquine to remove HLA class I molecules from the platelet surface. This technique was reported to enhance reactivity of HPA-1a (and HPA-3a) antibodies but resulted in significant cell death.

Kurata showed citric acid was superior to chloroquine and resulted in minimal cell death and, importantly, retention of the ability to detect platelet specific antibodies (Kurata *et al*, 1989; Kurata *et al*, 1990). However, other studies came to differing conclusions and subsequently it was shown that citric acid treatment of platelets had a detrimental effect on detection of HPA-5 antibodies (Decary, 1988; Lucas, 1990; Lown & Robinson, 1995). Thus, the differentiation of platelet-specific from HLA class I antibodies remained problematic when using intact platelets.

1.5.2 Techniques II: methods using solubilised membranes

In 1978, Kunicki and Aster used a combination of enzyme digestion, affinity chromatography and indirect radio-immunoprecipitation to identify that the HPA-1a antigen was on $\beta 3$ (Kunicki & Aster, 1978; Kunicki & Aster, 1979). This work was important in that it was the first time that a platelet alloantigen had been localised to a membrane protein and it provided a novel method for separating platelet-specific antibody reactivity from that of HLA class I antibodies. However, the use of such techniques was clearly impractical for use in routine detection of platelet alloantibodies.

McMillan developed a simplified immuno-chemical procedure, namely SDS-PAGE; this was an improvement on that used by Kunicki and Aster but it was still time-consuming and unsuited to large-scale studies (McMillan *et al*, 1982); .

Although ELISA assays had been used by a number of workers for the detection of platelet allo- and autoantibodies and for platelet cross-matching, these assays, like the PSIFT, detected both platelet-specific and HLA class I antibodies (Doughty *et al*, 1981; Gudino & Miller, 1981; Schiffer & Young, 1983; Tamerius *et al*, 1983; Taaning, 1985). Collins and Aster therefore developed an ELISA assay that utilised membrane proteins extracted from chloroquine-treated platelets (Collins & Aster, 1987). This assay simultaneously overcame the problems of complexity, speed of

performance, and of separation of platelet-specific from HLA class I reactivity. A further advantage was that it was up to two orders of magnitude more sensitive than the PSIFT. However, a major disadvantage was that it used membrane lysates that contained a range of proteins and antibody identification of mixtures of HPA-specific antibodies in such a pool of proteins potentially could be problematic.

1.5.3 Techniques III: glycoprotein-specific methods

Since HLA-stripping methods had not proved entirely reliable and use of solubilised whole platelet membranes did not easily resolve complex mixtures of antibodies, alternative methods that utilised immobilised purified, or individually captured, glycoproteins were developed.

In the USA, the antigen capture ELISA (ACE) and modified antigen capture ELISA (MACE) were developed. In the ACE assay, mAbs against platelet glycoproteins are immobilised on to wells of microtitre plates and used to capture specific glycoproteins from solubilised platelet membranes; human antibodies are then detected using an enzyme-labelled anti-human immunoglobulin and substrate system.(Woods, Jr. *et al*, 1984; Furihata *et al*, 1987).

The MACE assay, together with a similar assay utilising polystyrene microbeads, the 'protein immunobead assay', was developed to overcome the presence of anti-mouse antibodies, present in up to 40% of patient samples that could react with the mAbs used in the ACE assay and cause false-positive reactions (McMillan *et al*, 1987; Menitove *et al*, 1989; Hewitt & Burton, 1994; Clofent-Sanchez *et al*, 1996; Joiner *et al*, 1997; Klee, 2000; Leach & Aubuchon, 2003)

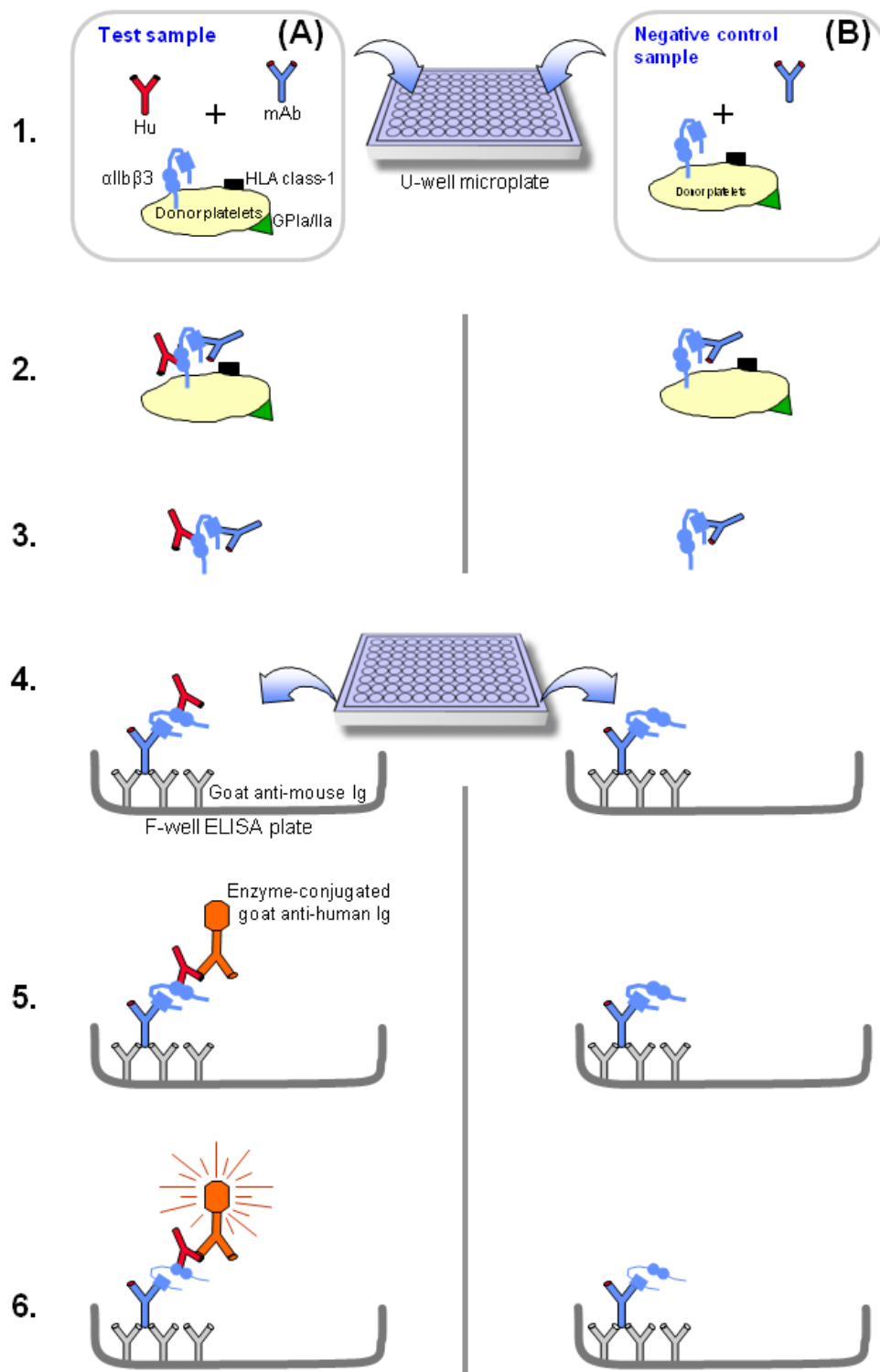
One interesting observation from the study of McMillan was that the incidence of platelet autoantibodies detected by the protein immunobead assay was more than double that identified by the ACE assay (McMillan *et al*, 1987). The authors concluded that such antibodies recognised epitopes on glycoproteins that changed

conformation when the glycoprotein was extracted from the platelet membrane and thus were not recognised by mAbs in the ACE assay.

Despite the ACE assay being shown to be relatively insensitive, giving low signal-to-noise ratios and prone to interference from anti-mouse antibodies, a commercial form of the assay was developed and is widely used in various formats today (Kiefel, 1992). Although the assay has been found to be rapid (Kaye & Allen, 1994; Kowalski *et al*, 1995d), each of the kit variants ('PAK-PLUS', 'PAK-1' and 'PAK-2') has been shown to have <100% sensitivity (Joiner *et al*, 1995; Kowalski *et al*, 1995c; Boehlen *et al*, 1998; Allen & Murphy, 1999). Furthermore, the problem of anti-mouse antibodies remained (Leach & Aubuchon, 2003). Thus, the use of purified, immobilised glycoproteins such as those used in these commercial kits can lead to false positive or false negative results and this has clearly been demonstrated in collaborative workshops (Porcelijn *et al*, 2008).

In 1987, Kiefel published a landmark paper describing a new assay, the Monoclonal Antibody Specific Immobilisation of Platelet Antigens (MAIPA) assay that has revolutionised detection and identification of platelet-specific antibodies, particularly when present as a mixture of specificities (Kiefel *et al*, 1987). In this assay, which is similar to the MACE assay, platelet glycoproteins are specifically captured from detergent-solubilised membranes using mAbs against the glycoprotein of interest, the main difference with MACE being that the mAbs are incubated with the platelets whilst they are intact. This assay has become the most important technique for differentiating platelet-specific and HLA class I antibodies in Europe and has become the gold standard glycoprotein capture method (Kaplan *et al*, 2007). The principle of the assay is shown in **Figure 1.5**.

FIGURE 1.5. Principle of the MAIPA assay. (1) Target platelets are sequentially incubated with test serum being investigated then, after washing of the platelets, with murine monoclonal capture antibody (blue cartoon antibody) directed against the glycoprotein being studied, e.g. $\alpha\text{IIb}\beta\text{3}$ (blue molecule). In (A) the test serum contains anti-HPA-1a (indicated by the red cartoon of an antibody) and in (B) no platelet specific alloantibodies are present. (2) After the second incubation a trimeric (A) or dimeric (B) complex is formed. (3) After a further wash phase the platelet membrane is solubilised in a non-ionic detergent releasing the trimeric (A) or dimeric (B) complexes into the fluid phase. (4) Particulate matter is removed by centrifugation then the lysates containing the complexes are added to the wells of a micro-ELISA plate previously coated with goat anti-mouse IgG antibody (grey antibody). (5) Unbound lysate is removed by washing and an enzyme-conjugated goat anti-human antibody added (red antibody). (6) Excess conjugate is removed by washing and a substrate solution added. Cleavage of the substrate, i.e. a colour reaction indicates binding of human antibody to the target platelets.



Adapted from: Allen *et al* (Allen *et al*, 2009).

Since publication of the original method a number of limitations of the assay and possible solutions to overcome them have been reported.

Firstly, it was observed that not all mAbs were suitable for use as capture mAbs and false-negative results could be obtained if the mAb was not carefully selected. Lown observed that mAbs against αIIb were generally more effective than anti- $\beta 3$ mAbs at capturing $\alpha IIb\beta 3$ and allowing detection of anti-HPA-1a antibodies (Lown *et al*, 1993). Morel-Kopp showed that false-negative autoantibody tests could occur due to blocking of the epitope recognised by the human antibody by the capture mAb and/or due to accessibility of the epitope recognised by the mAb (Morel-Kopp & Kaplan, 1993a).

Porcelijn demonstrated that some alloantibodies might fail to be detected when certain capture mAbs were used; he showed that 'Y2/51' inhibited binding of Mo^a (HPA-7bw), and Tib^a alloantibodies, 'VI-PL2' blocked binding of Tib^a antibodies and 'MBIX' blocked binding of Max^a (HPA-9bw) antibodies (Porcelijn *et al*, 1996). In two large international quality assurance exercises in which platelet alloantibodies were distributed, the choice of capture mAb was shown to have a significant effect on assay sensitivity (Allen *et al*, 1994; Wu *et al*, 2010). These data have led to the publication of a statement from the Working Party on Platelet immunology of the International Society of Blood Transfusion regarding selection and validation of reagents used in the MAIPA assay (Kaplan *et al*, 2007).

Secondly, as with the ACE assay, the presence of 'anti-mouse' antibodies could cause false-positive results when using the original method that simultaneously incubated the test sample, capture mAb and platelets. Two methods to overcome this problem have been reported. Morel-Kopp pre-incubated test sample with mouse IgG-coated agarose beads and then used the adsorbed sample in the MAIPA assay (Morel-Kopp & Kaplan, 1993b). An equally successful solution that has been widely

adopted is to sequentially incubate test sample with platelets then, (after washing the platelets), with mAb so that the human IgG anti-mouse antibodies in the test sample do not come into contact with the murine mAbs (Kiefel, 1992).

The MAIPA assay, in addition to being able to determine which glycoprotein is the target of platelet alloantibodies, is also more sensitive than the other widely used assay, the PSIFT (Dawkins, 1993; Allen *et al*, 1994; Porcelijn *et al*, 2008). This is particularly true for antibodies directed against membrane proteins that are present at very low copy number per platelet e.g. GPIa/IIa (HPA-5, HPA-13bw, HPA-18bw, HPA-25bw) and CD109 (HPA-15) which are present at around 1,000-2,000 copies per platelet, and failure to use the MAIPA assay may result in failure to detect clinically significant antibodies (Kiefel *et al*, 1988; Kiefel *et al*, 1989; Sutherland *et al*, 1991; Allen *et al*, 1992).

Other improvements to MAIPA assay sensitivity that have been described include use of alternative enzyme/substrate systems (Kiefel, 1992) and increased test sample volume to platelet number ratio (Porcelijn *et al*, 1994). The most recent improvement has been the reduction in assay time; this modified assay was evaluated in a number of laboratories and found to be as sensitive as the original version (Campbell *et al*, 2007). However, the use of this standardised method, together with key reagents supplied in an international workshop exercise did not eliminate inter-laboratory variation in the ability to detect platelet alloantibodies, suggesting that there are other, as yet unidentified factors, that influence assay sensitivity (Foxcroft *et al*, 2007).

1.5.4 Techniques IV: protein bead arrays

As discussed above, microbeads have successfully been used in place of ELISA plates in immunoassays for platelet antibodies for over 25 years. The use of bead arrays however is a more recent innovation and was first used for the detection of

HLA class I antibodies (Pei *et al*, 1999; Pei *et al*, 2003).

In the first described use of this assay for detection of platelet-specific antibodies, microbeads coated with rat anti-mouse IgG were used to capture human glycoproteins from platelets by means of mouse anti-human glycoprotein mAbs of different isotypes (Nguyen *et al*, 2004). The use of different antibody isotypes and of anti-IgG and anti-IgM conjugates permitted the simultaneous detection of six different antibody specificity/antibody class combinations. This assay, 'simultaneous analysis of specific platelet antibodies' (SASPA), showed good concordance with the MAIPA assay when alloantibodies were investigated but a large number of autoantibodies were not detected by SASPA (Nguyen *et al*, 2010).

Clearly, a limitation of this assay in terms of scaling up this assay to analyse multiple glycoproteins was the number of isotypes of IgG available. The Luminex® platform utilises two different dyes, combined at a range of concentrations within microbeads, to provide up to 500 simultaneous analyses. Chong used this system to couple recombinant $\beta 3$ fragments expressing HPA-1a or HPA-1b antigens to microbeads to screen a panel of 40 samples containing HPA and/or HLA class I antibodies (Chong *et al*, 2011). No false-positive results were obtained, all examples of anti-HPA-1a and HPA-1b were detected and titration studies showed that the assay was potentially an order of magnitude more sensitive than the MAIPA assay. Thus this system potentially allows rapid, sensitive and extensive testing for platelet antibodies.

1.5.5 Cell lines

In view of the limitation of assays that use glycoproteins immobilised on to microplates or microbeads with respect to epitope conformation, a number of groups have investigated using cell lines transfected with integrin cDNA to express the glycoproteins of interest since they should be able to express the glycoproteins in a more natural conformation.

Kroll used Chinese hamster ovary (CHO) cell lines to investigate expression of a number of low frequency HPAs whilst Hayashi, in order to avoid interfering reactivity from HLA class I antibodies, used the HLA class I lacking K562 cells to express the high-frequency antigen Nak^a (Kroll *et al*, 2005b; Hayashi *et al*, 2009). This latter cell line was shown to be able to detect antibody levels as low as 0.2 µg/mL. Hayashi was also able to express CD109 in the same cell line at a level 300 - 9,000-fold higher than on platelets and was able to detect HPA-15 antibodies at a level of 0.5 µg/mL (Hayashi *et al*, 2010). Thus, the use of cell lines is a sensitive method to detect alloantibodies against polymorphisms of platelet integrins.

1.6 Clinical conditions resulting from alloantigenic polymorphisms

Platelet antibodies directed against HPAs are causative of a number of clinical conditions.

Patients requiring regular platelet transfusions may fail to obtain the expected increment in platelet count following the development of HPA-specific antibodies, a condition known as refractoriness to platelet transfusion (RPT) (Hod & Schwartz, 2008). Although αIIbβ3 is frequently the target in this condition, the antibody specificity is usually HPA-1b (Schnaidt *et al*, 1996; Kiefel *et al*, 2001; Skouri *et al*, 2005; Fontao-Wendel *et al*, 2007). HPA-1a antibodies have been detected in multi-transfused patients (Uhrynowska & Zupanska, 1996; Kiefel *et al*, 2001) but only one case of RPT caused by HPA-1a antibodies has been documented (Pappalardo *et al*, 2001).

HPA-specific antibodies are responsible for the rare (in the UK) condition post-transfusion purpura (PTP). In this condition, which can occur after the transfusion of a range of blood products, not only are the transfused allogeneic platelets destroyed but also the recipients' own platelets (Taaning & Svejgaard, 1994). The precise

mechanism for this remains unclear although it has been shown that the transfusion provokes not only an increase in levels of previously existing alloantibody but also the development of autoantibodies against $\alpha\text{IIb}\beta\text{3}$ (Watkins *et al*, 2002b). Most cases of PTP have been caused by HPA-1a antibodies (Taaning & Svejgaard, 1994).

A clinically similar, but less severe, condition is caused by the passive transfusion of HPA-specific antibodies by plasma-containing blood products into an antigen-incompatible recipient; most cases have also been caused by HPA-1a antibodies (Price *et al*, 1999; Solenthaler *et al*, 1999; Kiefel *et al*, 2001).

HPA-specific antibodies have also been implicated in adverse events following transplantation. Panzer (Panzer *et al*, 1989) and Lucas (Lucas *et al*, 2010) reported cases where HPA-1b1b recipients of an allogeneic bone marrow transplant from a HPA-1a positive donor failed to achieve normal platelet counts for 10 months and over a year post-transplantation respectively, due to the suppression of the engrafted megakaryocytes by recipient antibodies; the recipients in the paper by Lucas *et al* were also refractory to platelet transfusion.

HPA-mismatch between donor and recipient may affect bone marrow graft survival (Balduini *et al*, 1999; Juji *et al*, 1999), whilst mismatch for HPA-4 or HPA-5 is implicated in rejection of solid organ grafts (Kekomaki *et al*, 1997; Kekomaki *et al*, 2001; Tsuno *et al*, 2012). A further complication of solid organ transplantation arises from the transfer of donor lymphocytes that may be capable of producing HPA-specific antibodies and causing RPT (West *et al*, 1999).

In a mouse model, Li *et al* showed that antibodies against platelet GPIb were able to cause recurrent miscarriage although these findings have not been confirmed in humans (Li *et al*, 2011).

Most importantly from the point of view of this project, the development of HPA-

specific antibodies can result in the condition known as neonatal alloimmune thrombocytopenia (NAIT), also known as feto-maternal alloimmune thrombocytopenia (FMAIT) and fetal-neonatal alloimmune thrombocytopenia (FNAIT).

1.7 NAIT

1.7.1 Cause

NAIT is caused by the development and subsequent transplacental transfer of maternal IgG antibodies against HPAs to the circulation of a fetus that has an HPA inherited from the father that is different to that of the mother. The antibody-coated platelets are removed from the circulation by the fetal reticulo-endothelial system; this can result in a variable degree of thrombocytopenia depending on a number of factors including the ability of the fetal marrow to compensate. Since fetal platelets express HPAs as early as 16 weeks gestation the condition may occur very early in pregnancy (Gruel *et al*, 1986).

1.7.2 Relative importance as a cause of neonatal thrombocytopenia

Thrombocytopenia (platelet count $<150 \times 10^3/\mu\text{L}$) occurs in 1 - 5% of infants at birth (Dreyfus *et al*, 1997; de Moerloose *et al*, 1998; Sainio *et al*, 2000). The incidence is increased in infants with complications arising from birth and in low-birthweight pre-term infants (Christensen *et al*, 2006; Sola-Visner *et al*, 2008).

It can occur as a result of physiological, infective, genetic, autoimmune or alloimmune causes and the timing of the onset of thrombocytopenia can be of help in determining the likely cause (Holzhauer & Zieger, 2011; Chakravorty & Roberts, 2012). The degree of thrombocytopenia is also useful in assigning a potential cause. Although severe isolated thrombocytopenia (platelet count $<50 \times 10^3/\mu\text{L}$) at birth is uncommon (0.1 - 0.2% of births) (Burrows & Kelton, 1993; Dreyfus *et al*, 1997; de

Moerloose *et al*, 1998; Sainio *et al*, 2000) the majority of such cases are due to alloimmunisation against HPAs (de Moerloose *et al*, 1998).

1.7.3 Unusual presentations of NAIT

Most cases of NAIT are detected post-delivery as a result of clinical observations of haemorrhagic symptoms such as purpura and petechiae, or are diagnosed incidentally following routine blood count. However, there have been a number of case reports of NAIT being diagnosed following observation of intracranial haemorrhage or hydrops fetalis during ultrasound scan (Montemagno *et al*, 1994; Murphy *et al*, 1996; Stanworth *et al*, 2001; Pova *et al*, 2007).

1.7.4 Antibody specificities

Despite the existence of many different antigenic SNPs of platelet glycoproteins (**Tables 1.2 – 1.4**), in Caucasians the overwhelming majority of cases of NAIT are caused by just two specificities, HPA-1a and HPA-5b, which account for approximately 75 - 80% and 10 - 20% cases respectively (Mueller-Eckhardt *et al*, 1989; Davoren *et al*, 2004; Ghevaert *et al*, 2007b). Although many case reports of NAIT due to HLA class I antibodies have been published, whether such antibodies are truly responsible or whether undetected HPA-specific antibodies are responsible is unclear (Tanaka *et al*, 2000; Thude *et al*, 2006; Gramatges *et al*, 2009; Starcevic *et al*, 2010). Rare cases due to ABO incompatibility have been reported (Curtis *et al*, 2008).

1.7.5 Genetic factors

Immunisation against the HPA-1a antigen has an extremely strong association with the presence of the HLA class II antigen HLA-DRB3*01:01 in the mother (Valentin *et al*, 1990). Williamson reported that 45 of 46 women who developed anti-HPA-1a during pregnancy were positive for this allele (odds ratio, OR, 140) and typing for DRB3*01:01 therefore had a high negative predictive value (99.6%) (Williamson *et al*,

1998). However, the positive predictive value was much lower (35%). In a much larger prospective antenatal study of almost 2,000 HPA-1b1b women, Killie showed that approximately 10% of women that developed HPA-1a antibodies lacked HLA DRB3*01:01 but the antibody levels in these women were lower (0.4 ± 0.3 i.u./mL) than those in women positive for HLA DRB3*01:01 (mean 10.3 ± 3.6 i.u./mL) (Killie *et al*, 2008). Recently, it has been shown that maternal compound heterozygosity for HLA DRB3*01:01 and DRB4*01:01 was associated with more severely affected infants (OR >700) (Loewenthal *et al*, 2012).

Genetic associations between the development of antibodies of specificities other than HPA-1a and other genetic markers are less clear. Semana showed that the immune response against HPA-5 antigens is linked to a range of HLA DR molecules that share the amino acid sequence Glu-Asp at positions 69 – 70, whilst Westman found an increased incidence of HLA-DRB1*15:01 in mothers with anti-HPA-6b compared to the general population although this did not reach statistical significance (Semana *et al*, 1996; Westman *et al*, 1997).

The development of HPA-1a antibodies has been shown to be unrelated to maternal ABO blood group (von dem Borne *et al*, 1981; Ahlen *et al*, 2012). However in the latter study of HPA-1a immunised mothers, the maternal ABO type was shown to be associated with the risk of severe NAIT in the infant. Mothers that were blood group O had a significantly lower risk of having a child with a platelet count $<50 \times 10^3/\mu\text{L}$ than those of blood group A (RR 0.43, CI: 0.25 - 0.75); the mechanisms for this were unclear.

1.7.6 Prospective studies of anti-HPA-1a mediated NAIT

In view of the relative rarity of NAIT, increasingly larger prospective studies have been undertaken in order to elucidate the natural history of anti-HPA-1a mediated NAIT.

Durand-Zaleski screened approximately 2,000 women (Durand-Zaleski *et al*, 1996). 52 women were typed as HPA-1b1b (2.5%) and anti-HPA-1a was detected in four i.e. 1 in 13 HPA-1b1b women or approximately 1 in 500 unselected women. The incidence of NAIT (platelet count $<150 \times 10^3/\mu\text{L}$) was 1 in 1,000.

A landmark study of almost 25,000 women by Williamson and a subsequent similarly sized study by Turner showed a similar incidence of the HPA-1b1b phenotype (1.7-2.5%) and 1 in 8 to 1 in 13 HPA-1b1b women were shown to have detectable anti-HPA-1a (Williamson *et al*, 1998; Turner *et al*, 2005). The incidence of severe thrombocytopenia reported by Williamson was 1 case per 1,100 births whilst moderate thrombocytopenia was more frequent at 1 case per 336 births. Turner reported only the incidence of newly identified cases (1 case per 2,325 births). Thus antibodies against HPA-1a and moderate thrombocytopenia are not infrequent findings during pregnancy.

The largest and most informative studies to date involved HPA-1a typing of over 100,000 women and follow-up of almost 2,000 HPA-1b1b women (Kjeldsen-Kragh *et al*, 2007; Killie *et al*, 2008). These studies provided a comprehensive analysis of the development of antibodies and changes in antibody levels throughout pregnancy.

1.7.7 Relationship between HPA-1a antibody level and pregnancy outcome

Prior to the advent of glycoprotein-specific techniques it was not possible to measure levels of HPA-1a specific antibody because the presence of HLA class I antibodies in a significant proportion of samples would potentially mask or add to the reactivity of HPA-specific antibodies in whole-platelet assays.

The first use of the MAIPA assay to assess HPA-1a antibody levels was by Dawkins who followed antibody levels in a small cohort of women throughout pregnancy (Dawkins, 1995). Using simply the optical density values he was able to show

decreasing levels of antibody through the pregnancy in all women. However, this study was limited by the small number of cases followed.

Williamson performed the first study of sufficient size to have the power to assess the relationship between HPA-1a antibody level and pregnancy outcome (Williamson *et al*, 1998). In this study, maternal samples obtained through pregnancy were titrated and tested in the MAIPA assay. The results showed that a titre of 1/32 or greater obtained during the third trimester of pregnancy was significantly associated with severe neonatal thrombocytopenia (positive predictive value 75%; negative predictive value 88%). They also reported that the presence of transient antibodies or antibodies detectable only in post-delivery samples were not associated with severe thrombocytopenia in the neonate.

Jaegtvik prepared an in-house anti-HPA-1a reference standard that could be used to prepare standard curves against which patient samples could be compared and an activity level derived (Jaegtvik *et al*, 2000). The use of this reference standard resulted in inter-assay and intra-assay variation of <10% and the authors were successfully able to show correlation between maternal antibody level and neonatal platelet count. Bertrand also used an in-house reference standard and investigated a new mathematical approach to analysis of the data with limited success since the new approach was unable to measure antibody levels below 50 AU/mL (equating to approximately 50 i.u./mL) (Bertrand *et al*, 2005).

Both of the above studies had limited applicability elsewhere because of the use of in-house reference standards. The development of a World Health Organisation (WHO) anti-HPA-1a high-potency standard of a designated antibody level (100 i.u./mL) (Allen *et al*, 2005) allowed for the first time, direct comparison of results between laboratories. The use of this reagent would, in theory, reduce variations observed when assessing antibody levels when using manual titrations and thus

potentially improve the predictive abilities of laboratory assays to the level observed in the study of Jaegtvik *et al.* However, the results obtained with three samples of varying HPA-1a antibody levels that were distributed in a National Institute for Biological Standards and Control (NIBSC) organised quality assurance exercise (hereafter referred to as NIBSC workshops) showed that standardisation of the calibration curve did not eliminate inter-laboratory variation and in one of the three samples distributed the reported antibody level ranged from 2 - 128 i.u./mL (Allen *et al.*, 2005).

Further doubts on the usefulness of antibody quantitation as a predictive tool was cast by Ghevaert who, in a study of 200 cases of anti-HPA-1a mediated NAIT, showed that the antibody level was not correlated with neonatal platelet count and by Sainio who observed very low antibody levels in mothers of severely affected infants (Ghevaert *et al.*, 2007a; Sainio *et al.*, 2012).

Two studies suggested that the type of assay and reaction conditions within the assay influenced the measured level of antibody. In a collaborative study, Bessos used an ELISA assay that utilised immobilised, purified $\alpha\text{IIb}\beta\text{3}$ to quantitate levels of anti-HPA-1a and compared their data to that obtained using a MAIPA assay that utilised intact platelets (Bessos *et al.*, 2008a). Antibody levels in the ELISA assay were on average 2.8 times those obtained in the MAIPA assay although this difference was not consistent between samples. Furthermore, whilst the MAIPA assay was able to show correlation between maternal antibody level and neonatal platelet count ($R^2 = 0.42$, $P = 0.002$), the ELISA assay failed to show correlation ($R^2 = 0.14$, $P = 0.121$). In the second study, Killie investigated the role of the mAb used to capture $\alpha\text{IIb}\beta\text{3}$ from platelets in the MAIPA assay and showed that different levels of maternal anti-HPA-1a would be obtained using different capture antibodies although the correlation between the antibody level and the platelet count was approximately the same (Killie *et al.*, 2010).

The large prospective study of Killie provided an explanation for the lack of correlation observed by Ghevaert (Killie *et al*, 2008). In Killie's study a correlation between maternal anti-HPA-1a level and neonatal platelet count was only observed in samples taken at 22 and 34 weeks gestation and no correlation was observed with samples collected, as in the Ghevaert study, at delivery.

Interestingly, Tiller showed that the level of maternal anti-HPA-1a significantly ($P = <0.001$) influenced the birth weight of the affected pregnancy but only in male children, with their weight being on average 530g lower than male infants born to antibody negative mothers (Tiller *et al*, 2012). A reduction in birth weight had also been observed by Mueller-Eckhardt *et al* (Mueller-Eckhardt *et al*, 1989).

These studies therefore suggested that a correlation between maternal antibody level and neonatal platelet count could be observed under some circumstances with some assays but that the correlation was less than perfect.

1.7.8 Undetectable antibodies

In various studies it has been reported that maternal HPA-1a antibodies are not detectable in some HPA-1b1b mothers that delivered infants with strong clinical indicators of NAIT (Mueller-Eckhardt *et al*, 1989; Dreyfus *et al*, 1997; Turner *et al*, 2005; Ghevaert *et al*, 2007b; Bakchoul *et al*, 2011) suggesting that current assays are not sufficiently sensitive.

Numerous international workshops have shown that the ability to detect platelet alloantibodies varies between laboratories and that this is dependent to a certain extent on the assays used (Metcalf *et al*, 1997; Panzer, 2001b; Bessos *et al*, 2005; Allen *et al*, 2007; Porcelijn *et al*, 2008; Wu *et al*, 2010). One major conclusion that can be drawn from these studies and highlighted in the report of Metcalfe was that no single assay, including the extremely sensitive MAIPA assay, is capable of detecting all examples of antibodies of a particular specificity (Metcalf *et al*, 1997).

One factor that has been shown to be important to the sensitivity of the MAIPA assay is the capture mAb, although the use of the same clone in different laboratories shows that this is not the only factor (Allen *et al*, 1994; Allen *et al*, 2007; Wu *et al*, 2010).

In view of the complexity of the MAIPA assay, it would perhaps not be surprising if variations other than the choice of mAb existed between laboratories but when a survey of the precise technical details was carried out as part of an international workshop the extent of assay variation was surprising (Bessos *et al*, 2005). In this exercise, which was distributed to 40 laboratories in 23 countries, no two laboratories used an identical assay. Thus reagent and technical variation might explain the variations in sensitivity. However, when key reagents for use in the MAIPA assay were provided to laboratories participating in a different international workshop exercise, the expected reduction in assay variation did not materialise (Foxcroft *et al*, 2007). This clearly suggests that there are additional in-house factors that influence assay sensitivity.

One potential reason for assay insensitivity might be elution of low affinity antibodies during the wash phases of current assays. This was investigated by a number of groups using surface plasmon resonance (SPR) technology, in which the interaction of antibody and target protein can be measured directly using target proteins immobilised onto sensor chips without the requirement of washing of that protein post-exposure to the antibody (Socher *et al*, 2009; Bakchoul *et al*, 2011; Peterson, 2012). Bakchoul was able to show that there was a strong correlation between the reactivity of the alloantibodies in the MAIPA assay and in SPR. Antibodies reactive in the MAIPA assay had high affinity values in SPR, whilst 7/14 samples that were undetectable by MAIPA were detectable in the SPR assay and all had low affinities. One obvious question is whether or not such low affinity antibodies are clinically important. Clearly this is the case since in the study of Socher, in two cases in which

the maternal antibodies were undetectable by MAIPA and had low affinities in SPR the neonates had severe thrombocytopenia. However, the clearance of platelets of sensitised with low affinity antibodies in a mouse model was slower than those sensitised with high affinity antibodies (Bakchoul *et al*, 2011).

One major question that arises from these studies is whether the low affinity observed is due to the antibodies being primarily directed at the similar integrin, $\alpha v \beta 3$ (see below), and thus whether assays using platelets as target are effectively only assessing the cross-reactivity (which may be of low-affinity) of such antibodies against $\alpha IIb \beta 3$.

1.7.9 Antenatal screening for NAIT

Since antenatal screening for platelet alloantibodies is not routinely performed, most cases of NAIT are detected clinically post-delivery; this clearly is too late for the affected infant. As discussed above, a number of large-scale studies have shown the feasibility of typing mothers for HPA-1a during pregnancy and screening for the presence of HPA-1a antibodies and a systematic review of screening programmes concluded that such programmes are likely to do more good than harm (Kamphuis *et al*, 2010). The question therefore arises as to why screening has not yet been implemented in the UK or elsewhere.

The WHO has published guidelines on the introduction of screening tests, describing a number of criteria that should be met before the introduction of a screening test. (Wilson & Jungner, 1968). These were reviewed in the context of NAIT (Williamson, 1998; Murphy & Williamson, 2000; Murphy *et al*, 2002d).

Screening for NAIT failed to meet a number of criteria; two of which are of direct relevance to this project:

“There should be a simple, safe, precise and validated screening test”.

“The distribution of test values in the target population should be known and a suitable cut-off level defined and agreed”.

These criteria were not met since the sensitivity and reliability of antibody detection were not sufficiently high, and quantitation of antibodies had not been assessed.

These factors have been important drivers for this project. Understanding why assay sensitivity is variable and why the correlation between antibody level and neonatal platelet count has varied between studies would allow us to develop more reliable assays and address both of these criteria. Assuming that other failings against the WHO criteria could be, or have been, addressed since the original reviews then screening for NAIT could potentially be introduced in England and the significant number of infants that are not diagnosed until affected by NAIT (approximately 2,000 cases with moderate, and 600 cases with severe, thrombocytopenia per annum) could be reduced and the health care burden on families and on the National Health Service reduced.

1.8 Placental integrin $\alpha v \beta 3$

At the interface between maternal and fetal circulation are placental syncytiotrophoblast cells which play a key role in transportation of many substances, including amino acids and IgG, across the placenta (Cleal & Lewis, 2008; Desforges & Sibley, 2010; Palmeira *et al*, 2012). These cells express the integrin $\beta 3$ chain in association with an αv -chain rather than αIIb as found on platelets (Vanderpuye *et al*, 1991); this complex is found on many other cells including endothelial cells, smooth muscle and monocytes and monocyte-derived macrophages (Byzova *et al*, 1998).

The mature αv chain is larger than αIIb at 1,018 amino acids versus 1,008 respectively but is otherwise very similar, with 36 - 38% identity and 54% homology (Fitzgerald *et al*, 1987a; Feuston *et al*, 2003) (**Appendix 2**). The observation that endothelial cells expressed $\alpha v \beta 3$, prompted Leeksma to investigate whether such

cells might also express HPA-1a epitopes since the $\beta 3$ chain was common to both $\alpha \text{IIb}\beta 3$ and $\alpha \beta 3$ and indeed that was found to be the case (Leeksma *et al*, 1986; Leeksma *et al*, 1987).

These findings are of relevance to NAIT. Fragments derived from syncytiotrophoblasts are continuously shed (from about seven weeks gestation) into the maternal circulation (Kumpel & Manoussaka, 2012). They therefore may be the stimulus for the production of HPA-1a antibodies.

Integrin $\alpha \nu \beta 3$ may also be important in aspects of NAIT other than as an immunising molecule. This integrin is important in angiogenesis and in adhesion of trophoblasts to endothelial cells during trophoblast invasion; compromised feto-placental angiogenesis can be a cause of intra-uterine growth restriction (IUGR) (Harris & Aplin, 2007; Cartwright *et al*, 2010). Blocking of $\alpha \nu \beta 3$ function by the binding of HPA-1a antibodies could therefore potentially interfere with angiogenesis and thus result in IUGR. This hypothesis was supported by a recent paper by Tiller who reported a significant difference in birthweight in male infants born to mothers with HPA-1a antibodies compared to those without (Tiller *et al*, 2012).

Since $\alpha \nu \beta 3$ is expressed on endothelial cells these might also be affected by the binding of HPA-1a antibodies and thus predispose to bleeding. A study by Radder was unable to show any activating effect on human umbilical vein endothelial cells (HUVECs) but van Gils was able to demonstrate adverse effects of HPA-1a antibodies on such cells, with both the spreading of growing cells and establishment of cell monolayers affected (Radder *et al*, 2004; van Gils *et al*, 2009).

These data therefore show that $\alpha \nu \beta 3$ potentially plays an important role in NAIT and that the interaction of HPA-1a antibodies with this integrin requires closer investigation.

1.9 Physiological effects of HPA-1a antibodies on platelets other than immune destruction

1.9.1 Effects on platelet function

Auto-, iso- and alloantibodies against various platelet membrane proteins may have inhibitory effects on platelet function or they may induce activation of platelets.

The first evidence that platelet iso-antibodies might inhibit platelet function was provided by Levy-Toledo who noted that an antibody isolated from a multi-transfused patient with Glanzmanns' thrombasthenia was able to bind to normal platelets and prevent agonist-induced aggregation (Levy-Toledano *et al*, 1978). The inhibited response was later shown to be due to blocking of fibrinogen binding (Lee *et al*, 1981). The development of autoantibodies against $\alpha\text{IIb}\beta 3$ in some patients with autoimmune thrombocytopenia can have a similar function-blocking effect (Greaves *et al*, 1983).

The observation that antibodies against $\alpha\text{IIb}\beta 3$ could inhibit platelet function prompted van Leeuwen to study the effects of HPA-1a alloantibodies on platelet function (van Leeuwen *et al*, 1984). Two examples of anti-HPA-1a plasma, obtained from mothers with NAIT-affected children, were shown to inhibit ADP and collagen-induced aggregation of HPA-1a positive (but not HPA-1a negative) platelets; this effect was also observed when using F(ab')_2 and Fab fragments. Studies on the kinetics of fibrinogen binding suggested that the HPA-1a antibodies were binding very close to the fibrinogen binding site. Since this study, others have used a number of techniques to study the effect of HPA-1a antibodies. Duncan used blocking of serotonin release to demonstrate that a PTP-derived example of anti-HPA-1a could block fibrinogen binding (Duncan & Rosse, 1986). Studies by Beadling and Kroll using a more extensive panel of anti-HPA-1a samples showed differences in the function-blocking abilities of HPA-1a antibodies depending on the sample source (Beadling *et al*, 1995; Kroll *et al*, 2005a). The majority (75 - 86%) of

examples of HPA-1a antibodies from PTP patients were able to block fibrinogen binding whilst only 2/43 NAIT-derived examples in the study of Kroll, and 0/9 examples in that of Beadling, were function-blocking. Thus it appeared that interference of platelet function did not contribute significantly to the pathology of NAIT. However the study of Frelinger showed that it was possible to block aggregation without blocking of fibrinogen binding (Frelinger, III *et al*, 1991).

Conversely, antibodies against platelet membrane proteins are able to activate platelets. A number of anti- $\alpha\text{IIb}\beta\text{3}$ mAbs have been shown to have this ability (Gulino *et al*, 1990; Frelinger, III *et al*, 1991). The level of response to binding of these antibodies was shown by Tomiyama to depend on polymorphisms and expression levels of platelet $\text{Fc}\gamma\text{RII}$ (Tomiyama *et al*, 1992). However, in the study of Frelinger it was shown that some mAbs were able to bind directly to $\alpha\text{IIb}\beta\text{3}$ and induce fibrinogen binding (and platelet activation) through a conformational change induced in the complex (Frelinger, III *et al*, 1991).

HPA-1a specific antibodies have been shown to be able to activate platelets through a $\text{Fc}\gamma\text{RII}$ mediated mechanism (Dettke *et al*, 2001). Whether or not HPA-1a antibodies are able to induce platelet activation through conformational change to $\alpha\text{IIb}\beta\text{3}$ (similar to that described above with the monoclonal antibodies) is not known. However, the PSI domain, on which the HPA-1a epitopes reside, is located at the hinge region of the folded integrin and binding of HPA-1a antibodies could theoretically induce or block conformational changes. After inside-out signalling, the swing-out of the hybrid and PSI domains is critical in order to allow the necessary conformational changes to the $\beta\text{-A}$ and $\beta\text{-propeller}$ domains that allow binding of fibrinogen (Xiao *et al*, 2004; Zhu *et al*, 2008; Provasi *et al*, 2009; Kamata *et al*, 2010). Haas showed that binding of a mAb that targeted aa's 95 - 105 in the hinge region was able to block fibrinogen binding and aggregation (Haas, 2008). Mould showed that binding of mAbs to the PSI domain were able to induce conformational changes

from resting to extended states in integrin $\alpha 5\beta 1$ (Mould *et al*, 2005).

Thus, binding of antibodies in the region of the HPA-1a epitopes can potentially have activating or inhibitory effects that may be important in the pathology of NAIT.

1.9.2 Effects on platelet production

The production of platelets from megakaryocytes involves the formation of extended branched filaments from the megakaryocyte called proplatelets; each megakaryocyte can produce up to 20 proplatelets and up to approximately 10^3 platelets (Hartwig & Italiano, Jr., 2003).

Megakaryocytes are known to express $\alpha IIb\beta 3$ and this is one of the earliest surface markers that can be detected, being found on CFU-MK cells at days five to six of culture (Rabellino *et al*, 1981; Vainchenker *et al*, 1982; Vinci *et al*, 1984; Chang *et al*, 2007). Expression of this integrin persists throughout the lifespan of the megakaryocyte including through the process of proplatelet formation. Indeed, the production of proplatelets has been shown to be dependent on the presence of $\alpha IIb\beta 3$ and its interaction with fibrinogen (Larson & Watson, 2006).

Takahashi used mAbs to dissect the stages at which different integrins were involved (Takahashi *et al*, 1999). An antibody against αIIb (clone 5B12) was shown to inhibit proplatelet formation but did not affect colony formation whereas Y2/51, which recognises a complex epitope on the PSI + hybrid domains of $\beta 3$, affected neither colony nor proplatelet formation. An antibody against $\alpha v\beta 3$ (clone 23C6) seemed to affect cell proliferation since the size of the colonies was reduced. However, others reported that a different anti- $\alpha v\beta 3$ antibody, LM609, and a different anti- $\alpha IIb\beta 3$ antibody, PG2, were both able to inhibit proplatelet formation. Thus it would appear that the epitope specificity of the antibody is important in terms of blocking abilities.

Conceivably therefore, human antibodies against $\alpha IIb\beta 3$ or against HPA-1a might

interfere with the production of platelets by megakaryocytes. Greinacher showed inhibition of megakaryopoiesis by drug-induced antibodies against $\alpha\text{IIb}\beta\text{3}$ and Warwick found that serum samples containing anti-HPA-1a from three mothers of NAIT-affected infants were able to specifically inhibit the growth of CFU-MK obtained from HPA-1a positive but not HPA-1a negative individuals (Warwick *et al*, 1994; Greinacher *et al*, 2009). However, in view of the limited number of samples investigated further studies are warranted.

1.10 Project Aims and Strategy

The sensitive detection of HPA-1a antibodies is critical for the diagnosis of NAIT and the ability to relate the level of maternal antibody to neonatal outcome (platelet count and/or intra-cranial haemorrhage) would potentially allow accurate identification of pregnancies that required active management of predicted NAIT.

From the above it can be concluded that, despite continued advances in diagnostic assays for platelet alloantibodies, there still remain significant deficiencies in antibody detection and quantitation and thus key criteria for the possible introduction of an antenatal screening programme for NAIT are currently not met.

This thesis explores the hypothesis that the main reason for the difficulty in detecting HPA-1a specific antibodies in some cases of NAIT, and for the variable correlation between maternal HPA-1a antibody level and neonatal platelet count, is that $\beta 3$ used for antibody detection is in an inappropriate conformation – either as a result of laboratory-induced artefacts, or as a result of antibodies being directed primarily at a $\beta 3$ integrin associated with a different α -chain to that on platelets i.e. $\alpha \beta 3$.

Using a logical, step-wise strategy, the aims of the thesis are firstly to define the extent of the problem of NAIT in the first ever national study in England (and North Wales and Northern Ireland). In the first phase of this study we assess the incidence, types of clinical presentation, relative importance of HPA-1a antibodies compared to other HPA specificities and the maternal and paternal HPA genotype distributions (and thus potential for non-detection of maternal HPA alloantibodies) of all cases of clinically suspected NAIT referred for investigation over a two year period to the only diagnostic laboratory for NAIT in England, the Platelet Immunology Reference Laboratory of NHS Blood and Transplant; the data are presented in **Chapter 3**.

The main hypothesis is then explored in the following two chapters where the effect

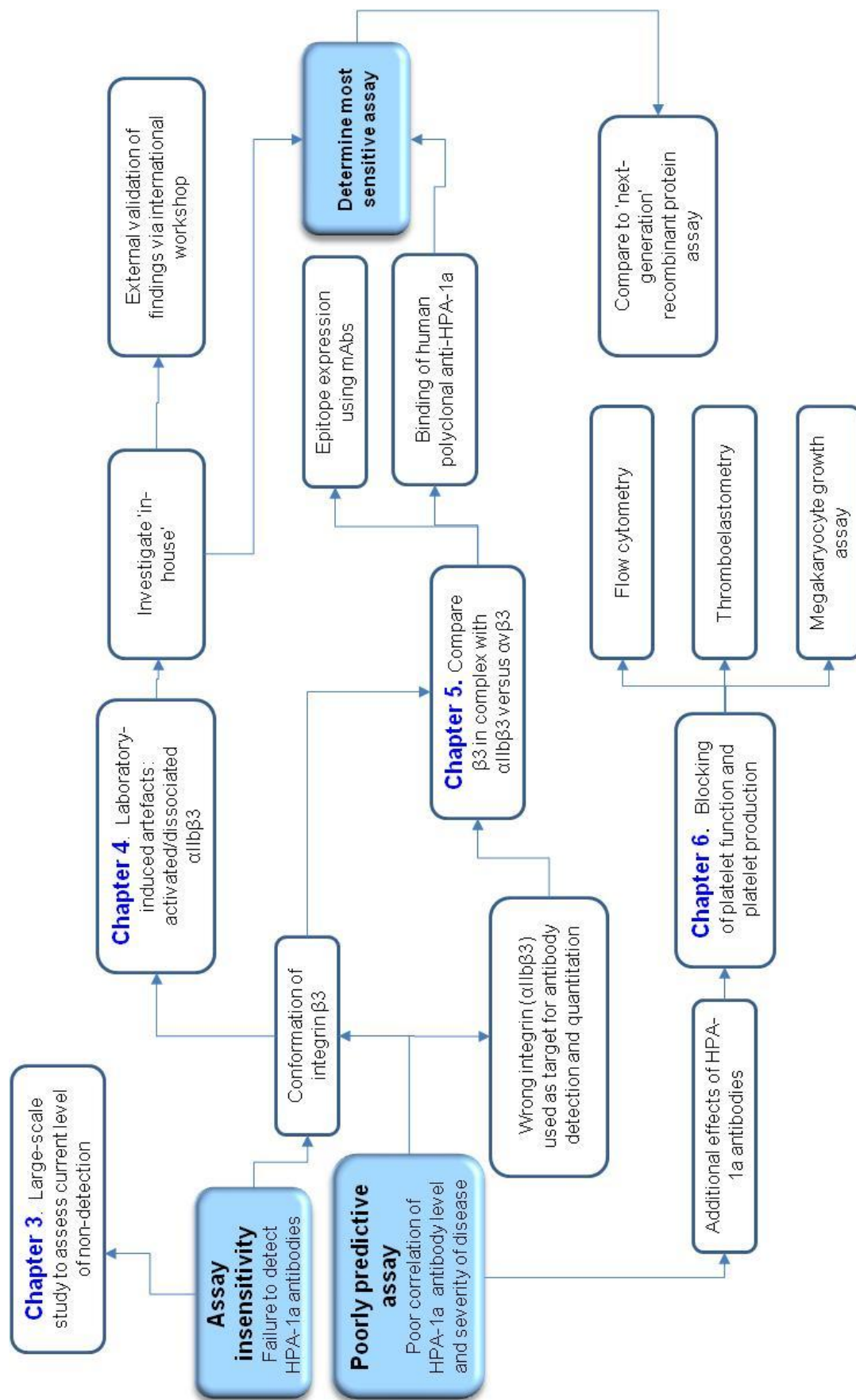
of integrin conformation is investigated.

In **Chapter 4**, the role of potential laboratory-induced artefacts (activation and dissociation of $\alpha\text{IIb}\beta 3$) is investigated and in **Chapter 5** the effect of alternate α -chains on $\beta 3$ and HPA-1a epitope conformation and antibody detection and quantitation is assessed.

In the last chapter the thesis explores potential effects of HPA-1a antibodies other than induction of thrombocytopenia, namely blocking of platelet function and platelet production, either of which might compound the effects of platelet destruction on the haemostatic abilities of the neonate.

A summary of the project goals and strategy is shown in **Figure 1.5**.

FIGURE 1.5. Project plan.



Chapter 2

Materials and Methods

2.1 Research Ethics

This project was approved by the Research and Development Committee of NHS Blood and Transplant.

Ethical approval was granted by Mid and South Bucks Rec (REC 06/Q1607/40) and Oxfordshire Rec A (08/H0604/166) Committees, and for the study of the epidemiology of NAIT by the London Multi-Centre Research Ethics Committee (Reference Numbers: 05/MRE02/83 and 06/MRE02/53).

All samples were pseudo-anonymised so that the identity of the donor/mother was unknown to the researcher.

2.2 Chemicals

Chemical	Supplier
Acetic acid	Sigma-Aldrich, UK
Acetone	Sigma-Aldrich, UK
ADP	Sigma-Aldrich, UK
β -mercaptoethanol	Sigma-Aldrich, UK
Bovine serum albumin (30% solution)	Lorne Laboratories, UK
Bromphenol blue	GE Healthcare, UK
CaCl_2	BDH, UK
Cell Dissociation Buffer	Invitrogen, UK
DMEM	Lonza, UK
DMSO	Sigma-Aldrich, UK
Dulbecco's PBS	PAA Laboratories Ltd, UK
EMEM	Lonza, UK
Fetal calf serum (heat inactivated)	PAA Laboratories Ltd, UK
Ficoll-Paque PLUS	GE Healthcare, UK
Glucose	Sigma-Aldrich, UK
Gly-Pro-Arg-Pro amide	Sigma-Aldrich, UK
Glycerol	Sigma-Aldrich, UK
Glycine	Sigma-Aldrich, UK
HCl	Merck, UK
HEPES	Lonza, UK
Hydrogen peroxide	Sigma-Aldrich, UK
IGEPAL CA-630	MP Biomedicals Inc, UK
KCl	Sigma-Aldrich, UK
L-glutamine	Sigma-Aldrich, UK
Methanol	BDH, UK
MgCl_2	Sigma-Aldrich, UK
NaCl	Sigma-Aldrich, UK
NaHCO_3	Sigma-Aldrich, UK
NaOH	BDH, UK
Na_2 -EDTA	Sigma-Aldrich, UK
OPD tablets	Dako, UK
Penicillin/streptomycin solution	Sigma-Aldrich, UK
Sodium carbonate/bi-carbonate capsules	Sigma-Aldrich, UK
Sodium dodecyl sulphate	Sigma-Aldrich, UK
Tris base	Sigma-Aldrich, UK
Triton X-100	Sigma-Aldrich, UK
Tween 20	Sigma-Aldrich, UK

2.3 Reagents

2.3.1 Fresh whole blood samples

Samples were obtained from volunteer plateletpheresis donors attending the Apheresis Unit of NHS Blood and Transplant, Oxford. Approximately 10 - 15 mL of blood was extracted from the sampling pouch (used to divert the first 50 mL of blood during the routine component donation process) and dispensed into 3.2% (0.109M) sodium citrate Vacutainer tubes (Becton Dickinson, UK). All donors were HPA-1a1a unless otherwise stated.

2.3.2 Serum and plasma samples

2.3.2.1 Blood donors – AB serum (negative controls)

Serum was obtained from blood donations collected into non-anticoagulated blood packs from non-transfused, blood group AB, male blood donors. The serum was screened for the presence of antibodies against red cell, white cell and platelet antigens and was used only if all assays were negative. Aliquots were stored at minus 40°C until required.

2.3.2.2 Blood donors – anti-HPA-1a

As part of a program to identify HPA-1b1b donors suitable for donating clinical products a number of donors were identified whose blood contained anti-HPA-1a. Citrated plasma from these donors was obtained, aliquoted and stored at -40°C until required. In some cases the plasma was re-calcified (**see 2.4.1**) prior to freezing.

2.3.2.3 NAIT cases

HPA-1b1b mothers whose infant(s) were suspected of having NAIT were identified using data queries run against the national NHSBT NAIT case management database ('NAITabase'). This database is used to record clinical details and summarised HPA antibody and HPA genotypes of mothers, fathers and infants in cases of suspected NAIT. Details of platelet counts and therapeutic products used

are also recorded.

Once identified, surplus serum or plasma samples were obtained from the NHSBT archives and pseudo-anonymised by allocating new sample identities (IDs) (in the format NR-xxxx) to each aliquot. Sample IDs were recorded in a new database together with the NAIT Case Number (in the format F-xxx) together with a limited amount of clinical information such as gestation when sample was collected, infant's platelet count at delivery. Insufficient data was recorded in the new database to permit identification of the original case but the Case Number would permit, if required, extraction of further clinical details from NAITabase by authorised NHSBT personnel. All samples were stored at -40°C until required.

2.3.3 Antibodies

Antibody	Mono (M) / Polyclonal (P)	Host species	Specificity	Label	Supplier
HIP8	M	Mouse	α IIb	PE	BD, UK
P4C10	M	Mouse	β 1	None	Millipore, UK
CRC54	M	Mouse	β 3	FITC	Abcam, UK
VI-PL2	M	Mouse	β 3	PE	BD, UK
SZ21	M	Mouse	β 3	PE	B-C, UK
Y2/51	M	Mouse	β 3	FITC	Inverness
PAC-1	M	Mouse	β 3 (activated)	FITC	BD, UK
P2	M	Mouse	α IIb β 3 complex	FITC	B-C, UK
PAB-1	M	Mouse	α IIb β 3 complex or α IIb or β 3	None	IBGRL, UK
PAB-6	M	Mouse	α IIb β 3 complex or α IIb or β 3	None	IBGRL, UK
5B12	M	Mouse	α IIb β 3 complex or α IIb or β 3	None	IBGRL, UK
LM609	M	Mouse	α v β 3	None	Millipore, UK
P5H9	M	Mouse	α v β 5	None	Millipore, UK
B9.12.1	M	Mouse	HLA class I	FITC	B-C, UK
19-7	M	Human	HPA-1a Type I	None	Prof W. Ouwehand, Cambridge, UK
23-15	M	Human	HPA-1a Type I	None	Prof W. Ouwehand, Cambridge, UK
B2	M	Human	HPA-1a Type II	FITC	IBGRL, UK

Antibody	Mono (M) / Polyclonal (P)	Host species	Specificity	Label	Supplier
B2	M	Human	HPA-1a Type II	None	IBGRL, UK
AK-4	M	Mouse	CD62P	FITC	BD, UK
581	M	Mouse	CD34	FITC	B-C, UK
Anti-mouse IgG	P	Goat		None	Stratech, UK
Anti-human IgG [F(ab') ₂ fragment]	P	Goat		HRP	Stratech, UK
AB7144F	P	Sheep	Fibrinogen	FITC	Millipore, UK

FITC fluorescein isothiocyanate; *PE* phycoerythrin; *BD* Becton Dickinson; *B-C* Beckman-Coulter; *IBGRL* International Blood Group Reference Laboratory

2.3.4 MAIPA assay reagents

Tris-buffered saline (TBS) x10 concentrate, pH 7.4

Tris base	24.2 g
NaCl	170 g
Made up to 2 L with de-ionised water pH adjusted to pH 7.4 with 5M HCl. Working solution prepared by diluting 1 in 10 with de-ionised water.	

TBS/BSA

TBS working solution containing 0.2% bovine serum albumin

TBS/BSA/EDTA

TBS/BSA containing 10mM Na₂-EDTA

Solubilisation buffer

Solution 'A' (0.15 M) NaCl	
NaCl	9.0 g
Dissolved in 1 L de-ionised water	
Solution 'B' 10 mM Tris	
Tris base	1.21 g
Dissolved in approximately 950 mL of isotonic saline (solution 'A' above, pH adjusted to 7.4 using 1M HCl.	
Triton X-100	5 mL
Added to solution "B" and stirred continuously until dissolved	
Made up to 1 L with solution 'A'.	

Wash buffer

TBS	1.21 g
NaCl	8.0 g
IGEPAL CA-630	5 mL
Tween-20	0.5 mL
CaCl ₂ (1 M)	0.5 mL
Made up to 1 L with de-ionised water. pH adjusted to 7.4 with 1M HCl or 1M NaOH	

Substrate solution

Ortho-phenylene-diamine	4 tablets
Hydrogen peroxide	5 μ L
Dissolved in 12 mL de-ionised water	

Coating buffer (0.05M carbonate/bi-carbonate buffer, pH 9.6)

One ampoule (containing pre-measured quantities of sodium carbonate and sodium bi-carbonate) dissolved in 100 mL de-ionised water.

2.3.5 SDS-PAGE reagents**‘x2 sample buffer’**

0.5M Tris-HCl, pH 6.8	2.5 mL
Glycerol (100%)	2 mL
β -mercapto-ethanol	0.4 mL
Bromphenol Blue	0.02 g
SDS	0.4 g
Made up to 10 mL with de-ionised water and frozen in aliquots until required.	

‘Running buffer’

Glycine	28.8 g
Tris-base	6.04 g
SDS	2 g
Made up to 2L with de-ionised water	

2.3.6 Tissue culture reagents**Supplemented medium**

DMEM or EMEM containing glucose (1 g/L) HEPES (25 mM) and sodium pyruvate (1 mM)	500 mL
L-glutamine	5 mL
Penicillin/streptomycin	5 mL
Heat-inactivated fetal calf serum	50 mL

2.3.7 Megakaryocyte culture reagents

All reagents were supplied as components of the MegaCult-C kit (Miltenyi Biotec Inc, UK) unless otherwise specified.

MACS-BSA solution

BSA	25 mL
Auto MACS Rinsing solution	475 mL

Fixing solution

Methanol (BDH, UK)	50 mL
Acetone (Sigma, UK)	150 mL
Cooled on ice for 30 mins prior to use.	

'Solution 1'. Tris-HCl buffer (0.05M, pH 7.6)

Solution 'A' (0.15 M saline)	
NaCl	8.766 g
Made up to 1L with de-ionised water.	
Solution 'B' (0.5 M Tris-HCl)	
Tris-HCl	78.8 g
Made up to 1L with de-ionised water and adjusted to pH 7.6 with 5M NaOH.	
9 parts of solution 'A' were mixed with 1 part solution 'B'.	

'Solution 2'. 5% human serum

Human serum	350 µL
'Solution 1'	6.65 mL

'Solution 3'. Primary antibody

Mouse anti-human αIIbβ3	25 µL
'Solution 2'	2.48 mL

'Solution 4'. 1% BSA

10% BSA	700 µL
'Solution 1'	6.3 mL

'Solution 5'. Secondary antibody

Biotin-conjugated, goat anti-mouse IgG (stock solution)	25 µL
'Solution 1'	225 µL
The resulting 'working solution' was diluted 1 in 15 (in solution '4') for staining	

'Solution 6'. Avidin-alkaline phosphatase conjugate

Avidin-alkaline phosphatase conjugate (stock solution)	10 µL
'Solution 4'	90 µL
The resulting 'working solution' was diluted 1 in 15 (in 'solution 4') for use.	

Alkaline phosphatase substrate

Prepared immediately before use. Three Tris buffer tablets were dissolved in 3 mL de-ionised water then 3 naphthol tablets added.

Counter-stain (Evans' blue)

10 drops of the 1% stock solution were added to 3 mL methanol.

2.3.8 ROTEM® *delta* assay reagents

Recombinant EXTEM reagent

Recombinant tissue factor	Concentration not specified by supplier
Phospholipids	Concentration not specified by supplier
Heparin inhibitor	Concentration not specified by supplier

Star-TEM reagent

CaCl ₂	200 mM in HEPES buffer, pH 7.4

2.3.9 Miscellaneous reagents

Modified HEPES-Tyrode's buffer

NaCl	138 mM
KCl	2.9 mM
NaHCO ₃	12 mM
MgCl ₂	0.4 mM
CaCl ₂	1 mM
Glucose	5.5 mM
BSA	0.2%
HEPES	5 mM

Acid elution buffer (acidified BSA)

Acetic acid	1 mL
30% BSA	5 mL
Made up to 100 mL with de-ionised water and pH adjusted to 2.8 with 1 N HCl	

2.4 Methods

2.4.1 Preparation of re-calcified plasma

To each 10 mL of citrated plasma was added 100 μ L 200 mM CaCl_2 and 100 μ L bovine thrombin (10 i.u./mL). The solutions were mixed thoroughly and left at 37°C for 1 hr. After removal of the clot the re-calcified plasma was frozen at -40°C until required.

2.4.2 Preparation of platelet-rich plasma (PRP)

Citrated samples were centrifuged (300 x *g*, 7 mins) and the PRP harvested into 12 x 75 mm plastic tubes. The platelet concentration was assessed using a Sysmex XE-2100 analyser (Sysmex, Wymbush, UK).

2.4.3 Preparation of washed platelet suspensions

PRP was centrifuged (10,000 x *g* for 1 min), the supernatant plasma discarded and the cell pellet re-suspended in 1 mL cold TBS/BSA/EDTA. The platelets were pelleted (10,000 x *g*, 1 min) then washed once more in TBS/BSA/EDTA. After removal of the supernatant, the platelets were re-suspended in TBS/BSA to a concentration of $2 \times 10^5/\mu\text{L}$.

2.4.4 Indirect Platelet Immunofluorescence Test (PIFT)

Test samples (serum/plasma) were thawed and centrifuged (13,000 x *g* for 1 min) prior to use. 25 μ L test sample was incubated with 5×10^6 platelets at 37°C for 60 mins. 1 mL cold TBS/BSA/EDTA was added and the tubes centrifuged (13,000 x *g*; 1 min). The supernatant was removed, the platelets re-suspended in 1 mL cold TBS/BSA/EDTA and centrifuged as above. After one further wash the cell pellet was re-suspended in 50 μ L diluted (1 in 25) FITC-conjugated anti-IgG (human or mouse as appropriate) and incubated in the dark at RT for 20 mins. 1 mL cold TBS/BSA was added and the cells analysed within 2 hrs on a BD LSRII flow cytometer using FACSDiva software. Platelets were gated on their forward and side scatter

characteristics and 10,000 events within this gate were analysed.

2.4.5 Standard MAIPA assay

50 μ L platelet suspension (at $2 \times 10^5/\mu$ L) was aliquoted into an appropriate number of 1.5 mL Eppendorf tubes followed by 25 μ L of test sample then incubated at 37°C for 40 minutes.

The platelets were pelleted by centrifugation (10,000 $\times g$ for 1 min), the supernatant removed and the cell pellet re-suspended in 1 mL of cold TBS/EDTA/BSA. After one further wash, the platelets were suspended in 40 μ L of appropriately diluted capture mAb and incubated at RT for 40 mins.

The platelets were washed three times in 1 mL volumes of cold TBS/EDTA/BSA. After removal of the supernatant the platelet pellet was suspended in 130 μ L solubilisation buffer and incubated at RT for 15 mins.

The lysates were centrifuged (10,000 $\times g$ for 5 mins) then 65 μ L of the supernatant added to each of two wells of flat-bottomed 96-well microplates previously coated with goat anti-mouse IgG (diluted 1/500 in coating buffer). The plates were incubated at 37°C for 40 mins then washed five times in wash buffer.

To each well was added 100 μ L of horseradish peroxidase conjugated goat anti-human IgG diluted 1/5,000. The plates were incubated at RT for 60 mins then washed six times in wash buffer. After removal of the supernatant fluid, 100 μ L of OPD substrate containing 0.0125% hydrogen peroxide was added and the colour allowed to develop for 10 mins.

The reaction was stopped by addition of 100 μ L of 0.5 M H_2SO_4 and the optical densities (OD) measured on a BioRad 680 microplate reader using Microplate Manager software. A result was deemed positive if the OD was >0.100 . This value had previously been determined by ourselves and others by extensive testing of

samples known to contain HPA alloantibodies and negative control samples and adjustment of MAIPA parameters to achieve maximum differentiation of results (Killie *et al*, 2008).

2.4.6 'Reverse MAIPA' assay

In this variation of the MAIPA assay the order of incubation of test sample and capture mAb was reversed:

50 μ L platelets suspension (at $2 \times 10^5/\mu$ L) was aliquoted into an appropriate number of 1.5 mL Eppendorf tubes followed by 40 μ L of appropriately diluted capture mAb; these tubes were then incubated at RT for 40 mins. The platelets were pelleted by centrifugation ($10,000 \times g$ for 1 min), the supernatant removed and the cell pellet re-suspended in 1 mL of cold TBS/EDTA/BSA. After one further wash, the platelets were suspended in 25 μ L of test sample and incubated at 37°C for 40 mins.

The platelets were washed three times in cold TBS/BSA/EDTA then, after removal of supernatant from the last wash, the cell pellet was lysed and the assay continued as described under 'standard MAIPA assay' above.

2.4.7 Assessment of cell surface integrin number and density

The number of integrin molecules on the surface of cell lines and platelets was assessed using a 'Biocytex Platelet Calibrator Kit' (Diagnostica Stago,UK) using the manufacturers' instructions.

2.4.7.1 Determination of saturating concentration of mAb

The Platelet Calibrator Kit requires that saturating amounts of anti- β 3 mAb are determined prior to assessment of number of integrin molecules; this was determined for both platelets and cell lines by titration in a flow cytometric assay.

Dilutions of VI-PL2 were prepared in reagent 'R1' to give a range of concentrations between 0.6 and 20 μ g/mL. Into 12 x 75 mm plastic tubes were aliquoted 100 μ L

mAb dilution and 5 μL cells; for the platelet assay, PRP was used, and for the cell line assay the cell suspension was adjusted to $5 \times 10^4/\mu\text{L}$ and 5 μL used. After thorough mixing, the cells were incubated at RT for 15 mins.

To each tube was added 3 mL of reagent 'R1' then the tubes centrifuged ($1,900 \times g$ for 5 mins). After removal of the supernatant, 100 μL of pre-prepared reagent 'R4' (70 μL 'R4' reagent plus 630 μL reagent 'R1') was added, the cells re-suspended thoroughly and incubated at RT for 15 mins. The cells were washed twice in 3 mL volumes of 'R1' then 500 μL 'R1' used to re-suspend the cells thoroughly prior to flow cytometric analysis as described above. Cells were gated on their forward and side scatter characteristics and 10,000 events within this gate were analysed.

A chart plotting mean fluorescence intensity (MFI) against antibody concentration was prepared and confirmation that the VI-PL2 was able to saturate all epitopes at a concentration of 5 $\mu\text{g/mL}$ was obtained (as required by the kit instructions).

2.4.7.2 Determination of number of surface integrin molecules

Cell suspensions were prepared by adding 150 μL reagent 'R1' to 50 μL cells at $5 \times 10^4/\mu\text{L}$, or 175 μL reagent 'R1' to 25 μL PRP.

20 μL of each diluted cell suspension were aliquoted into each of two 12 x 75 mm plastic tubes. To one set of tubes was added 20 μL VI-PL2 anti- $\beta 3$ and into the other 20 μL isotype control. All tubes were then incubated at RT for 10 mins.

A 'calibrator bead' tube was prepared by thoroughly vortexing the bead suspension (reagent 'R3') and then dispensing 40 μL into a 12 x 75 mm plastic tube.

To all tubes was added 20 μL reagent 'R4' (FITC-conjugated, polyclonal anti-mouse IgG). The tubes were vortexed thoroughly and incubated at RT for 10 mins. 2 mL reagent 'R1' was added and the cells analysed on a BD FACSDiva flow cytometer.

The relevant cell populations and the bead population were gated on forward and side scatter. The fluorescence intensity of the 'bright' bead population (population 4) was adjusted by altering the PMT voltage until the peak was in the fourth decade. Regions were created to encompass bead peaks individually and the MFIs of each bead peak and cell populations recorded.

The $\log_{10}$ of the number of antibody molecules bound per bead was plotted against the $\log_{10}$ of the bead MFI in MS-Excel and a straight line drawn using the 'LINEST' function. Using the derived correlation coefficient and the MFI, the antibody binding capacity i.e. number of molecules per cell was calculated.

2.4.8 Quantitation of anti-HPA-1a antibodies

Lyophilised aliquots of the WHO high-potency (100 i.u./mL) anti-HPA-1a reference standard '03/152' (NIBSC, UK) were carefully reconstituted with 1 mL de-ionised water.

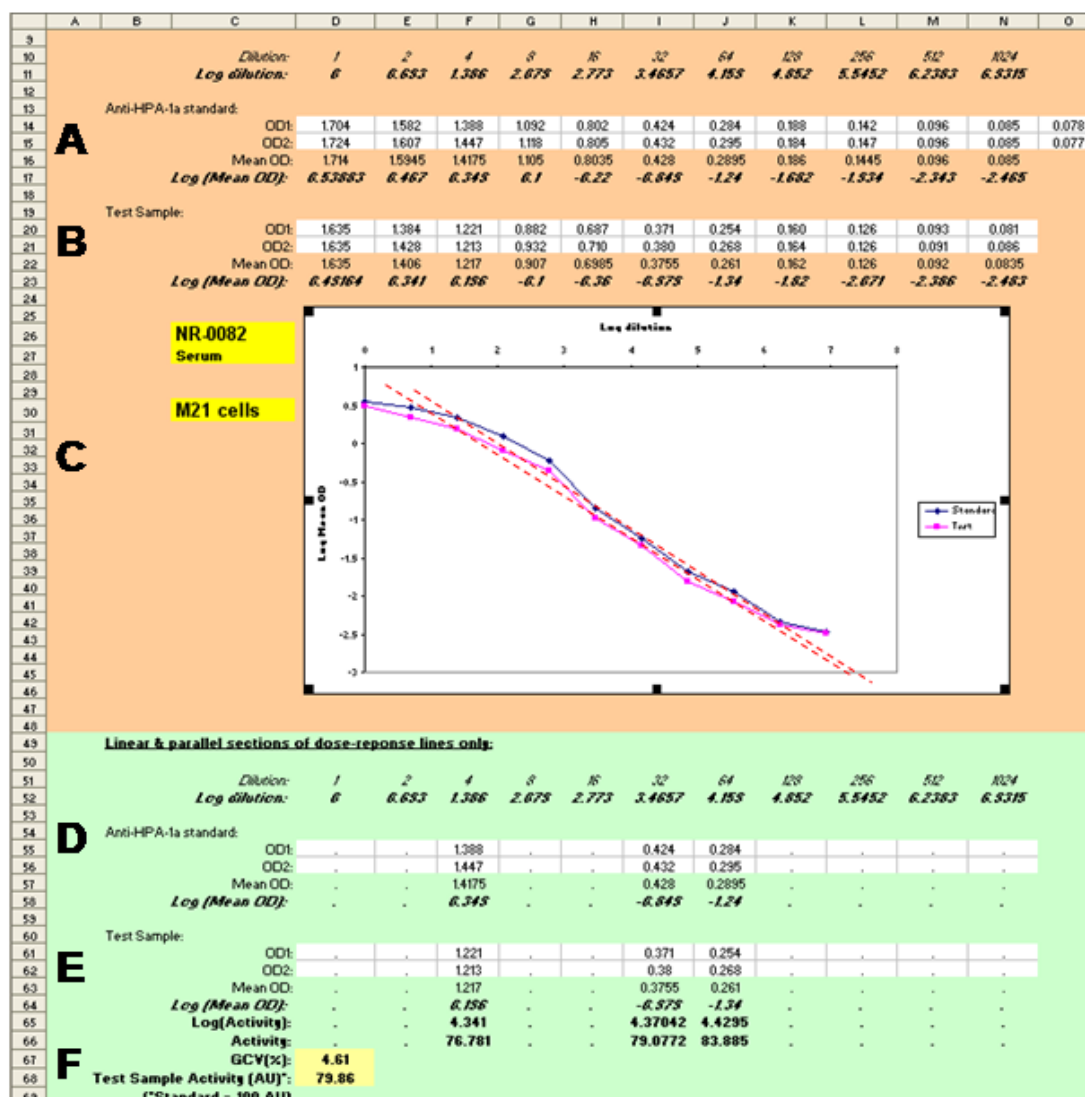
Doubling dilutions, from 1 in 2 to 1 in 1,024 of the standard and of test samples were prepared in TBS/BSA then tested in the standard MAIPA assay using anti- β 3 capture mAb 'VI-PL2' unless stated otherwise.

Anti-HPA-1a activity was calculated using the Microsoft Excel template and calculation method supplied by NIBSC (**Figure 2.1**).

FIGURE 2.1 Calculation of anti-HPA-1a level using NIBSC template. *The ODs of duplicate wells from each dilution of the reference standard are entered in section (A) and ODs of test sample dilutions in section (B). This generates the chart in section (C); the reference standard is indicated by the blue line and the test sample by the purple line.*

The curves are visually assessed for regions that are linear and parallel between reference and test samples and straight lines drawn (indicated by dashed red lines) between a minimum of three dilution points.

The ODs of the three selected dilutions of the reference standard are entered in section (D), and of the test in section (E). The calculated value for the test sample is indicated in section (F) together with the geometric coefficient variation of the sample; values <30% are considered to be acceptable.



2.4.9 Purification of IgG using Melon Gel IgG spin purification kit

Samples were de-salted using Zeba de-salt spin columns (Thermo-Scientific, UK) according to the manufacturers' instructions. The de-salted samples were harvested and used immediately or stored at -40°C until required.

Purified IgG was prepared from the de-salted samples using a negative selection gel matrix system 'Melon Gel' (Thermo-Scientific, UK) according to the manufacturers' instructions. The protein content of the purified IgG was assessed using a Nanodrop 2000 UV-Vis micro-volume spectrophotometer (Thermo Scientific, UK) then samples stored at -40°C until required.

Samples containing anti-HPA-1a were assessed for HPA-1a antibody level using the quantitative MAIPA assay (**section 2.4.8**).

2.4.10 Assessment of purity of purified IgG using SDS-PAGE

Dilutions of one random example (NR-0032) of purified IgG and dilutions of the original sample from which the IgG was purified were prepared in TBS buffer. 15 µL of each dilution was dispensed into 1.5 mL Eppendorf tubes and to each dilution was added 15 µL SDS-PAGE 'x2 Sample Buffer'.

The samples were placed in a heat block at 100°C for 5 mins then centrifuged (300 x *g* for 30 secs) to ensure all contents were in the base of the tubes.

In the first well of a pre-cast 'Criterion' 4 - 20% Tris-HCl 18-well gel (BioRad, UK) was placed 10 µL of Precision Plus Protein Kaleidoscope (BioRad, UK) and in the remaining wells 20 µL of sample dilutions. The gel was electrophoresed in Tris/Glycine/SDS buffer at 80V for 60 mins then the voltage increased to 100V and the samples run for a further 30 mins. The gel was removed carefully from the gel holder and placed into approximately 100 mL de-ionised water where it was agitated gently for 5 mins.

After removal of the water the gel was immersed in a fresh aliquot of de-ionised water and gently agitated for 5 mins. After one further wash, the gel was immersed in approximately 30 mL of Imperial Protein Stain (ThermoScientific, UK) and incubated at RT with gentle agitation for 2 hrs.

The protein stain was removed, 100 mL water added to the gel container and the gel gently agitated for 5 mins. The water was removed and replaced with a fresh aliquot and the gel left overnight at 4°C.

The gel was transferred onto blotting paper and placed in a gel dryer where it was allowed to dry for 2 hrs at 65°C. The dried gel was then scanned and annotated using an Epson Photo Perfection scanner and MS-PowerPoint.

2.4.11 DNA extraction from cell lines

DNA was extracted from cell lines using a Qiagen DNeasy kit (Qiagen, UK) according to the manufacturers' instructions using a total of 2.5×10^6 cells. The DNA content and purity was assessed using a NanoDrop 2000 Micro-Volume UV-Vis Spectrophotometer.

2.4.12 HPA typing of cells lines

HPA-1 genotyping of the cell lines was performed by Mr Rizwan Yusuf at NIBSC using the method of Metcalfe (Metcalfe & Waters, 1993).

2.4.13 Absorption and elution of HPA-1a antibodies

2.4.13.1 Heat elution

This was based on the method of Helmerhorst (Helmerhorst *et al*, 1982).

Cell pellets were prepared from 1×10^9 washed platelets or 1×10^7 M21 or M21-LIIB cells. To each pellet was added 500 μ L anti-HPA-1a D-0008 and the suspended cells incubated at 37°C for 30 mins. The cells were pelleted by centrifugation (13,000 x g, 2 mins), the supernatant removed and 1 mL cold TBS/BSA/EDTA added

to re-suspend the cells. The cells were again pelleted and subjected to one further wash. After removal of the supernatant from the last wash the cells were re-suspended in 500 μ L TBS/BSA and incubated at 56°C for 60 mins. The cells were pelleted as above and the supernatant (eluate) recovered; this was stored at -40°C until required.

2.4.13.2 Acid elution

This was based on the method of Kiefel (Kiefel *et al*, 2005).

Washed cell pellets (2×10^8 platelets, 1×10^7 M21 or M21-LIIB cells) were suspended in 200 μ L anti-HPA-1a D-0008 and incubated at 37°C for 30 mins. The cells were pelleted (13,000 $\times g$, 2 mins) and the supernatant removed and stored at -40°C for testing for the presence of anti-HPA-1a. The cells were washed five times in 200 μ L volumes of 0.85% NaCl then pelleted (13,000 $\times g$, 2 mins). To each pellet was added 160 μ L 0.85% NaCl and 40 μ L acid elution buffer. The re-suspended cells were incubated at RT for 10 mins then pelleted (13,000 $\times g$, 2 mins). 100 μ L of the supernatant (eluate) was removed and the pH neutralised by the addition of 9.6 μ L Tris solution (2.5M); this was stored at -40°C until required.

2.4.14 Molecular modelling

Three-dimensional modelling of integrins $\alpha\text{IIb}\beta 3$ and $\alpha\text{v}\beta 3$ was based on the crystal structures of $\alpha\text{IIb}\beta 3$ and $\alpha\text{v}\beta 3$. Modelling was performed using Discovery Studio 3.0 Visualizer (Accelrys Software Inc, USA) and the following files from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (Berman *et al*, 2000). Structure PDB ID: 3FCS ($\alpha\text{IIb}\beta 3$) (Zhu *et al*, 2008) and PDB ID: 3IJE ($\alpha\text{v}\beta 3$) (Xiong *et al*, 2009).

2.4.15 Luminex assay

The Luminex assay was performed by Dr Winnie Chong as described in Chong *et al* . (Chong *et al*, 2011).

Chapter 3

The project in context: a study of the epidemiology of NAIT in England, North Wales and Northern Ireland

3.1 Introduction

In Europe there are currently no antenatal screening programmes in place for the detection of platelet-specific alloantibodies or for the identification of the mothers most at-risk of having a NAIT-affected child i.e. mothers that are HPA-1b1b. The diagnosis of NAIT therefore is dependent on clinical recognition of the condition in the neonate and referral of appropriate samples for laboratory investigation. One problem with this approach is that in some cases, despite a strong clinical picture and HPA genotyping results that are compatible with a diagnosis of NAIT, maternal alloantibodies cannot be detected (Mueller-Eckhardt *et al*, 1989; Dreyfus *et al*, 1997; Turner *et al*, 2005; Ghevaert *et al*, 2007b; Bakchoul *et al*, 2011).

A number of studies have shown that the incidence of NAIT identified through clinical suspicion of the condition (and subsequent referral for laboratory diagnosis) is very much lower than the incidence observed in antenatal screening projects established as part of research studies (Davoren *et al*, 2002; Tiller *et al*, 2009). These data suggest that many cases of NAIT do not cause clinical symptoms, are not clinically recognised and/or are not investigated.

A detailed understanding therefore of the current referrals for investigation of NAIT is crucial for planning research or operational programmes and would be informative for discussions regarding possible introductions of screening programmes for NAIT.

We therefore established a large-scale study across the whole of England, North Wales and Northern Ireland. This two-year study had a number of goals.

The primary goal (*Study 1*) was to determine the incidence and outcomes of *clinically and laboratory confirmed* cases of NAIT as determined by reporting and case identification through three independent data sources, namely the British Paediatric Surveillance Unit (BPSU), the UK Obstetric Surveillance System (UKOSS) and the

national platelet immunology reference laboratory of NHSBT; the results of this study have been published (Knight *et al*, 2011).

The second goal (*Study 2*) was to study, in detail, all sample *referrals* for suspected NAIT to NHSBT. Importantly, it included cases where the pregnancy had not proceeded to term. In these cases, platelet counts on the neonate would probably not have been performed and the cases would not necessarily have been identified through UKOSS or BPSU reporting schemes (and thus be considered as confirmed cases of NAIT as in *Study 1*). Additionally, this analysis included a number of cases where the laboratory investigations ultimately showed the fetus or neonate was negative for the HPA antigen to which the mother had alloantibodies and thus the thrombocytopenia or haemorrhage observed in the fetus/neonate was not attributable to the maternal alloantibodies.

In those cases that did not proceed to term and in which fetal platelet counts were not performed it is likely that a proportion of cases included fetal loss due to NAIT and inclusion of such cases in the analysis of referrals was therefore thought to be useful. *Study 2* therefore had three goals.

Firstly, we aimed to assess whether there were specific clinical criteria that could be used to identify potential cases of NAIT, other than the typical presentation of thrombocytopenia at birth, which might in future be used to identify ‘at-risk’ groups and permit targeted diagnostic testing of groups of women in whom NAIT is under-recognised.

Secondly, we aimed to analyse in detail the serological data to determine the relative importance of the different HPA antibody specificities specifically in the population within England and North Wales.

Lastly, a number of studies have reported that in some cases of clinically suspected

cases of NAIT, HPA-specific alloantibodies cannot be detected and in particular there appears to be an excess of HPA-1b1b mothers in whom HPA-1a antibodies cannot be detected (Mueller-Eckhardt *et al*, 1989; Dreyfus *et al*, 1997; Turner *et al*, 2005; Ghevaert *et al*, 2007b; Bakchoul *et al*, 2011). We therefore aimed to analyse the molecular HPA typing data of all maternal and paternal samples in cases of clinically suspected NAIT where HPA-specific antibodies had not been detected in the maternal sample in order to determine in this large cohort whether there was an excess of mothers of particular HPA genotypes, and thus provide evidence to support the reported potential non-detection of antibodies in those cases.

These initial studies therefore establish the framework for the main topic of this thesis, with the expectation that HPA-1a antibodies would be shown to be the predominant cause of NAIT in England and North Wales. Since these antibodies have been shown to be variably detected in a number of international platelet immunology quality assurance studies (Allen *et al*, 2007; Porcelijn *et al*, 2008; Wu *et al*, 2010) and in studies of NAIT (Mueller-Eckhardt *et al*, 1989; Dreyfus *et al*, 1997; Turner *et al*, 2005; Ghevaert *et al*, 2007b; Bakchoul *et al*, 2011) an understanding and resolution of why detection is so variable will be important in any future discussions regarding introduction of antenatal screening in England.

3.2 Methods

Details on cases of laboratory-confirmed NAIT were obtained from two data sources, UKOSS (Knight *et al*, 2005) and BPSU (<http://www.rcpch.ac.uk/bpsu>), through a collaboration with the National Perinatal Epidemiology Unit (NPEU) (Oxford, UK; <https://www.npeu.ox.ac.uk/>). This unit undertakes a wide range of research designed to assess, inform and improve on care given to mothers and their family members during pregnancy, and during and immediately after birth.

In order to obtain data, BPSU distributes a notification form listing a range of rare conditions (the BPSU ‘Orange Card’) each month to approximately 3,200 consultant paediatricians or associate paediatric specialists in the UK and Republic of Ireland. For this particular study, clinicians were requested to report cases of NAIT falling into the following definition (as defined by the study guidelines):

“Any infant born during the study period with a documented maternal/fetal platelet antigen incompatibility, usually in the presence of maternal antibodies AND one of the following:

- cord platelet count at birth of $<50 \times 10^9/L$.
- Haemorrhagic complications before or after birth (e.g. intraventricular haemorrhage, GI bleed, bruising or petechiae).
- Antenatal therapy with maternal steroids, IVIg or fetal platelet transfusion.”

If clinicians returned an Orange Card indicating that they had seen a child falling into these case definitions, they received a questionnaire from the NPEU requesting confirmation of the diagnosis of NAIT and details on the infant. A follow-up questionnaire was distributed approximately one year after the birth of the affected infant in order to obtain information on the neuropsychological development of the infant.

UKOSS also uses a monthly mailing of notification forms, which are distributed to all senior obstetricians, midwives and anaesthetists in consultant-led maternity units in the UK. Receipt of a NAIT case notification at NPEU also resulted in the distribution of a questionnaire to the relevant clinician. Cases reported through this scheme included pregnant women with a previous child known to have been affected by NAIT or women known to have platelet alloantibodies identified through an alternative means e.g. previous screening studies for platelet antibodies, and known to be carrying an HPA-incompatible fetus.

In both BPSU and UKOSS schemes, clinicians were asked to return a 'nil' return if no cases were seen in order that we could be confident that all cases of NAIT occurring during the study period had been notified.

The Platelet Immunology Reference Laboratory of NHSBT in Bristol is the sole provider of a diagnostic service for NAIT (and other immunological conditions involving platelet allo- and autoantibodies) in England and North Wales. Clinical and laboratory details on all referrals of suspected NAIT to this laboratory are recorded by NHSBT staff in two databases, namely the 'HITS' database, a commercially designed database used to record details of samples received, tests requested and test results, and 'NAITabase', a Microsoft Access database written by myself and described in **Chapter 2**. Both databases are able to record whether the samples originated from the mother or other family member but 'NAITabase' is able to record more precisely the reason why an investigation was requested.

Database queries were designed to extract data from the two data sources using 'Business Objects 5i' (Version 5.1.7, Business Objects SA, USA) (HITS data) and Microsoft Access (Version 2003, Microsoft, USA) (NAITabase data). The queries were designed to identify all samples referred to NHSBT within the study period and where an investigation of *suspected* NAIT was requested, including samples

received from partners and children/fetuses for HPA typing studies.

The queries also identified cases where there was a potential risk for the individual to have a child affected by NAIT e.g. where the individual had a sister with an affected child, the individual had been identified as being HPA-1b1b as a blood donor and was now pregnant and so on.

For *Study 2*, the details in the 'Test Reason' field and the clinical details in all relevant fields in the extracted data were examined to determine the precise reason why samples had been referred for investigation. The cases were then allocated into one of seven categories:

(A) 'Proven history of NAIT in a previous pregnancy'. These were cases where NAIT had been diagnosed clinically and confirmed by laboratory investigation in a previous pregnancy; laboratory investigations were performed in the study period during or after a subsequent pregnancy to the index case.

(B) 'Possible history of (undiagnosed) NAIT in a previous pregnancy'. This group consisted of cases where clinical details supplied with the current referral suggested that a previous infant(s) might have suffered from NAIT but no laboratory investigations were performed at the time to confirm this, nor did the clinical details mention NAIT.

(C) 'Suspected NAIT in the current neonate'. These were typical presentations of NAIT where infants were found to be thrombocytopenic at birth.

(D) 'Cerebral abnormalities in the current fetus'. This group comprised cases where the mother was still pregnant and where the fetus had ventriculomegaly, hydrocephaly or other miscellaneous cerebral problems detailed in **Table 3.5.2**.

(E). Intrauterine growth retardation (IUGR). The referral forms on these cases

simply recorded 'IUGR' as the diagnosis.

(F). Other. This category included cases that did not fit into any of the above categories; see **Table 3.5.3** for details.

(G). Not known. No details were available in either of the NHSBT databases or on the original request form.

In all cases, only data necessary for the purposes of this study were retrieved and no person identifiable data were sought. Details of fields interrogated are shown in **Table 3.1**. Use of these fields allowed cross-referencing against UKOSS and BPSU data to confirm case identity without compromising data confidentiality.

No systematic attempt was made to contact clinicians to ascertain why they referred samples or the criteria they used to define particular clinical conditions e.g. IUGR, hydrocephaly as (a) this was beyond the resources of this project and (b) the goal of this phase of the project was to analyse the spectrum of referrals.

TABLE 3.1 Data fields used to cross-reference cases referred for investigation of suspected NAIT.

	Database	
	NAITabase	HITS
Fields used for case matching	• Maternal year of birth • Estimated date of delivery • Hospital • Actual date of delivery	• Maternal year of birth • Hospital • Date of delivery
Fields used for data validation	• Maternal HPA type • Mother's HPA antibody(ies) • Gravidity • Parity • Number of previous pregnancies • Gestation at delivery • Mode of delivery • Outcome • Presence of purpura/petechiae • Presence of ICH • Presence of GI bleed • Evidence of other bleeding problems • Details of other bleeding problems • Full recovery of infant • Neurological deficit in infant • Infant died • Cause of infant death • Maternal treatment during pregnancy • Blood products received by infant • Platelet counts of infant and date of count	• Maternal HPA type • Fetus/baby's HPA type

Comparison of the data obtained from the three data sources resulted in two lists: (1) Cases that could be matched across all three sources and (2) cases that had been 'identified' by only one or two of the sources; in these latter cases the data sources were re-interrogated using less strict criteria to detect non-exact matches e.g. a mother's date of birth that differed by one digit, with a delivery date in the same month as that in other data sources.

The database query tools were also modified to detect cases that might have been entered with an incorrect entry in the 'Test Reason' or other field that was used to retrieve data.

Once this process had been performed using a range of permutations and no matching cases retrieved, it was assumed that the case was not present in the relevant database.

A capture/recapture analysis was then performed. This process uses mathematical formulae to assess the extent of possible non-captured data when using two or more independent but overlapping data sources (Hook & Regal, 1995).

The total number of births during the study period was obtained from the Office for National Statistics for England and Wales (<http://www.statistics.gov.uk>), the General Register Office for Scotland and the Northern Ireland Statistics and Research Agency.

3.3 Results

Study 1

3.3.1 Incidence of clinically and laboratory confirmed NAIT

Across the three data sources a total of 173 cases were identified. Capture/re-capture analysis estimated a further 20 cases to have occurred during the study period. The incidence of NAIT across the UK was calculated to be 1 case per 8,080 births (95% confidence intervals: 6,993 and 9,346) (**Table 3.2**).

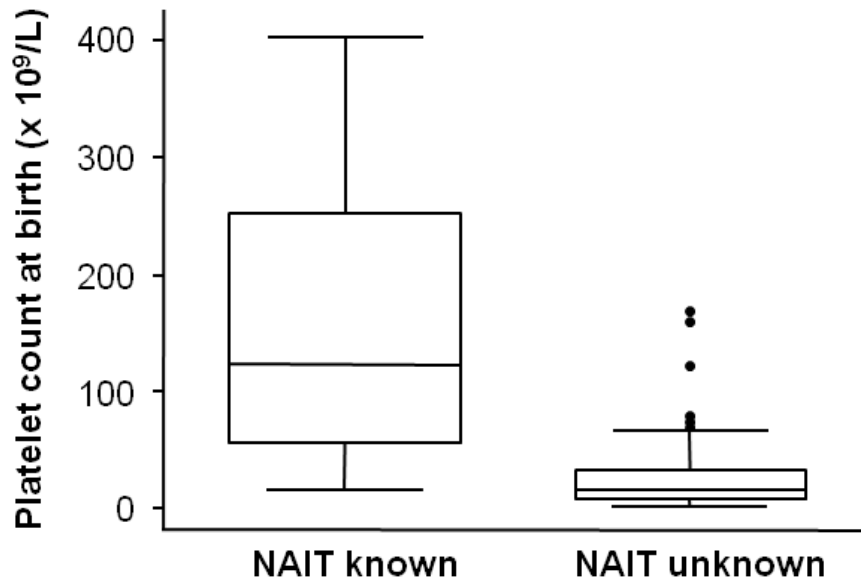
TABLE 3.2. Incidence of NAIT across the UK as determined by three data sources.

Number of reported cases	Number of additional cases estimated by capture/re-capture analysis	Total number of births	Incidence of NAIT
173	20	1,559,411	1 per 8,080 births

3.3.2 Severity of thrombocytopenia

The platelet counts in neonates that were diagnosed with NAIT only at delivery (and therefore had not received antenatal treatment for the condition) was significantly lower ($P = 0.001$) than that of neonates born to mothers having received treatment for NAIT, with median platelet counts (inter-quartile ranges) of 17 (9, 32) and 124 (56, 252) $\times 10^9/L$ respectively (**Figure 3.1**).

FIGURE 3.1. Severity of thrombocytopenia in *de novo* cases of NAIT versus cases identified at commencement of pregnancy and which received antenatal treatment.



Adapted from Knight et al, 2011.

3.3.3 Morbidity and mortality

The outcome of cases that were identified at delivery was significantly worse than that of cases in which antenatal treatment had been given.

Haemorrhagic symptoms were observed in 68/120 (67%) of previously undiagnosed cases and in 2/52 (5%) antenatally treated cases ($P = <0.001$).

Intra-cranial haemorrhage was observed in 22/120 (20%) of previously undiagnosed cases but only 2/52 (4%) cases that had been treated antenatally ($P = 0.014$).

Combining the data for any form of severe outcome (perinatal death, second trimester loss, termination and ICH or other related cerebral abnormality), 25/120 (22%) and 4/52 (8%) previously unknown versus antenatally managed cases respectively were affected ($P = 0.041$).

The developmental progress of infants was followed for up to one year; data was available on 116/146 (79%) live born infants reported through the UKOSS or BPSU schemes. Differences in numbers of affected cases were observed between cases identified at commencement of pregnancy versus those diagnosed post-natally but the differences were not significant (**Table 3.3**).

TABLE 3.3. Morbidity and mortality at age 1 year.

Characteristic	NAIT known at start of pregnancy	NAIT unknown or unrecognised	<i>P</i> *
Disability at age 1 year	0 (0%)	7 [#] (8%)	0.19
Infant death	0 (0%)	2 (2%)	0.59
Disability or death at age 1 year	0 (0%)	9 (10%)	0.11

* *Fisher's exact test.*

[#] *Two infants had severe global developmental delay and visual impairment, four had motor impairment, one of whom also had visual problems, and one had visual problems alone.*

Adapted from Knight et al (Knight et al, 2011).

These data show that early identification and antenatal treatment of NAIT results in less severe disease.

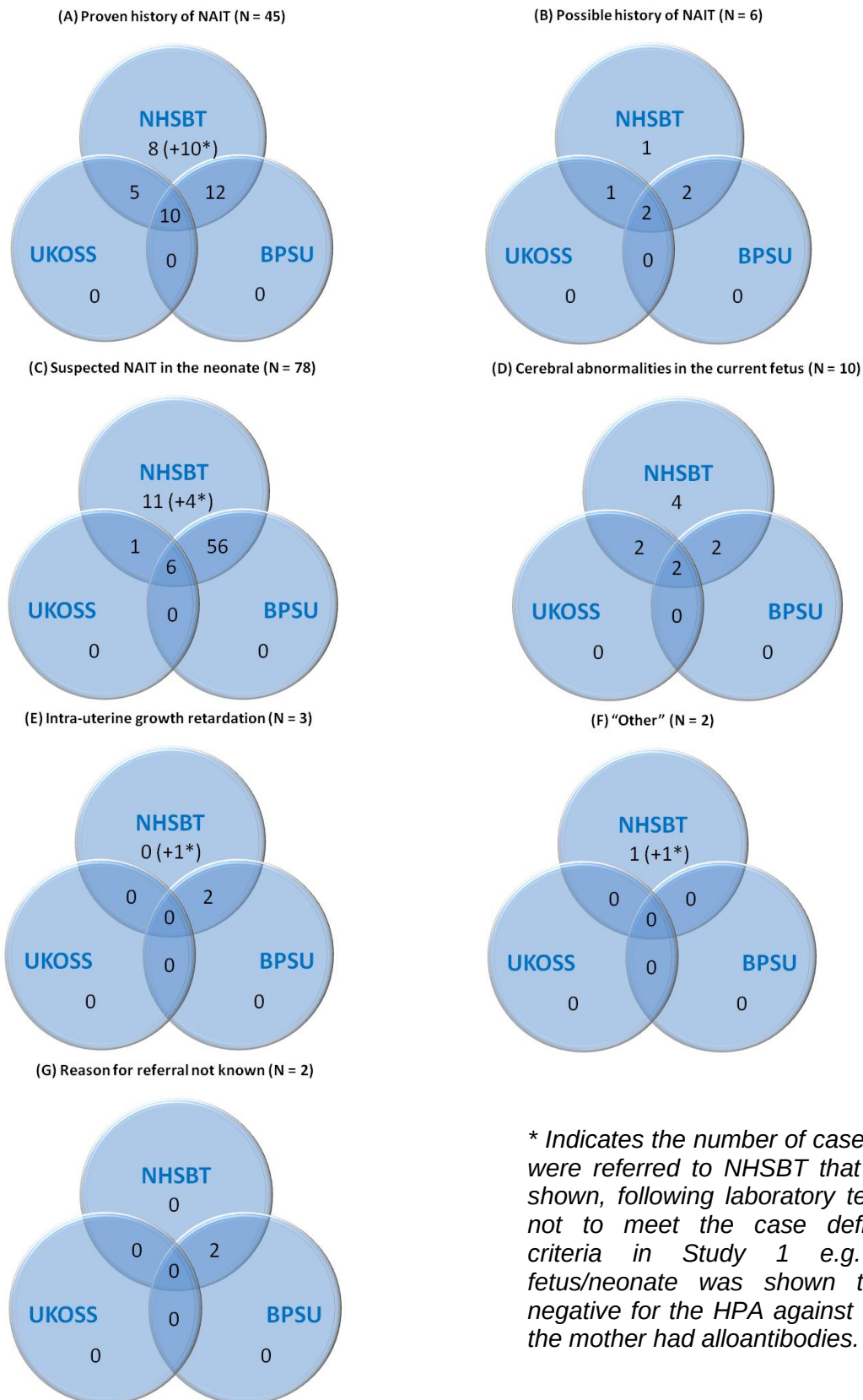
Study 2: Detailed analysis of referrals to NHSBT

In this study, which was a study of sample referrals for suspected NAIT, cases were identified that did not proceed to term or where the current fetus/neonate was shown to lack the HPA against which the maternal antibodies were directed; such cases would not be expected to be identified through the UKOSS or BPSU schemes and the numbers therefore differ to those in Study 1.

3.3.4 Analysis of clinical referral categories reporting through each of the three schemes

Figure 3.2 shows the number of cases reported by each scheme in each of the categories of referral. Since the referral categories were determined by analysis of NHSBT databases and inspection of test request forms referred to NHSBT, by definition there are no cases that were identified by the UKOSS and/or BPSU schemes and not by NHSBT.

FIGURE 3.2. Referrals for investigation of suspected NAIT to NHSBT: HPA antibody positive cases.



3.3.5 Incidence of cases where HPA-specific antibodies were detected

The total number of referrals for investigation of suspected NAIT received by NHSBT was 851.

HPA-specific antibodies were detected in 146 cases (17.2% of referrals) (**Figure 3.3, Table 3.5**). Forty five cases were known to have previously delivered infants diagnosed with NAIT (category '**A**' [**proven history of NAIT**']) and such cases are likely to be under active clinical management with samples being referred during subsequent pregnancies; excluding these cases therefore, HPA-specific antibodies were detected in 101/806 (12.5%) clinically detected, *de novo* cases where NAIT was suspected in the current fetus or neonate.

The largest referral group was clinical category '**C**' (**suspected NAIT in the current neonate**) where 365 samples were investigated, representing 42.9% of all referrals.

The other major category of referrals was cases referred because routine antenatal ultrasound scans had detected evidence of **cerebral abnormalities in the current fetus** (category '**D**'); 285 cases were investigated (33.5% of referrals).

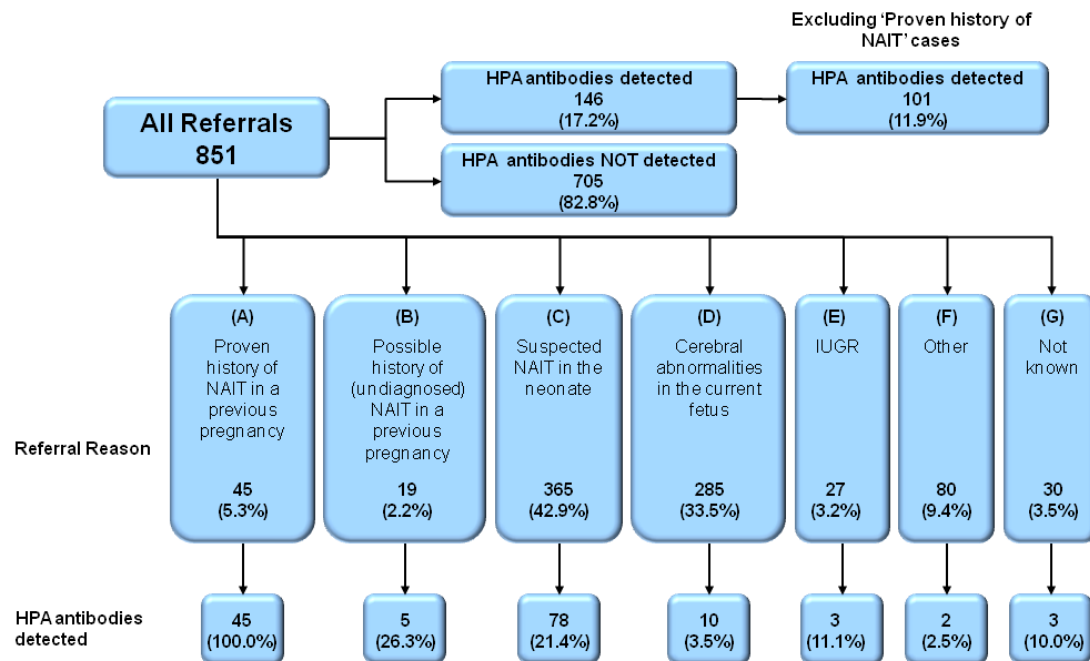
The remaining categories ('**A**': [**proven history of NAIT in a previous pregnancy**], '**B**' [**possible history of (undiagnosed) NAIT in a previous pregnancy**], '**E**' [**IUGR**], '**F**' [**other**], and '**G**' [**referral reason unknown**]) each accounted for <10% of referrals.

All (45) cases in category '**A**', (**proven history of NAIT in a previous pregnancy**) were found to have HPA-specific alloantibodies (**Figure 3.3, Table 3.5**).

The highest incidence of HPA-specific antibodies in *de novo* cases was in category '**B**', (**possible history of [undiagnosed] NAIT in a previous pregnancy**), where 5 of 19 (26.3%) cases were found to have HPA alloantibodies.

In category 'C' (**suspected NAIT in the current neonate**), the typical presentation of NAIT, 78/365 (21.4%) cases were found to have HPA-specific alloantibodies.

FIGURE 3.3. Analysis of referrals by clinical category and detection rates of HPA-specific antibodies.



The incidence of HPA-specific antibodies in pregnant women previously unscreened for platelet alloantibodies was calculated to be 1 per 13,771 births (7.3 cases per 100,000 births) (**Table 3.4**).

TABLE 3.4. Incidence of HPA-specific antibodies.

	Number of cases with HPA-specific antibodies	Total number of births	Antibody incidence
All referrals	145	1,390,899	1 case per 9,592 births
<i>de novo</i> referrals	101	1,390,899	1 case per 13,771 births

3.3.6 HPA specificities encountered

The single antibody specificity most frequently identified was HPA-1a, identified in 102/146 (69.9%) of cases where HPA-specific antibodies were detected (**Table 3.5**).

Excluding cases known to have HPA-specific antibodies in a previous pregnancy (category 'A' [**Proven history of NAIT**]), HPA-1a antibodies were identified in 70/101 (69.3%) referrals.

The second most frequently identified single antibody specificity was HPA-5b, accounting for 15.1% and 13.9% of all, and *de novo*, antibody-positive referrals respectively.

Including those cases where HPA-1a and HPA-5b antibodies were found in combination with other specificities, HPA-1a antibodies were detected in 74.3%, and HPA-5b antibodies in 19.8% cases.

TABLE 3.5 HPA specificities identified.

Clinical category of referral	No. referrals	No. Ab+ve	1a	1b	3a	4a	5a	5b	1a +5b	5b + 15b	15a	15b	N.K.
(A) Proven history of NAIT	45	45	32					8	4	1			
(B) Possible history of undiagnosed NAIT	19	5	3					1					1
(C) Suspected NAIT in current neonate	365	78	57	1	3	1	1	9	4	1		1	
(D) Cerebral abnormalities in current fetus	285	10	4				1	3	1		1		
(E) IUGR	27	3	2	1									
(F) Other	80	2	1					1					
(G) Not known	30	3	3										
TOTAL	851	146	102 (69.9%)	2 (1.4%)	3 (2.1%)	1 (0.7%)	2 (1.4%)	22 (15.1%)	9 (6.2%)	2 (1.4%)	1 (0.7%)	1 (0.7%)	1 (0.7%)
Excluding 'Proven history of NAIT'	806	101	70 (69.3%)	2 (2.0%)	3 (3.0%)	1 (1.0%)	2 (2.0%)	14 (13.9%)	5 (5.0%)	1 (1.0%)	1 (1.0%)	1 (1.0%)	1 (1.0%)

Detailed analysis of the clinical details supplied suggested that there were certain clinical sub-categories in which platelet alloantibodies were more likely to be detected although the number of cases in some subcategories was very small.

In category 'B' (**possible history of NAIT in a previous pregnancy**), subcategories 'B1' (abnormal fetal brain pathology noted in a previous pregnancy; fetus/baby was thrombocytopenic), and 'B2' (previous miscarriage and sister had baby with NAIT) had a high incidence of HPA-specific alloantibodies (**Table 3.5.1**).

TABLE 3.5.1. Detailed analysis of referral category 'B' – 'possible history of NAIT in a previous pregnancy' (*n* = 19).

Sub-category	Clinical features	No. referrals	HPA antibodies detected		
			No. cases	%	Specificity (No. cases)
B1	Abnormal fetal brain pathology noted in a previous pregnancy; fetus/baby was thrombocytopenic	2	2	100	1a (2)
B2	Previous miscarriage and sister had baby with NAIT	1	1	100	1a
B3	Previous IUD/stillbirth with abnormal brain pathology	4	1	25	5b
B4	Previous baby with thrombocytopenia	5	1	20	Unidentified
B5	Abnormal fetal brain pathology in a previous pregnancy; no platelet counts performed on baby	6	0	0	
B6	Previous child severely disabled	1	0	0	

In category 'D' (**cerebral abnormalities in the current fetus**), HPA-specific antibodies were detected in cases in three subcategories, 'D1' (possible or confirmed cysts), 'D2' (possible or confirmed haemorrhage [cerebellar / parenchymal / sub-arachnoid]) and 'D3' (hydrocephaly or ventriculomegaly) (**Table 3.5.2**).

TABLE 3.5.2. Detailed analysis of referral category ‘D’ – ‘cerebral abnormalities in the current fetus’ (*n* = 285).

Sub-category	Clinical features	No. referrals	HPA antibodies detected		
			No. cases	%	Specificity (No. cases)
D1	Possible or confirmed cysts	16	1	6.3	5b
D2	Possible or confirmed haemorrhage (cerebellar/parenchymal/subarachnoid)	23	1	4.3	1a
D3	Hydrocephaly or ventriculomegaly	226	8	3.5	1a (3); 5a (1); 5b (2); 1a+5b (1); 15a (1)
D4	Possible or confirmed tumour	3	0	0	
D5	Calcification in brain	1	0	0	
D6	Brain echogenic area. Asymmetric choroid plexus	1	0	0	
D7	Possible or confirmed holoprosencephaly	3	0	0	
D8	‘Brain abnormality’ (no details available)	11	0	0	
D9	Cerebral hypoplasia	1	0	0	

In category ‘F’ (**other**), HPA-specific antibodies were detected in mothers in two sub-categories, ‘F1’ (mother found to be HPA-1b1b as a blood donor or during a prospective NAIT study) and ‘F2’ (maternal AITP or other autoimmune disorder, or other family member with autoimmunity) (**Table 3.5.3**).

TABLE 3.5.3. Detailed analysis of referral category 'F' – 'other' (n = 80).

Sub-category	Clinical features	No. referrals	HPA antibodies detected		
			No. cases	%	Specificity (No. cases)
F1	Mother found to be HPA-1b1b as a blood donor or during prospective NAIT study	2	1	50.0	1a
F2	Maternal AITP or other autoimmune disorder, or other family member with autoimmunity	22	1	4.4	5b
F3	Miscarriage / stillbirth / IUD / NND	30	0	0	
F4	Has a female relative with infant affected by NAIT	9	0	0	
F5	Fetal hydrops	4	0	0	
F6	TOP for fetal abnormality or unspecified reason	2	0	0	
F7	Baby has infection	1	0	0	
F8	Late presentation of thrombocytopenia in baby	1	0	0	
F9	Genetic disorder in baby	2	0	0	
F10	Haematological disorder in mother/father of current infant	3	0	0	
F11	Spontaneous abortion after amniocentesis	1	0	0	
F12	Mum had RTA, resulting in fetal injury including dilated ventricles	1	0	0	
F13	Renal vein thrombosis, poor CTG	1	0	0	
F14	Ante-partum haemorrhage	1	0	0	

3.3.7 Obstetric history of mothers in whom platelet-specific alloantibodies were detected

Historic data suggests that up to 50% of cases of NAIT occurred in the first pregnancy (Mueller-Eckhardt *et al*, 1989; Williamson *et al*, 1998; Maslanka *et al*, 2003). Recently however, a large prospective screening study reported that <10% of cases occurred after the first pregnancy (Killie *et al*, 2008).

In order to determine whether immunisation occurred during the first pregnancy in any of the clinical categories in our study, the obstetric history fields in the databases and request forms were examined (**Table 3.6**). In this analysis; categories 'A' (proven history of NAIT in a previous pregnancy) and 'B' (possible history of [undiagnosed] NAIT in a previous pregnancy) were excluded since by definition, all cases in these categories had previously been pregnant.

TABLE 3.6. Rates of immunisation during first pregnancy in the various clinical categories.

	Previously pregnant				
	Yes		No		Not Known
Referral category	No. cases	%*	No. cases	%*	No. cases
C. Suspected NAIT in the current neonate	30	68.2	14	31.8	34
D. Cerebral abnormalities in the current fetus	7	77.8	2	22.2	1
E. IUGR	3	100	0	0.0	0
F. Other	0	0.0	0	0.0	2
G. Not known	2	100	0	0.0	1
TOTAL	42	72.4	16	27.6	38

* figure calculated as % of cases where information on previous pregnancies was available.

Immunisation of the mother during the first pregnancy occurred in 22.2% of cases in category 'D' (**cerebral abnormalities in the current fetus**) and in 31.8% cases in category 'C' (**suspected NAIT in the neonate**) and in 27.6% cases overall.

3.3.8 HPA genotypes of referrals

A number of lines of evidence suggest that platelet-specific antibodies may not be reliably detected in cases of NAIT. In the study of Dreyfus, maternal antibodies were not detected in 6/19 (32%) cases of NAIT in which there were parental HPA mismatches (Dreyfus *et al*, 1997). With respect to HPA-1a alloimmunisation, Ghevaert showed that amongst mothers of NAIT-affected infants there was an excess of mothers of HPA-1b1b genotype compared to a control group (5.5% versus 2.5% respectively) (Ghevaert *et al*, 2007b). Lastly, a number of groups have reported that amongst HPA-1b1b women with infants suspected of suffering from NAIT, approximately 4 - 17% do not have detectable HPA-1a antibodies (Mueller-Eckhardt *et al*, 1989; Turner *et al*, 2005; Tiller *et al*, 2009; Bakchoul *et al*, 2011).

Therefore, in order to investigate whether there were abnormal genotype distributions in this study (and thus possible non-detection of HPA antibodies), the HPA genotypes of maternal and paternal samples in those cases where alloantibodies were not detected were compared to a control (Caucasian) population using the chi-squared test.

Only genotype data on the HPA-1, -2, -3, -5 and -15 systems were available for analysis since NHSBT only routinely performs genotyping for HPA systems that have been shown to be clinically important in a Caucasian population.

Only partial genotype data were available in some cases, and in others no data were available; the numbers across **Tables 3.8.1 to 3.8.7** may therefore vary for each HPA system.

In the absence of published UK data, and to derive a statistically significant number, the control population gene frequencies (**Table 3.7**) were obtained from the EMBL-EBI website and were based upon approximately 15,000 individuals from Austria, Croatia, Czech Republic, France, Germany, Ireland, Poland and Spain (Metcalf *et al*, 2011).

TABLE 3.7. Expected Caucasian HPA gene frequencies derived from approximately 15,000 individuals.

HPA-1		HPA-2		HPA-3		HPA-5		HPA-15	
a	b	a	b	a	b	a	b	a	b
0.839	0.162	0.903	0.097	0.612	0.388	0.907	0.093	0.519	0.482

Allele frequencies in the NAIT population were obtained by direct counting (**Tables 3.8.1 to 3.8.7**) and in the control population by calculation from the above figures using the equation:

$$[\text{allele a}]^2 + [2 \times \text{allele a} \times \text{allele b}] + [\text{allele b}]^2 = 1$$

TABLE 3.8.1. HPA genotypes of maternal and paternal samples of cases where HPA-specific alloantibodies were not detected – all categories of referral

HPA	Mothers	Partners	Expected frequency	Mothers		Partners	
	<i>n</i>	<i>n</i>		Observed frequency	<i>P</i>	Observed frequency	<i>P</i>
1a1a	508	341	70.4	72.9	0.16	73.7	0.42
1a1b	163	114	27.2	23.4		24.6	
1b1b	26	8	2.6	3.7		1.7	
TOTAL	697	463					
2a2a	572	381	81.5	82.1	0.33	82.3	0.59
2a2b	112	75	17.5	16.1		16.2	
2b2b	13	7	0.9	1.9		1.5	
TOTAL	697	463					
3a3a	267	191	37.5	38.4	0.93	41.4	0.34
3a3b	327	197	47.5	47.0		42.7	
3b3b	102	73	15.1	14.7		15.8	
TOTAL	696	461					
5a5a	575	386	82.3	82.6	0.36	83.4	0.87
5a5b	119	74	16.9	17.1		16.0	
5b5b	2	3	0.9	0.3		0.6	
TOTAL	696	463					
15a15a	180	132	26.9	26.0	0.94	28.6	0.43
15a15b	348	211	50.0	50.3		45.8	
15b15b	164	118	23.2	23.7		25.6	
TOTAL	692	461					

No significant difference from the control population was observed in any of the HPA systems for either maternal or paternal samples.

In order to determine if any of the individual clinical categories of referral had abnormal genotype distributions that were masked by inclusion in the referral group as a whole, the genotype distributions were calculated for each clinical category (Tables 3.8.2 to 3.8.7).

TABLE 3.8.2. HPA genotypes of maternal and paternal samples of cases where HPA-specific alloantibodies were not detected – category ‘B’ ‘possible history of NAIT in a previous pregnancy’.

HPA	Mothers	Partners	Expected frequency	Mothers		Partners	
	<i>n</i>	<i>n</i>		Observed frequency	<i>P</i>	Observed frequency	<i>P</i>
1a1a	10	7	70.4	76.9	0.66	77.8	0.51
1a1b	3	1	27.2	23.1		11.1	
1b1b	0	1	2.6	0.0		11.1	
TOTAL	13	9					
2a2a	11	8	81.5	84.6	1.00	88.9	0.53
2a2b	2	1	17.5	15.4		11.1	
2b2b	0	0	0.9	0.0		0.0	
TOTAL	13	9					
3a3a	6	0	37.5	46.2	0.81	0.0	0.13
3a3b	6	7	47.5	46.2		77.8	
3b3b	1	2	15.1	7.7		22.2	
TOTAL	13	9					
5a5a	8	8	82.3	61.5	0.18	88.9	0.53
5a5b	5	1	16.9	38.5		11.1	
5b5b	0	0	0.9	0		0.0	
TOTAL	13	9					
15a15a	4	2	26.9	30.8	0.92	22.2	0.31
15a15b	7	7	50.0	53.8		77.8	
15b15b	2	0	23.2	15.4		0.0	
TOTAL	13	9					

TABLE 3.8.3. HPA genotypes of maternal and paternal samples of cases where HPA-specific alloantibodies were not detected – category ‘C’ ‘suspected NAIT in the current neonate’.

	Mothers	Partners	Expected frequency	Mothers		Partners	
	<i>n</i>	<i>n</i>		Observed frequency	<i>P</i>	Observed frequency	<i>P</i>
1a1a	205	168	70.4	71.9	0.64	76.4	0.32
1a1b	70	48	27.2	24.6		21.8	
1b1b	10	4	2.6	3.5		1.8	
TOTAL	285	220					
2a2a	243	176	81.5	85.3	0.46	80.0	0.70
2a2b	40	40	17.5	14.0		18.2	
2b2b	2	4	0.9	0.7		1.8	
TOTAL	285	220					
3a3a	110	88	37.5	38.7	0.91	40.2	0.82
3a3b	134	98	47.5	47.2		44.7	
3b3b	40	33	15.1	14.1		15.1	
TOTAL	285	219					
5a5a	238	189	82.3	83.8	0.79	85.9	0.26
5a5b	45	31	16.9	15.8		14.1	
5b5b	1	0	0.9	0.4		0.0	
TOTAL	284	220					
15a15a	73	51	26.9	25.9	0.97	23.3	0.56
15a15b	142	109	50.0	50.4		49.8	
15b15b	67	59	23.2	23.8		26.9	
TOTAL	282	219					

TABLE 3.8.4. HPA genotypes of maternal and paternal samples of cases where HPA-specific alloantibodies were not detected – category ‘D’ ‘cerebral abnormalities in the current fetus’.

HPA	Mothers	Partners	Expected frequency	Mothers		Partners	
	<i>n</i>	<i>n</i>		Observed frequency	<i>P</i>	Observed frequency	<i>P</i>
1a1a	206	105	70.4	75.5	0.26	70.0	0.70
1a1b	58	43	27.2	21.2		28.7	
1b1b	9	2	2.6	3.3		1.3	
TOTAL	273	150					
2a2a	228	129	81.5	83.5	0.53	86.0	0.27
2a2b	40	18	17.5	14.7		12.0	
2b2b	5	3	0.9	1.8		2.0	
TOTAL	273	150					
3a3a	108	69	37.5	39.6	0.87	46.0	0.11
3a3b	125	53	47.5	45.8		35.3	
3b3b	40	28	15.1	14.7		18.7	
TOTAL	273	150					
5a5a	225	122	82.3	82.4	0.36	81.3	0.96
5a5b	48	27	16.9	17.6		18.0	
5b5b	0	1	0.9	0.0		0.7	
TOTAL	273	150					
15a15a	73	53	26.9	26.8	0.99	35.3	0.15
15a15b	137	59	50.0	50.4		39.3	
15b15b	62	38	23.2	22.8		25.3	
TOTAL	272	150					

TABLE 3.8.5. HPA genotypes of maternal and paternal samples of cases where HPA-specific alloantibodies were not detected – category ‘E’ ‘IUGR’.

HPA	Mothers	Partners	Expected frequency	Mothers		Partners	
	<i>n</i>	<i>n</i>		Observed frequency	<i>P</i>	Observed frequency	<i>P</i>
1a1a	17	15	70.4	70.8	0.97	78.9	0.63
1a1b	6	4	27.2	25.0		21.1	
1b1b	1	0	2.6	4.2		0.0	
TOTAL	24	19					
2a2a	21	16	81.5	87.5	0.43	84.2	0.94
2a2b	2	3	17.5	8.3		15.8	
2b2b	1	0	0.9	4.2		0.0	
TOTAL	24	19					
3a3a	6	6	37.5	25.0	0.62	31.6	0.79
3a3b	14	11	47.5	58.3		57.9	
3b3b	4	2	15.1	16.7		10.5	
TOTAL	24	19					
5a5a	17	14	82.3	70.8	0.44	73.7	0.69
5a5b	6	5	16.9	25.0		26.3	
5b5b	1	0	0.9	4.2		0.0	
TOTAL	24	19					
15a15a	7	7	26.9	29.2	0.94	36.8	0.77
15a15b	11	9	50.0	45.8		47.4	
15b15b	6	3	23.2	25.0		15.8	
TOTAL	24	19					

TABLE 3.8.6. HPA genotypes of maternal and paternal samples of cases where HPA-specific alloantibodies were not detected – category ‘F’ ‘Other’.

	Mothers	Partners	Expected frequency	Mothers		Partners	
	<i>n</i>	<i>n</i>		Observed frequency	<i>P</i>	Observed frequency	<i>P</i>
1a1a	48	38	70.4	62.3	0.40	71.7	1.0
1a1b	24	14	27.1	31.2		26.4	
1b1b	5	1	2.6	6.5		1.9	
TOTAL	77	53					
2a2a	66	42	81.6	85.7	0.54	79.2	0.65
2a2b	11	11	17.4	14.3		20.8	
2b2b	0	0	0.9	0		0.0	
TOTAL	77	53					
3a3a	31	22	37.4	40.3	0.85	42.3	0.81
3a3b	33	22	47.5	42.9		42.3	
3b3b	13	8	15.1	16.9		15.4	
TOTAL	77	52					
5a5a	66	41	82.3	85.7	0.54	77.4	0.34
5a5b	11	10	16.8	14.3		18.9	
5b5b	0	2	0.9	0.0		3.8	
TOTAL	77	53					
15a15a	15	18	26.9	19.7	0.57	34.0	0.59
15a15b	39	22	49.9	51.3		41.5	
15b15b	22	13	23.2	28.9		24.5	
TOTAL	76	53					

TABLE 3.8.7. HPA genotypes of maternal and paternal samples of cases where HPA-specific alloantibodies were not detected – category ‘G’ ‘referral reason unknown’.

HPA	Mothers	Partners	Expected frequency	Mothers		Partners	
	<i>n</i>	<i>n</i>		Observed frequency	<i>P</i>	Observed frequency	<i>P</i>
1a1a	22	8	70.4	88.0	0.21	66.7	0.75
1a1b	2	4	27.2	8.0		33.3	
1b1b	1	0	2.6	4.0		0.0	
TOTAL	25	12					
2a2a	21	10	81.5	84.0	0.95	83.3	1.00
2a2b	4	2	17.5	16.0		16.7	
2b2b	0	0	0.9	0.0		0.0	
TOTAL	25	12					
3a3a	6	6	37.5	24.0	0.63	50.0	0.30
3a3b	15	6	47.5	60.0		50.0	
3b3b	4	0	15.1	16.0		0.0	
TOTAL	25	12					
5a5a	21	12	82.3	84.0	1.0	100.0	0.14
5a5b	4	0	16.9	16.0		0.0	
5b5b	0	0	0.9	0.0		0.0	
TOTAL	25	12					
15a15a	8	1	26.9	32.0	0.91	9.1	0.46
15a15b	12	5	50.0	48.0		45.5	
15b15b	5	5	23.2	20.0		45.5	
TOTAL	25	11					

No significant difference was observed in genotype frequency between the control and test populations in any of the HPA systems in any of the clinical categories.

3.3.9 Geographical distribution of previously undiagnosed HPA antibody positive cases

Samples requiring investigating for the presence of platelet alloantibodies in cases of suspected NAIT may be referred from specialist, centralised, fetal medicine units, general maternity units or blood banks or haematology departments associated with such units and it is possible that the clinical awareness and investigating policies for thrombocytopenia in a fetus/neonate vary between hospitals. In order to identify the sources and possible clustering of HPA antibody-positive cases, the location of the referring hospital in each case was plotted (**Figure 3.4**); confidence intervals for the incidence of cases with HPA-specific antibodies were calculated (**Table 3.9**).

FIGURE 3.4. Geographical location of HPA antibody-positive referrals. Each circle represents one case. The numerator indicates the number of HPA antibody-positive cases identified and the denominator is the total number of births during the study period. The final figure is the incidence of HPA antibody positive cases.

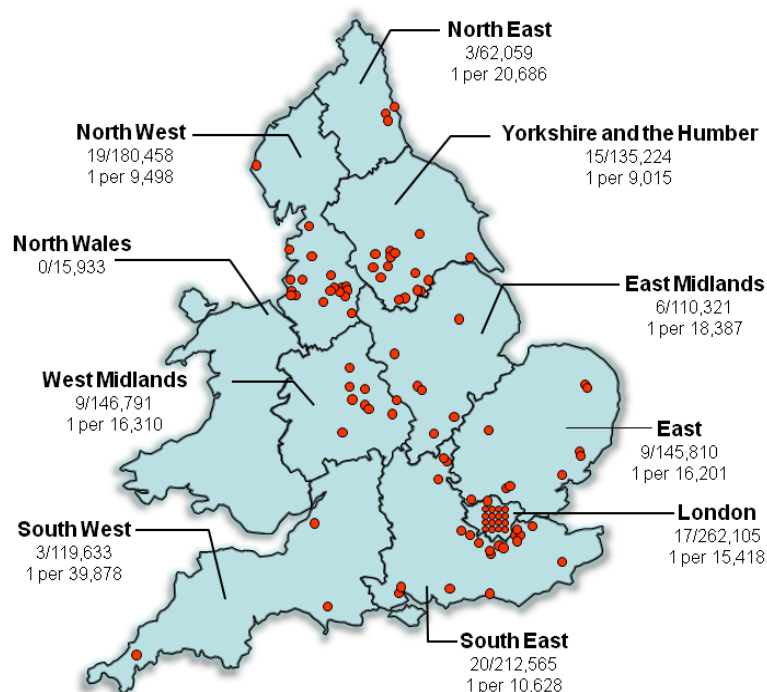


TABLE 3.9. Incidence of previously unknown HPA antibody-positive cases by health region.

Region	HPA antibody positive	Number of births	Incidence	95% confidence intervals	
				Lower	Upper
North East	3	62,059	1/20,686	1/7,078	1/100,305
Yorkshire / Humber	15	135,224	1/9,015	1/5,466	1/16,107
East Midlands	6	110,321	1/18,387	1/8,448	1/50,103
East	9	145,810	1/16,201	1/8,534	1/35,430
London	17	262,105	1/15,418	1/9,630	1/26,467
South East	20	212,565	1/10,628	1/6,882	1/17,400
South West	3	119,633	1/39,878	1/13,645	1/193,362
West Midlands	9	146,791	1/16,310	1/8,592	1/35,669
North West	19	180,458	1/9,498	1/6,082	1/15,775
Wales	0	15,933	na	na	na
ALL REGIONS	101	1,390,899	1/13,771	1/11,334	1/16,907

na: not applicable

Although the incidence varied widely between the Yorkshire and Humber and South West health regions the difference was not significant ($P = 0.27$).

3.4 Limitations of the study

The clinical data in NHSBT databases was derived from request forms that had been completed by a range of staff groups and was generally free text. Interpretation of this text into the clinical categories used here was also by a range of staff groups and it is likely therefore that variation in the quality of the data exists.

It was not possible to contact referring clinicians to obtain details of their clinical criteria or algorithms used to determine whether particular cases should be investigated for NAIT and it is likely that these varied across referring units; there may therefore be heterogeneity within the categories studied here.

Data entry into the clinical database was generally performed by one individual without validation by a second, and data extraction was also performed by only one individual; it is possible therefore that some errors are present.

Despite this study covering the whole of England, North Wales and Northern Ireland and extension of the study from one to two years, the incidence of NAIT was low, thus the power of the study to detect differences between groups was low. Further extension of the study was not possible.

3.5 Discussion and Conclusions

The main aim of this project is to understand why there have been difficulties in the detection and quantitation of HPA-1a antibodies since these deficiencies are some of the barriers to the introduction of antenatal screening for NAIT (Williamson, 1998; Murphy & Williamson, 2000; Murphy *et al*, 2002c). Some of these problems have been observed in a population within a defined geographical area within England where approximately 5% of HPA-1b1b mothers of apparently NAIT-affected children did not have detectable HPA-1a antibodies, and the measured level of anti-HPA-1a was unrelated to neonatal platelet count (Williamson *et al*, 1998; Ghevaert *et al*, 2007a; Ghevaert *et al*, 2007b). Thus, there appears to be a significant problem in the diagnosis and prediction of NAIT in (at least part of) England.

In this phase of the project, the largest ever study of NAIT in England and North Wales, we investigated whether the problem of non-detection of HPA antibodies, including HPA-1a, was more widespread. This study also gave us the opportunity to investigate two other important factors.

Firstly, to assess whether the outcome of pregnancies of newly diagnosed cases of NAIT i.e. non-antenatally treated, differed from those in which NAIT was predicted and thus actively managed in many cases.

Secondly, whether more unusual presentations of NAIT such as ventriculomegaly that have previously been published as isolated case reports represent a significant indicator for investigation of the mother for the presence of platelet specific antibodies (Petersen *et al*, 1986; Montemagno *et al*, 1994; Murphy *et al*, 1996; Povia *et al*, 2007).

This phase of the project therefore was expected to generate a significant amount of data regarding NAIT related directly to the population of England that would be potentially useful in future discussions regarding the introduction of an antenatal

screening program for NAIT, or other programmes targeted at particular 'at-risk' pregnancies.

Our data showed the incidence of clinically suspected NAIT to be 1 case per 8,080 births, a figure compatible with that found in similar studies and, as expected, much lower than that found in antenatal screening studies (Davoren *et al*, 2002; Tiller *et al*, 2009). Together with the finding of HPA antibodies in 17.2% of unselected referrals and 12.5% *de novo* referrals, as reported by others, these data show that our findings may have applicability elsewhere (Davoren *et al*, 2004; Ghevaert *et al*, 2007b; Tiller *et al*, 2009).

The subdivision of referrals into defined clinical categories allowed us to determine if there were particular factors that might, in the absence of an antenatal screening programme, influence the clinical decision to refer samples for investigation of suspected NAIT. Careful scrutiny of clinical details provided on request forms accompanying the blood samples allowed us to identify a group of mothers in whom there was a suggestion that previous children may have suffered from NAIT that had not been clinically recognised and/or had not been investigated. This category, (category 'B') was shown to have the highest incidence of HPA-specific antibodies with 5/19 (26.3%) mothers having detectable antibodies, the majority of which were HPA-1a specific. This is an important finding and suggests that NAIT is not always clinically recognised, perhaps partially explaining reported differences in incidence of NAIT between antenatal screening studies and studies that relied on clinical suspicion of NAIT (Durand-Zaleski *et al*, 1996; Davoren *et al*, 2002; Tiller *et al*, 2009).

Category 'C', 'suspected NAIT in the current neonate', highlighted the problem in clinical reporting of NAIT. In this category 11/78 cases (14.1%) in which the mothers were found to have HPA antibodies and where the neonates were shown to have the relevant HPA, were identified through referral of a blood sample to NHSBT, but were

not reported through the BPSU scheme. These data suggest that the unit referring the blood sample was a unit other than a paediatric unit; no follow-up was performed to confirm this but the author's experience would support this.

Interestingly, HPA-specific antibodies were found at relatively high frequency in both the IUGR (3/27, 11.1%) and 'cerebral abnormalities in the current fetus' categories (10/285, 3.5%); these figures are well in excess of the antibody incidence (0.62%) amongst multiparous blood donors reported by Dawkins (Dawkins *et al*, 1995).

The number of cases in the IUGR is probably too small to draw significant conclusions from although Tiller *et al*, in a much larger study, have shown that male infants born to mothers with HPA-1a antibodies have a birth weight that is 530g lower than a control group born to mothers with no detectable HPA antibodies (Tiller *et al*, 2012).

The data from the 'cerebral abnormalities in current fetus' is more informative in view of the larger numbers involved. Whilst isolated reports of NAIT presenting as hydrocephaly or ventriculomegaly have been published, this is the first report that quantifies the extent of this presenting feature (Petersen *et al*, 1986; Montemagno *et al*, 1994; Murphy *et al*, 1996; Spencer & Burrows, 2001; Pova *et al*, 2007). In view of the number and types of fetal medicine and hospital units involved it is likely that the criteria used to select such cases for investigation vary and thus it is difficult to draw firm conclusions regarding the need to screen such cases for HPA antibodies, however, these findings suggest that further controlled studies are warranted.

Most importantly, from the point of view of this project, HPA-1a antibodies were the most commonly encountered specificity, being detected as the only specificity in 102/146 (69.9%) of all cases, or 70/101 (69.3%) newly diagnosed cases, or in 76.0% and 74.3% cases in combination with other specificities respectively.

The frequency of HPA-1b1b mothers amongst the 'antibody negative' group was slightly higher (3.7%) than in the control population (2.6%) but this was not statistically significant. Detailed analysis showed the highest frequency (6.5%, 5/77) to be in category 'F' ('other'). Examination of the supplied referral details showed that in one case the mother had been typed as HPA-1b1b as a blood donor, and in two cases the mother's sister was HPA-1b1b with NAIT-affected children; these referrals therefore, were not for investigation of NAIT *per se*. Thus, we were not able to confirm a significant excess of HPA-1b1b mothers as shown in other studies. This may be due to the use of improved diagnostic assays or perhaps the control population does not accurately reflect the ethnic mix seen in the study population. Alternatively, it is possible that studies reporting an excess of HPA-1b1b antibody-negative mothers may have included more mothers that were blood donors or relatives of mothers with NAIT-affected children.

An important question that has arisen recently is whether immunisation against HPAs occurs predominantly after delivery. Early studies reported that 25 - 50% of women diagnosed with NAIT-affected children were primiparous, however, Killie *et al*, in the largest ever antenatal screening study, showed that only 7.5% women had detectable antibodies in the first pregnancy (Killie *et al*, 2008). Our data show that approximately 28% (16/58) women with NAIT-affected infants had not previously been pregnant and thus support earlier findings (Mueller-Eckhardt *et al*, 1989; Williamson *et al*, 1998; Maslanka *et al*, 2003). The reason for this discrepancy is unclear. Clearly this is an important factor in the context of antenatal screening, since the study of Killie suggests that most women become immunised following delivery and that antenatal screening may be less important, whilst other studies suggest that antenatal screening is necessary in order to identify the 25 - 50% women that are immunised. The finding that 6/13 primiparous women in the Norwegian study gave birth to infants with platelet counts $<50 \times 10^9/L$ would suggest

that it would be advantageous to identify immunised women as early as possible in order that appropriate treatment might be offered. Our data clearly show that antenatal treatment of NAIT has a beneficial effect. Of those cases that were not diagnosed until delivery, 67% experienced a haemorrhagic symptom compared to 5% receiving antenatal treatment ($P = <0.001$), whilst 20% versus 4% ($P = 0.014$) suffered from a ICH respectively (Knight *et al*, 2011). The median platelet count in non-antenatally treated infants was $17 \times 10^9/L$ whilst in those receiving treatment it was $124 \times 10^9/L$ ($P = <0.001$). The long term prognosis was also worse in non-antenatally treated than in treated infants with 9/88 (10%) and 0/24 respectively either becoming disabled (7 cases) or dying (2 cases) before 1 year. Thus, advanced knowledge of maternal immunisation would potentially allow amelioration of NAIT and the only mechanism by which this can occur is by introduction of antenatal screening. The lack of sensitivity and less than perfect predictive abilities of current assays currently preclude this in England.

Whilst this phase of the study has not shown an excess of HPA-1b1b mothers and thus deficiencies in antibody detection as shown in other studies, it has confirmed the importance of HPA-1a in the context of NAIT.

The remaining chapters therefore investigate in detail the serological and functional effects of HPA-1a antibodies in order to understand why HPA-1a antibodies have sometimes been difficult to detect, why it has proved difficult to demonstrate absolute correlation between maternal antibody level and neonatal platelet count, and to further investigate effects of HPA-1a antibodies on platelet function and platelet production.

3.6 Summary of findings in this chapter

- The incidence of NAIT in England, North Wales and Northern Ireland is 1 case per 8,080 births (95% CI: 6,993 and 9,346); this is the first time that a figure has been derived for this population.
- The incidence of HPA-specific antibodies in previously unscreened mothers was 1 per 13,771.
- HPA alloantibodies were detected in 17.2% unselected, and 12.5% *de novo* referrals.
- The highest incidence of HPA alloantibodies (other than in previously diagnosed cases), (26.3%) was in referrals where there was an obstetric history suggesting previously undiagnosed NAIT in siblings of the infant currently being investigated for suspected NAIT. These data suggest that (i) NAIT is not clinically diagnosed in a significant number of cases and (ii) that review of the maternal obstetric history in subsequent pregnancies may suggest a risk and therefore prompt testing for HPA antibodies in the current pregnancy. This is an extremely important finding that partially explains differences in the incidence of NAIT between antenatal screening studies and clinically suspected/referred cases.
- In cases of 'suspected NAIT in the neonate', 21.4% of mothers were found to have platelet-specific alloantibodies.
- Platelet alloantibodies were found at a moderately high incidence in cases referred because of IUGR (11.1%) (although case numbers were low), and 'cerebral abnormalities in the current fetus' (3.5%). These data suggest that these clinical presentations of NAIT may be more important than previously recognised.

- HPA-1a antibodies were the most commonly encountered specificity. These were present as the only HPA-specific antibody in 69.9% of all, or 69.3% of *de novo* cases. Including cases where other HPA-specific antibodies were present, they were detected in 76.0% of all, or 74.3% *de novo* cases.
- Immunisation of the mother occurred during the first pregnancy in 27.6% cases.
- No significant difference in HPA genotype frequency was observed between mothers referred for investigation of suspected NAIT in their neonate and a control population or in any of the other clinical categories of referral.
- The incidence of NAIT varied by health region (1 case per 9,015 to 1 case per 39,878 births) but these differences were not statistically significant.
- Antenatal treatment of NAIT results in significantly less severe disease.

3.7 Significance of these findings

These data confirm that HPA-1a antibodies are the most common cause of NAIT in England and North Wales thus confirming the validity of this project that focuses on HPA-1a antibodies.

We have clearly shown that early identification of 'at-risk' pregnancies e.g. by the use of antenatal screening has the potential to reduce the severity of disease.

The dissection of cases into *de novo* and repeat pregnancies provides useful information on the incidence of NAIT in a previously unscreened population.

The further dissection into specific referral categories identified that a significant number of NAIT cases are not identified clinically and that IUGR and cerebral abnormalities in the fetus are potentially important reasons for investigation of NAIT.

Chapter 4

Integrin $\alpha\text{IIb}\beta 3$
conformation I: effect of
integrin activation and
dissociation on HPA-1a
epitope expression

4.1 Introduction

The extracellular expression of a stable, functionally-competent and intact $\alpha\text{IIb}\beta 3$ complex is critically dependent on the presence of Ca^{2+} cations within the α - and β -chains (Nurden *et al*, 1987; Bajt & Loftus, 1994; Wilcox *et al*, 1994; Gidwitz *et al*, 2004; Murcia *et al*, 2008; Mansour *et al*, 2011; Raborn *et al*, 2011).

The chelating compound EDTA can bind over 60 divalent cations in a 1:1 ratio and has an extremely high affinity ($K = 2 \times 10^7 \text{ M}^{-1}$) for calcium (Sheppard & Henion, 1997a; Keowmaneechai & McClements, 2002). This compound is widely used as an anticoagulant and is frequently a component of buffers used in assays for platelet antibodies in order to prevent platelet aggregation [(Manoil *et al*, 1997) and personal observation of author from results of NIBSC workshops].

When used as an anticoagulant the final concentration of EDTA is typically 3 -7 mM (Sheppard & Henion, 1997b; Lowe *et al*, 2005) and when used in laboratory buffers around 10 mM (Nieminen & Kekomaki, 1992; Noris *et al*, 1995). Since EDTA binds calcium in a 1:1 ratio and the normal concentration of Ca^{2+} in blood is approximately 2 - 3 mM (Kapur *et al*, 2010), the concentration of EDTA in EDTA-anticoagulated blood or of platelets suspended in EDTA buffers is sufficient to reduce the calcium concentration to below the minimum level of 0.8 - 1.2 mM that is required to maintain the intact $\alpha\text{IIb}\beta 3$ complex (Kunicki *et al*, 1981). The dissociation of the $\alpha\text{IIb}\beta 3$ complex by EDTA however is also dependent on exposure time and temperature (Fitzgerald & Phillips, 1985).

Significant differences in the reagents used, and of the parameters within, the widely-used MAIPA assay were observed in an international workshop exercise including the use of EDTA-containing buffers by some, but not all, participating laboratories (Bessos *et al*, 2005). The ability to detect antibodies against $\alpha\text{IIb}\beta 3$ varied between laboratories participating in this and many other international workshops (Metcalf *et*

al, 1997; Panzer, 2001a; Bessos *et al*, 2005; Allen *et al*, 2007; Porcelijn *et al*, 2008; Wu *et al*, 2010).

We hypothesized that Ca^{2+} concentration might alter the conformation of the $\alpha\text{IIb}\beta 3$ integrin and thus the recognition of the HPA-1a epitope by alloantibodies. The aims of this phase of the project therefore were to determine:

- The effect of EDTA on the conformation of the $\alpha\text{IIb}\beta 3$ complex by using a panel of monoclonal antibodies, specifically including those employed routinely as capture antibodies in the MAIPA assay that is widely used for antibody detection by diagnostic laboratories.
- Whether conformational changes induced by EDTA in the $\alpha\text{IIb}\beta 3$ complex affect expression of the HPA-1a epitopes.
- If conformational changes affect the sensitivity of detection of HPA antibodies under research conditions and
- Whether the research findings are relevant to diagnostic laboratories and thus provide a possible explanation for the reported variable/non-detection of HPA-1a antibodies.

We also explore the possible effects of a further potential laboratory-induced artefact, namely platelet activation.

Activation of platelets may inadvertently be induced *in vitro* as a result of extracting platelets from whole blood (Parker *et al*, 1984; Michelson, 1996; Harrison, 2005). During the process of platelet activation, $\alpha\text{IIb}\beta 3$ undergoes a major structural re-organisation. In the resting state, the molecule exists as a compact 'closed' form with the headpiece located between the 'legs' or tailpieces whilst upon activation it transitions to an extended 'open' form (**Figure 1.3**) (Zhu *et al*, 2008; Eng *et al*, 2011).

In the resting form of $\alpha\text{IIb}\beta 3$, the PSI domain (containing the HPA-1a epitopes) is located at the genu of the molecule in a fold between the head and tail pieces and is partially obscured by the hybrid and EGF domains (Zhu *et al*, 2008). The transition from resting to activated state results in the swinging out of the hybrid domain and a concomitant substantial movement of the PSI domain (up to 7 nM away from the α -chain) since the interface between these two domains is rigid (Xiao *et al*, 2004; Provasi *et al*, 2009). This change in conformation is known to provide access to PSI domain epitopes that are not accessible in the closed structure (Honda *et al*, 1995b).

Whether the structural transformation affects the reactivity of platelet-specific alloantibodies, and in particular of HPA-1a antibodies, is unknown. Masters reported three examples of a platelet-specific antibody that recognised a polymorphism present only on platelets that had been immobilised on to plastic micro-ELISA plates, a process that may have induced platelet activation although activation status was not formally assessed by the authors (Masters & Taaning, 1998). These antibodies were undetectable by the MAIPA assay and thus localisation of the target epitopes to a particular integrin was therefore not possible. We have also detected a platelet-specific alloantibody in a patient who was refractory to platelet transfusion that was reactive only in an assay system (Capture-P®, Immucor, USA) in which platelets were immobilised on to plastic microtitre plates (Allen and others, unpublished data). This antibody failed to react in the MAIPA assay and thus the target glycoprotein also remains unknown.

Thus, the knowledge that platelet activation results in changes in PSI domain epitope accessibility and that HPA-1a antibodies are variably detected in international workshops, led us to hypothesize that capture mAbs used in the MAIPA assay and/or HPA-1a alloantibodies might vary in their recognition of activated versus non-activated platelets. We therefore explored this hypothesis

using a panel of HPA-1a antibodies, tested against ADP-activated and non-activated platelets.

4.2 Methods

4.2.1 Effect of exposure of integrin $\alpha\text{IIb}\beta 3$ complex to varying EDTA concentrations

The effect of EDTA on the $\alpha\text{IIb}\beta 3$ complex on platelets in whole blood was determined using a whole blood immunofluorescence assay.

Modified HEPES-Tyrode's buffer was prepared containing EDTA at a range of concentrations from 0.01 to 10 mM. Whole blood (5 μL) was incubated with appropriate EDTA buffer (55 μL) for 30 mins at 37°C then 10 μL PE-conjugated monoclonal antibody P2 (anti- $\alpha\text{IIb}\beta 3$ complex) or PE-conjugated isotype control was added and incubated at 37°C for a further 15 mins. 500 μL HEPES-Tyrode's buffer containing the same concentration of EDTA used in the primary incubation was added and fluorescence of the labelled platelets assessed by flow cytometry within two hours.

4.2.2 Effect of incubation temperature and incubation time of EDTA exposure on integrin $\alpha\text{IIb}\beta 3$

The above protocol was followed except that the primary incubation was at room temperature or 37°C and incubation times ranged from 0 mins (T_0) to 45 mins (T_{45}); EDTA was used at 1 mM in HEPES-Tyrode's buffer

4.2.3 Impact of exposure of PRP to EDTA on various epitopes on $\alpha\text{IIb}\beta 3$

To avoid conformational changes in $\alpha\text{IIb}\beta 3$ resulting from extraction of platelets from whole blood, a modified MAIPA assay that utilised PRP was developed. Prior to using PRP in this assay the effect of EDTA on platelets in PRP rather than in whole blood was studied.

Equal volumes of PRP were incubated with TBS ('mock treatment') or with TBS/EDTA (20 mM) stock solution (final EDTA concentration 10 mM) for 60 mins at 37°C; native PRP that had not been incubated at 37°C was used as a control.

The treated platelets (as PRP), together with untreated PRP, were used in immunofluorescent, flow cytometric assays without further manipulation (see below).

4.2.4 PRP immunofluorescence assays

For primary antibodies that were not directly conjugated to fluorochromes:

Aliquots of untreated, TBS- or EDTA-treated, PRP containing a total of 5×10^6 platelets were incubated with 25 μL test sample containing anti-HPA-1a, inert (AB serum) control or 10 μL mAb as appropriate.

After 60 mins incubation at 37°C , the platelets were washed three times (1 mL volumes) in cold TBS/BSA/EDTA. The platelets were re-suspended in 50 μL FITC-labelled anti-human or anti-mouse IgG secondary antibody as appropriate and incubated at RT for 20 mins. 1 mL cold TBS/BSA was added and fluorescence analysed within 2 hrs.

For fluorochrome-labelled primary antibodies:

Aliquots (5 μL) of PRP were incubated with 10 μL FITC- or PE-labelled antibody at RT for 20 mins then 1 mL cold TBS/BSA was added and fluorescence analysed within 2 hrs.

4.2.5 'PRP MAIPA' assay

To specifically study the effect of cation chelation on HPA-1a epitopes, a modification of the standard MAIPA assay was used since preparation of washed platelets by differential centrifugation could potentially induce confounding changes in the structure of $\alpha\text{IIb}\beta 3$ and thus potentially alter the conformation of HPA-1a epitopes.

In this 'PRP MAIPA' assay platelets were used in the form of platelet-rich PRP.

To prepare dissociated $\alpha\text{IIb}\beta 3$, PRP was incubated with TBS containing 20 mM EDTA (pH 7.2) at 37°C for 60 mins. The volume of EDTA solution added depended

on the platelet count of the PRP since the number of platelets to be used in each test was to be maintained at 1×10^7 in order to preserve the antigen:antibody ratio between experiments. The calculated EDTA final concentrations in the reaction tubes therefore ranged from 8 - 8.7 mM. Control, mock-treated platelets were prepared by incubating PRP with TBS.

The MAIPA assay was performed by using 200 μL volumes of PRP and 25 μL test sample. The remainder of the assay was as described for the standard MAIPA assay (Chapter 2.4.5).

4.2.6 Effect of EDTA on integrin conformation and mAb/polyclonal antibody binding during the MAIPA assay

To understand the dynamics of the changes occurring to $\alpha\text{IIb}\beta 3$ and to the HPA-1a epitope, together with that of human alloantibody and capture mAb binding, the volumes of the components of the PRP MAIPA (test sample, PRP and capture mAb) were scaled up by a factor of four to allow sub-samples to be removed for analysis at various stages of the assay. One example of anti-HPA-1a and one negative control were used.

A total of five experiments were performed; two using PAB-6 as capture mAb, two using 5B12 and one using PAB-1.

At the appropriate stage, one quarter of the reaction mix was removed for analysis. These aliquots were washed twice in TBS/BSA/EDTA then re-suspended to the original aliquot volume in TBS/BSA.

To perform flow cytometry, 5 μL of platelet suspension from the aliquot was incubated with P2 (10 μL), B2 (50 μL), anti-human IgG-FITC diluted 1 in 25 in TBS/BSA (50 μL) or anti-mouse IgG diluted 1 in 25 (50 μL) for 20 mins at RT. After the addition of 1 mL of cold TBS/BSA antibody binding was assessed using flow

cytometry.

4.2.7 Preparation of samples for inclusion in international quality assurance workshop exercise

Approximately 100 mL and 50 mL blood respectively was collected from two mothers of children affected by anti-HPA-1a mediated NAIT and divided equally between dry, non-anticoagulated and EDTA-anticoagulated tubes (Becton Dickinson, UK).

The non-anticoagulated samples were allowed to clot for 2 hrs at RT then all samples were centrifuged ($2,000 \times g$, 15 mins at RT). The serum and EDTA plasma were removed, aliquots removed for anti-platelet antibody and infectious marker (HB_sAg, HIV) testing and then stored at -40°C prior to transportation to NIBSC.

4.2.8 Activation of platelets

HPA-1a1a PRP was prepared, and platelet counts performed as previously described (**Chapter 2.4.2**). Assuming 1×10^7 platelets were required for each test within the MAIPA assay, sufficient PRP was decanted into a fresh 12 x 75 mm plastic tube for all tests to be performed. For each 100 μL volume of PRP, 1 μL Gly-Pro-Arg-Pro amide solution (100 mM) was added plus either 20 μL adenosine diphosphate (ADP) (200 μM in TBS) or 20 μL TBS. The tubes were then incubated at RT for 15 mins.

4.2.9 Assessment of platelet activation

To confirm activation of the platelets, four aliquots (each of 25 μL) were removed and incubated with fluorochrome-labelled mAbs (PAC-1, CD62P, VI-PL2 and isotype control) for 20 mins at RT. 2 mL cold TBS/BSA/EDTA was added and fluorescence analysed immediately.

4.2.10 Activated platelets MAIPA assay

The remaining PRP was washed twice in cold TBS/BSA/EDTA and the platelets re-

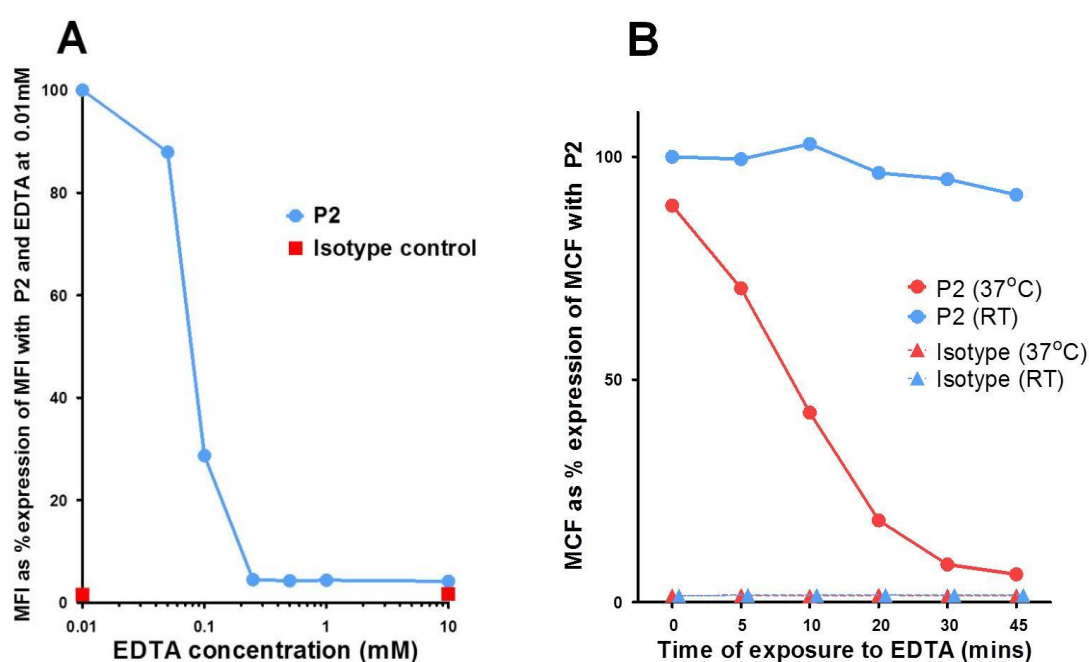
suspended in TBS/BSA at a concentration of $2 \times 10^5/\mu\text{L}$. The standard MAIPA assay (**Chapter 2.4.5**) was then performed using activated and non-activated platelets. A panel of 49 examples of anti-HPA-1a (32 NAIT-derived; 17 blood donor-derived) together with one negative control were used; each sample was tested on two occasions using platelets obtained from different blood donors.

4.3 Results

4.3.1 Effect of EDTA on integrin $\alpha\text{IIb}\beta 3$ on platelets in whole blood

Preliminary work by others within our group investigated the effect of EDTA concentration and the time of exposure to EDTA on the expression of the complex-dependent epitope on $\alpha\text{IIb}\beta 3$ recognised by the mAb 'P2' (Figure 4.1).

FIGURE 4.1. Effect of EDTA concentration (A), and temperature and time of exposure to 1 mM EDTA (B) on expression of the $\alpha\text{IIb}\beta 3$ complex.



Exposure to EDTA concentrations of ≥ 0.25 mM resulted in almost complete loss of the P2 epitope; exposure to concentrations between 0.05 mM and 0.25 mM resulted in partial loss.

EDTA-dependent loss of the P2 epitope was highly temperature sensitive. After 5 minutes exposure to EDTA at 37°C, P2 reactivity was only 70.5% of that at T_0 , whilst incubation for the same time period at RT resulted in approximately 99.5% residual activity of that at T_0 . After 45 mins incubation at 37° only 6.3% of the epitope

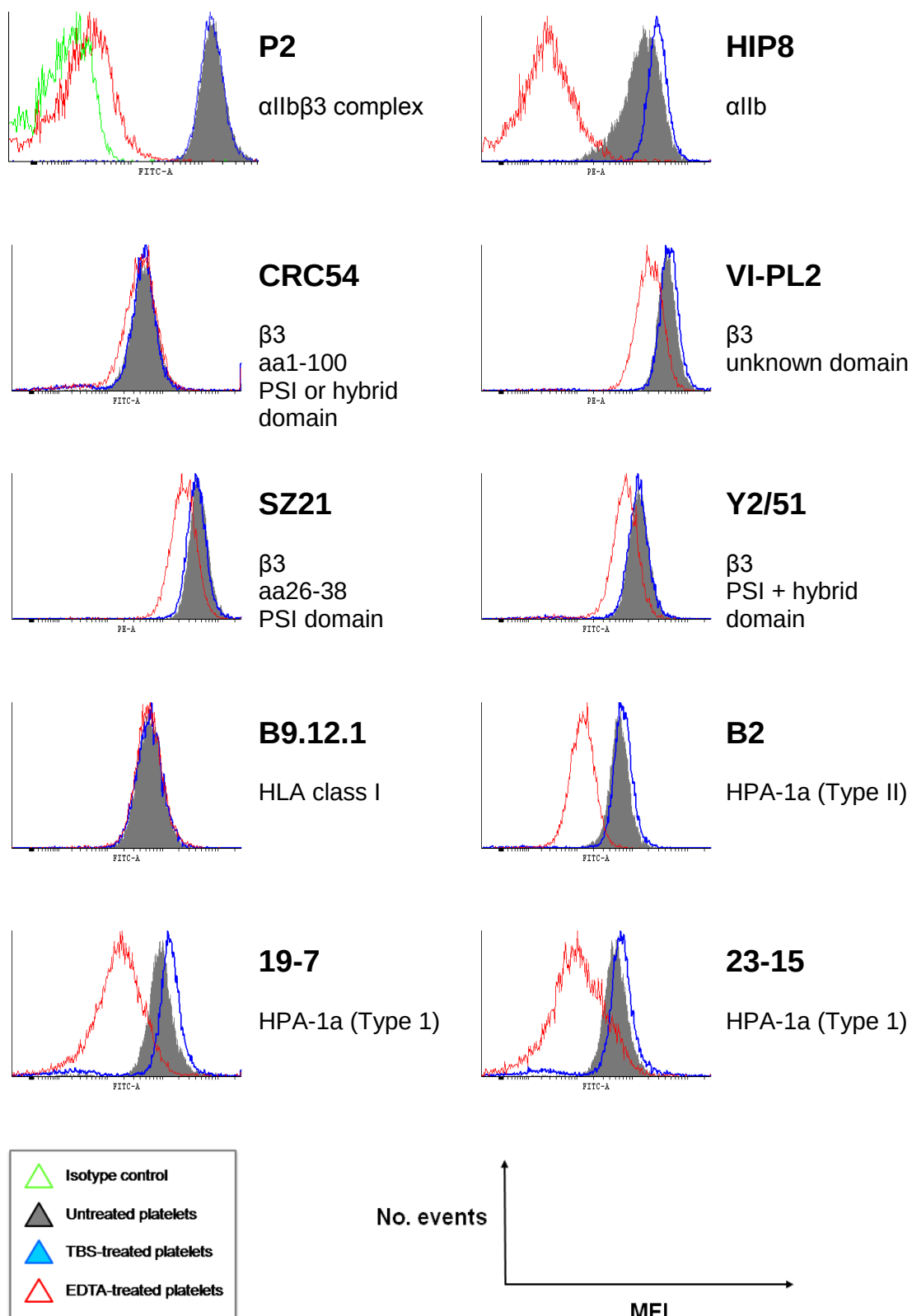
remained detectable compared to 91.5% following RT incubation.

These data showed that $\alpha\text{IIb}\beta 3$ is highly sensitive to EDTA disruption, and this effect is time, temperature and concentration dependent.

4.3.2 Precise effects of EDTA on integrin $\alpha\text{IIb}\beta 3$ and HPA-1a epitopes on platelets in PRP

A panel of mAbs was used to study the effect of EDTA on accessibility/conformation of αIIb , $\beta 3$, $\alpha\text{IIb}\beta 3$ complex and HPA-1a epitopes. A control antibody, B9.12.1 that recognises HLA class I, a non-cation dependent molecule was used as a negative control. Typical results are shown in **Figure 4.2** and results of replicate testing and statistical analysis of the data in **Table 4.1**.

FIGURE 4.2 Flow cytometric analysis of changes in mAb epitope accessibility following TBS- or EDTA-treatment of PRP. Clone names are shown in *bold typeface*.



The effect of cation chelation on epitopes on $\alpha\text{IIb}\beta 3$ recognised by mAb's varied widely.

Least affected was the epitope recognised by CRC54 which is located within the first 100 amino acids of $\beta 3$ (within PSI or hybrid domain) (TBS-treated PRP mean MFI: 5,166 SD: 1,019; EDTA-treated PRP mean MFI: 4,869 SD: 819, $n = 5$) ($P = 0.63$) (Table 4.1).

The epitopes recognised by P2 ($\alpha\text{IIb}\beta 3$ complex) and HIP8 (αIIb) were almost completely disrupted or inaccessible to the mAbs following EDTA treatment (P2 TBS-treated PRP mean MFI: 16,950 SD: 1,026; EDTA-treated PRP mean MFI: 831 SD: 173, $P = <0.0001$; HIP8 TBS-treated PRP mean MFI: 28,210 SD: 4,355; EDTA-treated PRP mean MFI: 821 SD: 252, $P = <0.0001$, $n = 5$).

Epitopes recognised by HPA-1a mAbs were also significantly affected by exposure of platelets to EDTA and MFIs were only 12.7 - 31.6% of the values of TBS-treated platelets.

Some epitopes appeared to be more accessible (or there was an increase in expression level) following mock (TBS) treatment of platelets at 37°C e.g. HIP8 (native PRP mean MFI 18,440 SD 4,491; TBS-treated PRP mean MFI 28,210 SD 4,355) ($P = 0.008$, $n = 5$).

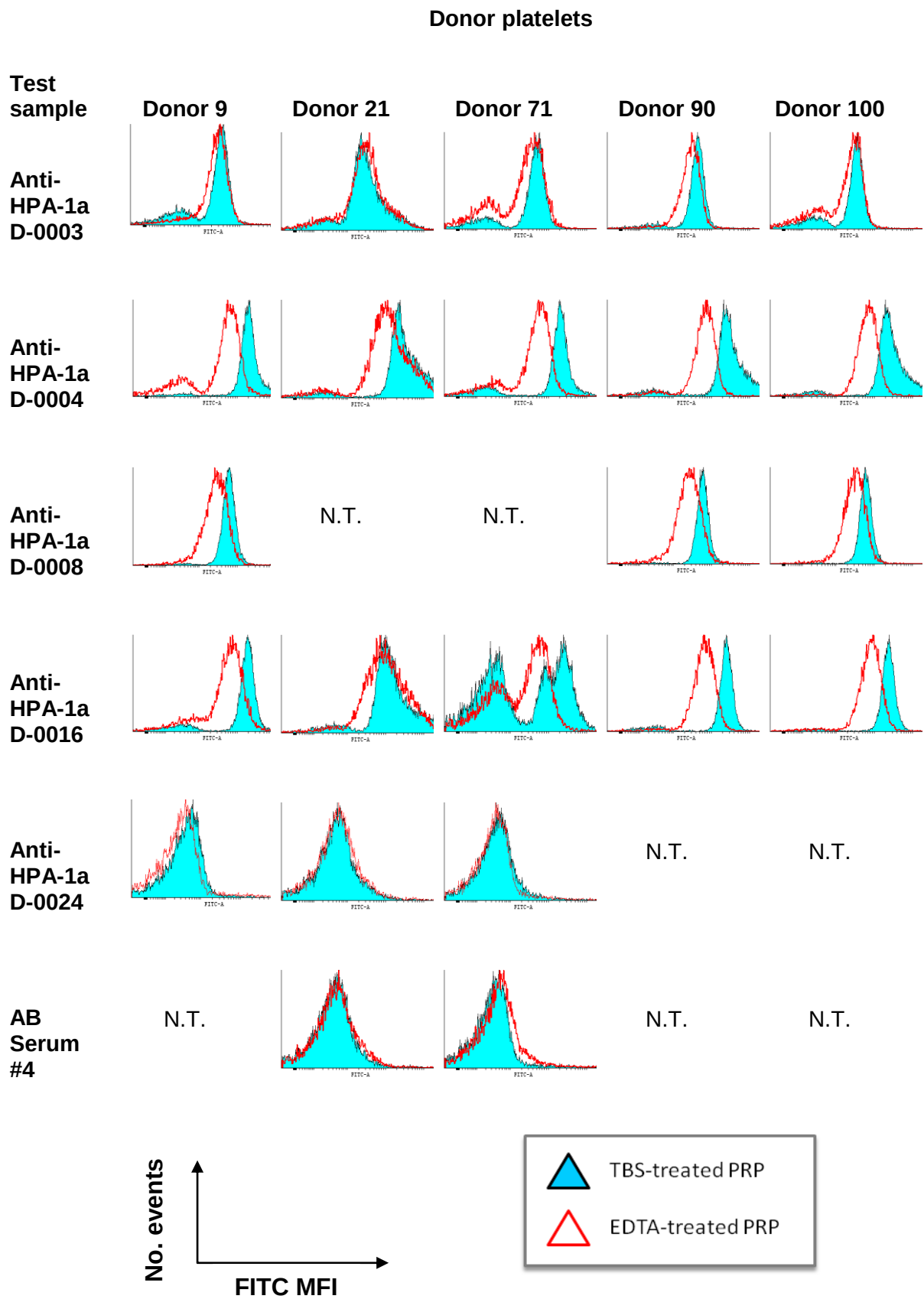
These data are potentially extremely important since some of the mAbs e.g. P2 and Y2/51 are used routinely in the MAIPA assay by some laboratories to capture $\alpha\text{IIb}\beta 3$ from platelets (Allen *et al*, 2007). The reduced binding of these mAbs to platelets exposed to EDTA will result in significantly less glycoprotein being captured by the goat anti-mouse IgG reagent in the MAIPA assay and as a consequence, less human alloantibody will be captured; this would result in reduced assay sensitivity.

Furthermore, these data show that the epitopes recognised by the human mAbs

against HPA-1a were also adversely affected by exposure of the platelets to EDTA.

In order to determine whether HPA-1a epitopes recognised by human polyclonal examples of HPA-1a antibodies were also affected, four examples of blood donor-derived anti-HPA-1a were tested against a range of HPA-1a donor samples (**Figure 4.3** and **Table 1**).

FIGURE 4.3 Flow cytometric analysis of changes in accessibility/conformation of HPA-1a epitopes recognised by human polyclonal antibodies following TBS- or EDTA-treatment of PRP. *Histograms showing the reactivity against native PRP have been omitted for clarity.*



Reactivity of anti-HPA-1a D-0003 was unaffected by EDTA treatment of the platelets and MFIs were not significantly different from TBS-treated platelets ($P = 0.28$, $n = 5$) (**Table 4.1**).

Anti-HPA-1a D-0004 and D-0008 gave consistently weaker reactions against all EDTA-treated samples compared to TBS-treated samples suggesting that these examples recognised epitopes that are sensitive to disruption by EDTA.

Anti-HPA-1a D-0016 gave weaker reactions against 4/5 EDTA-treated samples; the similar MFIs obtained against TBS- and EDTA-treated samples with one of the five donor samples suggest that antibodies against other, non-EDTA dependent, antigens are present e.g. HLA class I.

Increases in reactivity against TBS-treated platelets compared to native (RT incubated) PRP was observed with 2/4 examples of anti-HPA-1a (not shown in **Figure 4.3** for clarity of EDTA results) suggesting that incubation at 37°C increased the expression or accessibility of HPA-1a epitopes (sample D-0004, $P = 0.002$; sample D-0008 $P = 0.0001$, $n = 5$).

Sample D-0024 failed to react in this assay. Since detailed records were not available on each sample used in this project and the presence of antibodies was only recorded for the donor, it is possible that this particular sample was collected after the donor's antibody had disappeared from their circulation.

No non-specific (false-positive) results were observed when using a serum sample from a group AB, non-transfused male donor (AB#4).

TABLE 4.1. Reactivity by flow cytometry of monoclonal and polyclonal antibodies against TBS-, EDTA- and untreated platelets.

	Antibody	Specificity	Domain / amino acids	Platelet Treatment		
				Native PRP	TBS	TBS/EDTA
Monoclonal anti-$\alpha\text{IIb}\beta 3$ antibodies	P2	$\alpha\text{IIb}\beta 3$ complex	Unknown	17,870 $\pm$ 981	16,950 $\pm$ 1,026 ^{ns}	831 $\pm$ 173 ^{***}
	HIP-8	αIIb	Unknown	18,440 $\pm$ 4,491	28,210 $\pm$ 4,355 ^{**}	821 $\pm$ 252 ^{***}
	SZ21	$\beta 3$	PSI, 28-35	46,890 $\pm$ 4,472	43,240 $\pm$ 4,081 ^{ns}	35,200 $\pm$ 2,097 ^{**}
	Y2/51	$\beta 3$	PSI + hybrid	15,710 $\pm$ 3,757	14,660 $\pm$ 2,223 ^{ns}	9,300 $\pm$ 2,877 ^{**}
	CRC54	$\beta 3$	PSI or hybrid, 1-100	4,887 $\pm$ 695	5,166 $\pm$ 1,019 ^{ns}	4,869 $\pm$ 819 ^{ns}
	VIPL-2	$\beta 3$	Unknown	41,340 $\pm$ 6,376	48,700 $\pm$ 772 [*]	31,790 $\pm$ 1,087 ^{***}
	B2	HPA-1a type II	PSI + hybrid/EGF	6,995 $\pm$ 2,486	6,944 $\pm$ 2,576 ^{ns}	2,195 $\pm$ 1,160 ^{**}
Monoclonal anti-HPA-1a antibodies	23-15	HPA-1a type I	PSI	5,643 $\pm$ 631	8,573 $\pm$ 589 ^{***}	1,549 $\pm$ 367 ^{***}
	19-7	HPA-1a type I	PSI	11,280 $\pm$ 609	16,820 $\pm$ 1,315 ^{***}	2,144 $\pm$ 260 ^{***}
	B9.12.1	HLA class I	Unknown	6,410 $\pm$ 1,449	6,341 $\pm$ 1,408 ^{ns}	6,486 $\pm$ 1,570 ^{ns}
Other mAbs	D-0003	HPA-1a	Unknown	2,718 $\pm$ 343	4,404 $\pm$ 1,046 ^{**}	3,644 $\pm$ 1,044 ^{ns}
	D-0004	HPA-1a	Unknown	24,250 $\pm$ 4,904	34,360 $\pm$ 9,822 ^{ns}	10,420 $\pm$ 6,828 ^{**}
Polyclonal anti-HPA-1a antibodies	D-0008	HPA-1a	Unknown	3,621 $\pm$ 509	6,520 $\pm$ 352 ^{***}	4,029 $\pm$ 724 ^{***}
	D-0016	HPA-1a	Unknown	16,180 $\pm$ 4,275	22,370 $\pm$ 5,876 ^{ns}	10,510 $\pm$ 6,525 [*]
	AB#4	(none)	Not applicable	278 $\pm$ 174	523 $\pm$ 327	701 $\pm$ 273
Negative control serum						

Data are the means of the MFIs from 5 experiments $\pm$ SD except for antibodies 23-15 and 19-

7 where only four experiments were performed due to limited availability of the antibodies. *P* values were calculated using the unpaired *t*-test comparing TBS-treated platelets to untreated platelets or TBS/EDTA-treated to TBS-treated platelets; ns not significant (**p* = <0.05, ***p* = <0.01, *** *p* = <0.001).

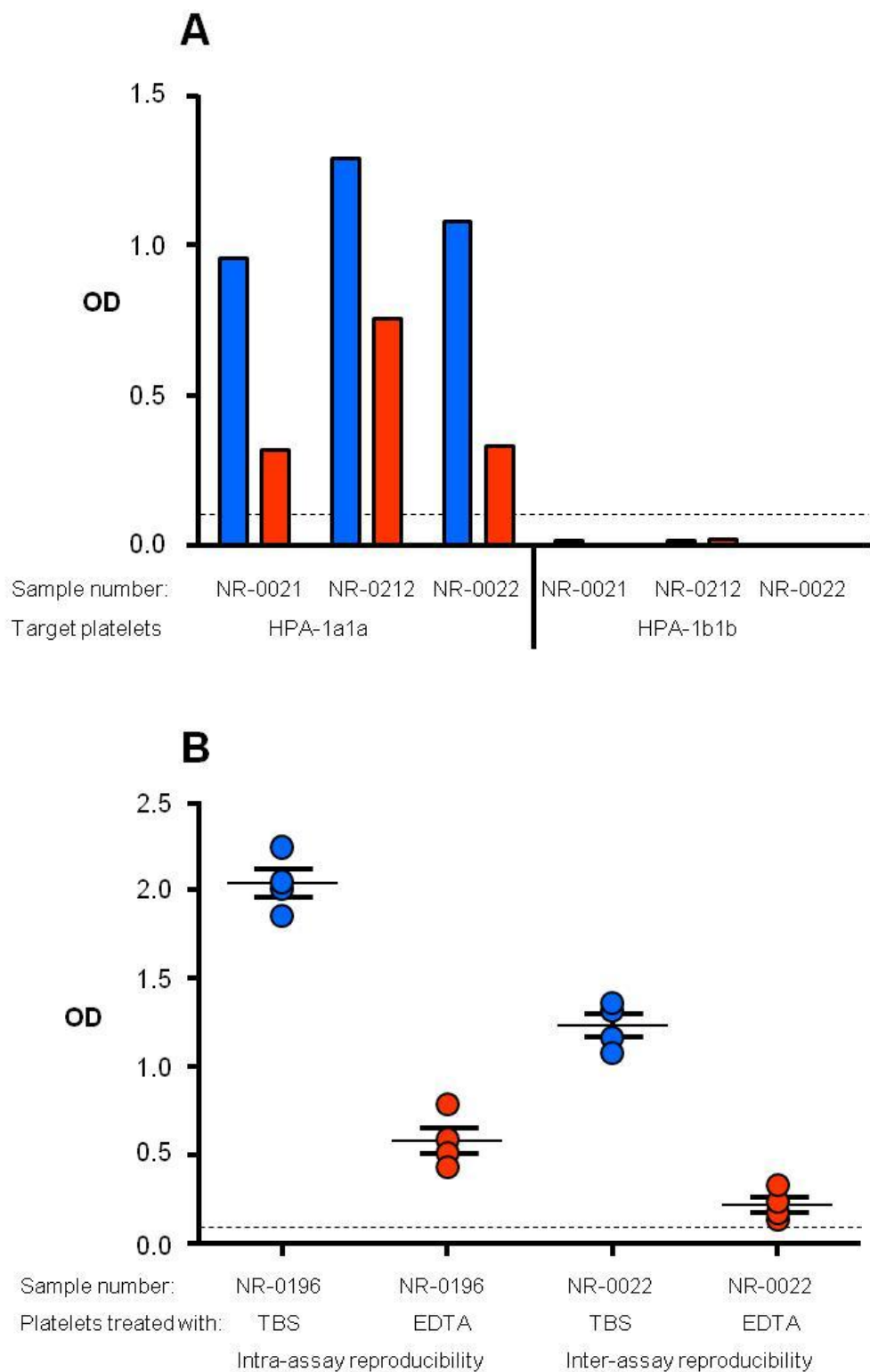
4.3.3 Effect of EDTA on sensitivity of the MAIPA assay

The MAIPA assay for the detection of HPA antibodies avoids the confounding reactivity caused by HLA class I antibodies that may be observed in Indirect PIFT assays. The MAIPA assay of Campbell *et al* was modified to avoid changes in the conformation of $\alpha\text{IIb}\beta 3$ induced during the extraction of platelets from whole blood (Campbell *et al*, 2007).

In this assay, PRP was used instead of platelets isolated from whole blood. This was first validated for specificity. Three examples of anti-HPA-1a were tested against HPA-1a1a and HPA-1b1b TBS- and EDTA-treated PRP (**Figure 4.4A**).

The assay was then validated for reproducibility. Two examples of anti-HPA-1a were tested four times within the same assay performed on the same day, and four times over four experiments against HPA-1a1a TBS- and EDTA-treated PRP (**Figure 4.4B**).

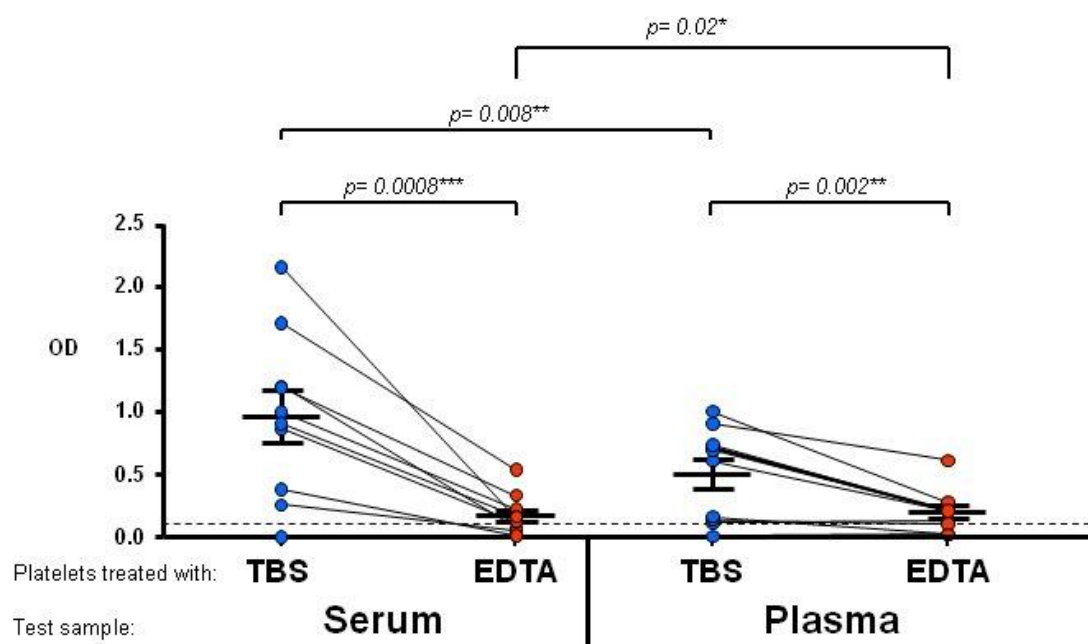
FIGURE 4.4 (A) specificity, and (B) reproducibility of the ‘PRP MAIPA’ assay. Blue bars represent TBS-treated PRP, red bars EDTA-treated PRP. The dashed line indicates the cut-off. In ‘B’ the bars show the mean and the SEM.



These data showed that the PRP MAIPA was specific and reproducible.

This assay was then used to study the effect of EDTA in either the test sample (i.e. EDTA plasma), or in buffer solutions (as used in some diagnostic laboratories) on ten examples of NAIT-derived anti-HPA-1a collected at the same venepuncture into dry (serum) tubes or into EDTA-anticoagulated tubes (**Figure 4.5**).

FIGURE 4.5 Effect of the presence of EDTA on the sensitivity of the MAIPA assay. The PRP MAIPA was performed as described above and used clone PAB-6 as capture mAb.



When using control buffer (TBS) treated PRP (blue circles), the use of EDTA plasma samples significantly ($P = 0.008$) reduced assay sensitivity compared to when using serum samples (serum: mean OD 0.969, SD 0.655; EDTA plasma mean OD 0.511, SD 0.365).

Treatment of PRP with EDTA buffer also significantly reduced assay sensitivity. This

reduction was observed both when using serum samples (EDTA-treated platelets mean OD 0.173, SD 0.164 compared to TBS buffer control mean OD 0.969, SD 0.655) ($P = 0.0008$), and when using EDTA plasma samples (EDTA-treated platelets mean OD 0.206, SD 0.167, versus TBS-buffer control mean OD 0.511, SD 0.365 ($P = 0.002$)).

4.3.4 Effect of EDTA on integrin conformation and mAb/polyclonal antibody binding during the MAIPA assay

To confirm that this reduction in sensitivity was due to both reduced human polyclonal antibody and reduced capture mAb binding, the integrity of the $\alpha\text{IIb}\beta 3$ complex and of the HPA-1a epitopes, and binding of mAb and polyclonal antibody was studied at various stages (Figure 4.6) of the PRP MAIPA assay by removing aliquots and testing them in flow cytometric assays (Figure 4.7)

FIGURE 4.6. Cartoon showing stages of MAIPA assay at which aliquots were removed for analysis.

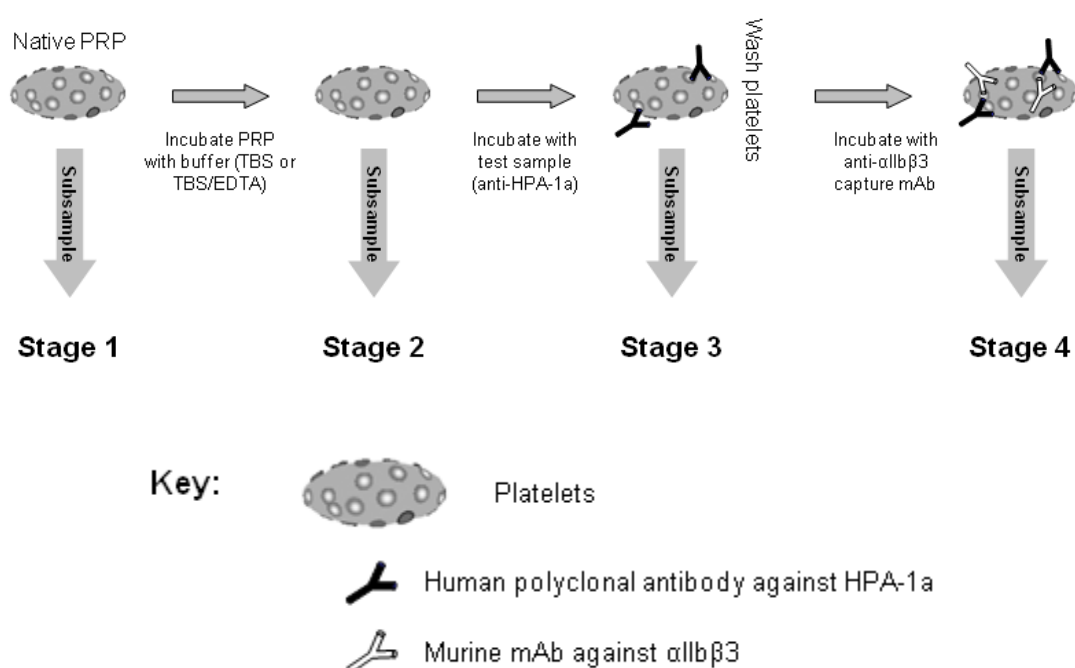
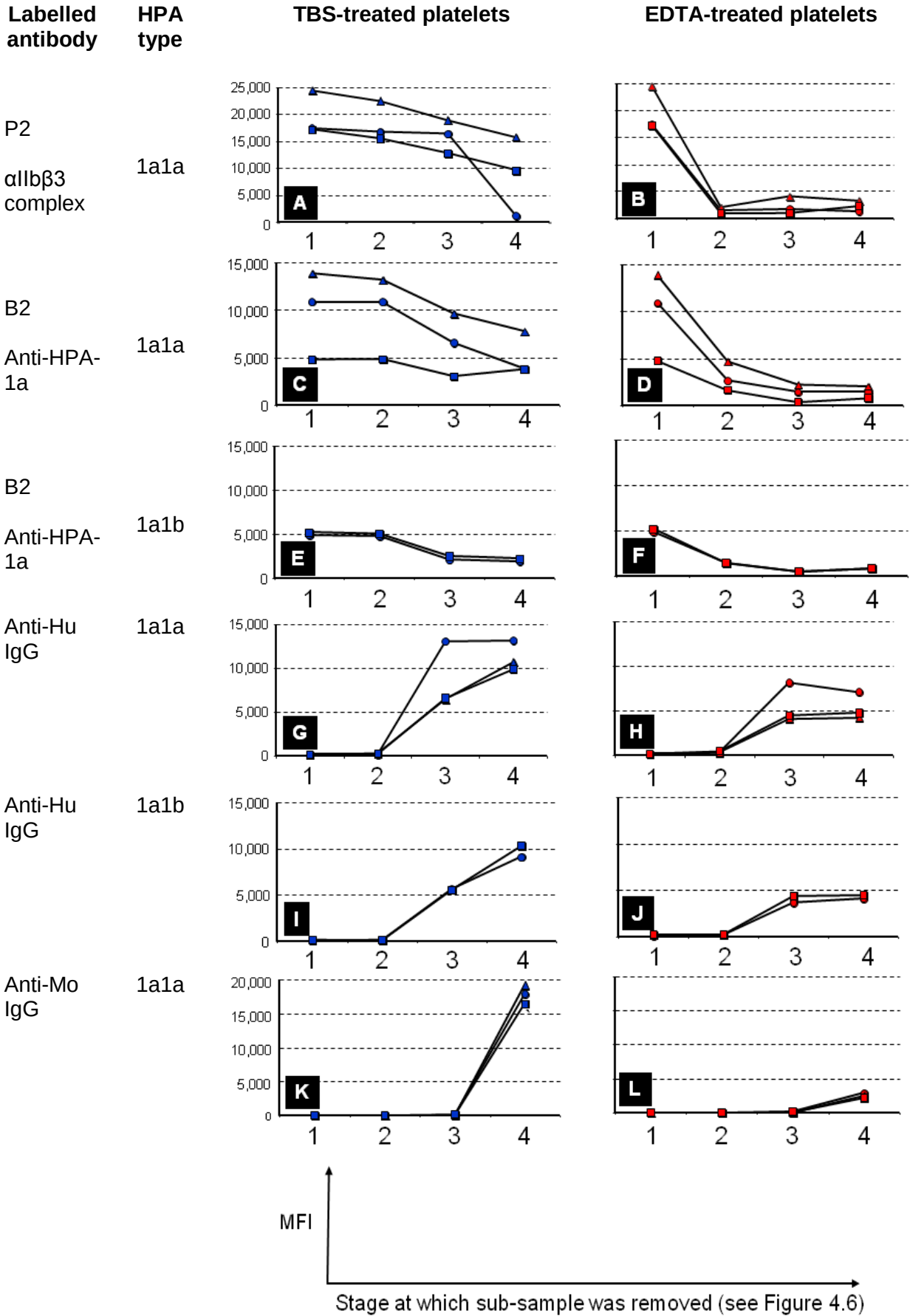


FIGURE 4.7 Flow cytometric analysis of integrin/epitope accessibility, and binding of human polyclonal and murine monoclonal antibodies in the MAIPA assay. PRP was treated with EDTA (red markers) or TBS buffer control (blue markers). Subsamples were taken throughout the PRP MAIPA assay and tested by flow cytometry with mAbs P2 and B2, goat anti-human and goat anti-mouse IgG. Three capture mAbs were used: PAB-1 [▲] in one experiment, PAB-6 [●] in two experiments and 5B12 [■] in two experiments. The results are those from testing one example of anti-HPA-1a in five experiments; the results of testing an inert, AB serum control are omitted for clarity but no binding of anti-human IgG conjugate was observed.



There are a number of conclusions that can be drawn from these data:

- The $\alpha\text{IIb}\beta 3$ complex undergoes a gradual conformational change (as shown by loss of reactivity with P2) throughout the assay when TBS control buffer was used (**Figure 4.7A**). The sudden loss of reactivity after incubation with the capture mAb PAB-6 (stage 4) suggests that these two clones bind close to each other.
- As shown previously, treatment of PRP with EDTA results in complete loss of P2 epitope. (**Figure 4.7B**, stage 2)
- HPA-1a epitopes are adversely affected by EDTA treatment, with a large reduction in binding of B2 observed after treatment of platelets with EDTA (**Figures 4.7D** and **4.7F**, stage 2).
- Binding of polyclonal anti-HPA-1a partially blocks binding of B2 anti-HPA-1a mAb (HPA-1a1a PRP: **Figures 4.7C** and **4.7D**, stage 3; HPA-1a1b PRP: **Figures 4.7E** and **4.7F**, stage 3).
- Less polyclonal HPA-1a antibody is bound to EDTA-treated (HPA-1a1a platelets: **Figure 4.7H**, stage 3; HPA-1a1b platelets **Figure 4.7J**) than to TBS-treated platelets (HPA-1a1a platelets: **Figure 4.7G**, stage 3; HPA-1a1b platelets **Figure 4.7I**).
- Considerably less capture mAb is bound to EDTA-treated (**Figure 4.7L**) than to TBS-treated platelets (**Figure 4.7K**).

Thus the conformational changes in $\alpha\text{IIb}\beta 3$ resulting from exposure to EDTA deleteriously affects binding of both polyclonal anti-HPA-1a and of mAbs used to capture the integrin resulting in compounded loss of sensitivity.

4.3.5 Validation of findings by international collaborative study

To investigate whether these findings could be replicated and thus were of relevance to diagnostic laboratories, permission was sought from NIBSC to include suitable samples in one of their bi-annual international workshops. In these exercises, participating laboratories are asked to perform HPA antibody screening, and to identify any HPA antibodies detected, in a number of coded samples. The participants are asked to provide a summary of their findings (HPA antibodies present or absent, and specificity) together with the results of each assay performed.

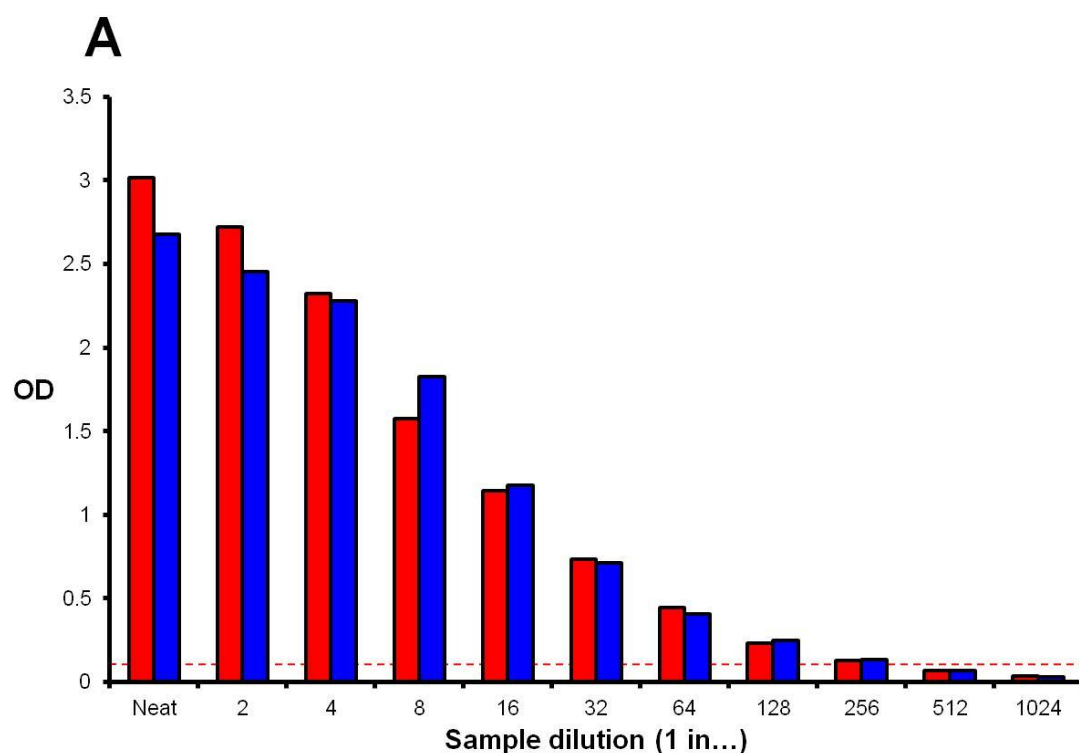
Access to the participants' raw data and to the full technical details of their MAIPA assay would be informative from the point of view of validating the findings of this project. Permission was therefore sought from the scheme organisers for both of these additional data sets and appropriate templates designed (**Appendix 4**).

4.3.6 Testing of samples for potential inclusion in the workshop

Having obtained permission to include suitable samples in workshop exercise 2011-B), two mothers whose children had been affected by anti-HPA-1a mediated NAIT were contacted and fresh serum and EDTA-anticoagulated samples collected.

All samples were titrated in the MAIPA assay using the high-potency anti-HPA-1a standard '03/152' as a reference (**Figure 4.8, Table 4.2**).

FIGURE 4.8. Assessment of serum and EDTA plasma samples containing anti-HPA-1a for potential inclusion of NIBSC international platelet immunology quality assurance scheme. Doubling dilutions of EDTA plasma (red bars) and serum samples (blue bars) were tested in the MAIPA assay using the anti- $\beta 3$ capture mAbs indicated. (A) NAIT case F1458, clone VI-PL2 as capture mAb. (B) Case F1272, capture mAb: VI-PL2. (C) Case F1272, capture mAb: PAB-6. The dashed line indicates the cut-off.



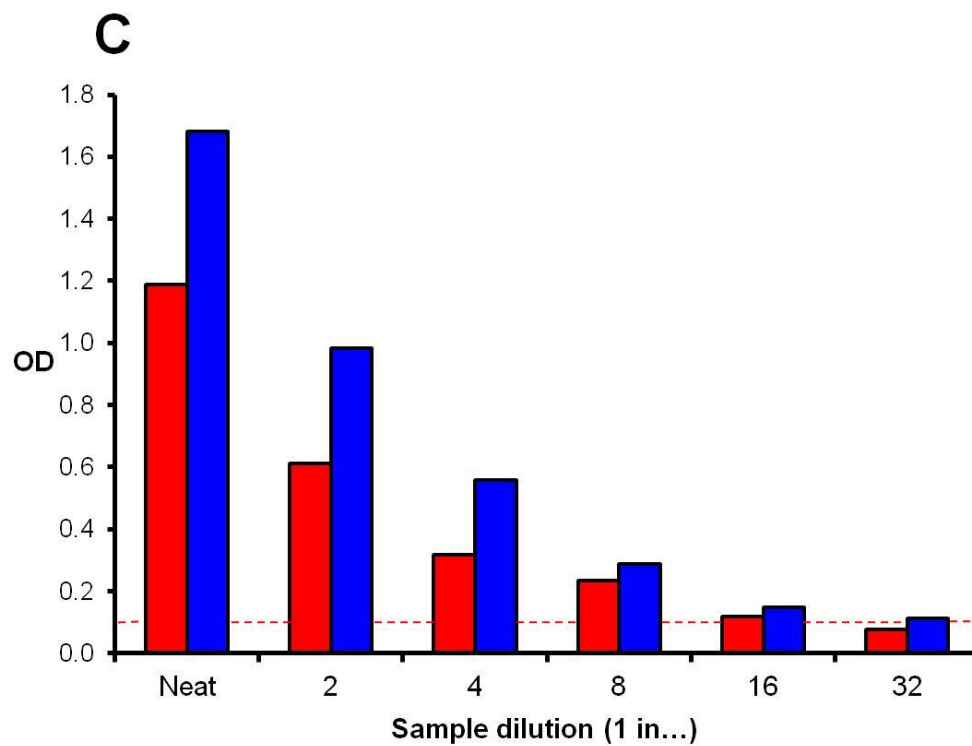
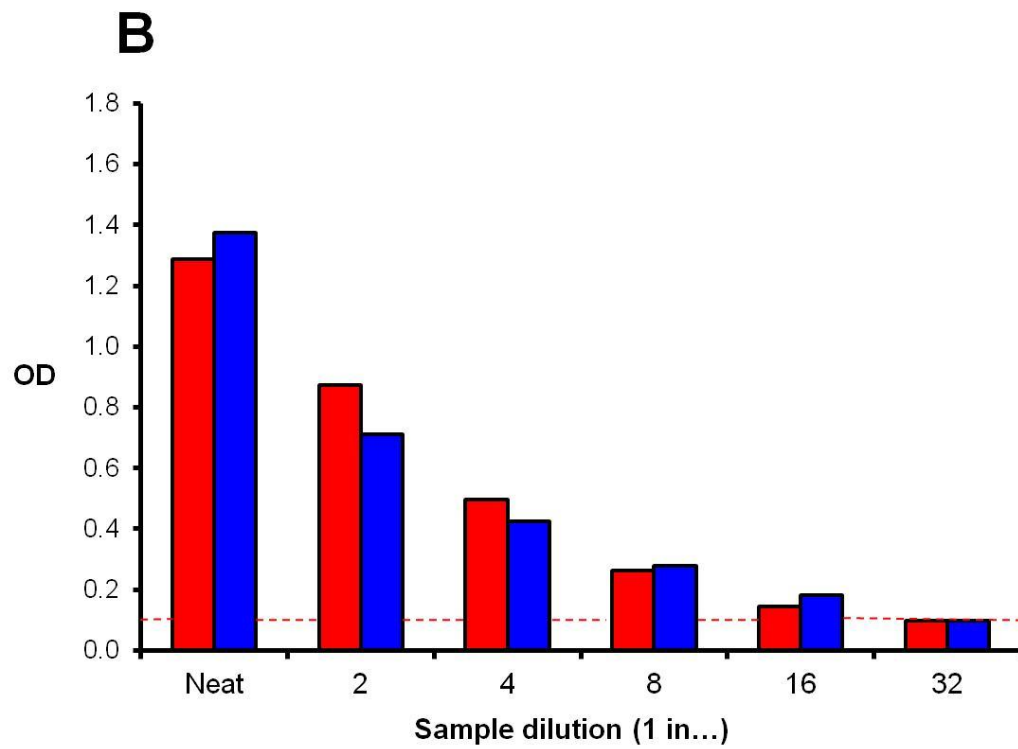


TABLE 4.2. Measured anti-HPA-1a levels in samples for potential inclusion in NIBSC international platelet immunology quality assurance exercise 2011-B.

NAIT Case Number	Sample Number	Material	Anti-HPA-1a level (i.u./mL)
F1458	NR-0259	Serum	208.9
	NR-0260	EDTA plasma	219.2
F1272	NR-0262	Serum	14.3
	NR-0261	EDTA plasma	16.3

The anti-HPA-1a from case F1458 was extremely potent and the EDTA plasma sample was as strongly reactive as the serum sample when using clone VI-PL2 as capture mAb (**Figure 4.8A**); this sample was therefore not submitted for inclusion in the exercise.

The anti-HPA-1a from case F1272 was deemed suitable for inclusion since there was ~30% reduction in OD in the undiluted EDTA plasma sample compared to the serum sample when using capture mAb PAB-6 (**Figure 4.8C**).

The material was distributed as Samples 1 (EDTA plasma) and 2 (serum) in exercise 2011-B. In order to allow participants to conserve material and focus their investigations on the integrin of relevance they were asked to test the samples only against $\alpha\text{IIb}\beta 3$ in glycoprotein-specific assays and Indirect PIFT if normally performed in their laboratory.

4.3.7 International workshop results

The data presented/reproduced here is with the permission of the scheme organisers and of the participating laboratories.

Thirty nine laboratories participated in Exercise 2011-B. The number of laboratories returning complete, relevant and objective data i.e. ODs or MFIs is shown in **Table 4.3**.

TABLE 4.3. Methodologies used in NIBSC Workshop Exercise 2011-B and assessment of data for validity.

Assay	Assay format	No. laboratories performing assay	No. laboratories returning complete and relevant raw data *
Indirect PIFT with flow cytometric analysis)	Not applicable	6	2
MAIPA	Not applicable	23	20
Antigen-Capture ELISA (ACE)	GTI PAK-12	19	19
ACE	GTI PAK-PLUS	6	5
ACE	GTI PAK-1	3	3

* Some participants did not return raw data and some returned data that was not suitable for analysis e.g. use of different capture mAbs when testing samples 1 and 2 thus preventing comparison of data.

4.3.7.1 Indirect PIFT results

Only two participating laboratories returned Indirect PIFT data.

One participant obtained similar results with both serum and EDTA plasma samples (mean MFIs 587 and 585 respectively).

The second participant reported enhanced reactivity (of the plasma sample compared to that of the serum sample (approximately 325% using HPA-1a1a platelets, (mean MFIs 14.7 and 4.53 respectively; approximately 777% using HPA-

1a1b platelets, mean MFIs 20.5 and 2.64).

This was an unexpected finding and did not correlate with earlier findings of this study or with the MAIPA data observed in this NIBSC exercise (see below). It is possible that the raw data for samples 1 and 2 may have been transposed at source but no attempt was made to contact the laboratory concerned to verify the data.

4.3.7.2 MAIPA assay results

Twenty participants that used the MAIPA assay, together with NIBSC and this study, returned data suitable for analysis. Some participants used more than one capture mAb; thus 24 data sets were available for analysis. The three data sets that were not suitable for analysis included one where different capture mAbs were used for samples 1 and 2, one where data from only HPA-1b1b platelets were provided, and one where the samples were diluted before use.

Twelve laboratories, together with NIBSC and this study, returned completed technique questionnaires (**Appendix 4**).

The raw data was analysed initially by calculating reactivity of the EDTA plasma sample as a percentage of that of the serum sample. To determine if EDTA plasma was affecting the assay a figure within 20% of that of the serum value was arbitrarily set as the level that might be expected from experimental error.

With HPA-1a1a platelets (**Figure 4.9A**), 8/24 (33.3%) data sets showed a moderate (>20% and <50%) reduction in reactivity of the EDTA plasma compared to serum.

Nine data sets (37.5%) showed a severe ($\geq 50\%$) reduction in reactivity of EDTA plasma compared to serum.

In total, 17/24 (70.8%) data sets showed a reduction in reactivity of >20% of EDTA plasma compared to serum.

The mean reduction in reactivity was 27.7% and the highest was 85.1% i.e. EDTA plasma gave ODs only 14.9% of that of serum.

Four data sets showed reactivity of EDTA plasma to be within $\pm 20\%$ of that of serum.

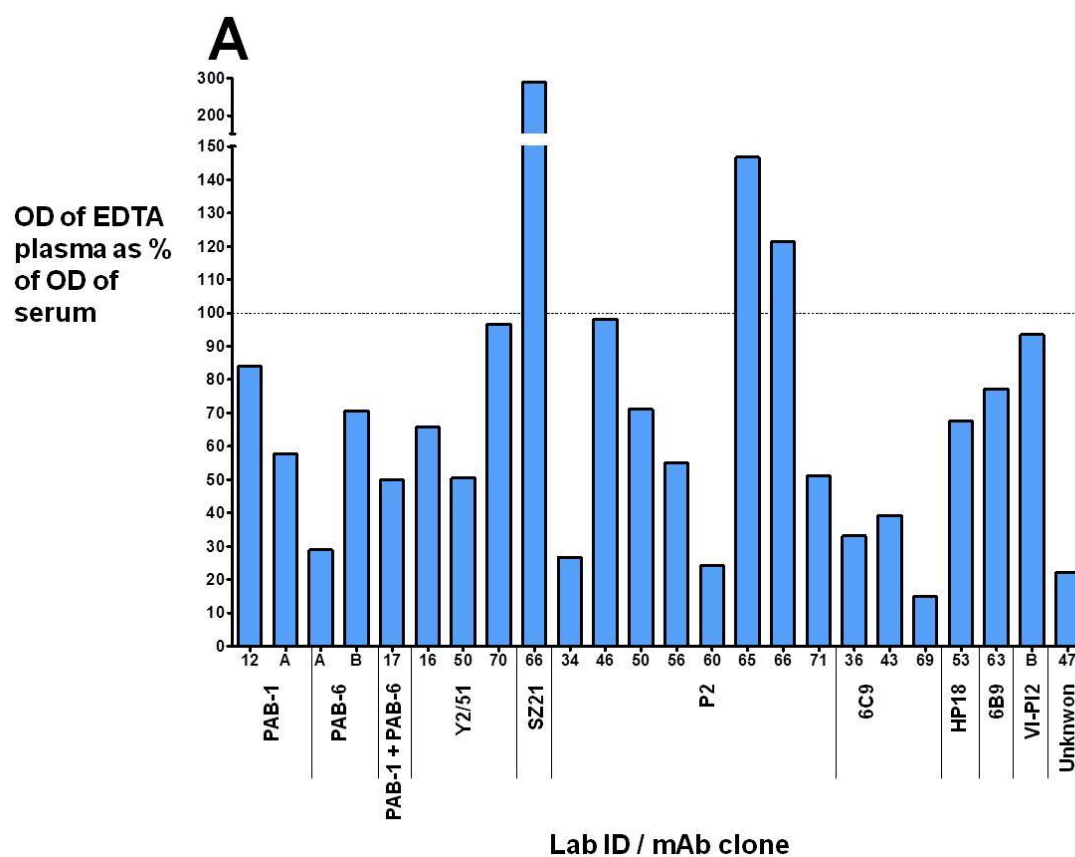
Three data sets showed ODs $>20\%$ higher with the EDTA plasma compared to the serum sample.

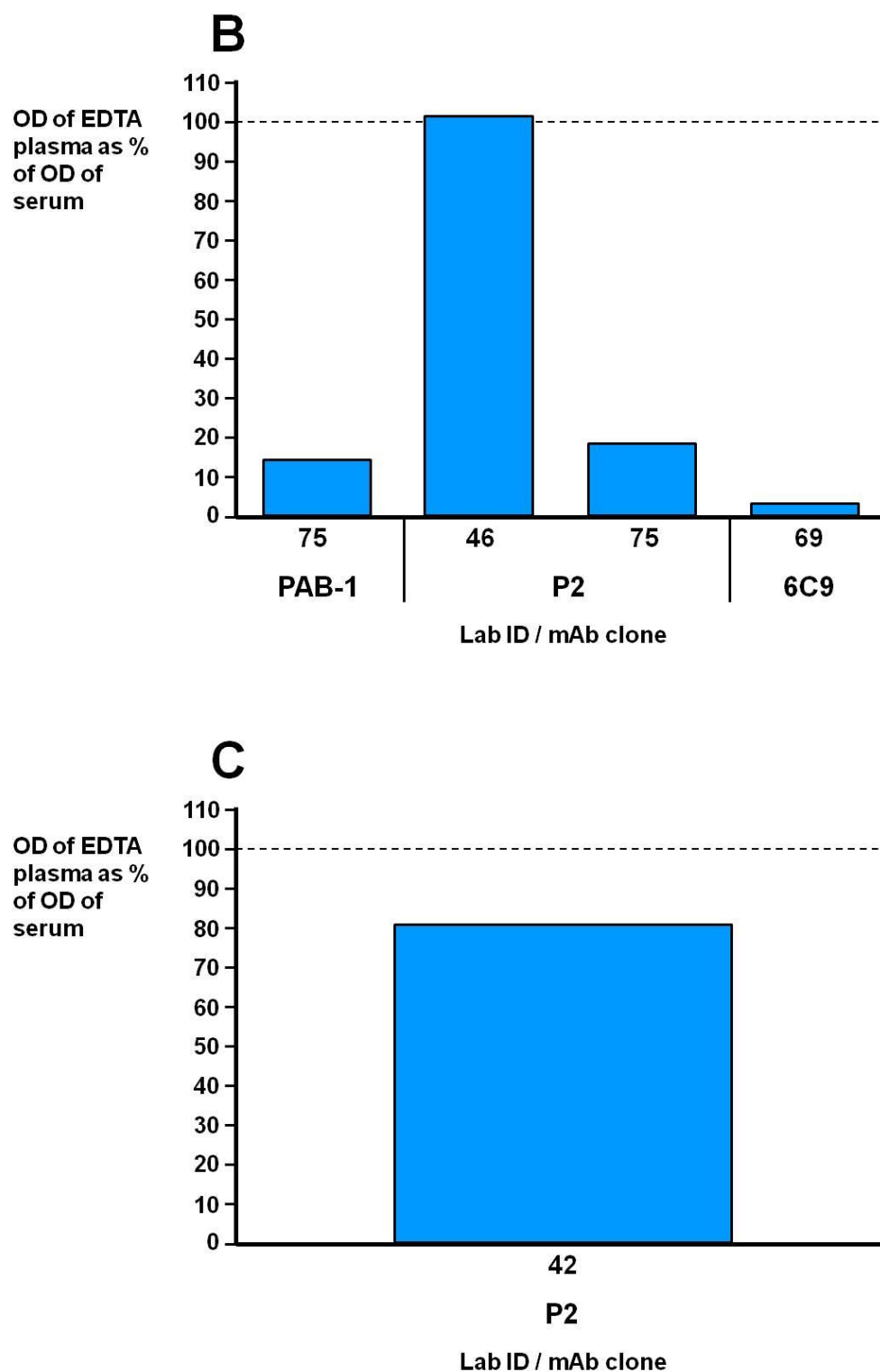
It was shown earlier (**Figure 4.2**) that the impact of EDTA on different $\alpha\text{IIb}\beta 3$ epitopes varied. The results with mAb 'P2' showed that the difference in reactivity of EDTA plasma was not related solely to the mAb clone used since some data sets obtained with P2 showed the reactivity of EDTA plasma to be within 20% of that of serum e.g. laboratories 46 and 66, whereas others e.g. laboratories 34 and 60, showed severe reductions in reactivity.

When using HPA-1a1b platelets (**Figure 4.9B**), the effect of EDTA was even more pronounced than when using HPA-1a1a platelets.

Three of the four data sets showed a severe reduction in reactivity (mean reduction 65.6%); in one laboratory the ODs of the EDTA plasma sample were only 3.4% of those of the serum sample.

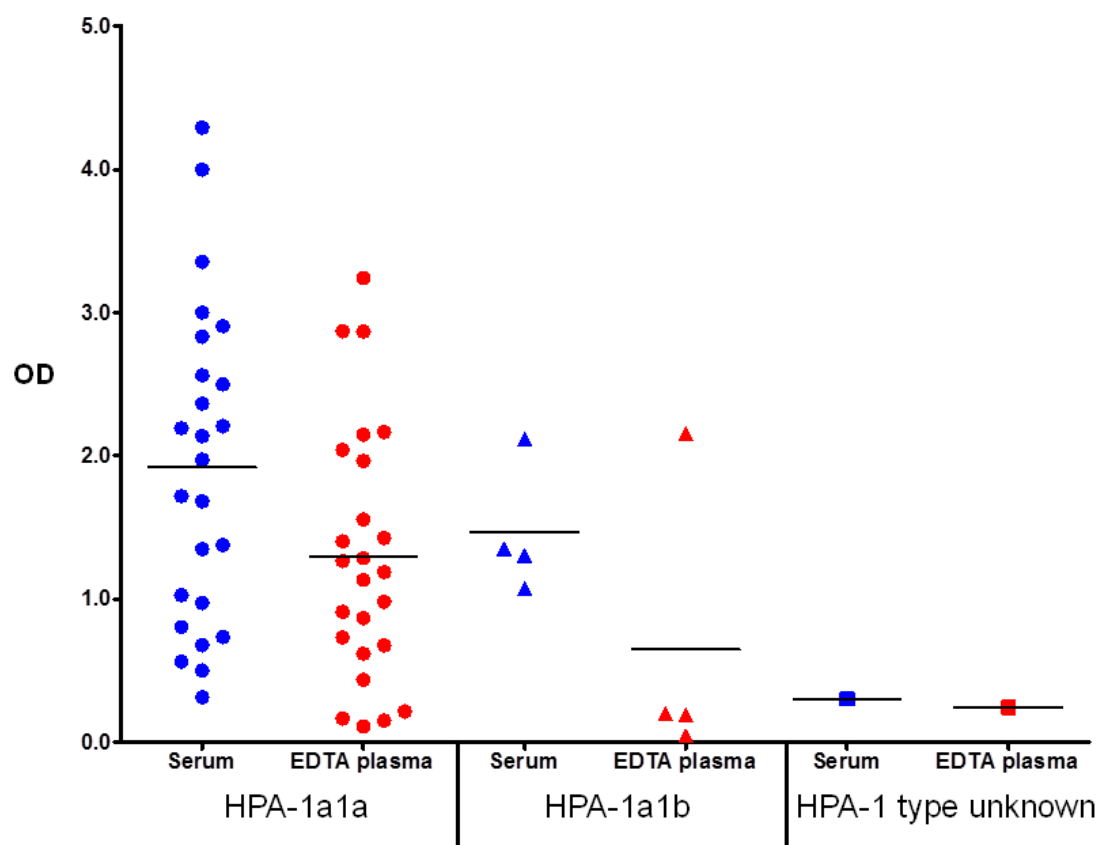
FIGURE 4.9. Comparison of reactivity of anti-HPA-1a in serum and EDTA plasma samples in the MAIPA assay. The ODs obtained with the EDTA plasma sample (Sample 1) were calculated as a percentage of the ODs of the serum sample (Sample 2) and are plotted on the 'y' axis. On the 'x' axis is shown the laboratory identifier code (Lab ID) and the name of the monoclonal antibody clone that was used to capture $\alpha\text{IIb}\beta 3$ from the membrane. **(A)** Results obtained when using HPA-1a1a platelets, **(B)** results using HPA-1a1b platelets and **(C)** results using platelets of unknown HPA-1a type.





The sensitivity of participants' assays was assessed by plotting raw ODs (**Figure 4.10**).

FIGURE 4.10. Sensitivity of participants' MAIPA assay as assessed by raw ODs. Each dot represents the mean OD of all results reported with a given test sample (plasma or serum) and test platelet sample combination. The horizontal bar represents the mean of all data within that set.

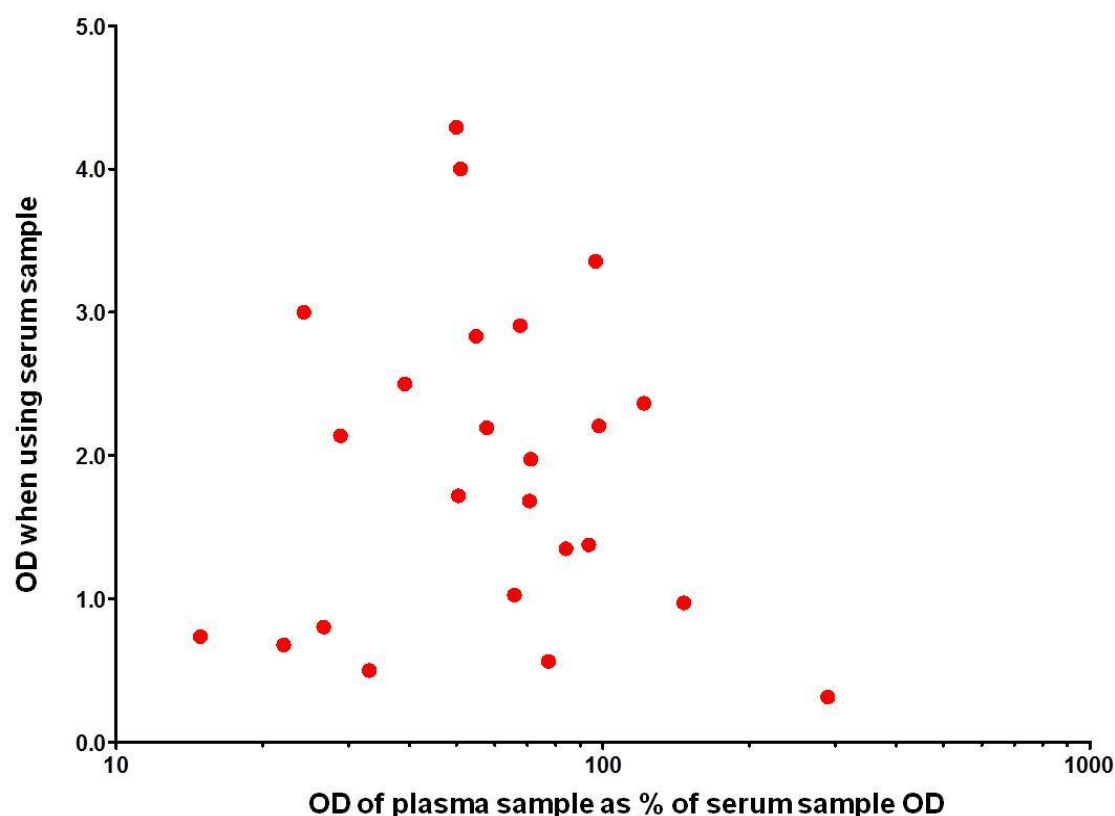


These data show a >10-fold range of ODs when using HPA-1a1a platelets. With serum samples, the ODs ranged from 0.314 to 4.292 (mean 1.922), and with EDTA plasma sample from 0.110 to 3.241 (mean 1.296); this difference was significant ($P = 0.001$, Wilcoxon matched pairs t-test).

From previous published data it is known that some laboratories use EDTA in their assay buffers together with an incubation temperature of 37°C. These conditions would potentially affect the reactivity of the serum sample (Bessos *et al*, 2005). This would potentially affect the calculated percentage reduction in activity of the EDTA plasma sample, thus the % activity figure might appear to be lower than in those

laboratories that did not use EDTA in their assay buffers. The results when using HPA-1a1a platelets were therefore analysed with respect to reactivity of the serum sample (Figure 4.11).

FIGURE 4.11. Relationship between strength of reactivity with serum sample and change in reactivity observed with EDTA plasma sample.



There was no correlation between the strength of reactivity of the serum sample and % reduction in reactivity of the EDTA plasma sample ($P = 0.28$, 2-tailed t-test) (Pearson correlation coefficient -0.23 , CI: -0.58 , 0.19).

Thus, the degree of difference between the EDTA plasma and serum samples was not related to the reactivity of the serum sample and thus to MAIPA assay sensitivity *per se*.

4.3.7.3 Antigen-Capture ELISA (ACE) results

In the ACE assay, there was a significant difference ($P = 0.003$, Wilcoxon matched pairs t-test) between the reactivity of the EDTA plasma and serum samples (**Figures 4.12 and 4.13**).

FIGURE 4.12. Comparison of reactivity of anti-HPA-1a in serum and EDTA plasma samples in the Antigen Capture ELISA assay using HPA-1a1a glycoprotein as target. The results show the ODs of the EDTA plasma sample calculated as a percentage of that of the serum sample. The histograms are shaded according to type of ACE assay used: blue: GTIPAK-12[®], orange: GTI PAK-1[®] and green: GTI-PAK-Plus[®].

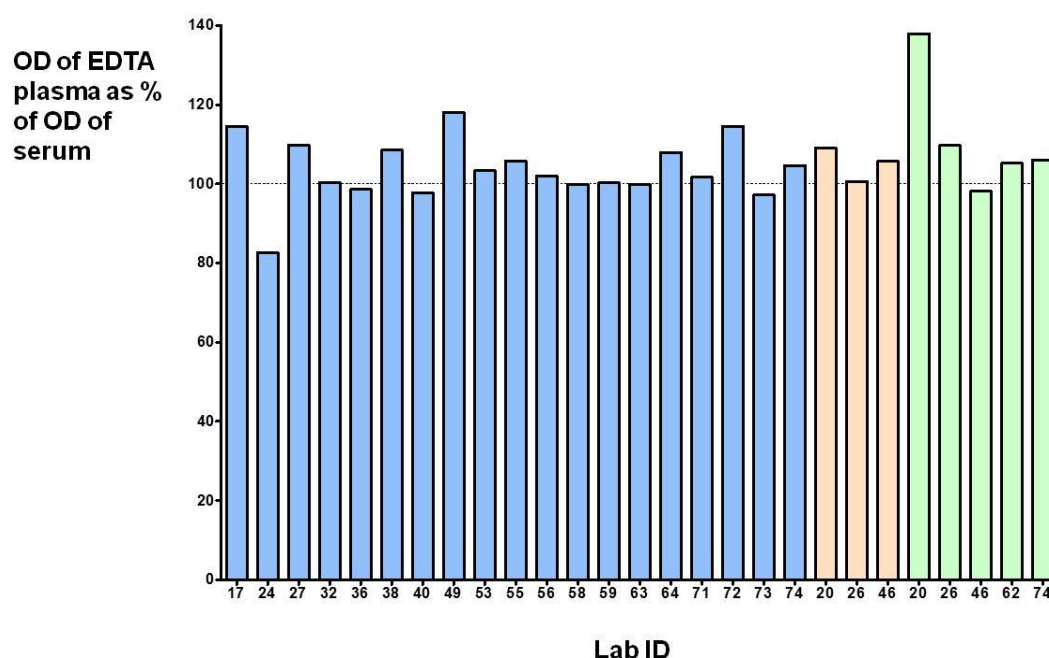
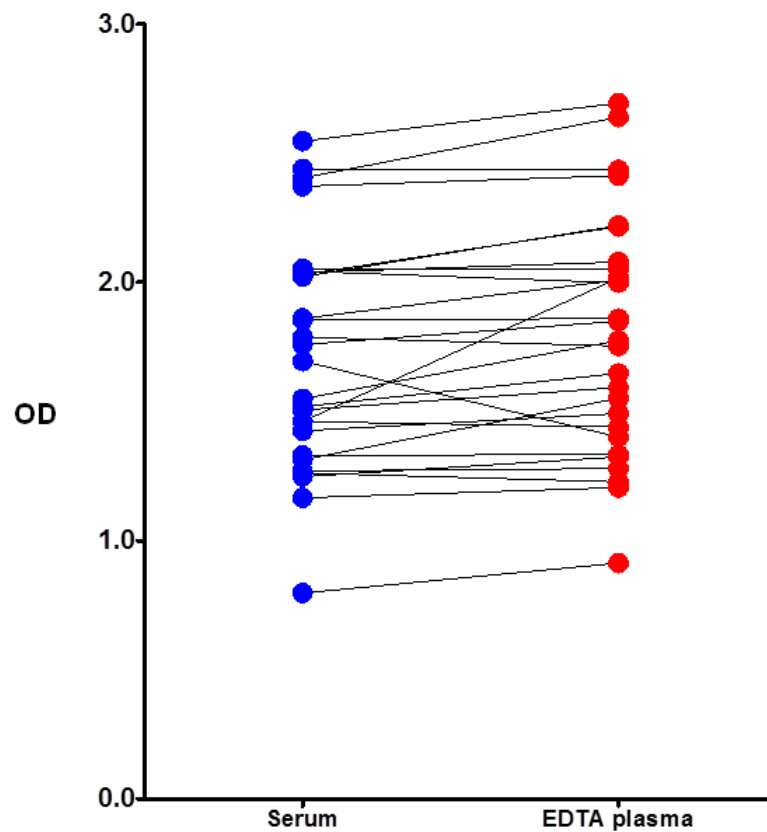


FIGURE 4.13. Optical densities obtained in the ACE assay. *Each dot represents the mean OD obtained in a participating laboratory.*



These data clearly showed:

- That the immobilised $\alpha\text{IIb}\beta 3$ in the ACE assay was not subject to the same deleterious effects of EDTA as seen in the MAIPA assay.
- Enhanced reactivity of the EDTA plasma sample. This may have been due to the presence of EDTA-dependent autoantibodies or other autoantibodies that were dependent on e.g. the pH of the medium to bind to epitopes that are cryptic under other assay conditions such as when using serum.

4.3.7.4 Participants' interpretation of their data

The main objective of the NIBSC quality assurance exercises is to evaluate whether participants are able to detect and identify platelet antibodies that may be present in the distributed samples; this included the samples under evaluation here.

Two of the 39 participants (5.1%) reported that sample 1 did not contain platelet-specific antibodies i.e. obtained false-negative results although they were able to report the presence of HPA-1a antibodies in sample 2.

One participant (2.6%) reported that the anti-HPA-1a in sample 1 was not detectable by the MAIPA assay but was reactive in the ACE assay; this participant reported anti-HPA-1a reactivity in sample 2.

In total therefore, 3/39 (7.7%) participants failed to detect this potent, NAIT-causing, antibody in the MAIPA assay when using the EDTA plasma sample.

Three participants (7.7%) reported that anti- $\alpha\text{IIb}\beta 3$ reactivity was present but were unable to determine that HPA-1a antibodies were present in sample 1; all three reported HPA-1a specificity in sample 2.

4.3.7.5 Analysis of participants' MAIPA assay methodologies

Since the MAIPA assay was originally published the assay has undergone numerous modifications (Kiefel *et al*, 1987; Clofent-Sanchez *et al*, 1996; Joiner *et al*, 1997; Campbell *et al*, 2007). These modifications have been applied to a varying extent in diagnostic laboratories and in an analysis of techniques following an international workshop organised by the International Society of Blood Transfusion it was found that amongst the 27 laboratories that returned their technique questionnaires there was an extremely wide variation in assay conditions and reagents used (Bessos *et al*, 2005) and no two participating laboratories used an identical assay (personal observations).

Since the results obtained in the MAIPA assay in this workshop exercise showed widespread differences in both serum and EDTA plasma reactivity between laboratories, even when the same capture mAbs were used, it would suggest that other parameters/conditions within the assay were affecting the results. The technique questionnaires distributed to participants were therefore collated and analysed (**Appendix 5**). Summarised data are shown in **Table 4.4**.

TABLE 4.4. Summarised details of techniques used by laboratories participating in WS 2011-B.

Assay parameter	No. participants	Comments
Anticoagulant used for blood used as source of platelets	• EDTA x7 • Citrate x7	Blood collected into EDTA and left at 37°C for 5 mins would show evidence of reversible dissociation of $\alpha\text{IIb}\beta 3$; left for >30 mins the dissociation would be irreversible. Potentially therefore the target integrin may not be recognised by the human alloantibody or murine capture mAb.
Solution used to store platelet suspension	• EDTA buffer x5 • NaCl x5 • Not stored x2 • Unknown x2	
Solution used to wash platelets prior to use	• EDTA buffer x11 • PBS/TBS x1 • NaCl x1 • Not washed x1	
Solution used to suspend platelets for use in assay	• EDTA buffer x6 • PBS/TBS x7 • NaCl x1	
Platelets used in suspension, or as cell pellet (and supernatant buffer removed prior to adding test serum/plasma)	• Suspension x7 • Pellet x7	Use of platelets suspended in EDTA buffer and incubation temperature of 37°C would potentially dissociate $\alpha\text{IIb}\beta 3$ complex.
Incubation temperature (serum/plasma and platelets)	• 37°C x14	If EDTA plasma samples are used, the $\alpha\text{IIb}\beta 3$ complex would be irreversibly dissociated after 30 mins.
Incubation time (serum/plasma and platelets)	• 30 mins x8 • 35 mins x1 • 40 mins x1 • 45 mins x2 • 60 mins x1 • 30-60 mins x1	All participants used a primary incubation time sufficiently long to irreversibly dissociate the $\alpha\text{IIb}\beta 3$ complex.
Solution used to wash platelets after primary incubation	• EDTA buffer x3 • TBS/PBS x5 • NaCl x6	
Diluent for monoclonal antibody	• EDTA buffer x3 • TBS/PBS x8 • None x3	Dilution of mAb in EDTA buffer and incubation temperature of 37°C would potentially dissociate $\alpha\text{IIb}\beta 3$ complex.

Assay parameter	No. participants	Comments
Incubation temperature (mAb and platelets)	• 37°C x12 • Room temp x1 • None x1*	Dilution of mAb in EDTA buffer and incubation temperature of 37°C would potentially dissociate $\alpha\text{IIb}\beta 3$ complex. * participant used simultaneous incubation of serum and mAb with platelets.
Incubation time (mAb and platelets)	• 30 mins x9 • 35 mins x1 • 40 mins x1 • 45 mins x2 • None x1*	* participant used simultaneous incubation of serum and mAb with platelets.

These data showed that:

- There was a wide variation of parameters used by participating laboratories and, as shown previously, no two laboratories used an identical assay (Bessos *et al*, 2005).
- There was widespread use of EDTA at various stages of the MAIPA assay.
- All laboratories used an incubation temperature of 37°C and an incubation time ≥ 30 mins during the primary (test sample) incubation phase; these conditions are sufficient to induce irreversible dissociation of $\alpha\text{IIb}\beta 3$ if EDTA were present.
- Three laboratories diluted the capture mAb in an EDTA-containing buffer, together with an incubation temperature of 37°C and incubation time ≥ 30 mins; these conditions are sufficient to induce irreversible dissociation of $\alpha\text{IIb}\beta 3$.
- Reduced reactivity of the EDTA plasma sample was observed with all capture mAbs suggesting that either the epitopes recognised by all of these mAbs were affected by EDTA treatment or that the reduced reactivity was due, in

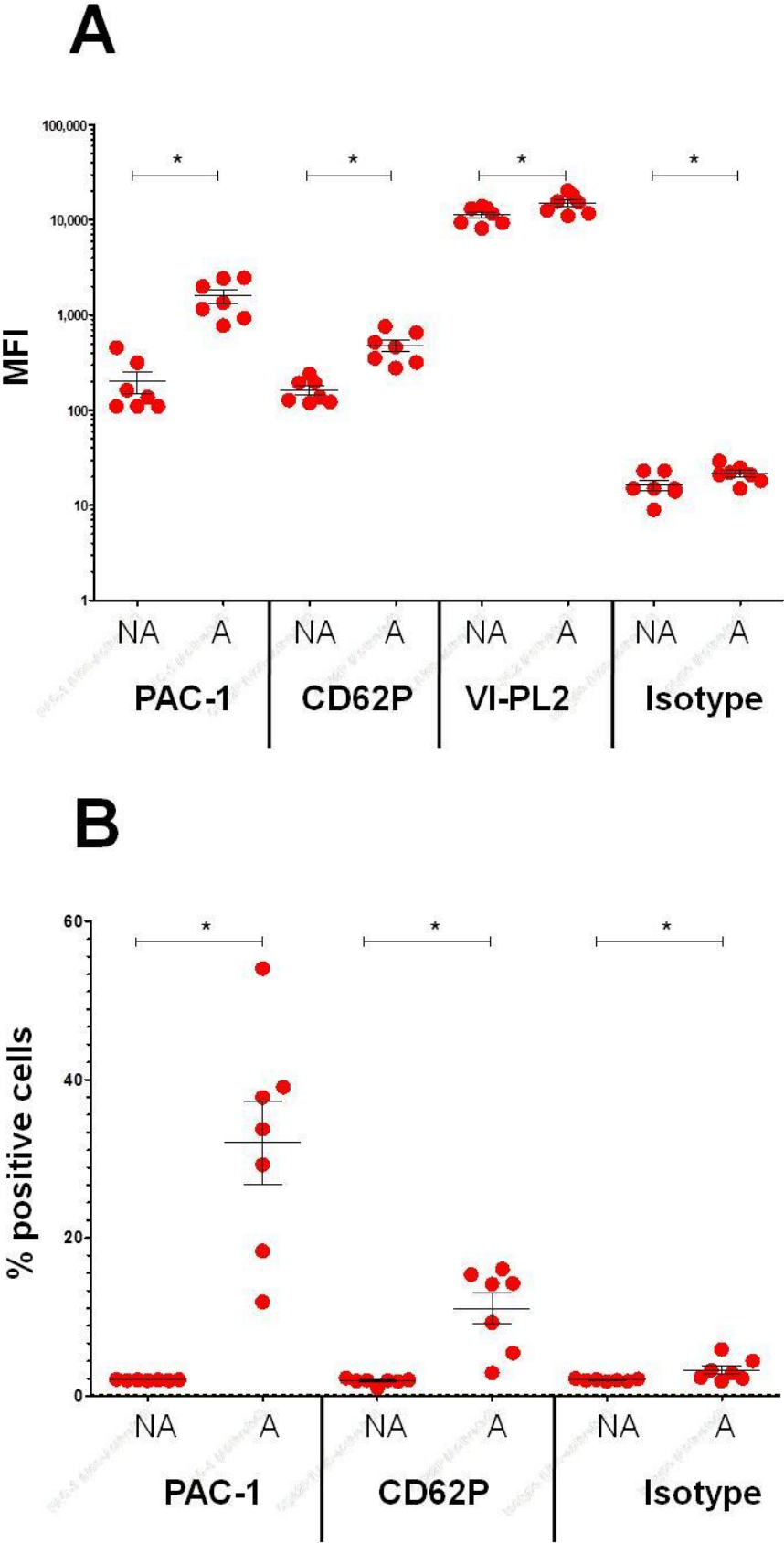
part, to reduced binding of human alloantibody (**Figure 4.3** and **4.7**, **Table 4.1**).

- From the limited data available in the returned questionnaires it was not possible to determine combinations of factors that affected assay sensitivity other than the use of EDTA-containing buffers as diluents for the platelets or capture mAb.

4.3.8 Activation of platelets

Platelets incubated in the presence of 10 μM ADP showed significantly higher markers of platelet activation (CD62P and PAC-1) compared to those incubated in the presence of TBS thus confirming activation of platelets and of $\alpha\text{IIb}\beta 3$. Expression of $\beta 3$ and binding of the isotype mAb was also increased (**Figure 4.14**).

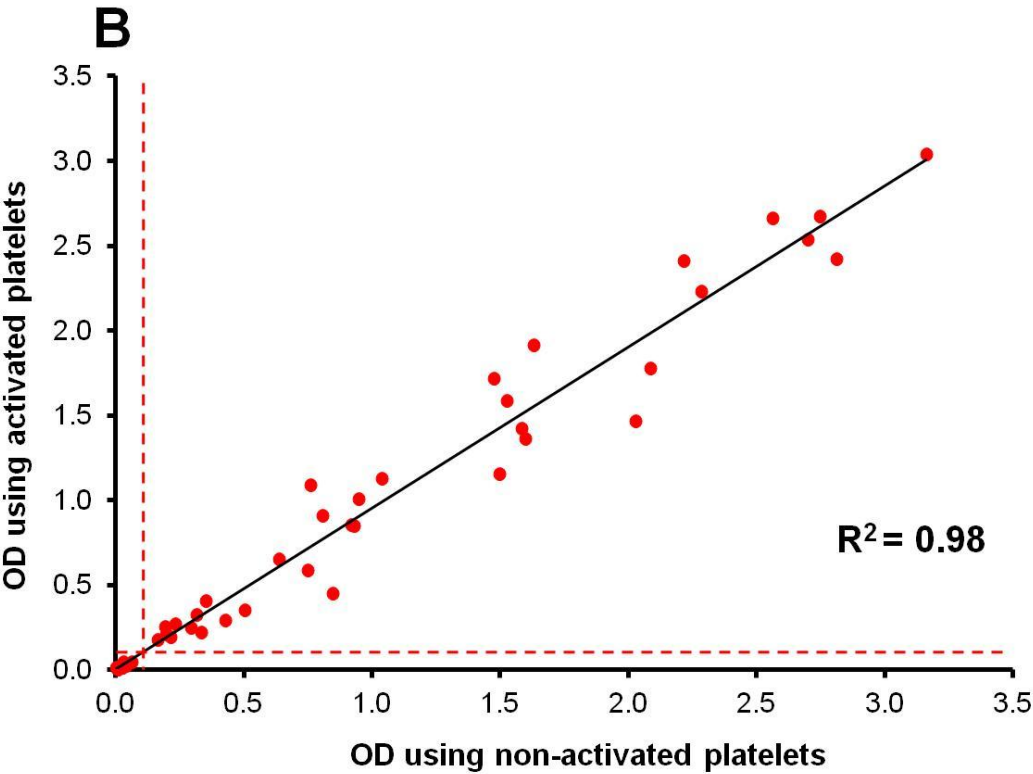
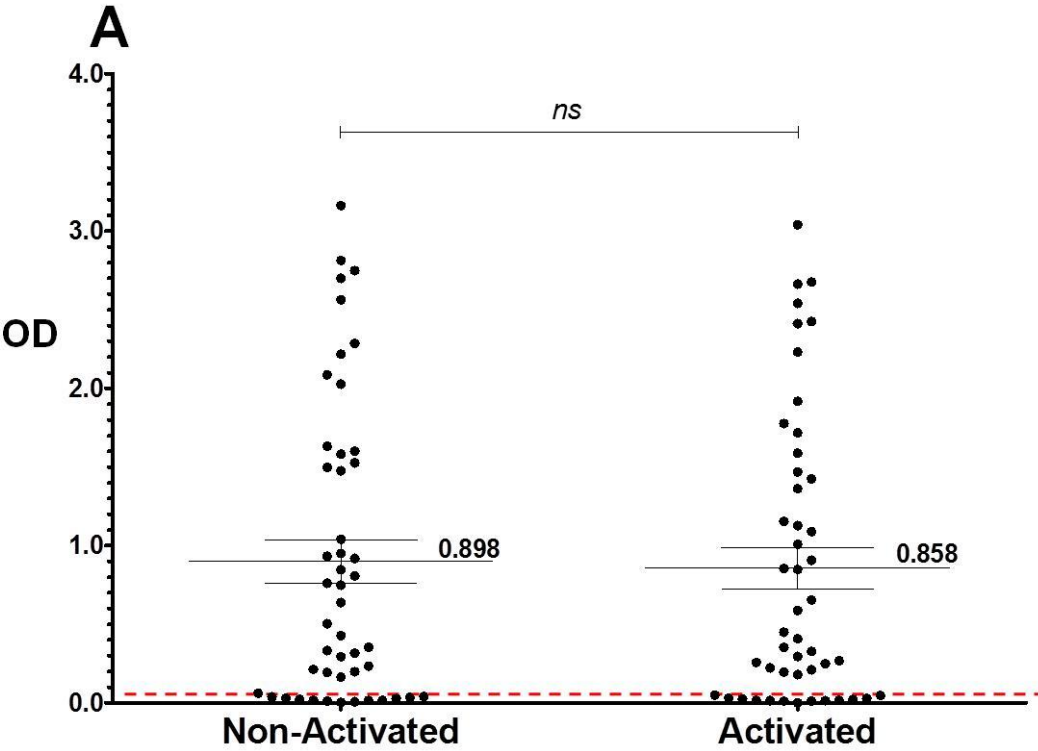
FIGURE 4.14. Confirmation of activation of platelets. *Platelets were incubated with 10 μM ADP or TBS as a negative control. The degree of platelet activation was assessed using fluorochrome-labelled mAbs against CD62P and the PAC-1 epitope. Clone VI-PL2 (anti- $\beta 3$) was used as a positive control and an irrelevant mouse IgG antibody as a negative control. (A) Mean fluorescence intensities of all aliquots of PRP treated with TBS (non-activated platelets, 'NA') or with ADP (activated platelets, 'A'). (B) Percent positive cells of all aliquots of PRP treated with TBS (non-activated platelets, 'NA') or with ADP (activated platelets, 'A'); data obtained when using anti- $\beta 3$ is not shown as the percent positive cells was 100% with both non-activated and activated platelets. To determine the % positive cells, a region was drawn containing 2% of cells in the non-activated control and the region then applied to the 'activated' tests with the relevant labelled mAb. The wide horizontal bar indicates the mean and the range bars the SEM. Significance was determined using the Wilcoxon matched pairs t-test (* $P = <0.05$, ** $P = <0.01$, ***, $P = <0.001$).*



4.3.9 MAIPA assay using activated platelets

ADP-activated platelets were used in the MAIPA assay to determine if samples known to contain anti-HPA-1a would show greater reactivity against activated integrin $\alpha\text{IIb}\beta 3$ than against non-activated integrin (**Figure 4.15**).

FIGURE 4.15. Reactivity of anti-HPA-1a samples in MAIPA assay using activated versus non-activated platelets. 49 examples of anti-HPA-1a and 1 negative control were tested in the MAIPA assay against non-activated (TBS-treated) or activated (ADP-treated) platelets. Two examples of anti-HPA-1a gave ODs that resulted in 'Over-Range' readings on the ELISA reader and are not included in these figures. **(A)** Scatter plot of individual examples of anti-HPA-1a. The wide horizontal bar indicates the mean OD of all examples (values shown in Bold typeface) and the shorter horizontal bars show the SEM. Significance was assessed using the Wilcoxon matched pairs *t*-test. **(B)** Correlation between ODs obtained when testing examples of anti-HPA-1a against non-activated versus activated platelets.



There was a high degree of correlation ($R^2 = 0.98$) between ODs obtained using ADP-activated and non-activated platelets (**Figure 6.2C**). There were no examples that were unreactive against non-activated platelets but which were reactive against ADP-activated platelets.

These data suggest that changes in the PSI domain occurring as a result of platelet activation do not affect expression of HPA-1a epitopes.

4.4 Discussion and Conclusions

In this chapter we have shown unequivocally that epitopes on $\alpha\text{IIb}\beta 3$ recognised by many capture mAbs used in the widely-used MAIPA assay are deleteriously affected by exposure to the cation chelator EDTA at 37°C; this results in reduced mAb binding and as a consequence, reduced capture of the integrin and thus of the attached human alloantibodies. Additionally we have shown that disruption of $\alpha\text{IIb}\beta 3$ by EDTA also affects the HPA-1a epitope(s), as demonstrated by binding of both monoclonal and human polyclonal antibodies. Thus, we have shown possible explanations for reduced sensitivity and/or failure to detect antibody by the Indirect PIFT (reduced alloantibody binding) and the MAIPA assay (reduced mAb and alloantibody binding) observed in some studies.

The structural integrity and function of $\alpha\text{IIb}\beta 3$ are known to critically depend on the presence of calcium and exposure to cation chelators such as EDTA has a time and temperature-dependent effect on these properties (Pidard *et al*, 1986; Gidwitz *et al*, 2004; Ma & Wong, 2007; Murcia *et al*, 2008). Brief (≤ 10 mins) exposure of the complex to EDTA at 37°C results in a reversible dissociation whilst prolonged exposure leads to irreversible dissociation; exposure at temperatures $\leq 25^\circ\text{C}$ results in significantly less dissociation (Rosa *et al*, 1984; Fitzgerald & Phillips, 1985). These findings potentially impact on assays used routinely by diagnostic laboratories for the detection of platelet antibodies against this integrin since EDTA is often present in laboratory buffers to prevent platelet aggregation, assays are often performed at 37°C and/or EDTA-anticoagulated plasma may be used (Manoil *et al*, 1997; Bessos *et al*, 2005). However, very little work has been done to investigate the reactivity of HPA-1a antibodies against EDTA-treated $\alpha\text{IIb}\beta 3$. Rosa obtained strong reactivity against $\beta 3$ in the presence of EDTA using one example of anti-HPA-1a in a Western blot (Rosa *et al*, 1984). However, this is of little relevance to routine diagnostic laboratories or to the work in this chapter since (a) western blotting is not

routinely used for the detection of HPA-1a antibodies and (b) the example of anti-HPA-1a used was derived from a case of PTP; such patients frequently develop autoantibodies in addition to alloantibodies and it is possible that these were responsible for the observed reactivity (Taaning & Tonnesen, 1999; Watkins *et al*, 2002b).

Our work with mAbs against different epitopes on $\alpha\text{IIb}\beta 3$ clearly shows that the majority show reduced binding to platelets that have been exposed to EDTA at 37°C. These findings are of direct relevance to the detection of HPA-1a antibodies since many of these clones have been used as capture mAbs in the MAIPA assay. Reduced binding of capture mAb will ultimately result in reduced detection of human alloantibodies bound to the integrin since less integrin will be captured. It is interesting to note that clone P2 has often been associated with failure to detect examples of anti-HPA-1a and its binding is particularly affected by EDTA (**Figure 4.2** and **Table 4.1**) (Allen *et al*, 2007). Our data also show the impact of temperature on the effect of EDTA. Chae and Park investigated the effect of EDTA treatment of platelets on the binding of clones HIP-8 and VI-PL2 and observed no significant difference in the binding of these antibodies in the presence of EDTA when using RT incubation; our data using these clones and a 37°C incubation highlighted the difference that temperature makes (Chae & Park, 2009).

We also showed that the reduction in binding to EDTA-treated platelets is not restricted to epitopes recognised by capture mAbs but also applies to both monoclonal and polyclonal examples of anti-HPA-1a. All three anti-HPA-1a mAbs exhibited significantly reduced binding (B2, $P = 0.006$, $n = 5$; 23-15 $P = <0.0001$, $n = 4$; 19-7, $P = <0.0001$, $n = 4$) whilst 3/4 polyclonal examples of anti-HPA-1a were similarly affected. Thus, sensitivity of the MAIPA assay would potentially be affected by both of these factors whilst the Indirect PIFT would potentially be affected by reduced binding of the human antibodies.

We investigated whether such a reduction in sensitivity might actually occur by using paired serum and EDTA plasma samples from ten cases of NAIT containing anti-HPA-1a and using a modified MAIPA assay that was designed to eliminate artefactual conformational changes in $\alpha\text{IIb}\beta 3$ that may be introduced by extraction of platelets from whole blood. This successfully showed significant reductions in sensitivity when using EDTA plasma versus serum ($P = 0.008$, mean OD = 0.511 and 0.979 respectively) in an assay system in which EDTA was not present, showing that sufficient free EDTA remained in the plasma sample to disrupt the $\alpha\text{IIb}\beta 3$ complex (**Figure 4.5**). Treatment of the platelets with EDTA (10 mM final concentration) buffer also resulted in reduced assay sensitivity when using serum samples ($P = 0.0008$, mean ODs 0.979 and 0.173) or EDTA plasma ($P = 0.002$, mean ODs = 0.511 and 0.206).

Thus, our hypothesis that EDTA chelation of cations by either anticoagulated blood sample or laboratory buffers might result in reduced assay sensitivity was shown to be correct.

We then sought to determine the relative binding of capture mAb and human polyclonal antibodies during the performance of the MAIPA assay. Using a modified assay in which subsamples were removed at various points we confirmed our earlier findings with respect to both types of antibody; reduced binding of capture mAbs was particularly notable (**Figure 4.7L**). Interestingly, the binding of the mAb P2 declined during the assay, even when using an assay system in which EDTA was absent (**Figure 4.7A**). There are a number of possible explanations for this. Firstly, the example of anti-HPA-1a used was collected into citrate (CPD). This anticoagulant also chelates calcium but has a lower affinity than EDTA (Phillips *et al*, 1997; Keowmaneechai & McClements, 2002); thus it is possible that the prolonged incubation at 37°C resulted in gradual removal of calcium from $\alpha\text{IIb}\beta 3$. Secondly, it is known that there is a natural recycling of $\alpha\text{IIb}\beta 3$ from the platelet surface to the

SCCS and this may have reduced the apparent level of complex (Wencel-Drake *et al*, 1986b; Wencel-Drake, 1990). Activation of $\alpha\text{IIb}\beta 3$ and externalisation of the internal pool of $\alpha\text{IIb}\beta 3$ by this mechanism may also account for the increased reactivity that we observed using some mAbs with platelets treated with TBS at 37°C compared to platelets kept at RT (**Figure 4.2**). Finally, it is known that patching and capping of integrins may occur after binding of monoclonal or polyclonal antibodies (Santoso *et al*, 1986; Santoso *et al*, 1987).

One possible criticism of our findings is that we used a modified version of the MAIPA assay whose performance may be different to versions used in diagnostic laboratories. We therefore included paired serum and EDTA plasma samples obtained from a mother whose children had been affected by anti-HPA-1a mediated NAIT in a NIBSC international workshop in which approximately 40 laboratories participated. This was an extremely informative exercise.

Pre-workshop analysis showed similar titration end-points for both serum and EDTA plasma samples (**Figures 4.8 B and C**); quantitation of the antibody also showed similar values at 14.3 and 16.3 i.u./mL respectively (**Table 4.2**). However, an approximate 30% reduction in reactivity was observed when using undiluted EDTA plasma compared to undiluted serum and using clone 'PAB-6' as capture mAb. These data show that dilution of the EDTA eliminates the difference between serum and EDTA plasma (presumably by reducing the level of EDTA to below the level sufficient to chelate calcium from $\alpha\text{IIb}\beta 3$). The data also show that quantitation values (i.u./mL) are not necessarily related to ODs observed in undiluted material; this is of relevance to the interpretation of published data regarding antibody level when assessed by OD or i.u./mL and is discussed in detail in **Chapter 5**.

The data returned from participants using the MAIPA assay largely confirmed the findings of this study.

Not all participants returned analysable data but of those returning valid data, 17/24 (70.8%) observed a moderate decrease in OD of the plasma sample compared to the serum sample (mean reduction 27.7%; range 0 - 85.1%) (**Figure 4.10**).

The reduction in reactivity was not related to the use of particular capture mAbs *per se* as demonstrated by the results with clone P2 where 3/8 participants using this clone reported no reduction in reactivity of the plasma sample.

Since the reactivity of the plasma was assessed as a percentage of that of the serum sample it was possible that assay insensitivity, for reasons other than the effect of EDTA, reduced the reactivity of the serum sample and thus calculated figures based on this would be erroneous; the relationship between serum reactivity, and plasma reactivity as a percentage of that of serum was therefore analysed. This showed that there was no correlation between these measurements.

A detailed analysis of the technique questionnaires returned by participants (**Table 4.4, Appendix 5**) showed that whilst many participants used EDTA, either as an anticoagulant for the blood from which platelets were extracted, or in buffer solutions, it was impossible to directly relate these factors to reactivity of the samples. The vast number of possible permutations of the MAIPA assay parameters probably means that it will not be possible to identify the impact of these factors using collaborative studies and require that one or two laboratories try and resolve this by changing single parameters at a time. There are also other potential factors that were not investigated by means of the questionnaire distributed to participants that would need to be taken into account e.g. whether calcium was added to any of the reagents used; this potentially would overcome the effects of EDTA and might restore previously disrupted $\alpha\text{IIb}\beta 3$ complex (White & Escolar, 2000).

Interestingly, one group has attempted to define the impact of use of different mAbs on the measured level of anti-HPA-1a (Killie *et al*, 2010). Their data show that whilst

there was correlation ($R^2 = 0.88$ and 0.90) in measured antibody level when using clones P2, PAB-1 and Y2/51, the actual levels varied randomly but significantly ($P = 0.029$) when comparing P2 and PAB-1 against Y2/51. All samples used in this study were plasma samples and our data provide an explanation. Clones P2 and PAB-1 are directed against the $\alpha\text{IIb}\beta 3$ complex and the epitopes are extremely affected by EDTA, whilst Y2/51 recognises an epitope on the PSI and hybrid domain and is less affected by EDTA treatment of the complex (**Figures 4.2 and 4.7, Table 4.1.**); taken together with the variable impact of EDTA on the reactivity of different examples of anti-HPA-1a (**Figures 4.3 and 4.5**) it is not surprising that different values were obtained. We explore these findings further in **Chapter 5**.

The results of using purified, immobilised $\alpha\text{IIb}\beta 3$, as used in the GTI antigen capture ELISA kits, were surprising. No participant reported a loss of reactivity of $\geq 20\%$ and statistical analysis showed that the EDTA plasma sample gave significantly higher ODs ($P = 0.003$) than the serum sample. These data show that, unlike membrane-bound $\alpha\text{IIb}\beta 3$, immobilised integrin is resistant to degradation by EDTA. This is supported by the findings of Bessos who compared their in-house ELISA assay that used purified, immobilised $\alpha\text{IIb}\beta 3$ with a conventional platelet MAIPA assay to measure the level of anti-HPA-1a in a panel of 26 plasma samples; they observed that the ELISA assay gave values approximately 2.8 times those of the MAIPA assay (Bessos *et al*, 2008a).

In our study, there are a number of possible explanations for the increased reactivity of the plasma sample in the ELISA assay including the presence of additional antibodies that recognise epitopes exposed by immobilisation of the integrin onto plastic (Masters & Taaning, 1998) that may be EDTA-dependent, or perhaps increased exposure of the HPA-1a epitope. However, the increased reactivity observed in the ELISA assay should not be interpreted that this assay is the technique of choice for detection of platelet antibodies since a number of studies

have shown its' limitations (Joiner *et al*, 1995; Kowalski *et al*, 1995b; Boehlen *et al*, 1998; Allen & Murphy, 1999).

Perhaps the most important finding from this workshop exercise was that 5.1% (2/39) of participants reported that the EDTA plasma sample did not contain HPA-1a antibodies whilst the serum sample did. One further participant failed to detect the anti-HPA-1a by the MAIPA assay. Thus 3/39 (7.7%) participants failed to detect the potent (approximately 16 i.u./mL) anti-HPA-1a in the EDTA plasma sample. The activity in this sample is well in excess of that reported to be able to cause severe NAIT and had indeed caused severe thrombocytopenia in one of the infants of the mother from whom the samples were obtained (Killie *et al*, 2008). These findings are of concern and suggest that use of EDTA anticoagulated samples may be a reason for non-detection of HPA-1a antibodies in some studies.

These findings are potentially of significance to studies of $\alpha\text{IIb}\beta 3$ in other applications e.g. immunophenotyping of leukaemia may involve the use of mAbs against αIIb and $\beta 3$ and the recommended anticoagulant is EDTA (Bain *et al*, 2002).

That cation chelation can impact on other integrins has been documented. Bournazos showed that the assessment of circulating platelet-leucocyte complexes, a process mediated by P-selectin / P-selectin glycoprotein ligand-1, was affected by anticoagulant and degree of cation chelation, and Maniecki showed that the measured levels of CD163 varied according to free calcium and mAb clone used (Bournazos *et al*, 2008; Maniecki *et al*, 2011). These findings may be of relevance to the study of alloantibodies against platelet integrins other than $\alpha\text{IIb}\beta 3$ since it is known that GPIa/IIa is also cation-dependant and thus detection of antibodies against HPA-5, -13 and -18 and -25 may be compromised by the use of EDTA (Takada & Hemler, 1989; Staatz *et al*, 1990).

Thus the findings in this chapter potentially explain a number of observations in the

literature regarding the non-detection of HPA-1a antibodies, their variable detection and perhaps even the results of some of the quantitation studies that used EDTA plasma samples (Jaegtvik *et al*, 2000; Killie *et al*, 2008). Our data suggest that the use of EDTA plasma samples in antibody screening (and quantitation) assays should either be avoided or, if this is not possible, then assays should be performed at temperature below 37°C.

We were unable to explain why some laboratories participating in the NIBSC workshop still obtained excellent results when using EDTA plasma samples and/or EDTA within their assay, together with an incubation temperature of 37°C and conversely poor results when apparently having no EDTA present but there may be other factors that we did not take into account (**Appendix 5**). However, we have clearly shown that conformation of $\alpha\text{IIb}\beta 3$ is affected by EDTA and this plays a major role in assay sensitivity. In **Chapter 5** we explore other aspects of integrin $\alpha\text{IIb}\beta 3$ that may impact on assay sensitivity.

We also explored potential differences in reactivity of HPA-1a antibodies against non-activated and activated $\alpha\text{IIb}\beta 3$ as a potential and previously unexplored reason for inter-laboratory differences in detection of HPA-1a antibodies.

The PSI domain of $\beta 3$, is partially obscured by its' location in the cleft between the EGF and hybrid domains of $\beta 3$ suggesting that accessibility of the HPA-1a epitopes in resting (closed) $\alpha\text{IIb}\beta 3$ is limited. An increase in accessibility might occur as a result of platelet activation since the PSI domain moves away from the core of the molecule by approximately 7 nm during this process, resulting in exposure of previously cryptic epitopes (Honda *et al*, 1995b; Xiao *et al*, 2004; Provasi *et al*, 2009).

We showed that there was no significant difference in reactivity of HPA-1a antibodies against resting versus activated $\alpha\text{IIb}\beta 3$. There are a number of possible interpretations for these findings.

Firstly, the platelets may not have been fully activated and thus the PSI domain remained partially obscured. The PAC-1 data would tend to disprove this hypothesis since the epitope recognised by this antibody is only expressed on activated $\alpha\text{IIb}\beta 3$ (Shattil *et al*, 1985; Blue *et al*, 2010). Our data show that there was a clear increase in the expression of PAC-1 epitope on ADP-activated platelets compared to control (TBS-treated) platelets as measured by the increase in MFI and in the number of cells expressing PAC-1 epitope (up to approximately 54%).

It is possible that activation of the platelets resulted in reduced expression and/or accessibility of the epitope recognised by the capture mAb 'VI-PL2' and thus reduced capture of $\alpha\text{IIb}\beta 3$ in the MAIPA assay. This was shown not to be the case by the use of fluorochrome-labelled VI-PL2 to assess $\beta 3$ expression following activation (**Figure 4.14A**).

The use of the peptide inhibitor Gly-Pro-Arg-Pro may have blocked the binding of HPA-1a antibodies. This was used in all assays so any blocking effect would have occurred with both non-activated and activated platelets and there is no reason to believe that this effect would be enhanced with activated platelets.

There is a possibility that the activated $\alpha\text{IIb}\beta 3$ molecules were internalised and replaced by non-activated molecules from the internal pool during the primary incubation phase of the MAIPA (with test antiserum) (Wencel-Drake *et al*, 1986a; Wencel-Drake, 1990). No experiments were performed to confirm this.

Lastly, there is a possibility is that despite the movement of the PSI domain away from the tail domains, the HPA-1a epitopes still remain partially facing the tail domains and thus accessibility to HPA-1a alloantibodies remains restricted. Our molecular model (**Chapter 5**) of $\alpha\text{IIb}\beta 3$ suggests that the HPA-1a epitopes are on the external surface and this was confirmed by Landau (Landau & Rosenberg, 2010).

In conclusion therefore these data suggest that differences in the ability to detect HPA-1a antibodies are not related to the activation status of platelets used in immunoassays.

4.5 Summary of findings in this chapter

- Using experimental conditions we showed that exposure of $\alpha\text{IIb}\beta 3$ to 10 mM EDTA (a concentration used routinely in diagnostic laboratories) affects epitopes recognised by many mAbs including those used as capture mAbs in the MAIPA assay.
- The degree to which mAb binding was affected was determined by epitope specificity.
 - HIP8 (anti- αIIb) and P2 (anti- $\alpha\text{IIb}\beta 3$ complex) were most severely affected.
 - Y2/51 (PSI + hybrid domain), SZ21 (PSI domain) and VI-PL2 (unknown domain) were affected to a lesser extent.
 - CRC54 (within amino acids 1-100, PSI and hybrid domains) was unaffected by cation chelation.
 - Binding of three anti-HPA-1a mAbs was adversely affected by EDTA treatment of platelets.
- EDTA treatment of platelets had no effect on HLA class I epitopes.
- Exposure of platelets to 10 mM EDTA affected the binding of some examples of human polyclonal anti-HPA-1a in the Indirect PIFT assay.
- A modified version of the MAIPA assay and paired serum and EDTA plasma samples collected at the same venepuncture showed that use of:
 - Platelets exposed to EDTA buffer severely decreased the sensitivity of the assay compared to platelets exposed to control (TBS) buffer when using serum samples.

- Blood collected into EDTA compounded this effect.
- Stage-by-stage analysis of the MAIPA assay demonstrated that the reduction in sensitivity was mediated both by reduced binding of human allo-anti-HPA-1a and of capture mAb.
- Inclusion of serum and EDTA plasma samples containing anti-HPA-1a in a NIBSC workshop showed that our experimental findings could be replicated in laboratories providing a diagnostic service. Specifically, it showed that use of EDTA plasma could severely affect detection of anti-HPA-1a when using the MAIPA assay depending on which anti- $\beta 3$ capture mAb was used and other, as yet unidentified, factors.
- It also demonstrated that use of immobilised, purified $\alpha\text{IIb}\beta 3$ such as that used within commercial ACE kits produced by Gen-Probe GTi Diagnostics (USA) was unaffected by the use of EDTA plasma.
- No significant difference was observed in the strength of reactivity or detection of the panel of anti-HPA-1a samples against activated platelets compared to non-activated platelets.

4.6 Significance of these findings

Many studies of platelet alloantibodies, including prospective studies of NAIT, have been based on the results of testing samples collected into EDTA. Furthermore, as shown here EDTA is frequently present in buffers used in the MAIPA assay used in many diagnostic laboratories.

Our data conclusively show that the use of EDTA-anticoagulated samples or the use of EDTA-containing buffers can, depending on assay conditions, adversely affect detection of HPA-1a antibodies and is of direct relevance to diagnostic laboratories worldwide.

The use of platelets heterozygous for HPA-1a compounded the adverse effects of EDTA and resulted in approximately 97% loss of sensitivity.

The antibody used in the NIBSC workshop was a potent example and had caused NAIT. Despite this, 5.1% participating laboratories failed to detect this antibody when using the EDTA plasma sample; one further participant only detected the antibody in the ACE assay and not in the MAIPA assay. In total therefore 7.7% participants failed to detect this potent antibody in the MAIPA assay. It is highly likely therefore that weaker examples of anti-HPA-1a would be missed by more laboratories if EDTA plasma samples were used. Thus, these findings provide one possible explanation for the reported non-detection of antibodies in some cases of NAIT.

The impact of EDTA-induced conformational changes of $\alpha\text{IIb}\beta 3$ on the detection of other alloantibodies or of autoantibodies in the MAIPA assay was not studied but it is likely that that their detection also would be affected because of the reduced binding of the capture mAb, irrespective of the effects of EDTA on the epitope recognised by these human antibodies.

The findings in this chapter are also of relevance to other areas of integrin biology

that use mAbs against αIIb or $\beta 3$ e.g. leukaemia immunophenotyping (Bain *et al*, 2002), or studies of integrin GPIa/IIa (VLA-2) since stability of this molecule is also dependent on the presence of Mg^{2+} and Ca^{2+} (Staatz *et al*, 1990; Smith *et al*, 2000).

Finally, our data suggest that HPA-1a epitopes are not affected significantly by activation of $\alpha\text{IIb}\beta 3$. The detection of some examples of anti-HPA-1a in some laboratories and not in others therefore is probably not related to activation status of the integrin.

Chapter 5.

Integrin $\alpha\text{IIb}\beta 3$

conformation II: effect of α -
chains on HPA-1a epitope
expression

5.1 Introduction

A number of studies have reported that HPA-1a antibodies cannot be detected in the maternal blood in up to 20% of cases of clinically diagnosed NAIT and this is supported by the finding of an excess of HPA-1b1b mothers amongst referrals for investigation of suspected NAIT (Kaplan *et al*, 1988; Williamson *et al*, 1998; Bussel *et al*, 2005; Tiller *et al*, 2009).

In **Chapter 4** we showed that one possible explanation for the non-detection of HPA-1a antibodies was the use of assays rendered insensitive as a result of the use of the cation chelator, EDTA (Allen *et al*, 2012).

A second potential reason why HPA-1a alloantibodies may not be detectable is because they are of low-affinity and are eluted from the platelet membrane during the wash phases of the MAIPA and other assays (Socher *et al*, 2009; Bakchoul *et al*, 2011; Peterson, 2012). In the study of Bakchoul, analysis of 14 'MAIPA negative' samples by surface plasmon resonance (SPR), which measures antibody affinity directly and does not involve wash phases, showed the presence of anti-HPA-1a in seven examples. In a similar study, 11/61 samples contained anti-HPA-1a that were undetectable by MACE and flow cytometric assays (Peterson, 2012).

In this chapter we investigate a further possible reason, namely potential differences in HPA-1a epitopes resulting from the association of the $\beta 3$ chain with an alternative α -chain (αv). We hypothesised that the stimulating antigen in NAIT is the placental syncytiotrophoblast integrin $\alpha\text{v}\beta 3$ and thus the resulting HPA-1a antibodies have higher affinity for this molecule than for platelet $\alpha\text{IIb}\beta 3$ used in routine assays for the detection of HPA-1a antibodies. This would explain the apparently low affinity of some examples of HPA-1a antibodies for $\alpha\text{IIb}\beta 3$ as shown by Socher, Bakchoul and Peterson (Socher *et al*, 2009; Bakchoul *et al*, 2011; Peterson, 2012).

In this study we used a melanoma cell line 'M21', that has been shown to express $\alpha\text{v}\beta 3$ but not $\alpha\text{IIb}\beta 3$, as a model for placental syncytiotrophoblasts (Felding-Habermann *et al*, 2002). A companion cell line, M21-LIIb, was used as a direct comparator for the expression of $\alpha\text{IIb}\beta 3$ in an otherwise identical cellular environment. These M21-LIIb cells were derived from M21-L cells, a line derived from M21 cells, that lack mRNA for αv and as a consequence does not express $\beta 3$ on the cell surface (Cheresh & Spiro, 1987). The transfection of αIIb cDNA into M21-L cells restored expression of $\beta 3$ as part of the $\alpha\text{IIb}\beta 3$ complex; $\alpha\text{v}\beta 3$ was shown not to be expressed (Felding-Habermann *et al*, 1996). Due to limited availability of M21 and M21-LIIb cells, the establishment of cell culture and initial experiments to determine optimal experimental conditions were performed using an alternative melanoma cell line 'C32' that was more readily available and which also expresses $\alpha\text{v}\beta 3$ (Knudsen *et al*, 1988).

We compare the use of $\alpha\text{IIb}\beta 3$ versus $\alpha\text{v}\beta 3$ as targets for detection of HPA-1a antibodies by characterising the relative expression levels of both molecules on platelets and on cell lines. We then developed and validated assays using cell lines to detect HPA-1a antibodies, and finally compare the sensitivity of both molecules for the detection and quantitation of HPA-1a antibodies.

5.2 Methods

5.2.1 Culture of melanoma cell lines

All cell lines were cultured in T150 flasks at 37°C in 5% CO₂ in air in supplemented Dulbecco's minimal essential medium (DMEM) and passaged every 3-4 days.

Adherent C32 and M21 cells were detached by removal of culture medium followed by a single wash in Dulbecco's PBS then addition of 2 mL Cell Dissociation Buffer (CDB) to the cell monolayer. The cells were allowed to detach (with gentle frequent agitation) for a maximum of 10 mins after which 18 mL supplemented medium was added. The cells were pelleted by centrifugation (300 x *g*, 10 mins at 4°C), re-suspended in 10 mL supplemented medium then approximately 1.0 - 1.5 x 10⁶ cells (depending on expected culture period) added to each T150 flask containing 39 mL supplemented medium.

Non-adherent M21-L11b cells were passaged by harvesting all supernatant fluid and centrifugation (300 x *g*, 10 mins at 4°C). The pelleted cells were re-suspended in supplemented medium and grown in fresh flasks as described above.

All cultures were assessed for the presence of *Mycobacterium* species using a 'MycoAlert' kit (Lonza, UK).

After three cell passages the yield of C32 cells was not as high as expected from figures quoted in the literature. The American Type Culture Collection (ATCC, USA) suggests that such cells are cultured in Eagles Minimal Essential Medium and this was used (supplemented) for all subsequent cell passages.

Cells were passaged a maximum of 20 times.

5.2.2 Cryopreservation of cell lines

Surplus cells were cryopreserved in DMSO (10%) in FCS.

5.2.3 Effect of Cell Dissociation Buffer on integrin expression

Platelets (5×10^7) were pelleted from PRP and the pellet re-suspended in 200 μL CDB or TBS. Each suspension was incubated at 37°C for a fixed time period from 0 - 60 mins and then pelleted by centrifugation ($10,000 \times g$, 1 min). The platelets were washed once in cold TBS/BSA/EDTA then re-suspended in 100 μL cold TBS/BSA. To 5 μL platelet suspension was added 10 μL P2-FITC or 50 μL B2-FITC. After 20 mins incubation at RT, 1 mL cold TBS/BSA was added and fluorescence analysed on a Becton-Dickinson LSR II flow cytometer using FACSDiva software. As a control to demonstrate maximum dissociation of $\alpha\text{IIb}\beta 3$, one aliquot of platelets was incubated with 200 μL 10 mM EDTA for 60 mins.

5.2.4 Analysis of cell surface integrins by flow cytometry

2.5×10^5 cells or 5×10^6 platelets in a volume of 50 μL were incubated with 10 μL appropriate antibody at RT for 30 mins. One mL of cold TBS/BSA was added to those tubes containing conjugated primary antibodies and fluorescence analysed immediately as above.

To tubes containing unlabelled primary antibodies was added 1 mL cold TBS/BSA/EDTA. The cells were pelleted by centrifugation ($13,000 \times g$, 1 min) then washed once more. The pelleted cells were suspended in 50 μL secondary antibody, incubated at RT for 20 mins then 1 mL cold TBS/BSA added and fluorescence analysed immediately as above.

5.2.5 Development of Indirect Immunofluorescence and MAIPA assays using cell lines to detect HPA-1a antibodies

Due to the limited availability of material from NAIT-derived examples of anti-HPA-1a, the initial development of the cell line assays used donor- and PTP-derived examples.

The Indirect cell line immunofluorescence assay was performed as for the Indirect

PIFT (**Chapter 2.4.4**) but using 2.5×10^5 cells per assay.

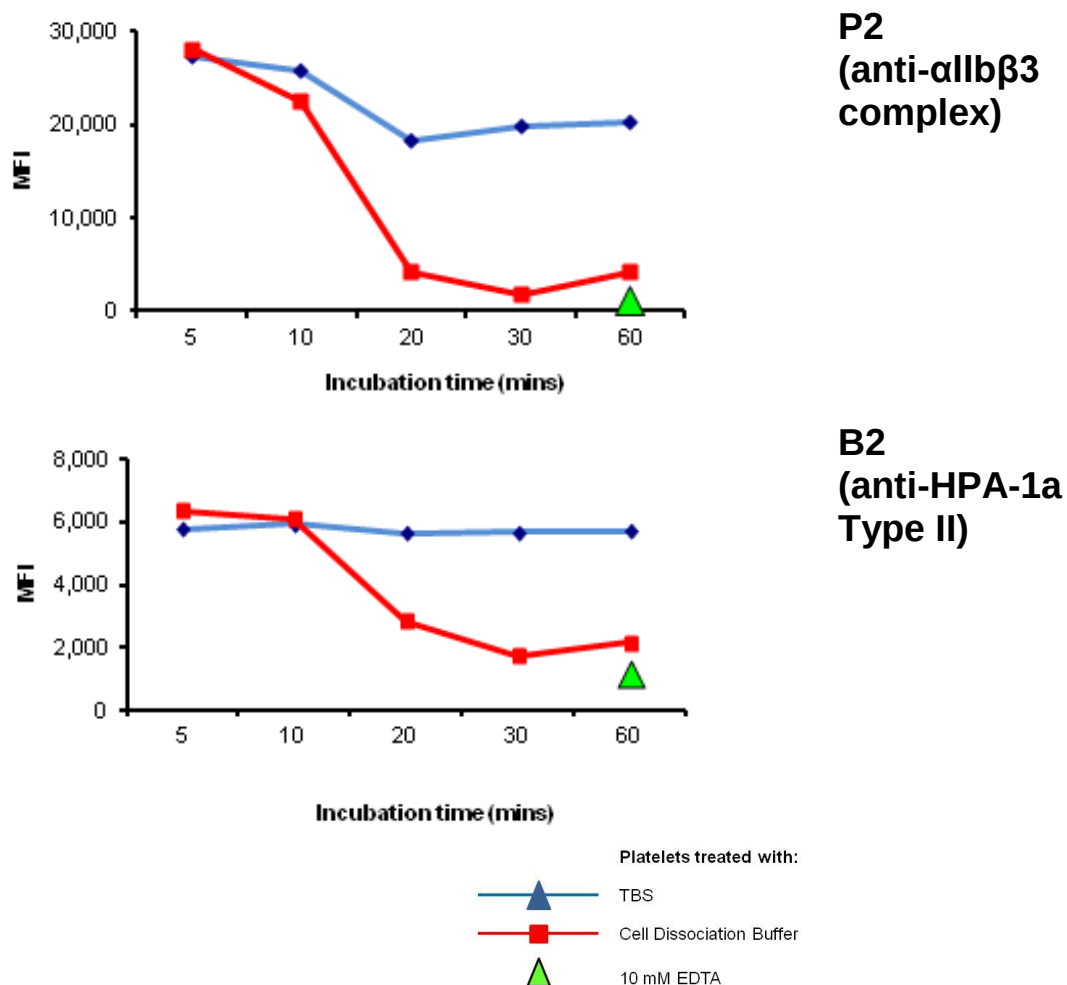
The 'MAIPA' assay using cell lines was performed using the same protocol as for the platelet MAIPA assay (**Chapter 2.4.5**) except for the number of cells used (determined in initial experiments).

5.3 Results

5.3.1 Effect of Cell Dissociation Buffer on integrin expression

Since the CDB used to detach M21 and C32 cells from the culture flasks contains EDTA (at a concentration unspecified by the supplier) and this detachment takes place at 37°C, the possibility that CDB might alter the conformation of $\alpha\text{IIb}\beta 3$ was considered. Since the effect of cation chelation on platelet $\alpha\text{IIb}\beta 3$ had been extensively studied previously using the mAbs P2 (anti- $\alpha\text{IIb}\beta 3$ complex) and B2 (anti-HPA-1a Type II) the effect of CDB on surface integrin complexes was studied using platelets as a model (Figure 5.1).

FIGURE 5.1. Effect of incubation of platelets in cell dissociation on expression of the $\alpha\text{IIb}\beta 3$ complex and HPA-1a epitopes.



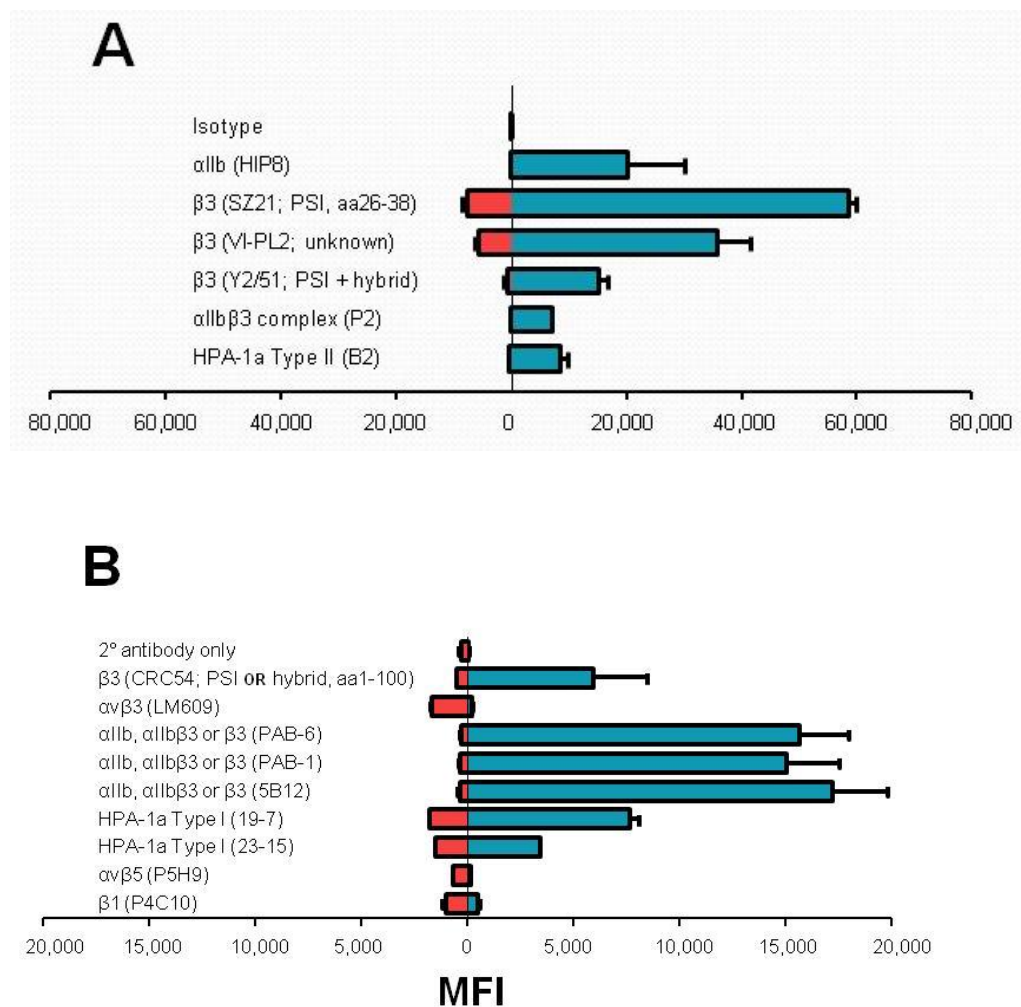
CDB had only limited impact on P2 and B2 epitopes until approximately 10 mins incubation, thereafter a rapid loss of reactivity was seen.

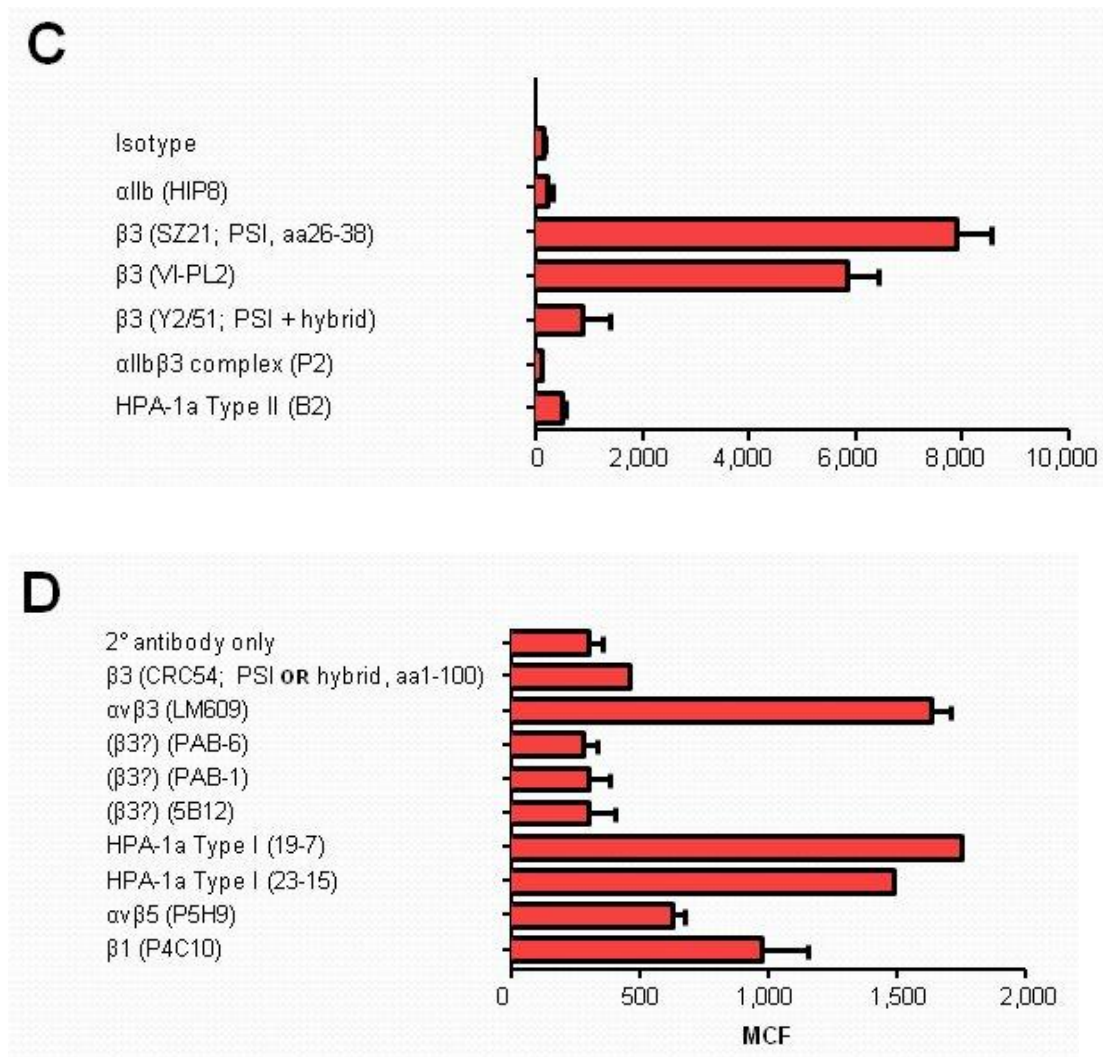
Ten minutes was therefore the maximum period to which M21 or C32 cells were exposed to CDB during cell passaging.

5.3.2 Integrin expression on C32 cells

The expression of β 3 and HPA-1a epitopes on C32 cells was investigated. In addition, since the cell lines would ultimately be used in the MAIPA assay, where co-precipitation of integrins has been reported to occur under some circumstances, not only the expression of α v β 3 but also the expression of integrins related to α v or β 3 (Figure 1.1) were investigated (Figure 5.2).

FIGURE 5.2. Integrin expression on C32 cells compared to platelets. *Integrin expression was assessed by flow cytometry using directly (A and C) or indirectly labelled mAbs (B and D). Red bars, C32 cells; blue bars, platelets. Figures A and B have equivalent scales for MFI for both C32 cells and platelets so that the relative expression levels can be seen; re-scaled data for the C32 cells is shown in Figures C and D. Data are the mean of three experiments; error bars show the SEM.*





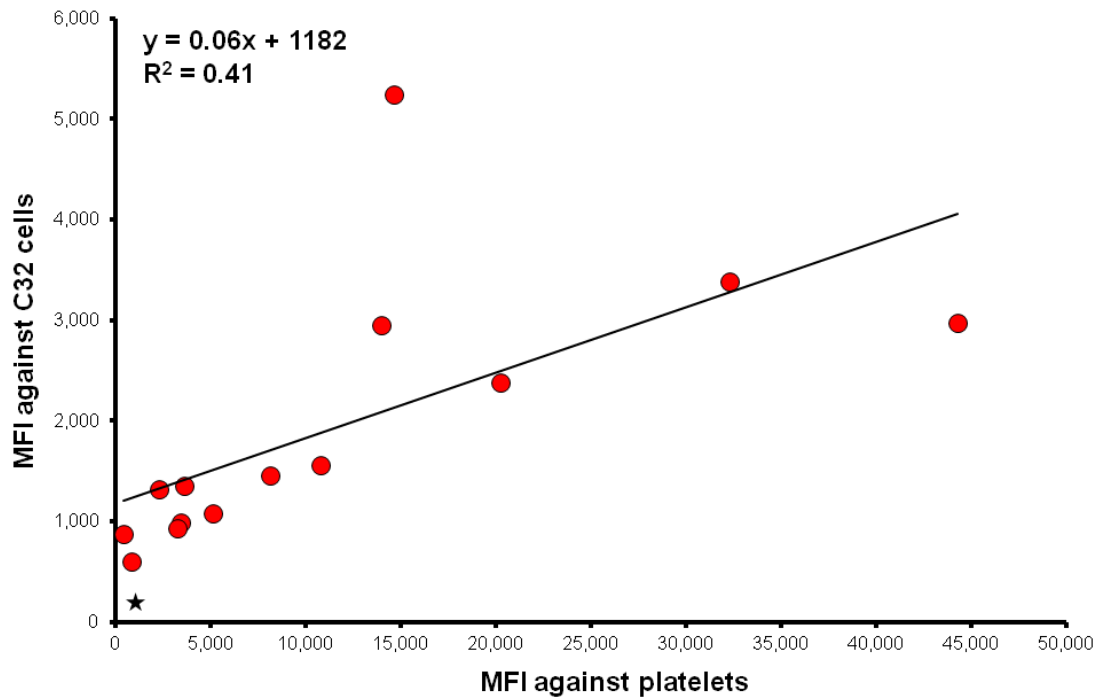
Integrin β 3 was present on C32 cells but at a much lower level compared to platelets.

Reactivity of C32 cells with LM609, and lack of reactivity with P2, confirmed that this cell line expressed β 3 as a constituent part of the α v β 3 complex and not as part of the α IIb β 3 complex.

The C32 cells also reacted with the three anti-HPA-1a mAbs thus confirming that these cells express HPA-1a epitopes.

To confirm that the HPA-1a epitopes were recognisable by polyclonal antibodies, a panel of fourteen blood donor-derived antibodies and one negative control (group AB serum) was tested against C32 cells and platelets in an Indirect Immunofluorescence assay (**Figure 5.3**).

FIGURE 5.3. Reactivity of human polyclonal HPA-1a antibodies against $\alpha\text{v}\beta 3$ -expressing C32 cells compared to platelets in the Indirect Immunofluorescence assay. Each circle represents the averaged MFI value from two experiments for one example of anti-HPA-1a; the black star is the averaged MFI of the negative control.



All examples of anti-HPA-1a were reactive against the C32 cells although there was less than perfect correlation with platelet reactivity ($R^2 = 0.41$). The negative control was negative against both platelets and C32 cells.

These data showed that human polyclonal HPA-1a antibodies were able to recognise HPA-1a epitopes on $\alpha\text{v}\beta 3$ on C32 cells.

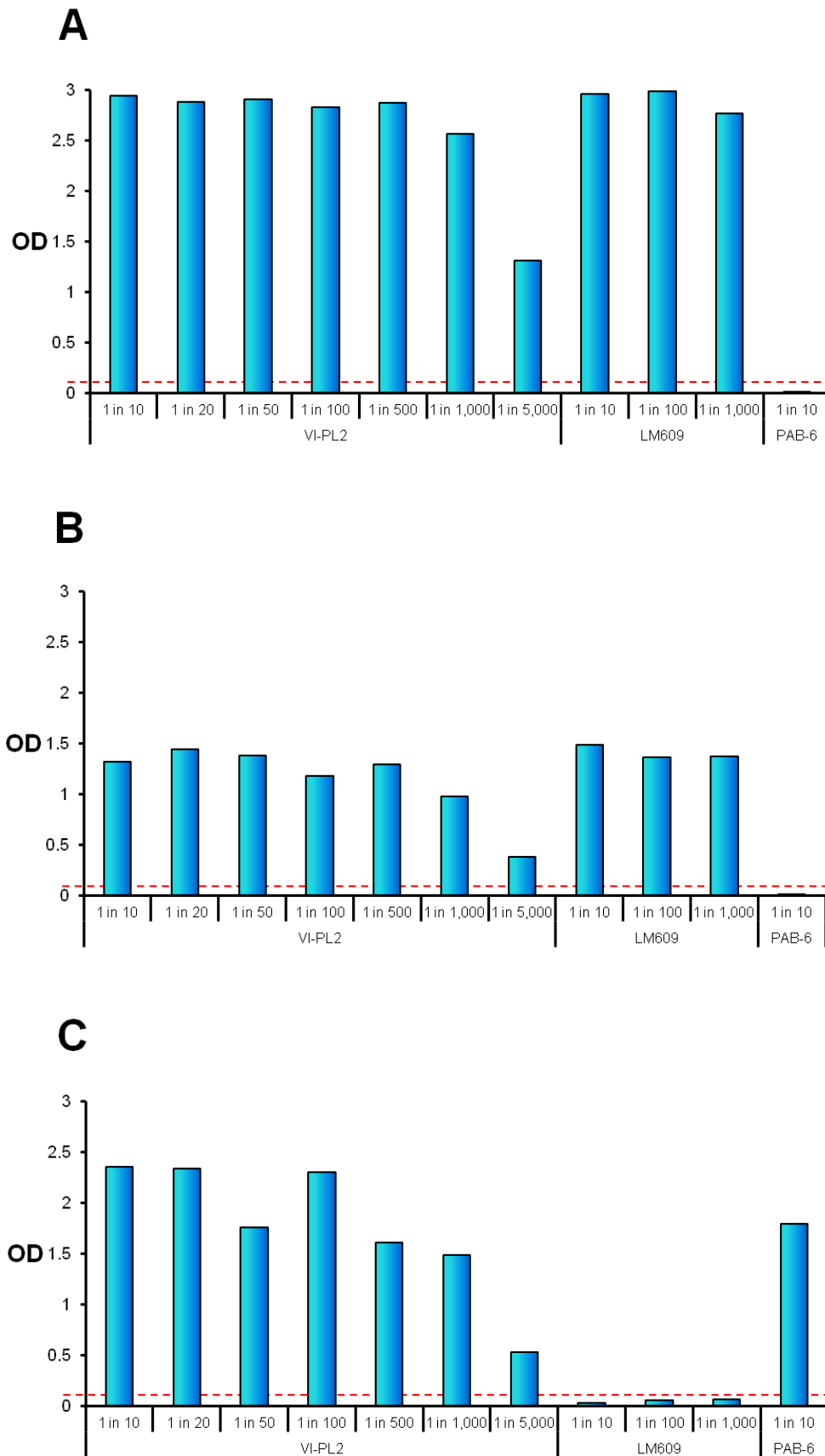
5.3.3 Development of the cell line MAIPA assay

Since the Indirect Immunofluorescence assay uses intact platelets and thus will detect antibodies against other cell surface proteins such as HLA class I that might give false positive results when investigating HPA-1a antibodies, the possibility of adapting the MAIPA assay for use with the melanoma cell lines (monoclonal antibody-specific immobilisation of melanoma antigens, 'MAIMA') was investigated (Kiefel *et al*, 1987; Campbell *et al*, 2007).

MAIMA assays were performed using the same protocol as the platelet MAIPA assay except for the number of cells used. C32 cells were tested at a total of 1×10^5 and 5×10^5 cells per reaction, suspended in 50 μL TBS/BSA to determine the optimum number of cells, and capture mAbs 'VI-PL2' and 'LM609' were titrated to determine optimum dilutions.

Samples expected to give a positive result (anti-HPA-1a D-0016) and negative results (AB Serum #4) were included. Results were compared to those obtained in a platelet MAIPA assay using 1×10^7 platelets per reaction and capture mAb 'PAB-6' (Figure 5.4).

FIGURE 5.4. Optimisation of cell number and capture mAb dilution for MAIMA assay. (A) C32 cells at 5×10^5 per reaction, (B) C32 cells at 1×10^5 /reaction and (C) platelets at 1×10^7 /reaction. Capture mAb and dilutions are on the x axis and OD on the y axis. The cut-off (0.100 OD units) is indicated by the dashed red line. Only the results of testing the anti-HPA-1a are shown for clarity; values obtained with the negative control were all close to zero and are not shown. Results are the averaged ODs from two experiments.



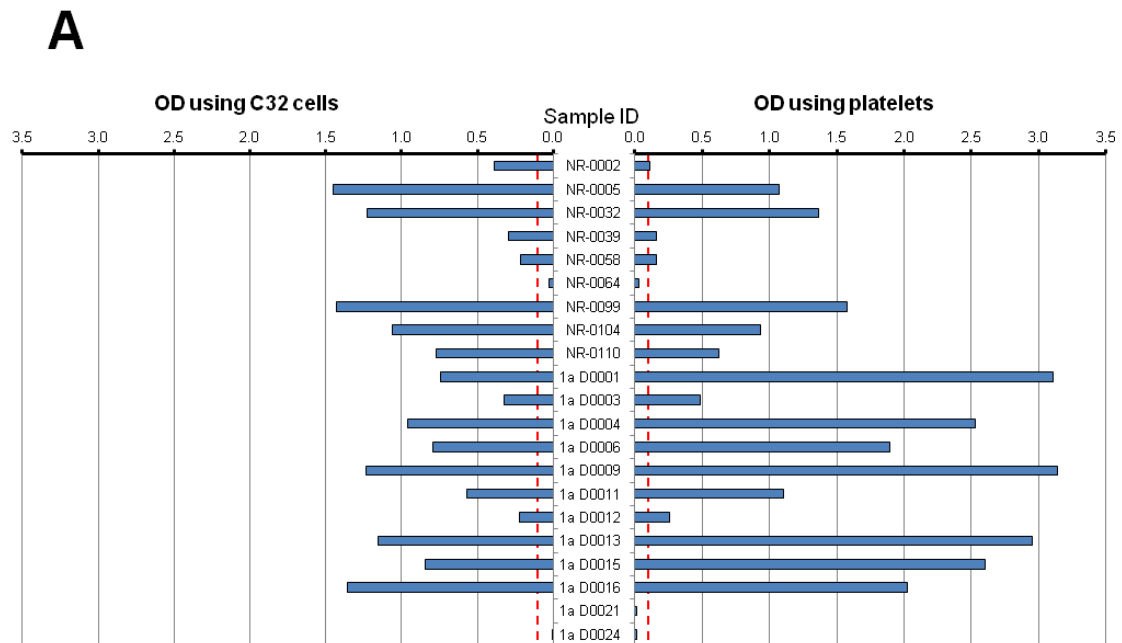
Working dilutions of 1 in 50 for 'VI-PL2' was selected as optimal as it was on the plateau of the curve. Since the protein concentration of LM609 was twice that of VI-PL2 (1 mg/mL versus 0.5 mg/mL respectively), a working dilution of 1 in 100 for LM609 was selected.

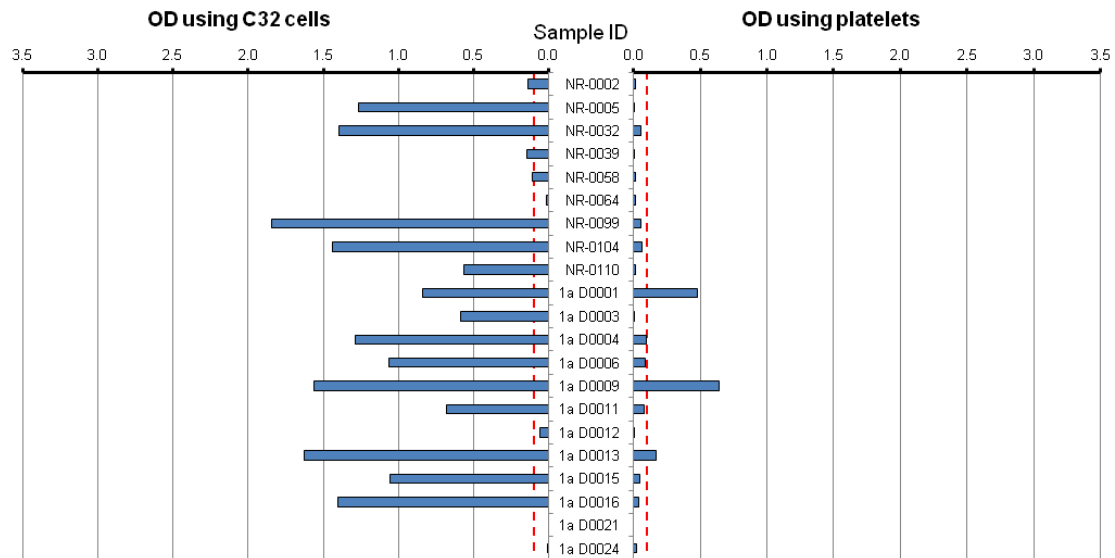
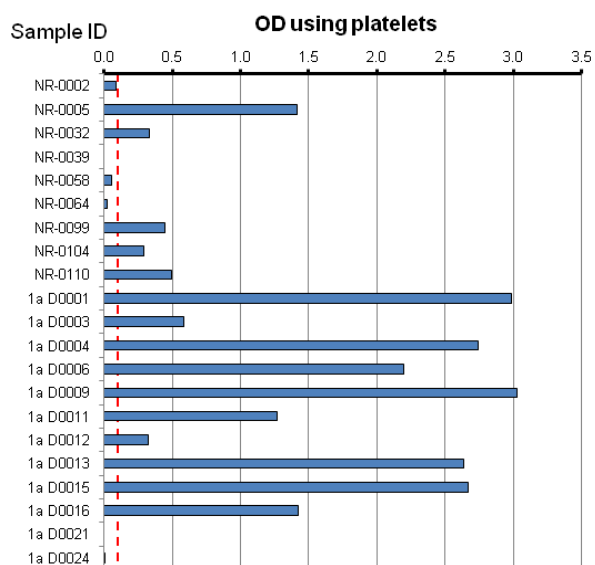
Use of 5×10^5 C32 cells gave higher ODs than when using 1×10^5 cells with both capture mAbs at all dilutions. The size of the cell pellet at 5×10^5 cells was considerable and it was considered that there was a potential for cell loss during the wash stages of the assay resulting in poor reproducibility when using this number of cells. The data was therefore plotted graphically and the number of C32 cells that gave an OD similar to that of platelets when using VI-PL2 at 1 in 50 was selected for further experiments; this was 2.5×10^5 cells per reaction.

5.3.4 Validation of C32 cell line MAIMA for detection of HPA-1a antibodies

To confirm that the C32 MAIMA assay was effective in detecting different examples of anti-HPA-1a, a panel of 21 examples of donor, PTP and NAIT-derived examples of anti-HPA-1a were tested, using capture mAbs VI-PL2 and LM609 against both C32 cells and platelets, and using PAB-6 against platelets as a control (**Figure 5.5**).

FIGURE 5.5. Reactivity of anti-HPA-1a samples against C32 cells and platelets in MAIMA and MAIPA assays. Results using of using three capture mAbs are shown, (A) VI-PL2, (B) LM609 and (C) PAB-6. Results are the averaged ODs from two experiments. The dashed red line indicates the cut-off.



B**C**

Some examples of anti-HPA-1a (NR-0064, D-0021 and D-0024) were unexpectedly unreactive against both C32 cells and platelets using any of the capture mAbs.

Unfortunately the sample archive database did not include detailed results on samples from the same individuals collected at different times; it is possible therefore that these samples were been taken subsequent to when HPA-1a antibodies were first identified in these individuals and the antibodies having 'disappeared' from their

circulation. Alternatively it is possible that reactivity was lost due to repeated freeze-thawing of samples prior to being transferred to the sample archive used in this project.

It was notable that some examples of anti-HPA-1a were more strongly reactive against $\alpha\text{v}\beta 3$ -expressing C32 cells than against platelets when using capture mAb VI-PL2 e.g. NR-0005, NR-0032 and NR-0039. However, other examples were less reactive e.g. 1a D-0001, 1a D-0004 and 1a D-0013 (**Figure 5.5A**).

Only three examples of anti-HPA-1a were detected when capturing $\alpha\text{v}\beta 3$ from platelets using LM609; two of these were PTP-derived examples and one was donor-derived (**Figure 5.5B**).

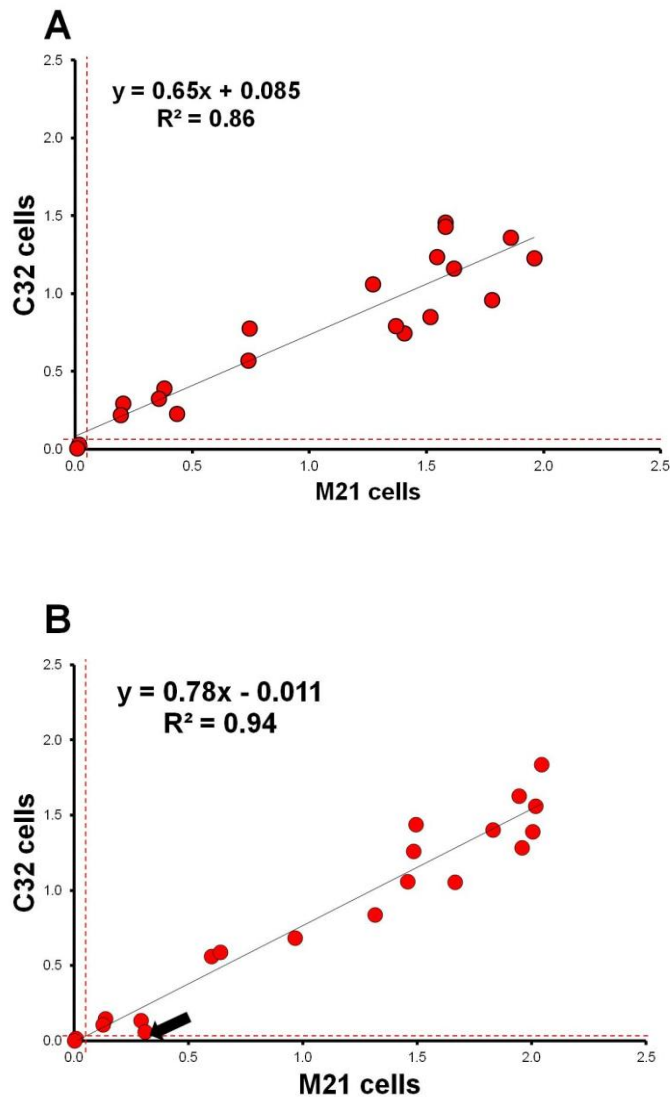
Two examples of anti-HPA-1a (NR-0039 and NR-0058), detectable when using C32 cells (**Figure 5.5A**) and platelets (**Figure 5.5B**) when using clone VI-PL2, were not detectable when using PAB-6 as capture mAb when using platelets (**Figure 5.5C**); both of these examples were weakly reactive when using C32 cells and LM609 as capture mAb.

These data clearly indicated that $\alpha\text{v}\beta 3$ -expressing C32 cells could be used in the MAIMA assay to detect HPA-1a antibodies. However, the data also showed that use of $\alpha\text{v}\beta 3$ versus $\alpha\text{IIb}\beta 3$, and the use of different capture mAbs, resulted in differences in the ability to detect different examples of anti-HPA-1a.

5.3.5 Comparison of $\alpha\text{v}\beta 3$ -expressing C32 cells and $\alpha\text{v}\beta 3$ -expressing M21 cells for detection of HPA-1a antibodies by MAIMA

The panel of examples of anti-HPA-1a used above was then used to determine the suitability of the $\alpha\text{v}\beta 3$ -expressing M21 cell line as target for the detection of anti-HPA-1a (Figure 5.6).

FIGURE 5.6. Comparison of C32 and M21 cell lines as targets in the MAIMA assay for detection of HPA-1a antibodies. (A) Using anti- $\beta 3$ clone VI-PL2 as capture mAb. (B) Using anti- $\alpha\text{v}\beta 3$ clone LM609 as capture mAb. Results are averaged ODs from two experiments.



There was a high degree of correlation between C32 and M21 cell lines when using both capture mAbs suggesting that HPA-1a epitopes on both cell lines were very similar. However, one example of anti-HPA-1a was undetectable when using C32 cells and clone LM609 as capture mAb; this was reactive when using clone VI-PL2 (**Figure 5.6B**, arrowed).

5.3.6 Integrin profile of M21 and M21-LIIB cell lines

The data thus far demonstrated that cell lines could successfully be used in the MAIMA assay but that there appeared to be differences in detection of HPA-1a antibodies when using platelet α IIb β 3 compared to α v β 3 on the C32 cell line.

These differences were explored in more detail using cell lines that had been derived from the same origin but which expressed either α v β 3 (M21 cells) or α IIb β 3 (M21-LIIB cells).

The expression of α IIb β 3 and α v β 3 integrins on these cell lines was determined using a panel of mAbs and flow cytometry (**Table 5.1**).

TABLE 5.1. Integrin profile of M21, M21-LIIB cells and platelets

Antibody	Specificity	Domain / amino acids	Label	MFI		
				M21	M21-LIib	Platelets
Labelled primary antibodies						
Isotype control	na	na	FITC	83 ± 13	100 ± 16	35 ± 10
HIP-8	αIIb	Unknown	PE	67 ± 21	2,623 ± 497	9,815 ± 1,839
SZ21	β3	PSI, 28-35	PE	7,970 ± 340	7,400 ± 1,746	57,785 ± 4,228
Y2/51	β3	PSI + hybrid	FITC	987 ± 81	1,010 ± 224	13,959 ± 794
VI-PL2	β3	Unknown	PE	1,871 ± 343	2,098 ± 465	15,819 ± 997
P2	αIIbβ3 complex	Unknown	PE	76 ± 7	474 ± 94	3,319 ± 240
B2	HPA-1a type II	PSI + hybrid	FITC	296 ± 88	381 ± 76	4,346 ±144
Labelled secondary antibodies						
Anti-mouse IgG	na	na	na	129 ± 31	219 ± 18	57 ± 22
Anti-human IgG	na	na	na	105 ± 3	163 ± 49	94 ± 8
LM609	αvβ3	Unknown	na	1,217 ± 94	194 ± 14	179 ± 53
P5H9	αvβ5	Unknown	na	123 ± 12	208 ± 36	84 ± 48
PAB-1	αIIb, αIIbβ3 or β3	Unknown	na	130 ± 33	1,122 ± 123	20,250 ± 3,726
PAB-6	αIIb, αIIbβ3 or β3	Unknown	na	122 ± 14	1,275 ± 103	21,610 ± 3,201
5B12	αIIb, αIIbβ3 or β3	Unknown	na	105 ± 21	1,123 ± 24	21,836 ± 2,178
P4C10	β1	Unknown	na	146 ± 28	267 ± 15	74 ± 35
23-15	HPA-1a type I	PSI	na	712 ± 29	855 ± 31	3,370 ± 267
19-7	HPA-1a type I	PSI	na	1,046 ± 119	1,092 ± 36	7,484 ± 878

na: not applicable. Data are the means of the MFIs from three experiments $\pm$ SD.

As shown with the C32 cell line, expression of $\beta 3$ was considerably lower on both M21 and M21-LIIB cell lines than on platelets.

In order to determine whether there were differences in accessibility or conformation

of the integrin domains on the cell lines compared to platelets, the MFI of each mAb against the cell lines was calculated as a percentage of the MFI obtained with that particular mAb against platelets (**Table 5.2**).

TABLE 5.2. Epitope expression on cell lines relative to platelets

Antibody	Specificity	Domain / amino acids	Label	MFI as % of platelet MFI		
				M21	M21-LIib	C32
Labelled primary antibodies						
HIP-8	α Iib	Unknown	PE	0.7	26.7	0.5
SZ21	β 3	PSI, 28-35	PE	13.8	12.8	13.5
Y2/51	β 3	PSI + hybrid	FITC	7.1	7.2	5.8
VI-PL2	β 3	Unknown	PE	11.8	13.3	12.5
P2	α Iib β 3 complex	Unknown	PE	2.3	14.3	0.8
B2	HPA-1a type II	PSI + hybrid	FITC	6.8	8.8	5.9
Labelled secondary antibodies						
PAB-1	α Iib, α Iib β 3 or β 3	Unknown	na	0.6	5.5	2.4
PAB-6	α Iib, α Iib β 3 or β 3	Unknown	na	0.6	5.9	2.2
5B12	α Iib, α Iib β 3 or β 3	Unknown	na	0.5	5.1	2.5
23-15	HPA-1a type I	PSI	na	21.1	25.4	43.0
19-7	HPA-1a type I	PSI	na	14.0	14.6	22.8

All cell lines have approximately similar expression levels of β 3 and HPA-1a epitopes to each other. However, some epitopes e.g. those recognised by clones SZ21, 23-15 and 19-7 (PSI domain), appeared to be present at a relatively higher level (or are more accessible on these cell lines than on platelets) compared to epitopes recognised by clones such Y2/51 and B2 which recognise compound epitopes involving the PSI/hybrid/EGF domains of β 3.

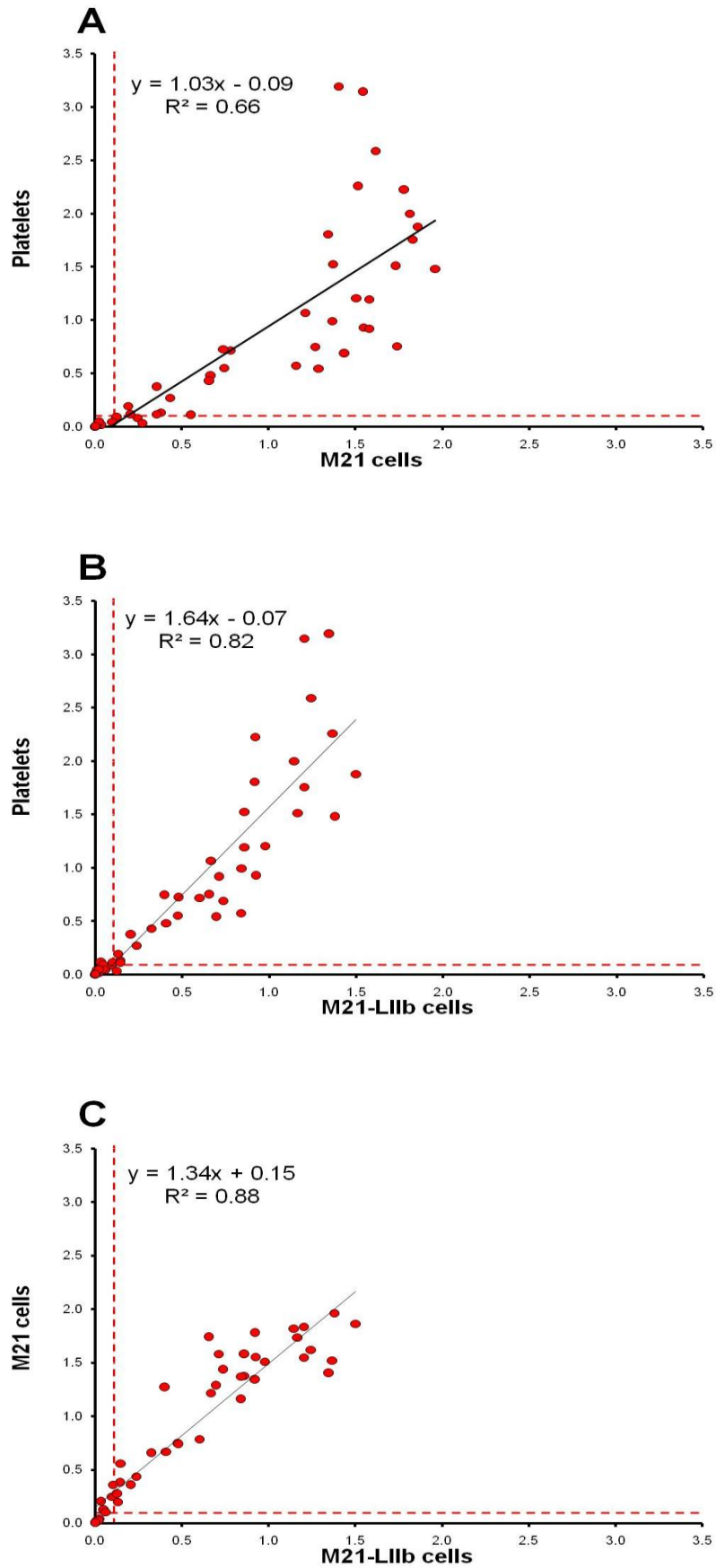
5.3.7 Differences in expression of HPA-1a epitopes induced by different α -chains

The M21 and the M21-LIIb cell lines were used to screen an extensive panel of donor, PTP and NAIT-derived examples of anti-HPA-1a using the MAIMA assay and platelets as a comparator; two different capture mAbs were used with each cell type. All NAIT cases were collected post-delivery.

5.3.7.1 Inter-cell comparison using VI-PL2 as capture mAb

We directly compared the reactivity of the panel of anti-HPA-1a's against $\alpha\text{IIb}\beta 3$ and $\alpha\text{v}\beta 3$ by using the same capture mAb, VI-PL2 (anti- $\beta 3$) (**Figure 5.7**).

FIGURE 5.7. Results of testing a panel of donor- and NAIT-derived examples of anti-HPA-1a in the MAIMA and MAIPA assays using VI-PL2 as capture mAb. (A) *Comparison of reactivity against platelets compared to M21 cells; (B)* *platelets compared to M21-LIIb cells and (C)* *M21 cells compared to M21-LIIb cells. Each circle represents the mean OD of one example of anti-HPA-1a that was tested twice. Both axes show the ODs obtained against the cell type indicated and in each graph have been scaled to the same maximum OD to allow comparison across cell types. The dashed red line indicates the cut-off.*



When using the same capture mAb, the highest ODs were seen with platelet samples. The ODs against cell lines achieved a maximum of only approximately 1.5 OD units against M21-LIIB cells and 2.0 against M21 cells compared to >3.0 when using platelets. These findings agree with those observed when using mAbs and suggest that saturation of integrin molecules occurs when using cell lines.

A number of samples gave ODs below the cut-off with one cell type but above the cut-off with another; these were deemed to be 'false-negative' results with the cell type where the OD was below the cut-off. The number of these false negative results varied depending on the cell type.

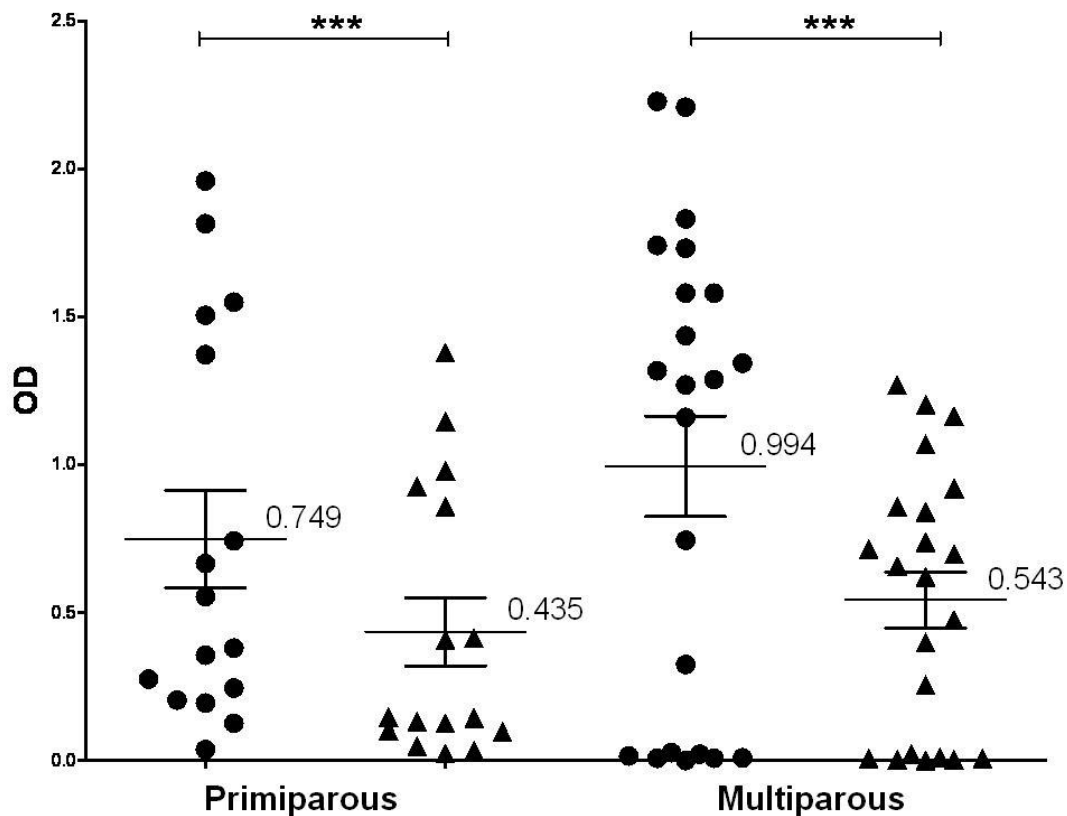
The highest correlation of reactivity was between M21 and M21-LIIB cells ($R^2 = 0.88$) (**Figure 5.7C**); the poorest correlation was between platelets and M21 cells ($R^2 = 0.66$) (**Figure 5.7A**).

Significantly, M21 cells expressing $\alpha\text{v}\beta 3$ showed higher ODs than M21-LIIB cells expressing $\alpha\text{IIb}\beta 3$ ($P = <0.0001$, Wilcoxon matched pairs t-test). On average, ODs using M21 cells were 1.34 times higher than when using M21-LIIB cells. These results were consistent with our hypothesis that $\alpha\text{v}\beta 3$ is the immunising antigen in NAIT and that HPA-1a antibodies preferentially recognise this molecule.

However, the panel of anti-HPA-1a samples also included donor- and PTP-derived examples where the individuals may have been immunised by blood transfusion. A further analysis was therefore performed using data on NAIT-derived examples only.

Since it might be expected that primiparous women (who would not normally have been exposed to platelet $\alpha\text{IIb}\beta 3$ until after delivery) might have HPA-1a antibodies with a more restricted recognition of $\alpha\text{IIb}\beta 3$ than multiparous women (exposed to $\alpha\text{IIb}\beta 3$ as a result of feto-maternal haemorrhage occurring post-delivery), the data was also examined with respect to maternal parity (**Figure 5.8**).

FIGURE 5.8. Reactivity of maternal HPA-1a antibodies in the MAIMA assay against $\alpha\text{v}\beta 3$ (M21 cells) versus $\alpha\text{IIb}\beta 3$ (M21-LIIB cells) and influence of maternal parity. Each of the 38 samples of anti-HPA-1a was tested twice and are shown as the averaged value. Results using M21 cells are shown as (●) and M21-LIIB cells as (▲). The horizontal bars indicate the mean (actual value above bar) and SEM. Significance was calculated using the Wilcoxon matched pairs t-test (* $P = <0.05$; ** $P = <0.01$; *** $P = <0.001$).



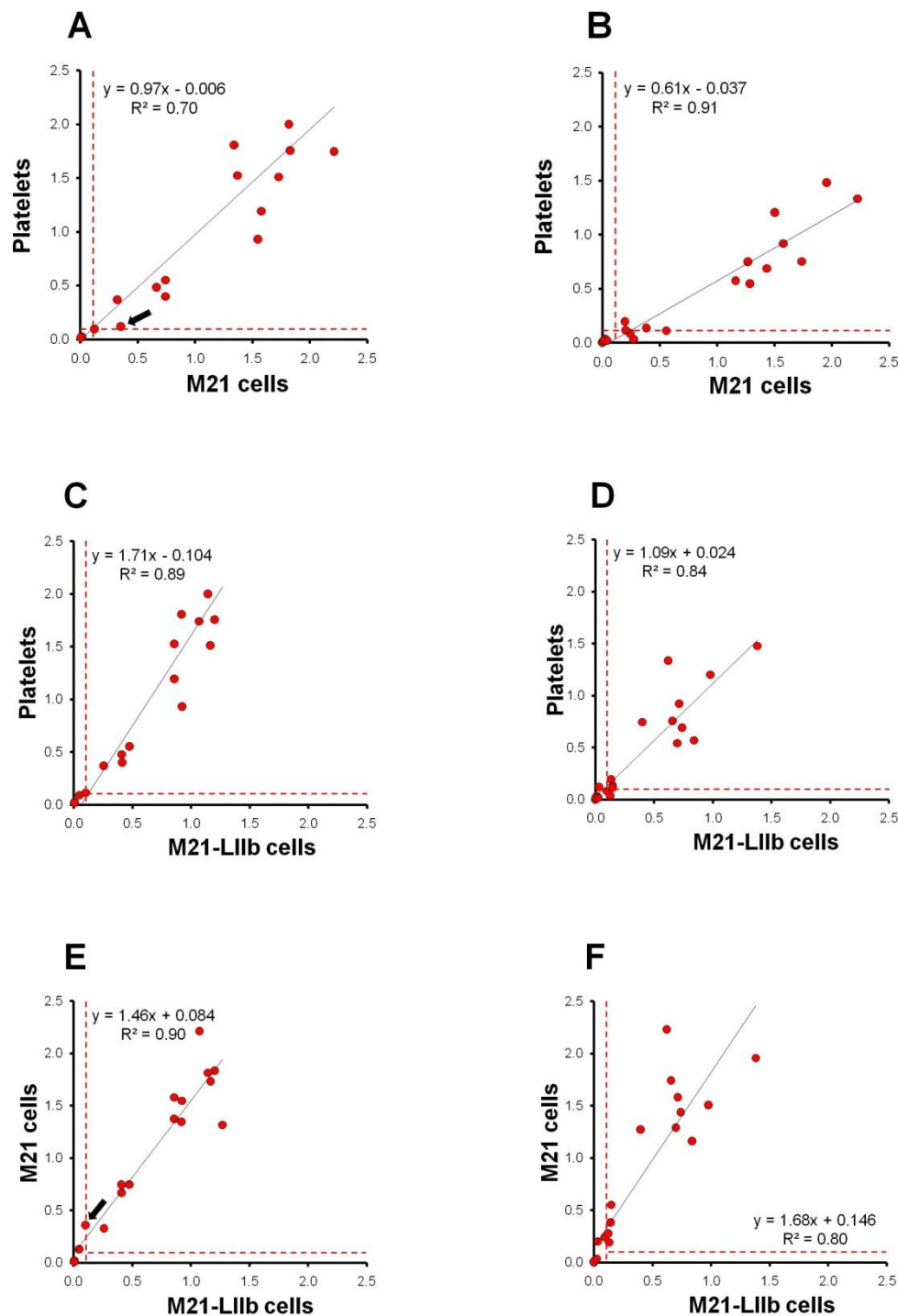
Reactivity of HPA-1a antibodies against $\alpha\text{v}\beta 3$ (M21) was significantly higher than against $\alpha\text{IIb}\beta 3$ (M21-LIIB), both in samples from primiparous and multiparous women. These data therefore also were consistent with our hypothesis that $\alpha\text{v}\beta 3$ is the immunising antigen in NAIT.

However, since it was shown in **Chapter 2** that chelation of cations from $\alpha\text{IIb}\beta 3$ affected reactivity of HPA-1a antibodies and it was known that the panel of NAIT-derived samples consisted of both serum and EDTA samples, the impact of sample type on reactivity against the cell lines was investigated (**Figure 5.9**).

FIGURE 5.9. Impact of sample type on reactivity of NAIT-derived HPA-1a antibodies against platelets, M21 and M21-LIIB cell lines in the MAIPA and MAIMA assays. All samples were from newly-diagnosed cases and had been collected at or around delivery. Results are the mean of two experiments.

Serum samples
($n = 18$)

EDTA plasma samples
($n = 20$)



These data showed a number of effects related to both cell and sample type.

When using serum samples, there was generally a good correlation between results obtained using platelets ($\alpha\text{IIb}\beta 3$) and M21 cells ($\alpha\text{v}\beta 3$). However, one example of anti-HPA-1a (NR-0056) reacted clearly against M21 cells but only weakly against platelets (**Figure 5.9A**, arrowed). This sample also reacted weakly against M21-LIIB cells (**Figure 5.9C**) suggesting that it preferentially recognised an epitope on $\alpha\text{v}\beta 3$.

The maximum difference in reactivity of serum samples was between platelets and M21-LIIB cells where platelet ODs were 1.71 times those of M21-LIIB cells (**Figure 5.9C**).

There was a strong correlation ($R^2 = 0.90$) between anti-HPA-1a reactivity against M21 and M21-LIIB cells (**Figure 5.9E**) but the M21 cells had superior sensitivity (ODs 1.46 times those of M21-LIIB cells).

When using EDTA plasma samples there was excellent correlation between platelet and M21-LIIB cell reactivity (**Figure 5.9D**). However large differences in the relationships between anti-HPA-1a reactivity against platelets and M21 cells (**Figure 5.9B**), and between M21 cells and M21-LIIB cells (**Figure 5.9F**) were observed. In the former, reactivity against platelets was only 0.61 times that of reactivity against M21 cells. In the latter, reactivity against M21 cells was 1.68 times that of M21-LIIB cells. In **Figures 5.9B and 5.9F** it can be seen that there are five samples that give considerably weaker reactions against platelets and M21-LIIB cells respectively than against M21 cells. This showed that $\alpha\text{IIb}\beta 3$ on cell lines was susceptible, like $\alpha\text{IIb}\beta 3$ on platelets, to cation chelation resulting in loss of assay sensitivity.

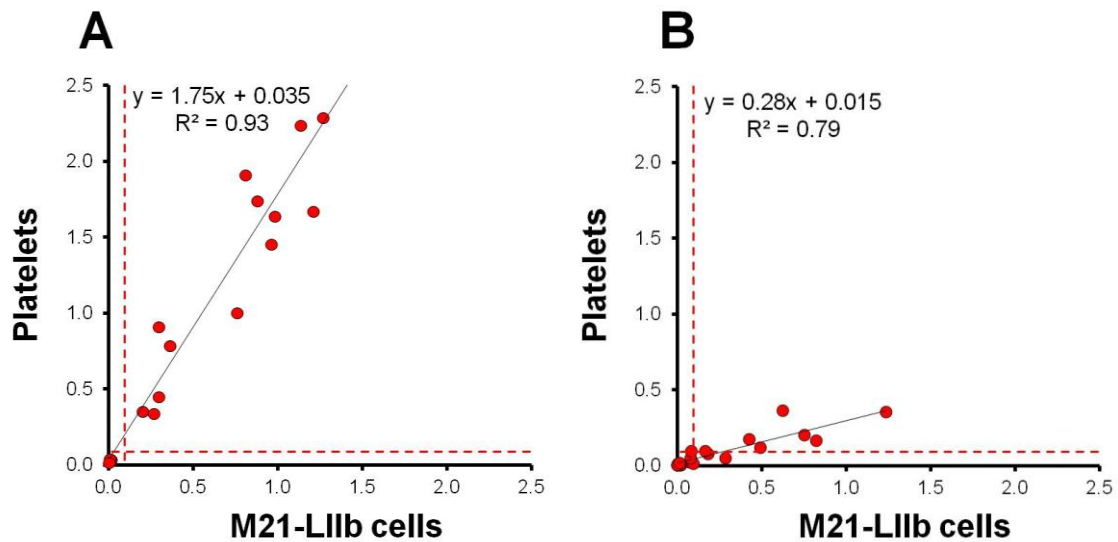
5.3.7.2 Comparison of reactivity of M21-LIIB cells and platelet reactivity using an alternative capture mAb, (PAB-6)

To confirm that the increased reactivity against platelets compared to M21-LIIB cells was not an artefact induced by the use of clone VI-PL2, a different capture mAb (PAB-6) was used to test the NAIT-derived examples of anti-HPA-1a used above (Figure 5.10).

FIGURE 5.10. Impact of sample type on reactivity of NAIT-derived HPA-1a antibodies against platelets and M21-LIIB cell lines when using clone PAB-6 as capture mAb in the MAIPA and MAIMA assays. All samples were from newly-diagnosed cases and had been collected at or around delivery. Each circle represents the mean OD of one example of anti-HPA-1a that was tested twice.

Serum samples
($n = 18$)

EDTA plasma samples
($n = 20$)



The difference between serum and EDTA plasma reactivity was extremely marked when using clone PAB-6.

When using serum samples, the ODs when using platelets were approximately 1.75 times those of M21-LIIb cells (**Figure 5.10A**). However, when using EDTA plasma samples the ODs when using platelets were only 0.28 times those of M21-LIIb cells (**Figure 5.10B**); these compare to figures of 1.71 times and 1.09 times respectively when using clone VI-PL2 (**Figures 5.9C and 5.9D**)

These data confirmed the sensitivity of platelet $\alpha\text{IIb}\beta 3$ to cation chelation and the variability of the effect that this has on different capture mAbs.

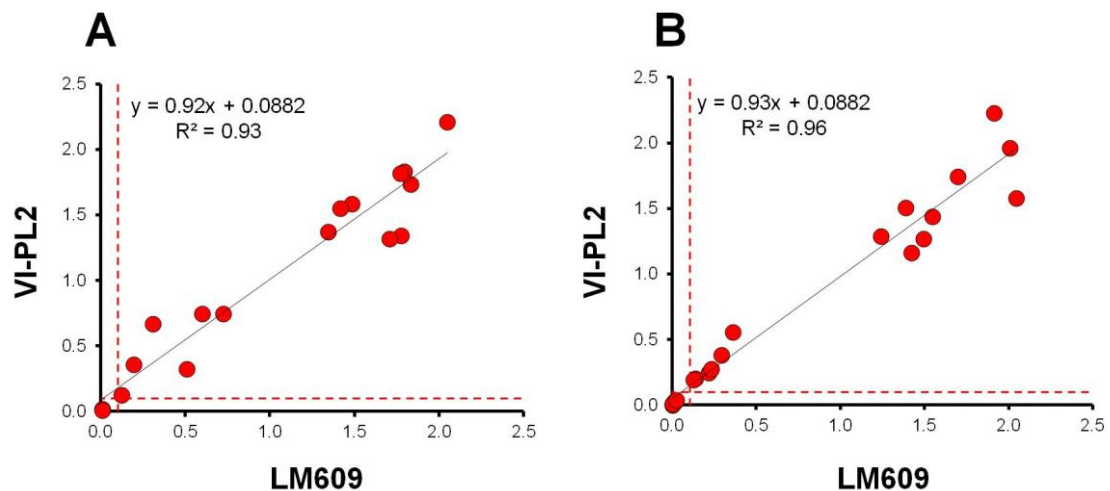
5.3.7.3 Comparison of reactivity of anti-HPA-1a antibodies against M21 cells when using different capture mAbs

Finally, the resistance of $\alpha\text{v}\beta 3$ to cation chelation and the impact of using different capture mAbs was investigated using the same panel of NAIT-derived examples of anti-HPA-1a (Figure 5.11).

FIGURE 5.11. Effect of cation chelation and capture mAb on detection of HPA-1a antibodies using integrin $\alpha\text{v}\beta 3$ on M21 cells using the MAIMA assay. All samples were from newly-diagnosed cases and had been collected at or around delivery. Each circle represents the mean OD of one example of anti-HPA-1a that was tested twice.

Serum samples
($n = 18$)

EDTA plasma samples
($n = 20$)

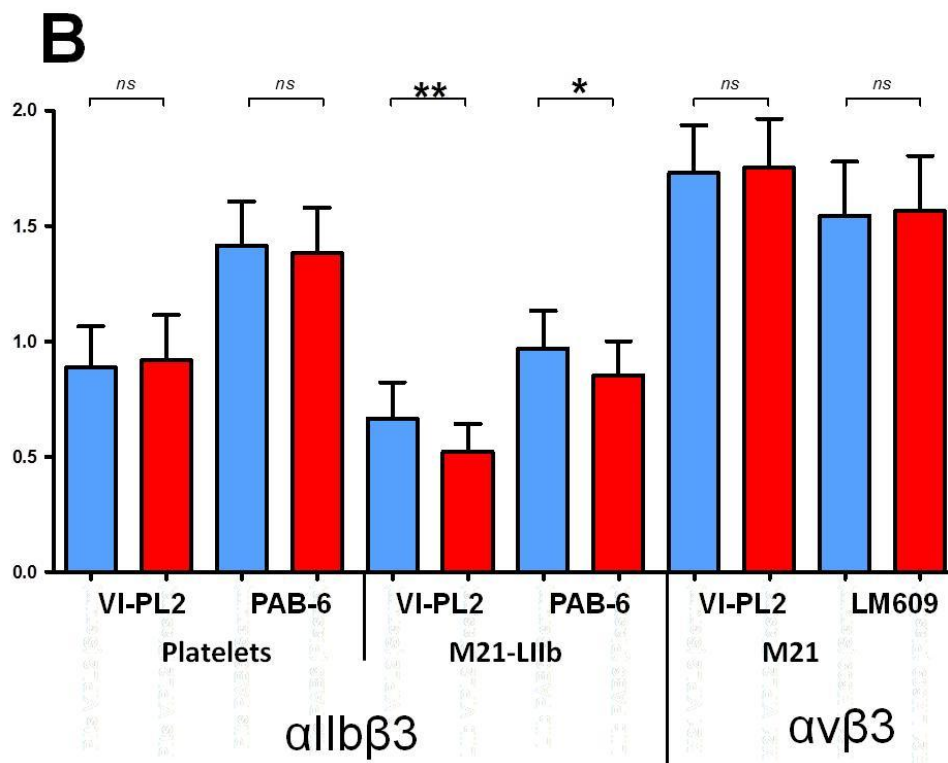
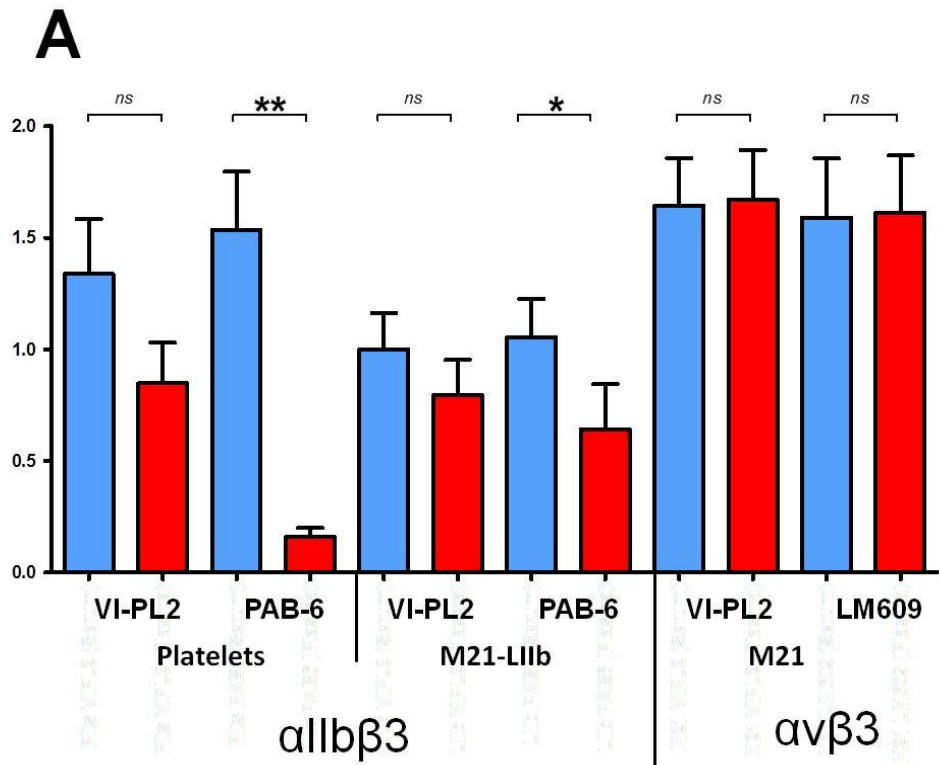


These data clearly show that the sample type or capture mAb have no effect on detection of HPA-1a antibodies when using $\alpha\text{v}\beta 3$ -expressing M21 cells.

5.3.8 Confirmation that the test sample affects assay sensitivity

In order to confirm that it was the EDTA plasma used in the primary incubation that was affecting $\alpha\text{IIb}\beta 3$, a modified version of the MAIPA assay was used. In this assay ('reverse MAIPA') the order of test sample and mAb incubations were reversed; this was used in parallel with the standard version. Serum and EDTA plasma samples containing anti-HPA-1a collected from the same venepuncture were used (**Figure 5.12**).

FIGURE 5.12. Effect of sample type and integrin α -chain on sensitivity of MAIPA/MAIMA assays. *Nine pairs of serum (blue) and EDTA plasma (red) samples (obtained at the same venepuncture) were tested by the standard (A) and 'reverse' MAIPA/MAIMA assays (B) assay in which the order of incubation of test sample and capture mAb with target cells was reversed. Target cell and capture mAb are shown on the x axis and OD on the y axis. Error bars are the standard error of the mean. P values were calculated using the Wilcoxon matched pairs t-test (*P = <0.05; **P = <0.01; ***P = < 0.001).*



These data clearly show a reduction in ODs when using EDTA plasma samples and $\alpha\text{IIb}\beta 3$ as target in the standard MAIPA assay; this difference was statistically significant when using PAB-6 as capture mAb and either platelets ($P = <0.01$) or M21-LIIb cells ($P = <0.05$) as target.

Using the 'reverse MAIPA' (**Figure 5.16B**), no significant difference in ODs between serum and EDTA plasma samples was observed against platelets with either capture mAb.

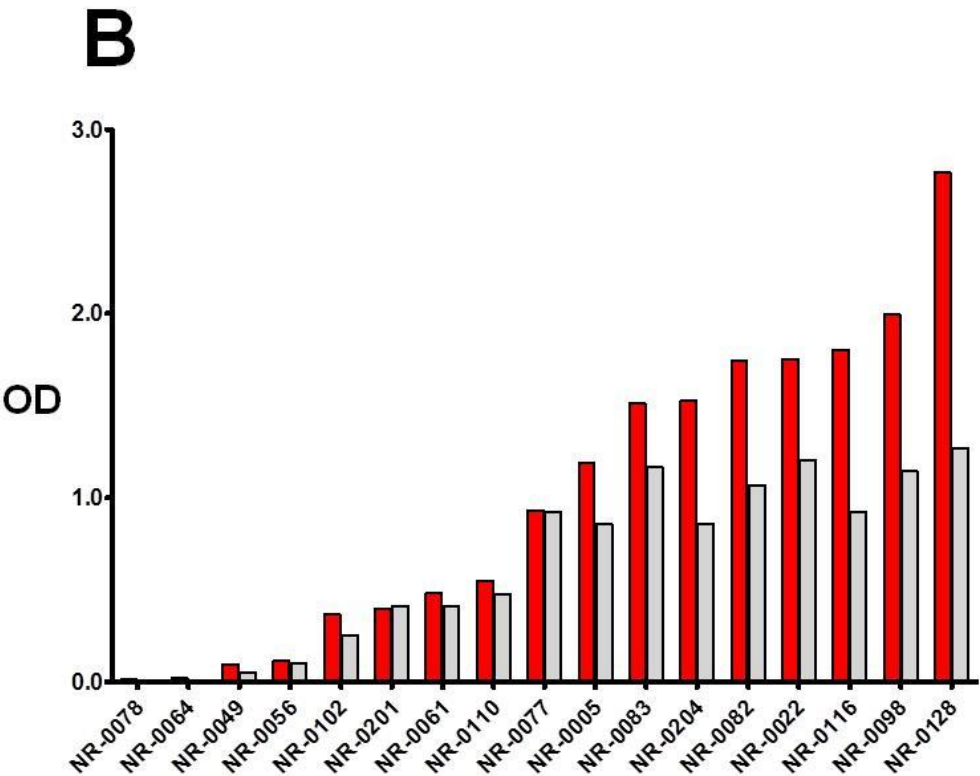
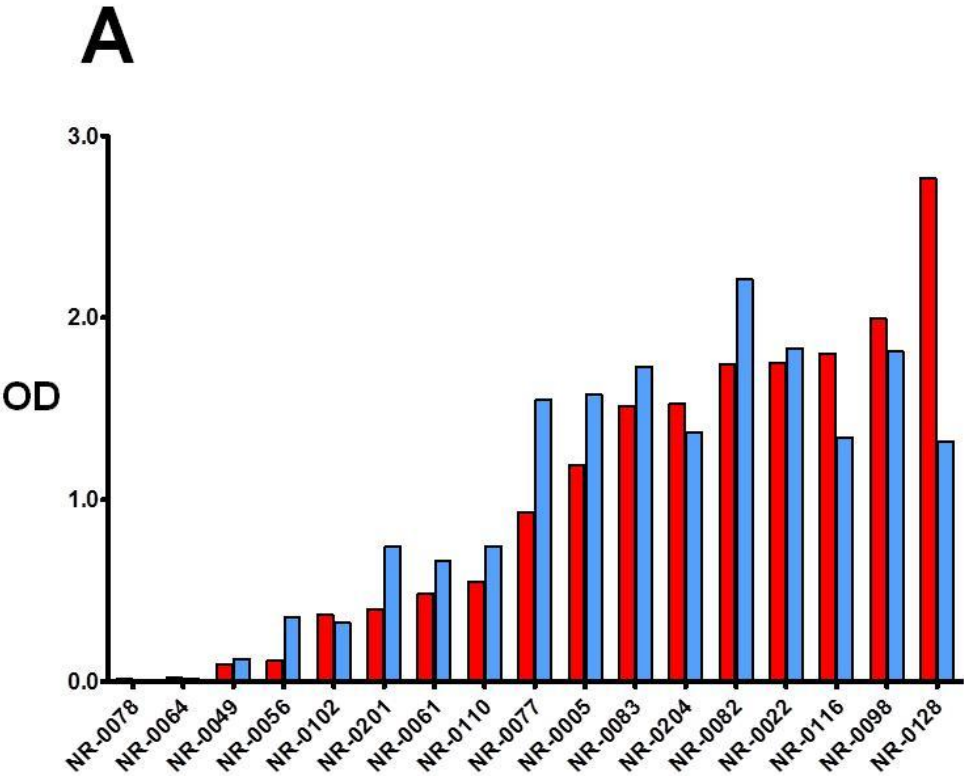
Interestingly, there was still a significant difference between serum and EDTA plasma sample ODs when using M21-LIIb cells in the 'reverse MAIPA' but not when using platelets or M21 cells.

As expected, no significant differences in ODs were observed between serum and EDTA plasma samples when using $\alpha\text{v}\beta 3$ as target using either clone VI-PL2 or LM609.

5.3.9 Comparison of sensitivity of $\alpha\text{IIb}\beta 3$ and $\alpha\text{v}\beta 3$ as targets for detection of HPA-1a antibodies

Serum samples from a number of HPA-1a immunised mothers gave higher ODs against M21 ($\alpha\text{v}\beta 3$) cells than against platelets suggesting that $\alpha\text{v}\beta 3$ may be a more sensitive target for the detection of HPA-1a alloantibodies than $\alpha\text{IIb}\beta 3$. We explored this hypothesis by directly comparing histograms of M21 cell versus platelet data, and M21-LIIb versus platelet data when using clone VI-PL2 (as this was the only clone reactive with all cell types) (**Figure 5.13**).

FIGURE 5.13. Effect of integrin alpha-chain on the sensitivity of the MAIPA/MAIMA assays for the detection of HPA-1a antibodies. Seventeen serum samples originally found to contain anti-HPA-1a by the diagnostic laboratory were tested in the MAIPA and MAIMA assays using clone VI-PL2 as capture mAb. **(A)** Comparison of M21 cells ($\alpha\text{v}\beta 3$) (blue bars) and platelets ($\alpha\text{IIb}\beta 3$) (red bars) and **(B)** comparison of M21-LIIb cells ($\alpha\text{IIb}\beta 3$) (grey bars) and platelets. The x axis indicates the sample identification number and the y axis the OD; results are organised by increasing OD against platelets from left to right.



When the OD using platelets was less than approximately 1.8 OD units the majority of samples gave higher ODs against M21 cells than against platelets. However, when the ODs in the platelet MAIPA assay were >1.8 OD units the reactivity against M21 cells was lower than that of platelets. This suggested that either cell lines generally or the M21 cell line ($\alpha\text{v}\beta 3$) in particular were more sensitive than platelets for the detection of low levels of anti-HPA-1a but at high levels of HPA-1a antibody, saturation of integrin molecules on M21 cells but not on platelets occurred.

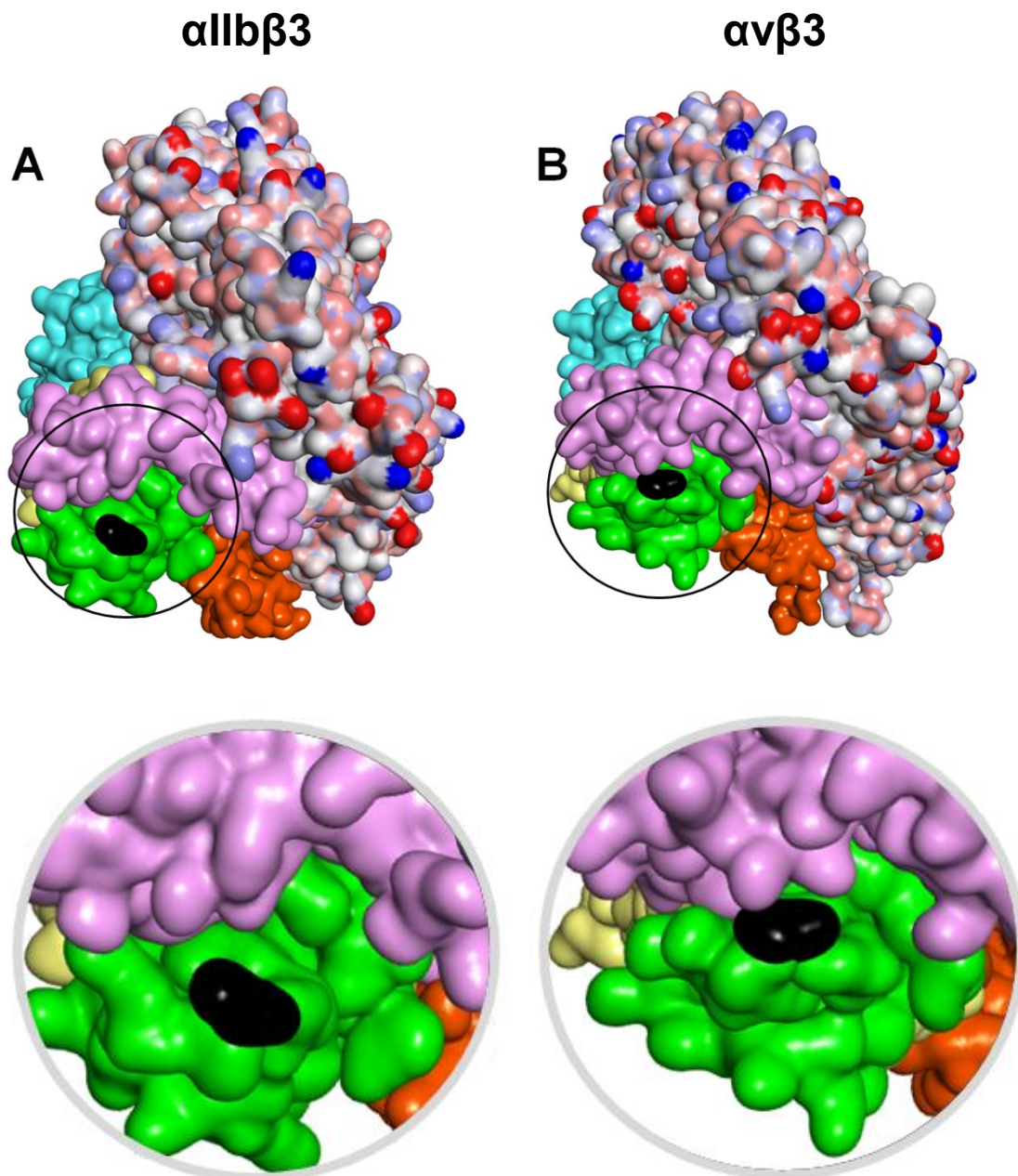
Analysis of the results comparing $\alpha\text{IIb}\beta 3$ on platelets against $\alpha\text{IIb}\beta 3$ on M21-LIIb cells showed that the reactivity against platelets was higher or the same as M21-LIIb cells with all examples of anti-HPA-1a tested (**Figure 5.13B**).

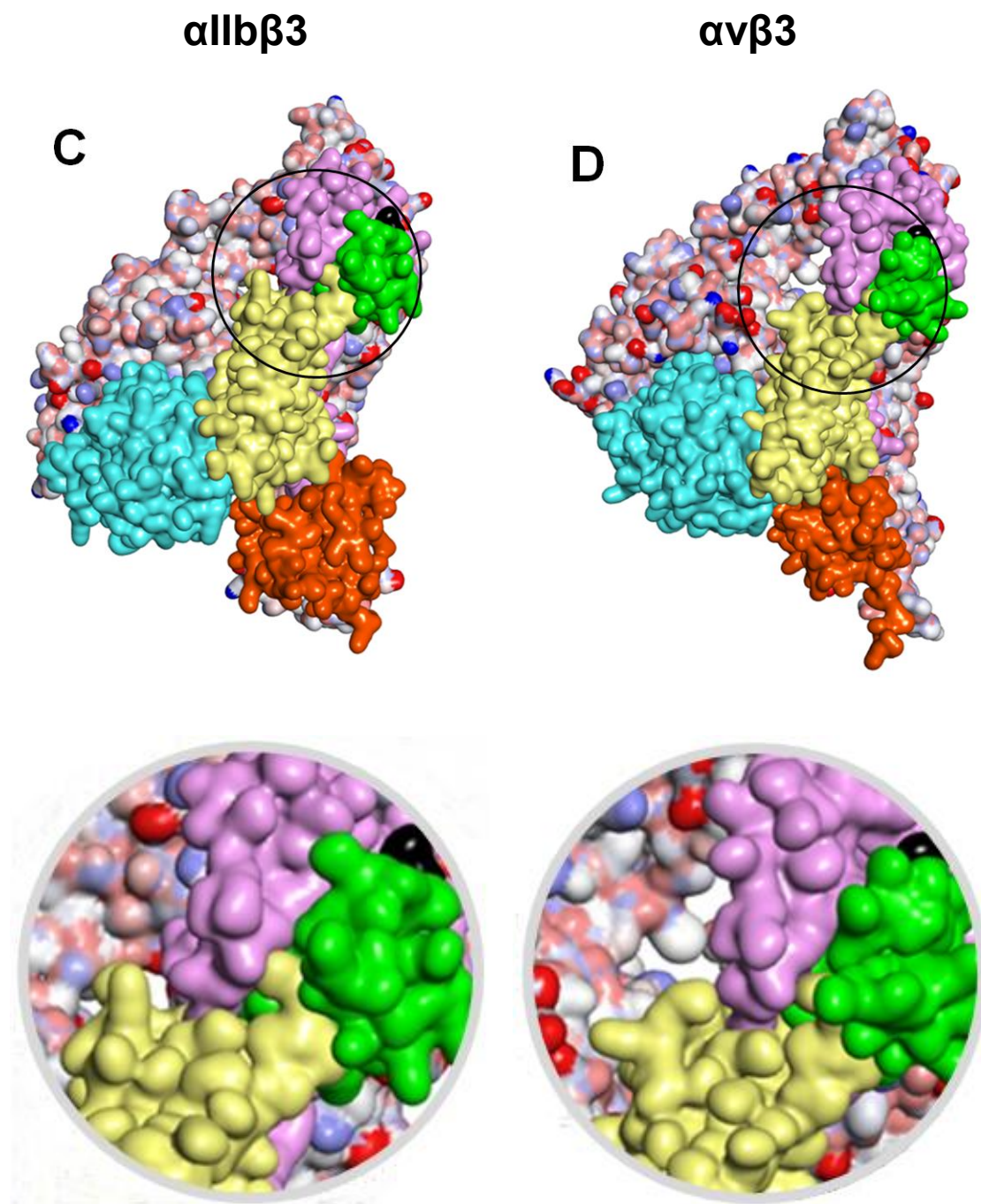
These data therefore suggested that the higher sensitivity of the M21 cell MAIPA at low antibody levels was related to the integrin type and not simply the use of a cell line compared to platelets.

5.3.10 Molecular modelling of $\alpha\text{IIb}\beta 3$ and $\alpha\text{v}\beta 3$

The above data suggested that differences might exist in the conformation of $\beta 3$ when associated with αv compared to when associated with αIIb , and/or that accessibility of particular mAb epitopes varied between the two integrins. Molecular modelling (**Chapter 2**) of the two integrins was therefore performed using Discovery Studio 3.0 Visualizer (Accelrys Software, USA) (**Figure 5.14**).

FIGURE 5.14. Molecular modelling of integrins $\alpha\text{IIb}\beta 3$ and $\alpha\text{v}\beta 3$. The α -chains of $\alpha\text{IIb}\beta 3$ (**A and C**) and $\alpha\text{v}\beta 3$ (**B and D**) are shown with surfaces rendered according to amino acid charge, and the β -chains are coloured according to domain. The PSI domain is coloured green with the location of the HPA-1 polymorphism in black. Other $\beta 3$ -domains are: hybrid (yellow), EGF (purple), β -TD (red) and β -A (blue). In **Figures A and B** the complexes are orientated to highlight differences in the PSI domain; in **Figures C and D** the molecules have been rotated to show the hybrid domain. The figures in circles in the lower panels show a magnified view of the area circled in the main figures.





These models clearly show that the different α -chains impose structural differences in the β -chain.

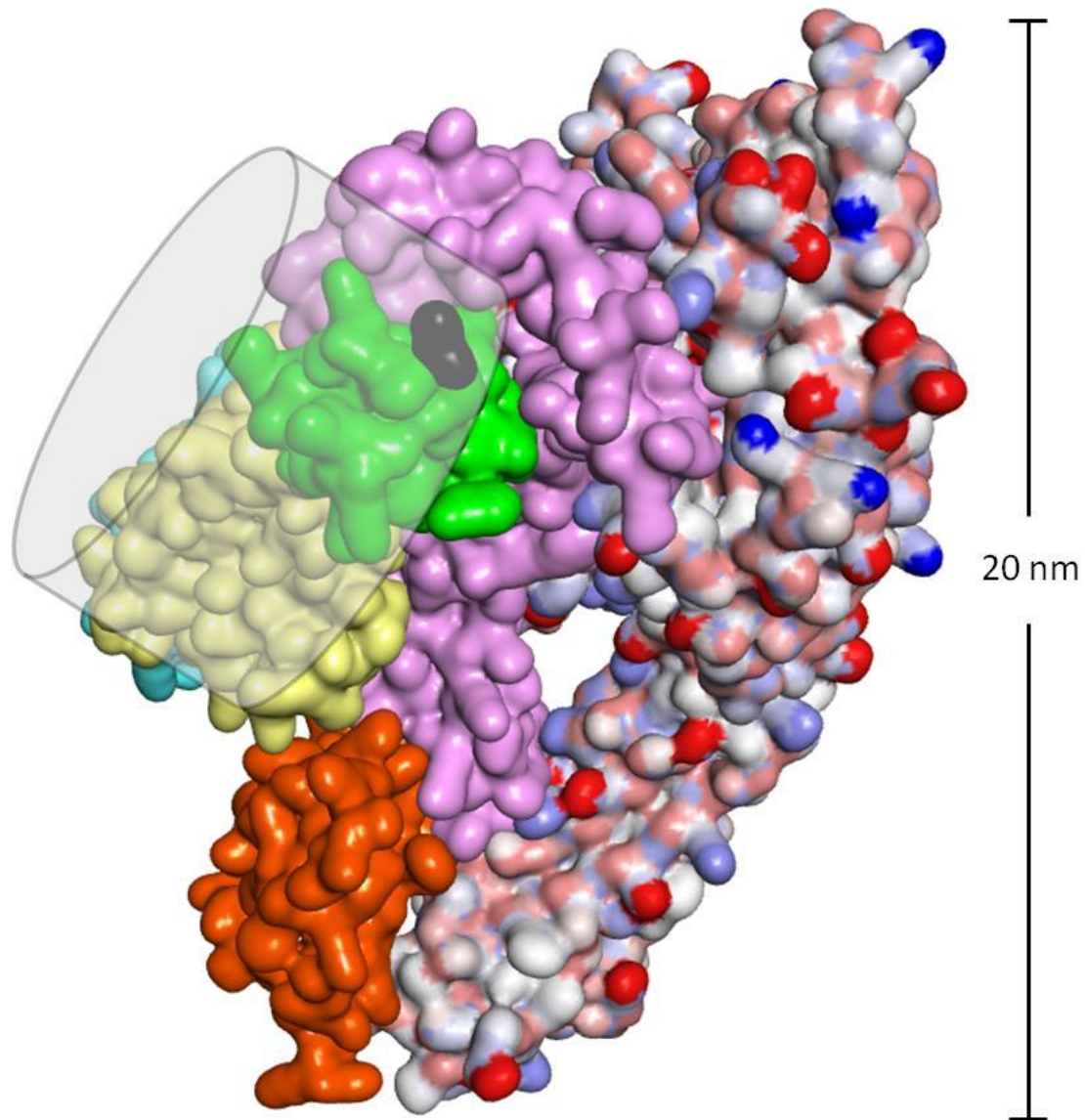
In **Figures 5.14A** and **5.14B** it can be seen that there are differences in the PSI (green) and EGF domains (purple) that could potentially affect the binding of HPA-1a

'Type I' antibodies that recognise epitopes only on the PSI domain. Charge differences in the α -chain would also potentially affect binding of HPA-1a antibodies depending on precise epitope specificity and angle of binding.

Figures 5.14C and 5.14D demonstrate further changes in orientation of amino acids in the PSI and EGF domains between the two integrins and also show major alterations in the hybrid domain (yellow). Since HPA-1a 'Type II' antibodies recognise epitopes dependent on PSI and hybrid/EGF domains, these additional changes in the conformation in the hybrid and EGF domains might also lead to differences in the reactivity of such antibodies against $\alpha\text{v}\beta 3$ compared to $\alpha\text{IIb}\beta 3$.

We investigated the relationship between the size of a IgG Fab fragment and the HPA-1a epitope and $\beta 3$ domains (**Figure 5.15**).

FIGURE 5.15. Size of $\beta 3$ domains of $\alpha\text{IIb}\beta 3$ in relation to IgG Fab fragment. *The domains are coloured as described above. The grey cylinder represents a Fab fragment of IgG. The relative size of the Fab compared to the domains is based on the schematic in Mould et al (Mould et al, 2005).*

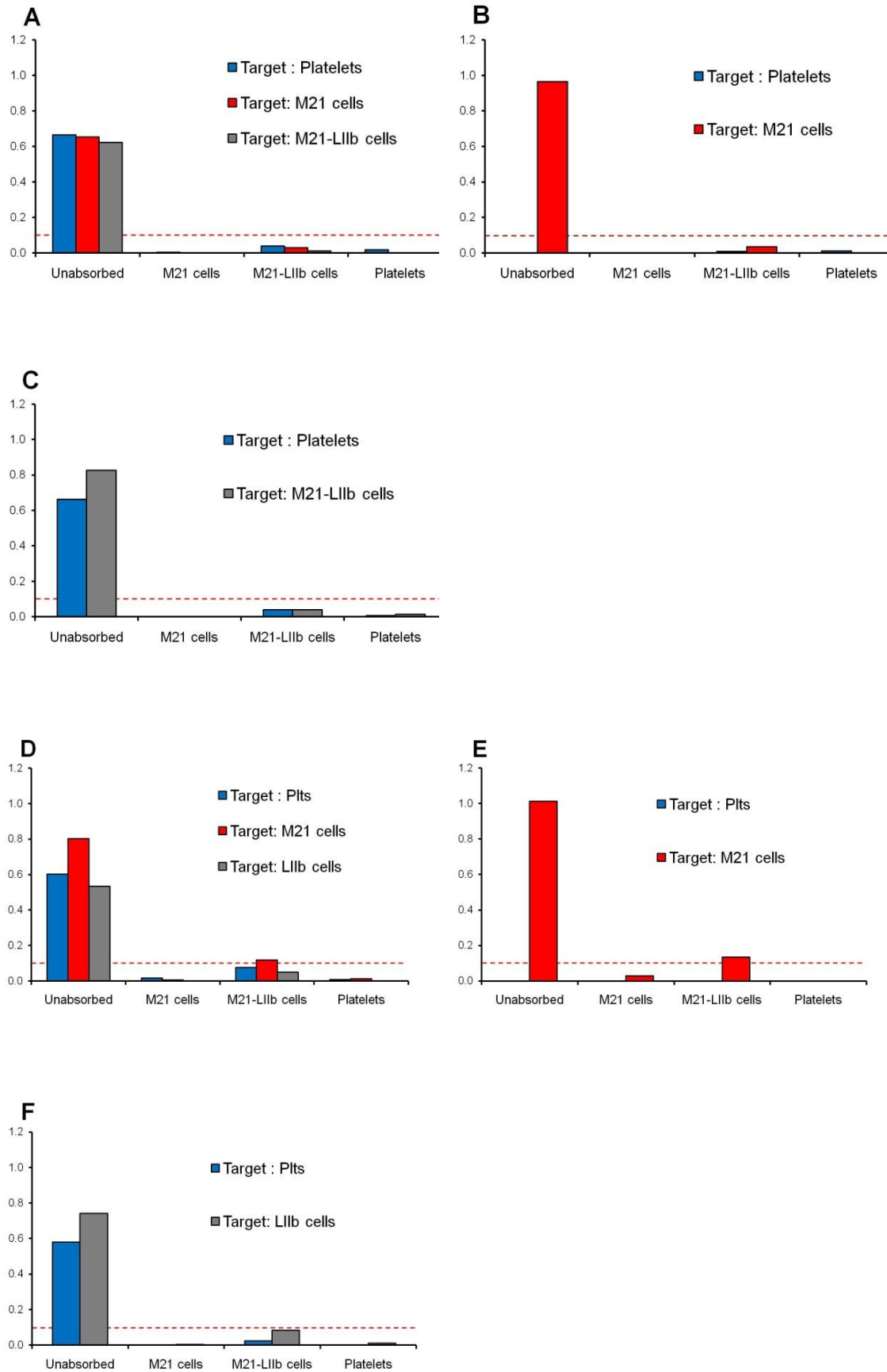


The Fab fragment is sufficiently large to span the HPA-1a epitope, the PSI domain and parts of other domains (e.g. EGF); changes in the orientation of $\beta 3$ resulting from association with different α -chains potentially would therefore severely impact on antibody binding

5.3.11 Absorption/elution experiments to demonstrate differences in HPA-1a epitopes between $\alpha\text{IIb}\beta 3$ and $\alpha\text{v}\beta 3$

Since $\alpha\text{v}\beta 3$ seemed to be more sensitive than $\alpha\text{IIb}\beta 3$ for the detection of some examples of anti-HPA-1a and structural differences had been demonstrated by molecular modelling, absorption/elution experiments were performed using cell lines and platelets (**Figure 5.16**).

FIGURE 5.16. Absorption/elution experiments of anti-HPA-1a against platelets, M21 and M21-LIIb cells. *One example of anti-HPA-1a (sample D-0008) that was moderately strong (ODs approximately 0.6 - 0.7) and was available in sufficiently large quantity was absorbed against platelets, M21 and M21 LIIb cells and the absorbed sample tested against the three cell types using capture mAbs (A) VI-PL2, (B) LM609 and (C) PAB-6 in the MAIPA/MAIMA assays. Eluates prepared from these cells using the heat elution method were also tested in the MAIPA assay using capture mAb clones VI-PL2 (D), LM609 (E) and PAB-6 (F). The original, unabsorbed sample is included as a comparison. The x axis indicates the cell type used to prepare the eluate and the y axis the OD.*



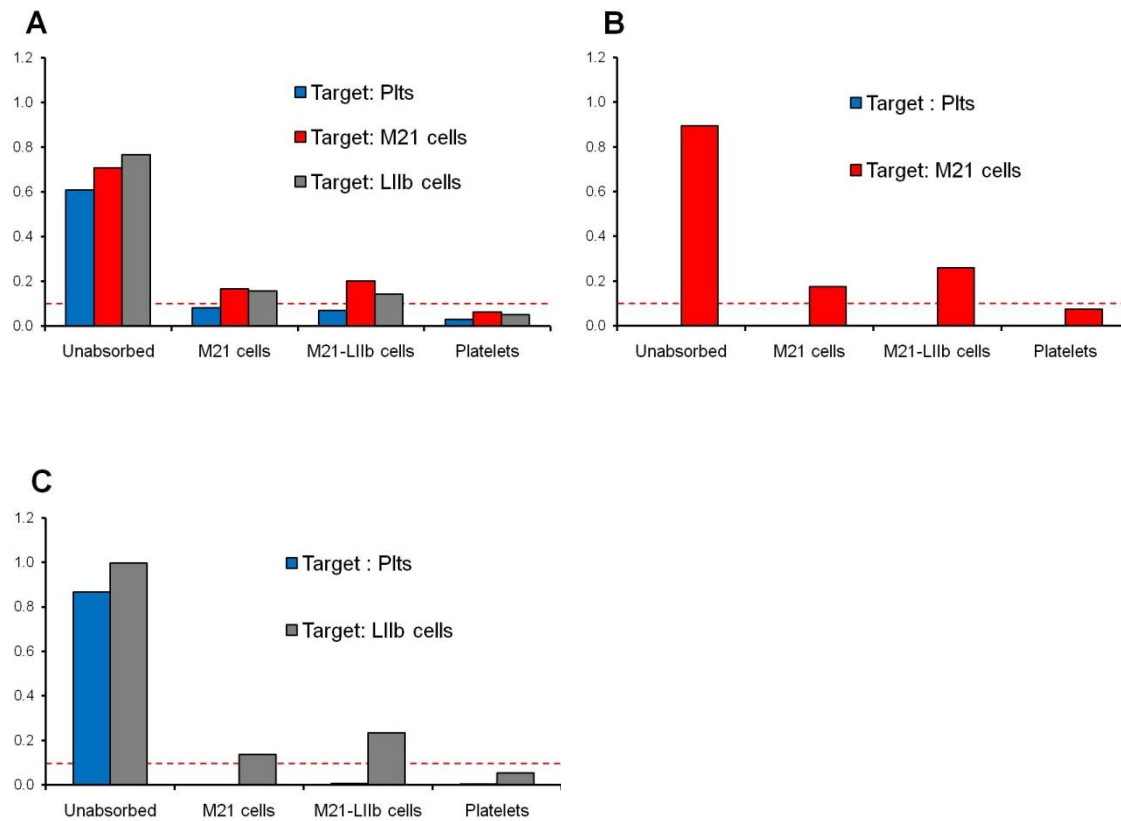
Absorption of the sample using any of the cell types resulted in complete absorption of anti-HPA-1a and testing of the absorbed sample showed no ODs above cut-off against any of the cell types with any of the capture mAbs used (**Figures 5.16 A-C**).

The eluates prepared from M21 cells and platelets did not contain detectable HPA-1a antibody activity. An eluate prepared from M21-LIIb cells showed reactivity above cut-off only against M21 cells (using VI-PL2 or LM609 as capture mAbs) (**Figures 5.16 D and E**).

Very weak, but below cut-off, reactivity was observed in the eluate from the M21-LIIb cells when using VI-PL2 as capture mAb and either platelets or M21-LIIb cells as target, and when using clone PAB-6 and M21-LIIb cells as target (**Figures 5.16D, F**).

Since the activity in the eluates was weaker than expected, a further experiment using acid elution was performed (Kiefel *et al*, 2005) (**Figure 5.17**).

FIGURE 5.17. Acid elution of HPA-1a antibodies. *Eluates were prepared using acid elution and tested using clones VI-PL2 (A), LM609 (B) and PAB-6 (C) in the MAIPA/MAIMA assays. The unabsorbed sample is included as a comparison. The x axis indicates the cell type used to prepare the eluate and the y axis the OD.*



Higher ODs were obtained using this method, with eluates prepared from both M21 and M21-LIIB cells reacting with M21 and M21-LIIB cells. No anti-HPA-1a activity could be detected in eluates prepared from platelets.

These data suggested that HPA-1a epitopes on M21 and M21-LIIB cells are similarly recognised by this particular example of anti-HPA-1a and these experiments did not therefore confirm the increased sensitivity of M21 cells for detection of HPA-1a antibodies or the existence of sub-populations of HPA-1a antibodies able to recognise $\alpha\text{IIb}\beta 3$ or $\alpha\text{v}\beta 3$ preferentially. Clearly, further experiments need to be performed using examples of NAIT-derived anti-HPA-1a that showed either enhanced reactivity with $\alpha\text{v}\beta 3$ or no difference in reactivity. However, these were not possible due to limited availability of sample material.

5.3.12 Effect of sample type and target integrin on measured levels of HPA-1a antibody

Having shown differences in reactivity of HPA-1a antibodies between M21 and M21-LIIb cells and platelets in a screening assay i.e. measurement of ODs (**Figure 5.9**), the effect of sample type and target cell in a quantitative assay was investigated using NAIT-derived examples of anti-HPA-1a.

Since the binding of clone PAB-6 was shown to be grossly affected by the presence of EDTA, (**Figure 5.10B**) clone VI-PL2 was used as capture mAb throughout these experiments.

Using paired serum and EDTA plasma samples collected at the same venepuncture the titration curves with some samples appeared almost identical between serum and plasma samples with all three cell types whereas with others the slope and shape of the curve varied widely. An example of the former is shown in **Figure 5.18**, and an example of the latter is shown in **Figure 5.19**.

FIGURE 5.18. MAIPA/MAIMA titration curves of an example of anti-HPA-1a where the titration curve appears to be minimally affected by sample type. Serum and EDTA plasma samples from the same case (F0892) and the NIBSC high-potency anti-HPA-1a standard '03/152' were titrated in doubling dilutions from neat to 1 in 1024 (i.e. from 100 i.u./mL to 0.1 i.u./mL in the NIBSC standard) and tested in the MAIPA/MAIMA assays. The x axis shows the base10 log of the dilution and the y axis shows the base10 log of the mean ODs obtained from duplicate wells. The high potency standard is shown in blue and the test sample in red. The calculated activity in the test sample is shown in **bold italic font**.

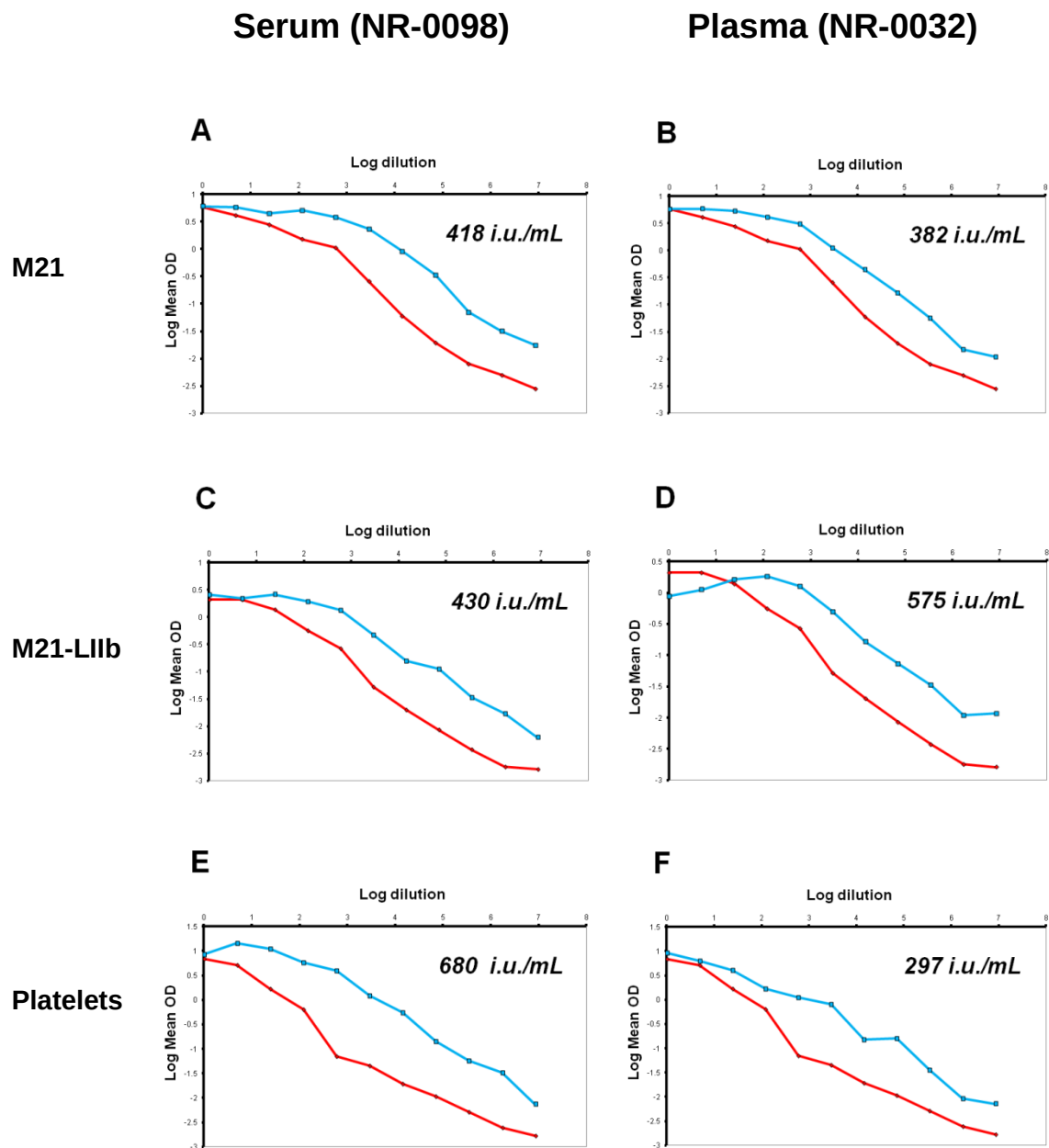
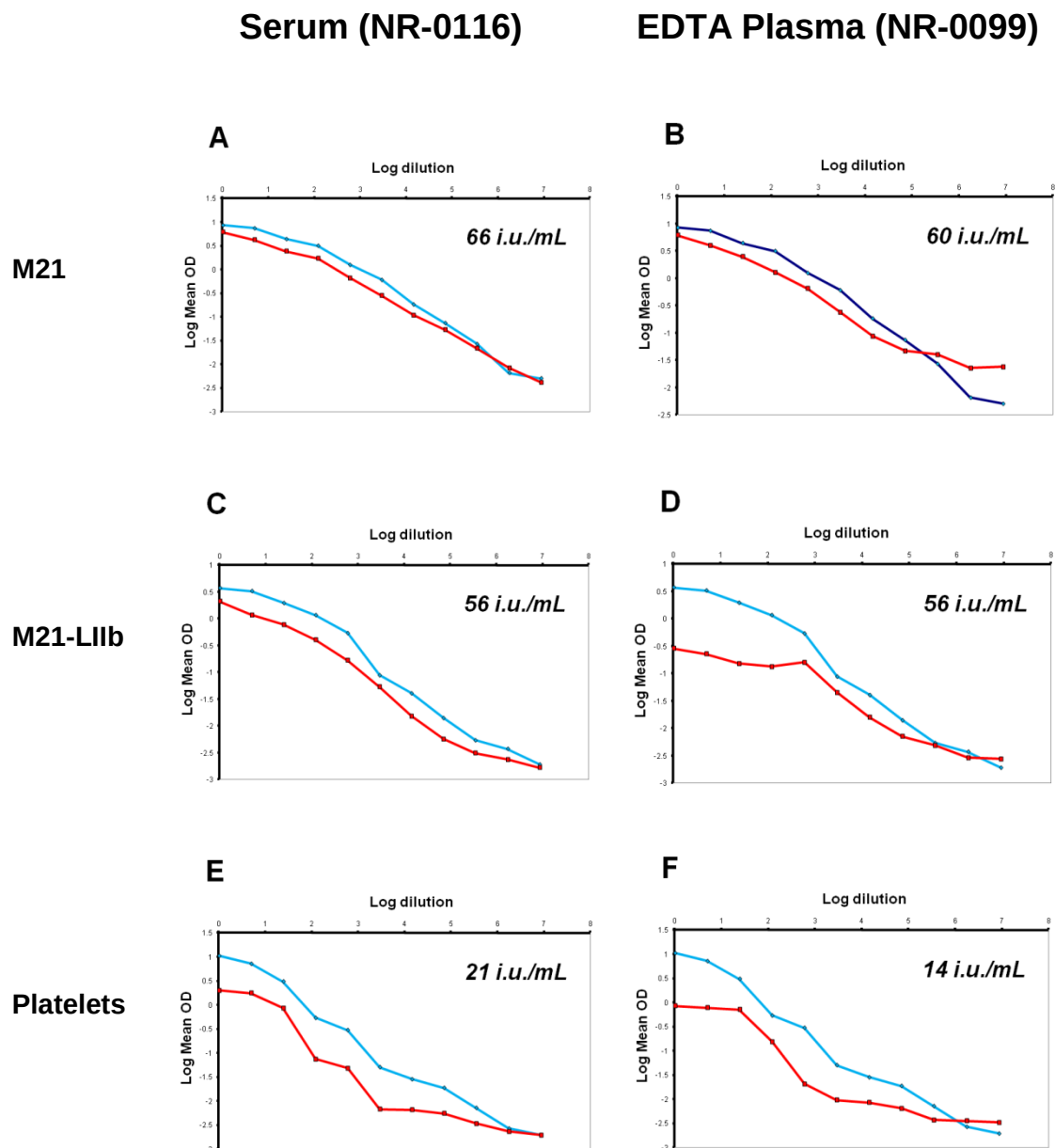


FIGURE 5.19. MAIPA/MAIMA titration curves of an example of anti-HPA-1a whose titration curve is grossly affected by sample type. Serum and EDTA plasma samples from the same case (F0975) and the potent anti-HPA-1a standard '03/152' were titrated in doubling dilutions from neat to 1 in 1024 (equivalent to 100 iu/mL to 0.1 iu/mL in standard) and tested in the MAIPA/MAIMA assays. The x axis shows the base10 log of the dilution and the y axis shows the base10 log of the mean ODs obtained from duplicate wells. The high potency standard is shown in blue and the test sample in red.



The results with paired samples NR-0116 and NR-0099 demonstrate the reduction in ODs when testing undiluted sample previously observed when using EDTA plasma compared to serum samples and $\alpha\text{IIb}\beta 3$ as target. However, the reduction in ODs with undiluted sample does not necessarily translate into differing levels of anti-HPA-1a compared to serum since the activity when using M21-LIIB cells was identical in both serum and plasma (56 i.u./mL) (**Figures 5.19C and D**).

Conversely, samples NR-0098 and NR-0032 demonstrate that despite having similar OD values in undiluted serum and plasma when using platelets as target, the calculated activity was very different (680 versus 297 i.u./mL respectively) (**Figures 5.18 E and F**).

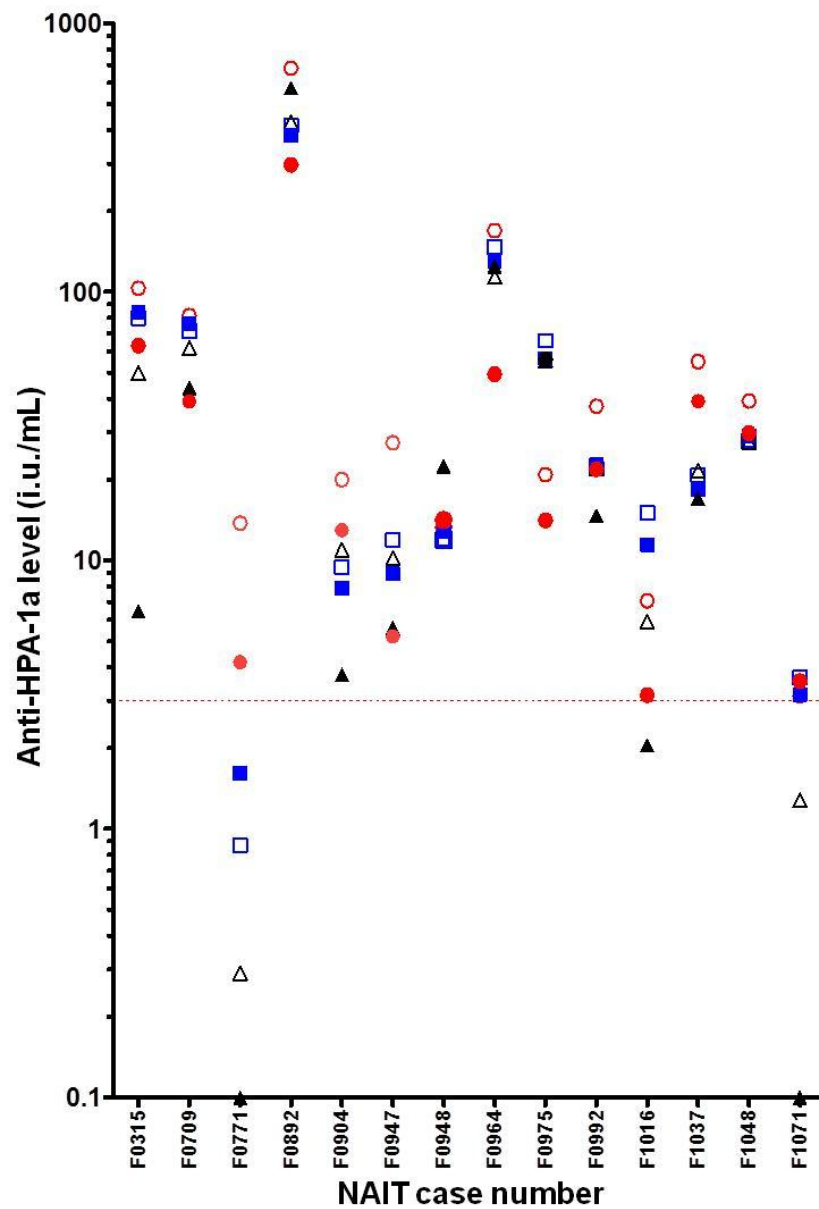
These data suggest that OD in undiluted samples is not a reliable indicator of antibody potency.

In order to explore the relationship between anti-HPA-1a levels measured using serum or EDTA plasma and different integrins/cell types a panel of fourteen paired samples were titrated in the quantitative MAIPA assay (**Table 5.3, Figure 5.20**).

TABLE 5.3. Quantitation of anti-HPA-1a in serum versus EDTA plasma samples using the MAIPA/MAIMA assays. *The level of anti-HPA-a in fourteen paired serum and EDTA plasma samples was assessed in a quantitative MAIPA assay using the NIBSC high-potency anti-HPA-1a standard '03/152' as a reference. Antibody levels were calculated as described in Chapter 2.*

NAIT Case No.	Sample ID (serum/EDTA plasma)	Calculated level of anti-HPA-1a (i.u./mL)					
		Serum			EDTA plasma		
		M21	M21-LIIB	Platelets	M21	M21-LIIB	Platelets
F0315	NR-0082/NR-0073	80	50	103	84	6	63
F0709	NR-0022/NR-0021	71	62	82	76	44	39
F0771	NR-0049/NR-0039	1	0	14	2	0	4
F0892	NR-0098/NR-0032	418	430	680	382	575	297
F0904	NR-0127/NR-0119	9	11	20	8	4	13
F0947	NR-0092/NR-0089	12	10	27	9	6	5
F0948	NR-0105/NR-0126	12	14	13	13	22	14
F0964	NR-0083/NR-0075	147	113	169	130	125	49
F0975	NR-0116/NR-0099	66	56	21	60	56	14
F0992	NR-0077/NR-0090	22	22	38	23	15	22
F1016	NR-0061/NR-0084	15	6	7	11	2	3
F1037	NR-0004/NR-0104	21	22	55	18	17	39
F1048	NR-0005/NR-0103	28	28	39	29	30	30
F1071	NR-0056/NR-0002	4	1	3	3	0	4

FIGURE 5.20. Effect of sample type and target cell on measured level of anti-HPA-1a. The dashed red line is the level at which severe thrombocytopenia is likely in the fetus/neonate (Killie et al, 2008).



Key:

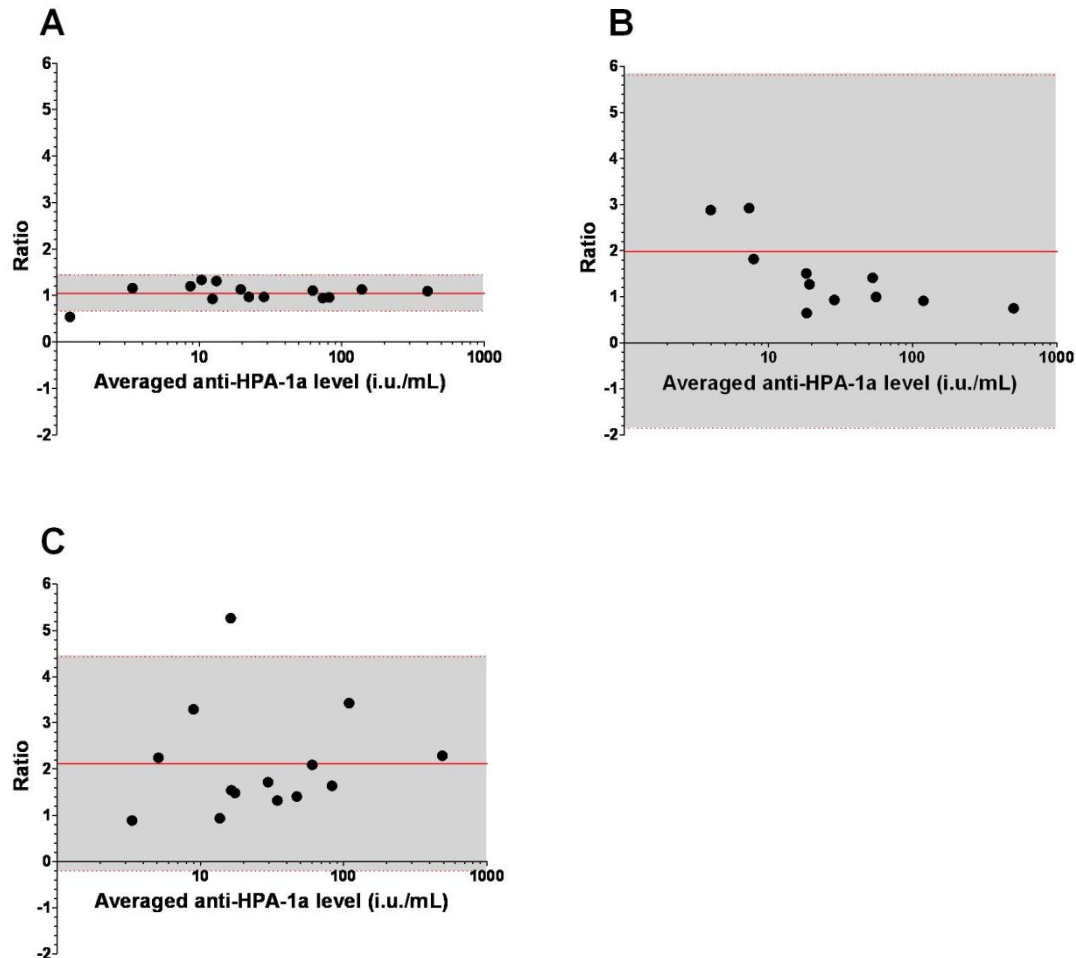
	Serum	EDTA plasma
M21		
M21-LIIB		
Platelets		

To assess the differences in calculated level of HPA-1a antibody, the activity when using EDTA plasma was calculated as a percentage of that obtained when using serum; results are shown in **Table 5.4** and as Bland-Altman plots in **Figure 5.21**.

TABLE 5.4. Measured level of anti-HPA-1a in EDTA plasma as percentage of level in paired (same venepuncture) serum sample. *The level of anti-HPA-1a in fourteen paired serum and EDTA plasma samples was assessed in a quantitative MAIPA assay using the NIBSC high-potency anti-HPA-1a standard '03/152' as a reference. Antibody levels were calculated as described in Chapter 2 then the level in EDTA plasma calculated as a percentage of that in serum from the same individual.*

NAIT Case No.	Sample ID (serum / EDTA plasma)	Level of anti-HPA-1a in EDTA plasma as percentage of level in serum		
		M21	M21-LIIB	Platelets
F0315	NR-0082 / NR-0073	105	13	61
F0709	NR-0022 / NR-0021	106	71	48
F0771	NR-0049 / NR-0039	185	0	30
F0892	NR-0098 / NR-0032	91	134	44
F0904	NR-0127 / NR-0119	83	34	65
F0947	NR-0092 / NR-0089	75	55	19
F0948	NR-0105 / NR-0126	108	155	107
F0964	NR-0083 / NR-0075	89	110	29
F0975	NR-0116 / NR-0099	91	100	68
F0992	NR-0077 / NR-0090	103	66	58
F1016	NR-0061 / NR-0084	76	35	45
F1037	NR-0004 / NR-0104	88	79	71
F1048	NR-0005 / NR-0103	103	107	76
F1071	NR-0056 / NR-0002	86	0	113
Mean		99	69	60

FIGURE 5.21. Bland-Altman plots of levels of anti-HPA-1a measured in paired (same venepuncture) serum and EDTA plasma samples. (A) M21 cells, (B) M21-LIIB cells and (C) platelets. The x axis shows the averaged value of anti-HPA-1a level measured in serum and EDTA plasma samples; the y axis shows the bias of serum versus EDTA plasma and is shown as a ratio of the antibody levels. The solid red line shows the mean bias and the shaded grey area show the 95% confidence intervals.



Using M21-LIIB cells (**Figure 5.21B**) the mean bias (red line) between serum and EDTA plasma was 1.98 (SD: 1.96) and the mean calculated anti-HPA-1a activity in EDTA plasma 69% of that of serum (**Table 5.4**) .

When using platelets as target, the mean bias between serum and EDTA plasma was 2.11 (SD: 1.18) (**Figure 5.21C**), with a mean anti-HPA-1a activity in EDTA

plasma 60% of that calculated when using serum (**Table 5.4**).

The use of integrin $\alpha\text{v}\beta 3$ reduced the variation in measured anti-HPA-1a level between serum and EDTA plasma significantly, with a mean bias between serum and EDTA plasma of 1.05 (SD: 0.20) (**Figure 5.21A**) and the measured level in EDTA plasma 99% of that of serum (**Table 5.4**).

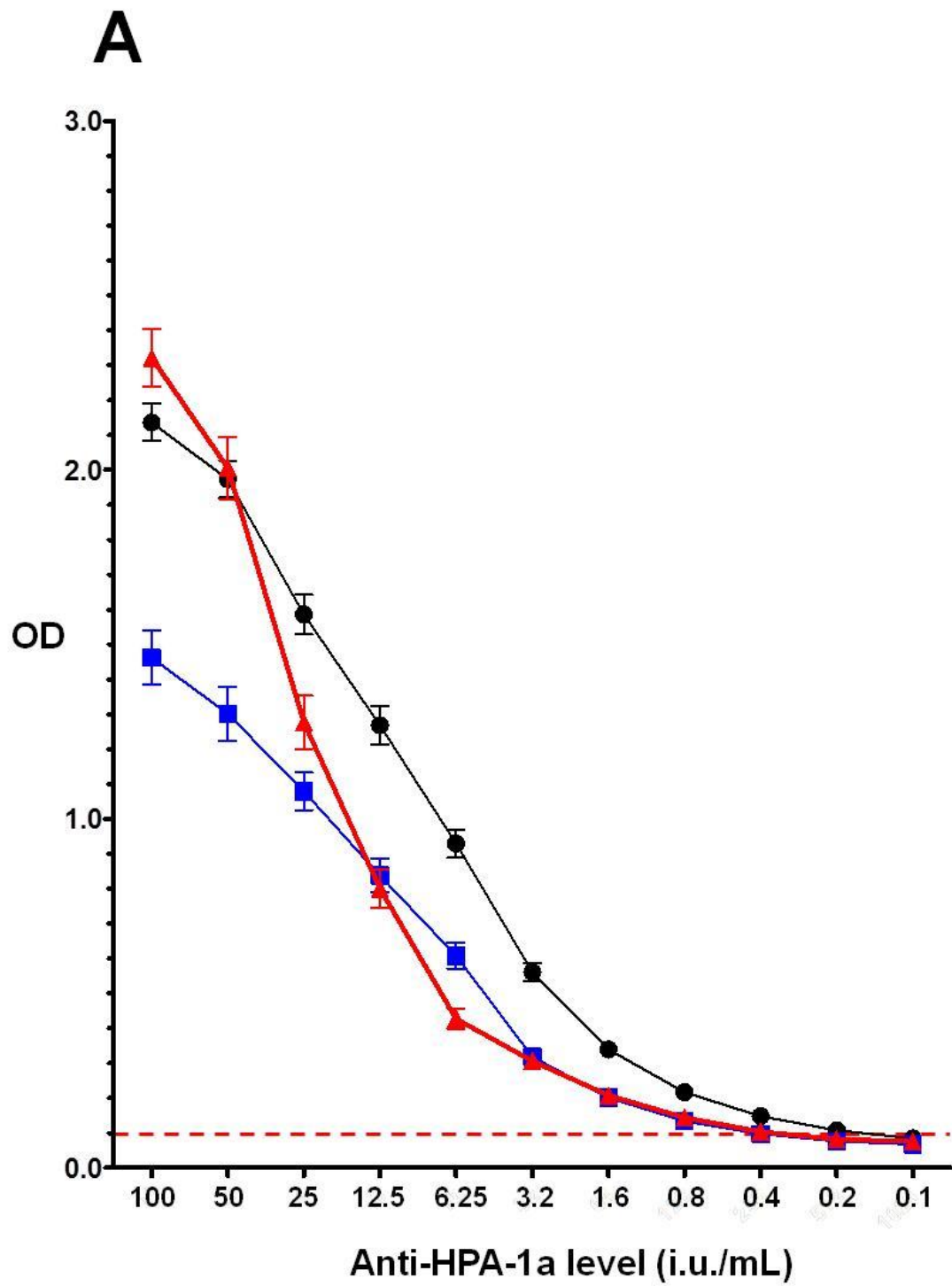
These data show that the type of α -chain with which the β -chain is associated, and the environment in which the integrin is situated affects not only the ODs obtained with different examples of anti-HPA-1a but also the measured level of antibody.

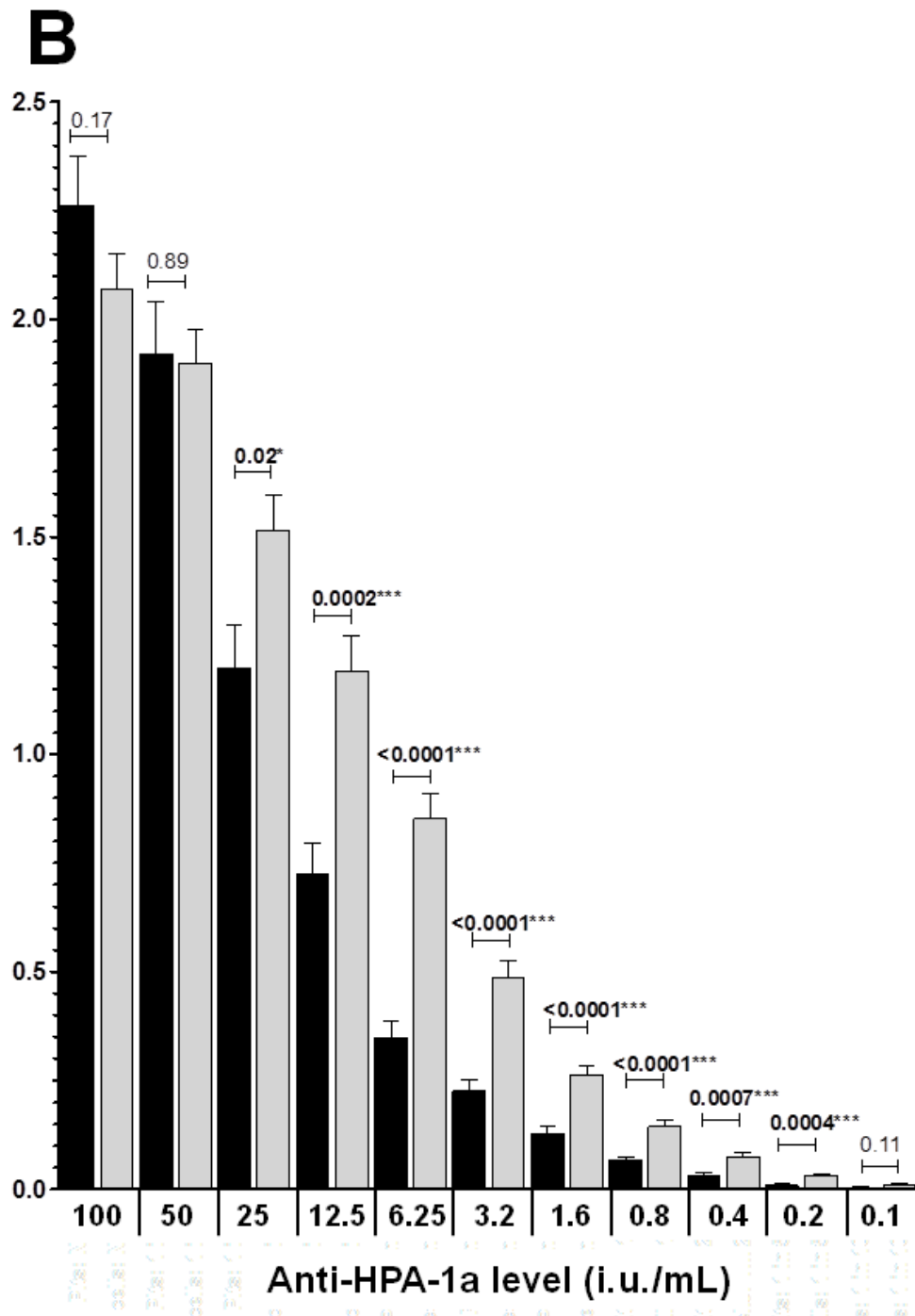
Most importantly, these data show that the measured level of anti-HPA-1a can differ significantly between serum and EDTA plasma when using $\alpha\text{IIb}\beta 3$ as target. The degree of variation however is not consistent and varies between different examples of anti-HPA-1a obtained from different individuals.

5.3.13 Effect of integrin density and integrin α -chain on reactivity of anti-HPA-1a in the MAIPA/MAIMA assays

It was observed during the above studies that the titration curves of the high-potency anti-HPA-1a standard NIBSC '03/152' were very different between the three cell types. The data obtained with this standard in all 28 titration experiments was collated and the curves resulting from this pooled data are shown in **Figure 5.22A** and a statistical analysis of M21 cell versus platelet data in **Figure 5.22B**.

FIGURE 5.22. Titration of NIBSC high-potency anti-HPA-1a standard '03/152'. (A) Doubling dilutions of the standard were prepared and tested in the MAIPA/MAIMA assays using clone VI-PL2 as capture mAb against M21 (black line), M21-LIIb (blue line) and platelets (red line). The x axis indicates the reciprocal of the dilution of the high-potency standard and the y axis shows the OD. The error bars show the mean and SEM of 28 experiments. The dashed red line indicates the cut-off. (B) Statistical analysis of data obtained when testing the high-potency standard against M21 cells (grey bars) and platelets (black bars). The x axis shows the concentration of anti-HPA-1a in i.u./mL and the y axis the OD. P values were calculated using the Wilcoxon matched pairs t-test (*P = <0.05; **P = <0.01; ***P = <0.001).





At high levels (>50 i.u./mL) of anti-HPA-1a the $\alpha\text{v}\beta 3$ -expressing M21 cells appeared to be less sensitive than platelets although this difference was not statistically significant (**Figure 5.22B**).

Using a cut-off of 0.100 OD units, the sensitivity of the platelet MAIPA was similar to

that of others at 0.4 i.u./mL (Sainio *et al*, 2012); the M21-LIIB MAIMA assay had the same sensitivity.

Importantly, the M21 MAIMA assay was significantly more sensitive than the platelet MAIPA assay with a detection limit of 0.2 i.u./mL ($P = 0.0004$ at 0.2 i.u./mL) and was four-fold greater than that of Sainio *et al* (Sainio *et al*, 2012).

At levels of HPA-1a antibody between 25 and 0.2 i.u./mL the M21 cells were significantly ($P = <0.05$) more sensitive than platelets.

5.3.14 Assessment of integrin number and density on cell lines

Since the use of M21 cells showed superior sensitivity for detection of low levels of HPA-1a antibody compared to platelets and M21-LIIB cells, integrin number and density was investigated as a possible reason (**Table 5.5**).

TABLE 5.5. Integrin density on cell lines and platelets and calculated number of integrin molecules per test. *The number of integrin molecules was estimated using the Biocytex Platelet Calibrator Kit' (Diagnostica Stago,UK) (Chapter 2). Surface areas were calculated using the cell diameter obtained using the Vi-CELL (Beckman-Coulter) (M21 and M21-LIIB cells) or published data (platelets) and assuming a spheroid shape for the cells using the formula $SA = 4\pi r^2$ where SA = surface area, V = volume, and r is the radius of the cell.*

Cell type	Number integrin molecules per cell	Surface area of cell (μm^2)	Integrin density (No. molecules per μm^2)	Number of cells per test	Number of integrin molecules per test
Platelets	49,067	25	1,978	1×10^7	4.9×10^{11}
M21	2,940	669	4	2.5×10^5	7.4×10^8
M21-LIIB	3,531	797	4	2.5×10^5	8.8×10^8

M21 and M21-LIIB cell lines expressed similar numbers of integrin molecules and had similar integrin density yet had very different titration curves suggesting that the conformation of the $\beta 3$ chain (and HPA-1a epitopes) was different between these two cell lines. The titration curve of the M21-LIIB line was more similar in gradient to that of platelets than M21 cells at levels <50 i.u./mL but was different at levels >50 i.u./mL suggesting that integrin number and/or density directly affected the OD.

Calculations based on number of integrin molecules and cell size showed that the total number of integrin molecules used per test when using platelets was approximately three orders of magnitude higher than when using M21 cells; this would suggest that the platelet MAIPA would be more sensitive than the M21 MAIMA assay yet the reverse was true at levels of anti-HPA-1a <50 i.u./mL.

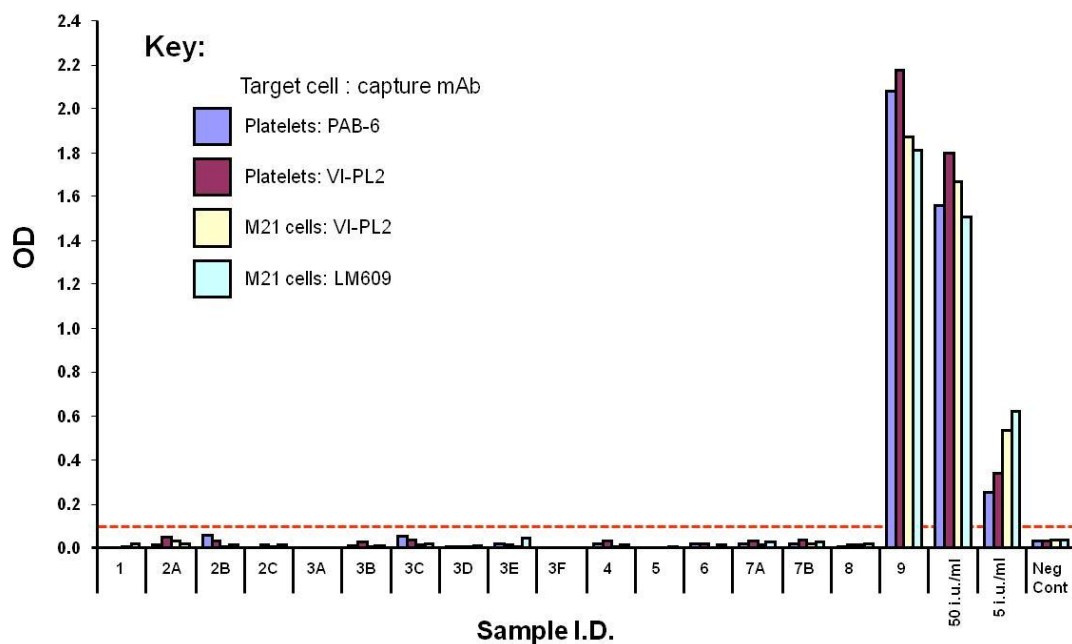
5.3.15 Comparison of $\alpha\text{v}\beta 3$ (M21 cells) and $\alpha\text{IIb}\beta 3$ (platelets) for detection of 'difficult to detect' HPA-1a antibodies

The superior sensitivity of the M21 cells for detection of 'weak' examples of anti-HPA-1a when compared to platelets (**Figure 5.13**) suggested that use of these cells might detect the presence of antibody in some HPA-1b1b mothers in whom antibodies had not been detected when using platelets.

A search of 'NAITabase' was performed and this identified a number of HPA-1b1b mothers in whom HPA-1a antibodies had not been detected by the investigating laboratory but review of clinical data suggested that NAIT might be a diagnosis. In two cases (cases 3 and 9) there was a high clinical suspicion of NAIT, with both cases having had previous children affected by NAIT; other cases had a low index of suspicion.

Blinded samples from these cases were obtained and tested in the platelet MAIPA and the M21 MAIMA assays (**Figure 5.23**).

FIGURE 5.23. Assessment of sensitivity of cell lines versus platelets for detection of ‘difficult to detect’ examples of anti-HPA-1a. Samples were screened against M21 cells and platelets using two different capture mAbs. Strong positive (50 i.u./mL) and weak positive (5 i.u./mL) anti-HPA-1a controls were prepared by diluting sample NR-0083 in TBS/BSA; a sample from a group AB non-transfused, male, blood donor was used as a negative control. The sample identifier is shown on the x axis. Where a number of samples were available from the same case these are identified by letters after the number.

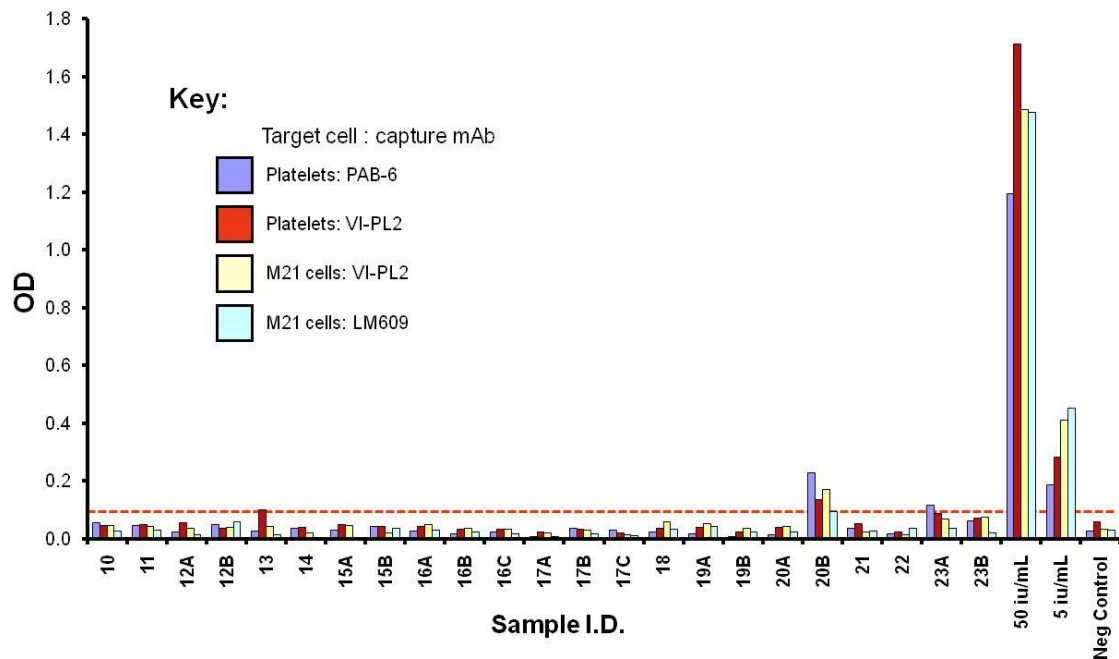


Antibodies could only be detected in the sample from case 9; these were detectable using both M21 cells and platelets and using either capture mAb. When the laboratory supplying these samples supplied the (coded) identity of the samples it was revealed that this sample was not the original sample that had been tested negative by that laboratory but a sample taken subsequently and in which the reference laboratory had also detected HPA-1a antibodies. These data therefore could not confirm that M21 cells were able to detect HPA-1a antibodies that were undetectable using conventional assays in the NHSBT reference laboratory.

Subsequent to this initial study, a number of additional cases of cases of NAIT in

infants of HPA-1b1b mothers were identified in which antibodies were not detected in the initial post delivery sample by the reference laboratory but where antibodies were detected some weeks post-delivery; these were also investigated (**Figure 5.24**).

FIGURE 5.24. Assessment of sensitivity of MAIMA versus MAIPA for detection of ‘difficult to detect’ examples of anti-HPA-1a. Samples were screened against M21 cells and platelets using two different capture mAbs. Strong positive (50 iu/mL) and weak positive (5 iu/mL) anti-HPA1a controls were prepared by diluting sample NR-0083 in TBS/BSA; a sample from a group AB non-transfused, male, blood donor was used as a negative control. The sample identifier is shown on the x axis; where a number of samples were available from the same case these are identified by letters after the number.



No examples of anti-HPA-1a reactive with M21 cells but unreactive against platelets were identified amongst these 14 cases. The data therefore did not confirm the superior sensitivity of the $\alpha\text{v}\beta 3$ assay.

5.3.16 Sensitivity of MAIMA compared to next-generation recombinant protein assays

Recent developments in recombinant protein technology have led to the production of a number of recombinant fragments of $\beta 3$ that express HPA-1a (Stafford *et al*, 2008). Some of these have been shown to be potentially suitable for the detection of HPA-1a antibodies and work is currently in progress within NHSBT to develop a bead-based assay ('Luminex') using these fragments that is suitable for high-throughput, rapid and sensitive screening for HPA-1 antibodies (Chong *et al*, 2011).

In view of the fact that this study has demonstrated that M21 cells are better able to detect low levels of HPA-1a antibodies compared to platelets a collaboration was undertaken with Chong *et al* to investigate the relative sensitivities of these new assays.

Sixty four samples, including serum, EDTA plasma, citrate plasma and re-calcified citrate plasma was blinded and tested in the cell line MAIMA, the Luminex assay and the platelet MAIPA as a comparison (**Tables 5.6** and **5.7**).

TABLE 5.6. Comparison of Luminex, platelet MAIPA and cell line MAIMA assays. The Luminex data are ratios calculated as follows (MFI for r-HPA-1a/MFI for inert bead)/(negative control MFI/negative control MFI for inert bead); cut-off value is 2.00. The MAIPA/MAIMA data are ODs; cut-off value is 0.100. Green-shaded cells indicate a positive result; red-shaded cells indicate a negative result. All samples (except negative controls) had been recorded in the sample archive database as having been obtained from HPA-1b1b individuals and that anti-HPA-1a was present.

Sample ID	Material	Assay						
		Luminex	MAIPA/MAIMA					
			VI-PL2			PAB6		LM609
		NBG ratio	Platelets	M21 cells	M21-LIIB cells	Platelets	M21-LIIB cells	M21 cells
AB 3	Serum	0.62	0.005	0.008	0.001	0.003	0.000	0.000
AB 5	Serum	0.97	0.003	0.012	0.003	0.004	0.000	0.005
AB HE	Serum	1.11	0.017	0.001	0.000	0.018	0.004	0.000
	Mean	0.90	0.008	0.007	0.001	0.008	0.001	0.002
1a-D0003	Citrate	13.80	0.378	0.358	0.205	0.388	0.355	0.639
1a-D0004	Citrate	4.62	2.226	1.779	0.922	2.470	1.201	1.959
1a-D0008	Citrate	32.23	0.716	0.783	0.602	0.677	0.782	1.008
1a-D0013	Citrate	41.80	2.588	1.617	1.243	1.968	1.591	1.944
1a-D0015	Citrate	5.87	2.362	1.747	1.500	2.381	1.755	1.736
1a-D0016	Citrate	16.01	1.876	1.858	1.501	1.771	1.535	1.832
1a-D0017	Citrate	0.51	0.047	0.017	0.001	0.096	0.012	0.014
1a-D0018	Citrate	1.21	0.048	0.138	0.091	0.076	0.020	0.118
1a-D0024	Citrate	1.25	0.005	0.007	0.001	0.002	0.011	0.000
	Mean	13.03	1.138	0.923	0.674	1.092	0.807	1.028
NR-0001	EDTA	8.24	0.084	0.245	0.097	0.021	0.056	0.218
NR-0002	EDTA	4.45	0.133	0.380	0.144	0.049	0.285	0.292
NR-0003	EDTA	2.48	0.034	0.275	0.034	0.014	0.093	0.231
NR-0021	EDTA	5.88	0.663	2.279	0.950	0.118	0.171	2.223
NR-0023	EDTA	1.10	0.017	0.008	0.003	0.018	0.000	0.009
NR-0031	EDTA	0.56	0.000	0.000	0.000	0.005	0.000	0.000
NR-0032	EDTA	6.90	1.481	1.959	1.379	0.354	1.236	2.006
NR-0039	EDTA	18.11	0.118	0.204	0.033	0.002	0.018	0.137
NR-0040	EDTA	24.09	0.573	1.160	0.840	0.048	0.077	1.421
NR-0046	EDTA	1.02	0.005	0.007	0.009	0.009	0.007	0.003
NR-0058	EDTA	5.65	0.193	0.194	0.133	0.098	0.083	0.127
NR-0073	EDTA	44.16	1.335	2.228	0.620	0.098	0.163	1.914
NR-0075	EDTA	59.17	0.690	1.436	0.738	0.175	0.422	1.547
NR-0084	EDTA	5.98	0.112	0.554	0.147	0.003	0.016	0.359
NR-0090	EDTA	3.45	1.204	1.505	0.979	0.362	0.621	0.503
NR-0099	EDTA	42.10	0.920	1.580	0.715	0.201	0.750	2.045
NR-0103	EDTA	2.49	0.677	1.707	1.248	0.264	1.595	1.681
NR-0104	EDTA	47.35	0.748	1.270	0.400	0.120	0.492	1.494
NR-0125	EDTA	0.59	0.000	0.002	0.000	0.003	0.000	0.000
NR-0156	EDTA	0.63	0.018	0.019	0.004	0.003	0.000	0.005
NR-0157	EDTA	1.49	0.000	0.002	0.000	0.000	0.000	0.000

		Assay						
		Luminex	MAIPA/MAIMA					
			VI-PL2			PAB6		LM609
Sample ID	Material	NBG ratio	Platelets	M21 cells	M21-LIIB cells	Platelets	M21-LIIB cells	M21 cells
	Mean	13.61	0.499	0.944	0.471	0.109	0.338	0.900
1a-D0001	RP	14.03	3.193	1.404	1.346	2.617	1.573	1.317
1a-D0005	RP	5.25	1.067	1.211	0.667	1.407	0.944	1.383
1a-D0009	RP	11.18	3.146	1.544	1.204	2.821	1.602	2.017
1a-D0012	RP	4.11	0.222	0.442	0.244	0.305	0.232	0.321
NR-0137	RP	1.15	0.007	0.000	0.001	0.002	0.005	0.001
NR-0145	RP	58.26	1.348	0.835	0.487	0.763	0.394	0.793
NR-0149	RP	12.06	0.247	0.593	0.357	0.179	0.219	0.403
NR-0150	RP	22.50	2.174	1.101	0.901	2.759	1.134	1.514
NR-0151	RP	1.24	0.010	0.004	0.004	0.008	0.000	0.001
NR-0152	RP	1.18	0.032	0.000	0.002	0.002	0.001	0.003
NR-0153	RP	1.15	0.009	0.002	0.001	0.009	0.000	0.000
NR-0154	RP	1.51	0.092	0.255	0.128	0.233	0.123	0.188
	Mean	11.14	0.962	0.616	0.445	0.925	0.519	0.662
1a-D0006	Serum	5.86	0.992	1.368	0.843	1.451	1.060	1.458
1a-D0010	Serum	12.12	0.432	0.657	0.325	0.724	0.455	0.738
1a-D0011	Serum	34.15	0.727	0.740	0.480	1.091	0.764	0.967
NR-0005	Serum	1.90	1.193	1.580	0.859	1.455	0.960	1.483
NR-0022	Serum	5.75	1.755	1.831	1.203	2.288	1.267	1.795
NR-0056	Serum	3.99	0.109	0.383	0.122	0.271	0.278	0.220
NR-0061	Serum	5.40	0.481	0.666	0.409	0.337	0.269	0.306
NR-0064	Serum	2.11	0.022	0.016	0.007	0.038	0.007	0.008
NR-0077	Serum	1.93	0.930	1.549	0.926	0.175	0.757	1.414
NR-0078	Serum	1.34	0.000	0.000	0.000	0.012	0.000	0.000
NR-0082	Serum	32.30	1.743	2.209	1.070	2.236	1.137	2.048
NR-0083	Serum	13.67	1.511	1.732	1.165	1.670	1.208	1.832
NR-0098	Serum	5.19	1.999	1.815	1.145	1.740	0.880	1.771
NR-0102	Serum	51.13	0.318	0.281	0.275	0.373	0.285	0.365
NR-0110	Serum	10.07	0.511	0.881	0.426	0.755	0.342	0.686
NR-0116	Serum	8.61	1.678	1.792	0.510	1.679	0.808	2.207
NR-0128	Serum	10.22	2.216	1.544	1.029	2.568	1.201	1.987
NR-0201	Serum	3.12	0.346	0.550	0.280	0.506	0.289	0.628
NR-0204	Serum	19.44	1.485	1.356	0.845	1.622	0.845	1.295
	Mean	12.02	0.915	1.119	0.658	1.037	0.695	1.080

Citrate: citrated plasma; EDTA: EDTA plasma; RP: re-calcified citrated plasma.

TABLE 5.7. Summarised data of impact of sample type on assay results

Sample type	Assay	Cell type	Capture mAb	True positive	False positive	True Negative	False negative
Serum (n=22)	Luminex	na	na	18	0	4	0
	MAIMA	M21	VI-PL2	17	0	4	1
	MAIMA	M21	LM609	17	0	4	1
	MAIMA	M21-LIIB	VI-PL2	17	0	4	1
	MAIMA	M21-LIIB	PAB-6	17	0	4	1
	MAIPA	Platelets	VI-PL2	17	0	4	1
	MAIPA	Platelets	PAB-6	17	0	4	1
EDTA plasma (n=21)	Luminex	na	na	15	0	6	0
	MAIMA	M21	VI-PL2	15	0	6	0
	MAIMA	M21	LM609	15	0	6	0
	MAIMA	M21-LIIB	VI-PL2	12	0	6	3
	MAIMA	M21-LIIB	PAB-6	9	0	6	6
	MAIPA	Platelets	VI-PL2	13	0	6	2
	MAIPA	Platelets	PAB-6	7	0	6	8
Citrate plasma (n=9)	Luminex	na	na	6	0	1	2
	MAIMA	M21	VI-PL2	7	0	1	1
	MAIMA	M21	LM609	6	0	1	2
	MAIMA	M21-LIIB	VI-PL2	6	0	1	2
	MAIMA	M21-LIIB	PAB-6	6	0	1	2
	MAIPA	Platelets	VI-PL2	6	0	1	2
	MAIPA	Platelets	PAB-6	6	0	1	2
Re-calcified citrate plasma (n=12)	Luminex	na	na	7	0	4	1
	MAIMA	M21	VI-PL2	8	0	4	0
	MAIMA	M21	LM609	8	0	4	0
	MAIMA	M21-LIIB	VI-PL2	8	0	4	0
	MAIMA	M21-LIIB	PAB-6	8	0	4	0
	MAIPA	Platelets	VI-PL2	8	0	4	0
	MAIPA	Platelets	PAB-6	8	0	4	0

na: not applicable

As shown previously, EDTA plasma was the least suitable material for use in the MAIPA assay when using $\alpha\text{IIb}\beta 3$ as target with a number of false-negative results obtained. Using $\alpha\text{IIb}\beta 3$ on platelets, 8/15 (53%) samples tested falsely negative when using PAB-6 as capture mAb, and 2/15 (13%) when using VI-PL2 as capture mAb.

Using $\alpha\text{IIb}\beta 3$ on M21-LIIb cells, 6/15 (40%) and 3/15 (20%) samples tested falsely negative when using PAB-6 and VI-PL2 as capture mAbs respectively.

The detection of anti-HPA-1a using $\alpha\text{v}\beta 3$ (M21 cells) or recombinant $\beta 3$ fragments was unaffected by the use of EDTA plasma.

Citrated plasma also resulted in a number of false-negative assays. However, re-calcified citrated plasma and serum were suitable for use in all assays. The Luminex assay detected one example (NR-0064) of anti-HPA-1a in a serum sample that was not detected by any other method. This sample gave only a very weak fluorescence signal against the rHPA-1a protein (data not shown).

One citrated sample (1a D-0018) was detectable only in the MAIMA assay using M21 cells and either LM609 or VI-PL2 as capture mAb. This sample had been distributed in a NIBSC workshop (Exercise 2002-B) and had been detected by 25/33 (76%) of laboratories and was thus deemed to be a 'false negative' in all other assays.

One further sample (1a-D0017) that tested negative in all assays was concluded to be falsely negative in all assays since it had been tested twice in NIBSC workshops (Exercises 2001-A and 2003-A) and 58 - 69% of laboratories were able to detect anti-HPA-1a in it.

Twelve samples tested negative in all assays; these were interpreted as 'true negative'.

The sensitivity and specificity of the various assays using the different sample types were calculated including, (**Table 5.8**) and excluding (**Table 5.9**) EDTA plasma samples.

TABLE 5.8. Comparison of the sensitivity and specificity of a next-generation recombinant protein assay (Luminex) and cell line assays for the detection of HPA-1a antibodies.

Assay	Cell type	Integrin	Capture mAb	Specificity (%)	Sensitivity (%)
Luminex	na	$\beta 3$	na	100	93.9
MAIMA	M21	$\alpha\text{v}\beta 3$	VI-PL2	100	95.9
MAIMA	M21	$\alpha\text{v}\beta 3$	LM609	100	93.9
MAIMA	M21-LIIB	$\alpha\text{IIb}\beta 3$	VI-PL2	100	89.8
MAIMA	M21-LIIB	$\alpha\text{IIb}\beta 3$	PAB-6	100	81.6
MAIPA	Platelets	$\alpha\text{IIb}\beta 3$	VI-PL2	100	89.8
MAIPA	Platelets	$\alpha\text{IIb}\beta 3$	PAB-6	100	75.5

TABLE 5.9. Comparison of the sensitivity and specificity of a next-generation recombinant protein assay (Luminex) and cell line assays for the detection of HPA-1a antibodies, excluding EDTA plasma samples.

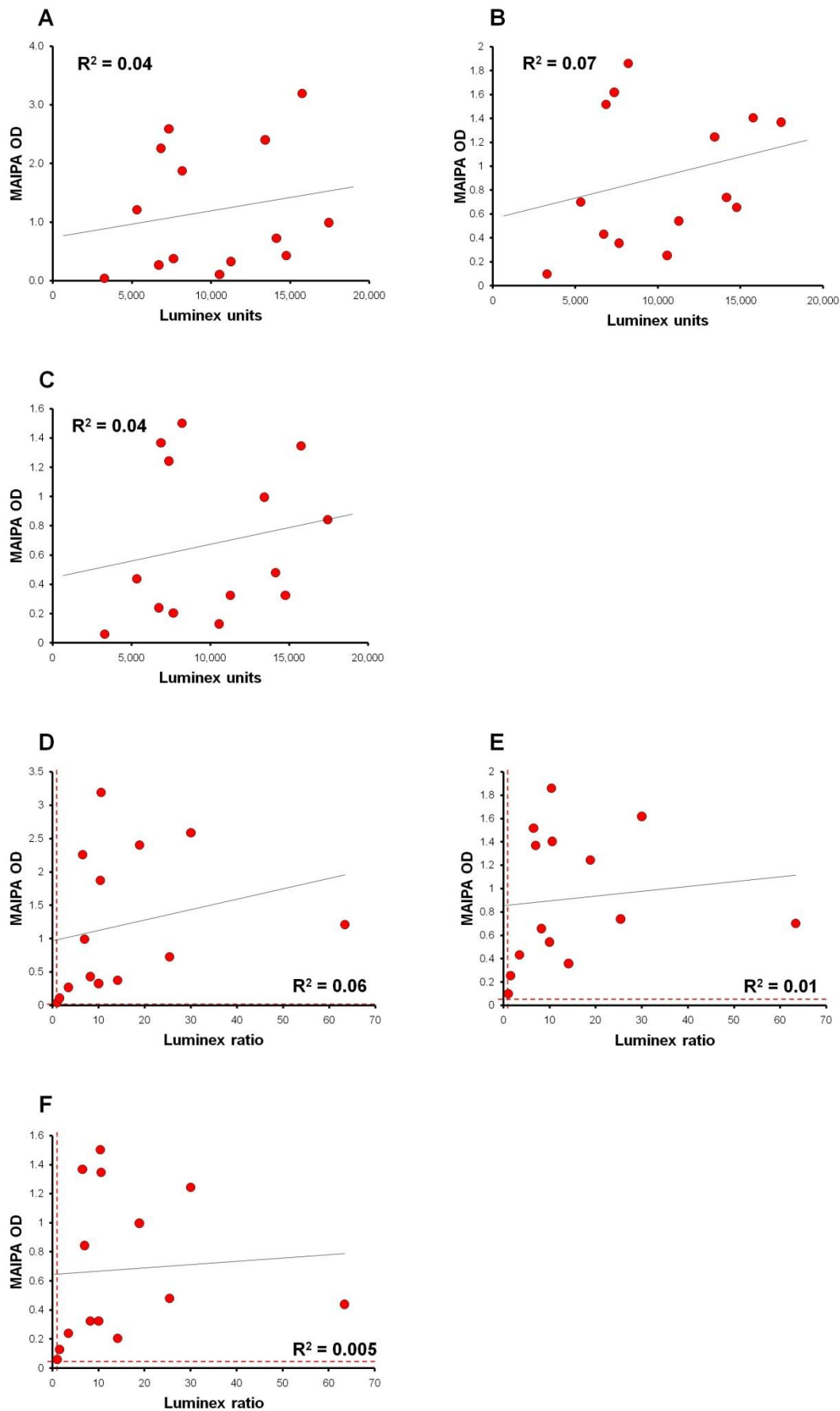
Assay	Cell type	Integrin	Capture mAb	Specificity (%)	Sensitivity (%)
Luminex	na	$\beta 3$	na	100	91.2
MAIMA	M21	$\alpha\text{v}\beta 3$	VI-PL2	100	94.1
MAIMA	M21	$\alpha\text{v}\beta 3$	LM609	100	91.2
MAIMA	M21-LIIB	$\alpha\text{IIb}\beta 3$	VI-PL2	100	91.2
MAIMA	M21-LIIB	$\alpha\text{IIb}\beta 3$	PAB-6	100	91.2
MAIPA	Platelets	$\alpha\text{IIb}\beta 3$	VI-PL2	100	91.2
MAIPA	Platelets	$\alpha\text{IIb}\beta 3$	PAB-6	100	91.2

5.3.17 Correlation of strength of anti-HPA-1a reactivity against $\alpha\text{IIb}\beta 3$, $\alpha\text{v}\beta 3$ (MAIPA/MAIMA assays) and recombinant $\beta 3$ (Luminex)

A number of lines of evidence in this chapter suggest that both integrin conformation and density play a role in the ability to detect different examples of anti-HPA-1a.

The Luminex assay uses only recombinant $\beta 3$ chains immobilised on to microbeads and thus the HPA-1a epitopes might be expected to be orientated differently to those epitopes on $\alpha\text{v}\beta 3$ or $\alpha\text{IIb}\beta 3$; the correlation between strength of reactivity against dimeric integrin ($\alpha\text{v}\beta 3$ or $\alpha\text{IIb}\beta 3$) used in the MAIPA/MAIMA assays and monomeric $\beta 3$ chain used in the Luminex assay was therefore investigated (**Figure 5.25**).

FIGURE 5.25. Correlation between reactivity of examples of anti-HPA-1a against dimeric integrin ($\alpha\text{v}\beta 3$ or $\alpha\text{IIb}\beta 3$) and against monomeric $\beta 3$ chain only. A panel of anti-HPA-1a samples (serum, citrated plasma and re-calcified citrated plasma) was tested in the MAIPA/MAIMA assays using anti- $\beta 3$ clone VI-PL2 as capture mAb, and in the Luminex assay. **Figures A-C** show the correlation between MAIPA OD values and raw data obtained by Luminex; **Figures D-F** show MAIPA ODs plotted against Luminex ratios. **Figures A** and **D** show ODs in the MAIPA assay when using platelets, **Figures B** and **E** data obtained when using M21 cells and **Figures C** and **F** M21-L11b cell line results. The dashed red lines in **Figures D-F** indicate the cut-off values; raw data values are not normally used in the Luminex assay so no cut-off lines are shown.



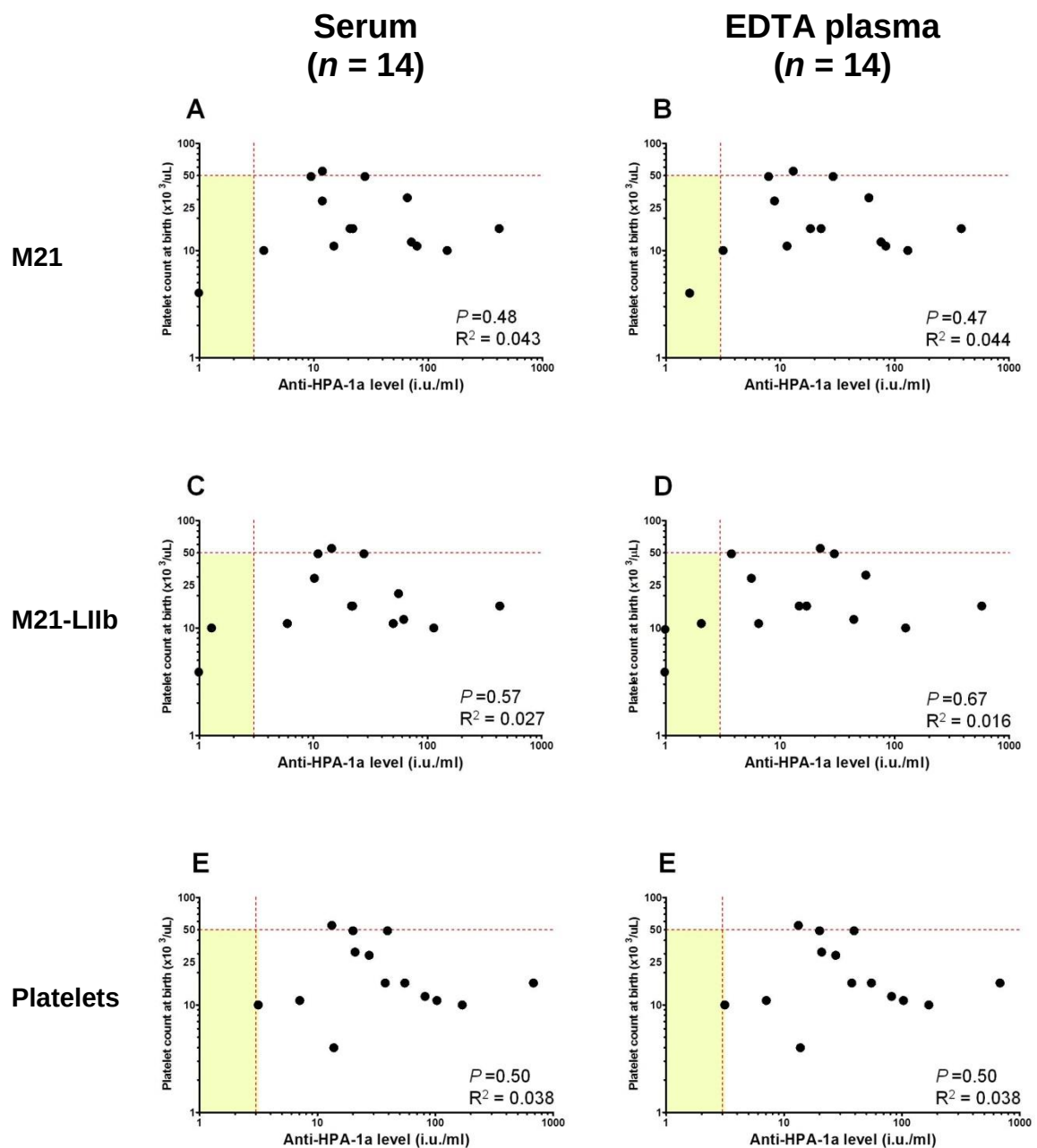
There was very little correlation obtained between reactivity against intact integrin and isolated $\beta 3$, supporting the hypothesis that the conformation of $\beta 3$ is important for recognition by HPA-1a antibodies.

5.3.18 Relationship between measured level of anti-HPA-1a using different integrins/cell types and neonatal platelet count

Conflicting data has been published regarding the relationship between the measured level of HPA-1a antibodies with the platelet count of the neonate at delivery (Bertrand *et al*, 2006; Ghevaert *et al*, 2007b; Killie *et al*, 2008; Bessos *et al*, 2008a; Bertrand *et al*, 2011).

Since this study has shown that the sample type, target integrin and cell type affect both the OD value obtained in screening assays and the measured level of HPA-1a antibodies, the relationship between anti-HPA-1a level and neonatal platelet count was explored in a limited number of samples tested against M21 and M21-LIIB cell lines and platelets, using both serum and EDTA plasma samples (**Figure 5.26**).

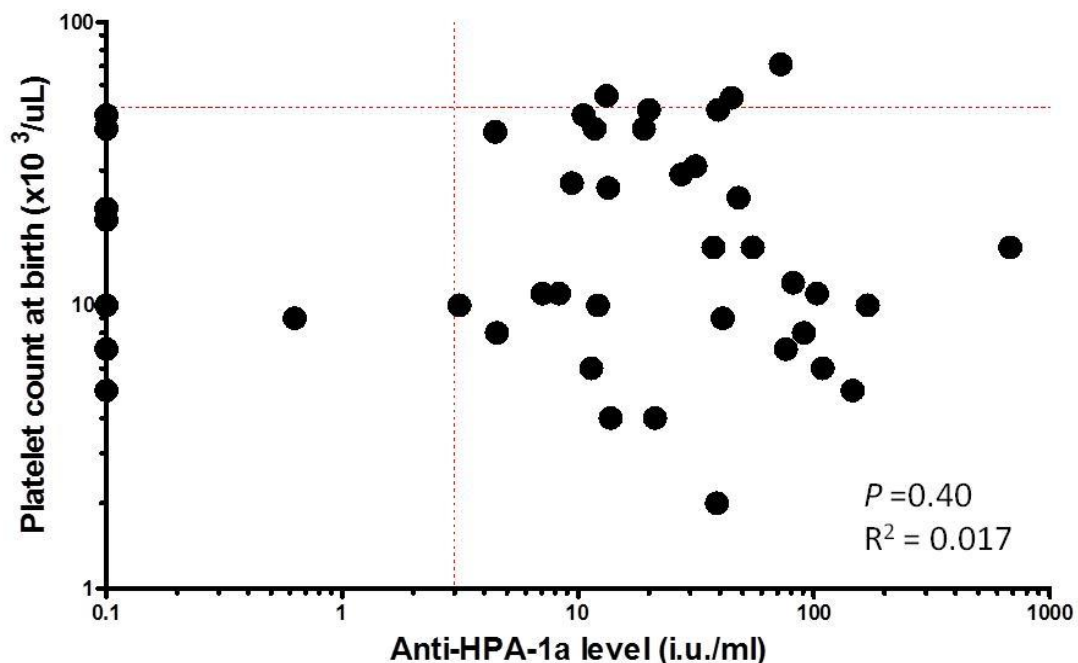
FIGURE 5.26. Relationship between measured level of anti-HPA-1a and neonatal platelet count when using cell lines or platelets as target. The measured level of anti-HPA-1a in both serum and EDTA plasma samples from the same case were plotted against the neonatal platelet count at delivery. All cases were newly diagnosed i.e. the mother or fetus in the current pregnancy had not received any form of treatment. P and R^2 values were calculated using the Pearson two-tailed t -test. The vertical dashed red line indicates the antibody level above which severe thrombocytopenia is likely and the horizontal dashed red line is set at the threshold of severe thrombocytopenia ($50 \times 10^3/\mu\text{L}$).



No significant correlation was observed between the level of anti-HPA-1a and the neonatal platelet count with any of the cell types.

Since the best correlation between HPA-1a antibody level and neonatal platelet count appeared to be obtained when using serum samples tested against platelets, an extended panel of serum samples was tested (**Figure 5.27**).

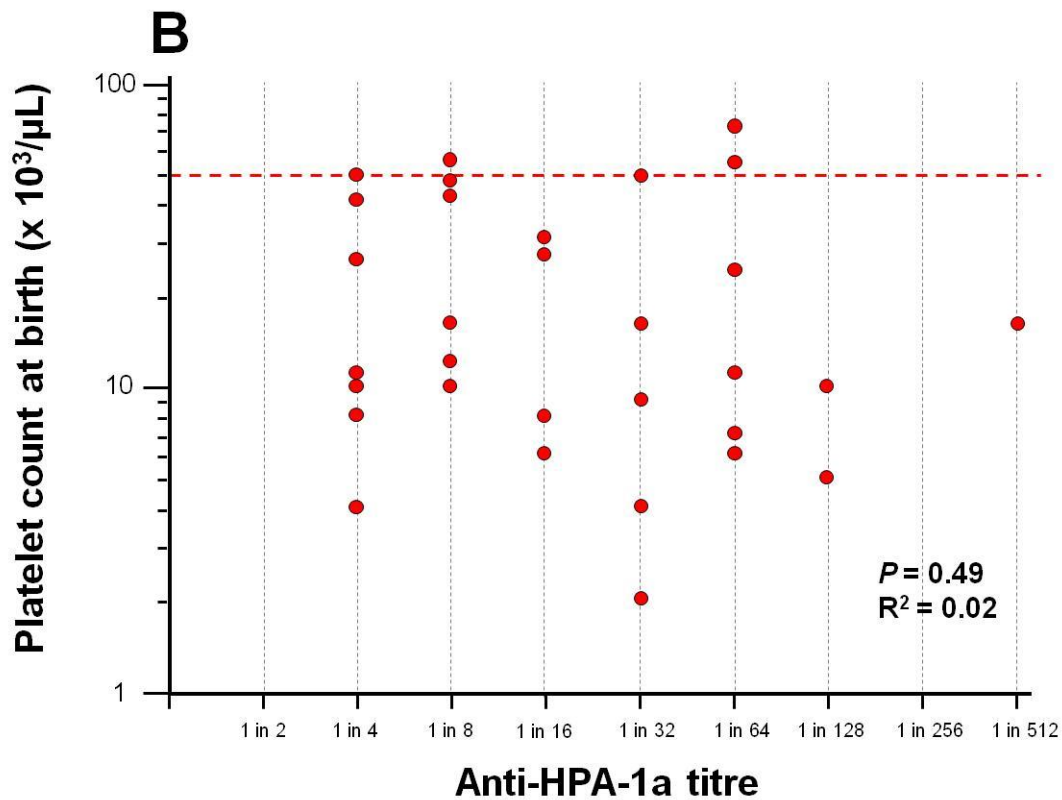
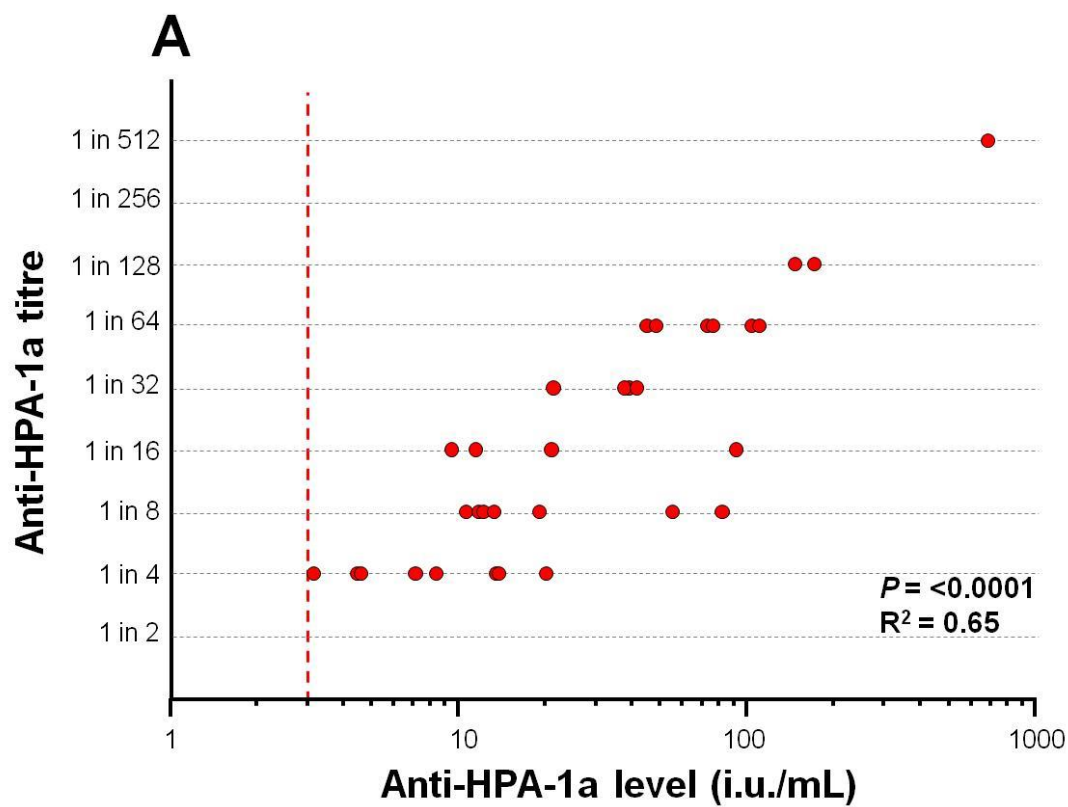
FIGURE 5.27. Relationship between measured level of HPA-1a antibodies using serum samples tested against platelets. A panel of 43 serum samples from mothers having delivered full-term infants was used to investigate the relationship between maternal HPA-1a antibody level and neonatal platelet count. All cases were *de novo* cases i.e. neither mother or fetus had received treatment for NAIT during pregnancy. All samples were collected at, or near, delivery.



Using a threshold of 3 i.u./mL and a platelet count of $50 \times 10^3/\mu\text{L}$ as predictor and measure of severe thrombocytopenia respectively, the sensitivity of the assay was 76.9%; specificity could not be calculated as there were no cases where the neonatal platelet count was above $50 \times 10^3/\mu\text{L}$ and the HPA-1a antibody level <3 i.u./mL. This sensitivity is lower than that reported by Killie in a much larger study (93%) but similar to that found by Bertrand *et al* (81.2%) who used a threshold of >28 i.u./mL as a predictor of the fetal response to maternal therapy for NAIT (Killie *et al*, 2008; Bertrand *et al*, 2011).

Prior to the availability of the NIBSC high-potency standard anti-HPA-1a, antibody titres were used as a predictor of severity of thrombocytopenia, with titres of ≥ 1 in 32 in the third trimester having a positive predictive value of 75% (Williamson *et al*, 1998). The relationship between antibody titre and antibody potency was therefore investigated (**Figure 5.28A**), together with the relationship between antibody titre and neonatal platelet count at birth (**Figure 5.28B**).

FIGURE 5.28. Relationship between anti-HPA-1a titre and potency, and between antibody titre and neonatal platelet count at delivery. (A) Samples were assigned a potency calculated with reference to the NIBSC high-potency standard anti-HPA-1a NIBSC 03/152. The titre was the last of the doubling dilutions of test sample that resulted in an OD in the MAIPA assay of >0.100 when tested against HPA-1a1a platelets. The dashed red line is the potency at which severe thrombocytopenia is likely to occur (Killie *et al*, 2008). (B) Correlation between anti-HPA-1a titre and neonatal platelet count. The dashed red line is set at the threshold of severe thrombocytopenia ($50 \times 10^3/\mu\text{L}$). R^2 and P values were calculated using the non-parametric Spearman two-tailed test.



There was good correlation between antibody titre and antibody potency ($R^2 = 0.65$, $P = <0.0001$). However, for any given antibody titre a large range of potency values were observed e.g. with a titre of 1 in 4 the potency ranged from 3.1 to 20.0 i.u./mL.

The antibody titre and neonatal platelet count correlated poorly in this series.

5.4 Limitations of the study

There are two limitations to this study. Firstly, all of the examples of anti-HPA-1a used here were originally detected primarily using the MAIPA assay and using platelet $\alpha\text{IIb}\beta 3$ as target. There is therefore perhaps a bias of antibodies reactive against this molecule; screening a previously untested cohort of samples would provide a more realistic comparison of sensitivities. However, screening of the 23 samples from HPA-1b1b mothers with suspected NAIT where antibody screening assays using platelets were negative partially mitigated against this bias.

Secondly, as stated above, all of the samples were collected after the delivery of an affected infant and thus studies of the use of $\alpha\text{v}\beta 3$ to measure antibody levels through pregnancy was not possible.

5.5 Discussion and Conclusions

This chapter highlights that the detection and measurement of levels of anti-HPA-1a antibodies is far more complex than previously thought.

The use of a cell line expressing $\alpha\text{v}\beta 3$ (as a model for syncytiotrophoblasts), together with a derivative of this line that expressed $\alpha\text{IIb}\beta 3$, was extremely informative and showed that there is a complex relationship between the conformation and density of target integrin, the precise specificity of different examples of anti-HPA-1a and the apparent 'level' of those antibodies when assayed by the widely-used MAIPA assay. Differences in the conformation of the $\beta 3$ chain and thus the HPA-1a epitopes, either as result of *in vitro* conditions or as a result of association with alternate α -chains, have a significant impact on the reactivity of HPA-1a antibodies both in terms of detection and of measurement of antibody levels.

One of the hypotheses for this project was that the stimulus for the production of maternal antibodies against HPA-1a is syncytiotrophoblast particles rather than platelet $\alpha\text{IIb}\beta 3$, as suggested by Kumpel *et al* (Kumpel *et al*, 2008). We hypothesised that the $\beta 3$ chain and the HPA-1a epitopes might be orientated differently when associated with αv compared to when associated with αIIb and this might then affect the binding, detection and measured levels of anti-HPA-1a.

We successfully showed differences in $\beta 3$ chain and HPA-1a epitope accessibility between platelet $\alpha\text{IIb}\beta 3$ and $\alpha\text{v}\beta 3$ on our syncytiotrophoblast model cell line through the use of a panel of mAbs. It might be argued that these findings are not relevant to syncytiotrophoblasts. However, Vanderpuye showed that $\beta 3$ expression on placental microvilli was 4 - 8 fold less than on platelets and this agrees with our findings where we observed $\beta 3$ reactivity on M21 cells to be 7 - 21% of that of platelets, suggesting that our use M21 cells as a model is valid in terms of integrin expression levels and/or epitope accessibility (Vanderpuye *et al*, 1991).

We showed that the degree of reactivity of the mAbs against cell lines compared to platelets was related to their epitope specificity; antibodies against the $\beta 3$ PSI domain (e.g. SZ21) were more strongly reactive than antibodies against compound epitopes that depended on other $\beta 3$ domains e.g. Y2/51 suggesting that either conformational differences exist between platelet $\alpha\text{IIb}\beta 3$ and $\alpha\text{v}\beta 3/\alpha\text{IIb}\beta 3$ on cell lines and/or that epitope accessibility is different. Interestingly, both Vanderpuye and Kumpel failed to demonstrate reactivity of Y2/51 with microvilli using immunohistochemistry suggesting that the epitope is extremely conformation sensitive and that false negative results may be obtained in some circumstances (Vanderpuye *et al*, 1991; Kumpel *et al*, 2008).

Our data with anti- $\beta 3$ mAbs thus suggested that HPA-1a antibodies might react differently with cell lines depending on whether they were of Type I or Type II.

Using three anti-HPA-1a mAbs this was indeed shown to be the case, with the two Type I antibodies (23-15 and 19-7) showing reactivity against cell lines approximately 14 - 21% of that of platelets, whilst the Type II antibody B2 showed approximately 7% reactivity compared to that of platelets (**Table 5.2**).

It might therefore be expected that polyclonal HPA-1a antibodies might also show differential reactivity against $\alpha\text{IIb}\beta 3$ or $\alpha\text{v}\beta 3$ depending on whether they were Type I or II. The observation of Stafford that Type I antibodies can be detected in the majority of examples of NAIT-derived anti-HPA-1a, whilst Type II antibodies are present in only a small proportion, supports our hypothesis that the stimulating molecule is syncytiotrophoblast $\alpha\text{v}\beta 3$ (Stafford *et al*, 2008). Fragments of these cells are continually shed into the maternal circulation during pregnancy and since the PSI domain is more accessible, and the hybrid domain less accessible, on this molecule most antibodies might be expected to primarily recognise the PSI domain (Kumpel & Manoussaka, 2012). Conversely, since exposure to platelet $\alpha\text{IIb}\beta 3$ would occur only

in those cases where feto-maternal haemorrhage occurred (approximately 15% pregnancies), it would be expected that only a very small proportion of primigravidae would develop Type II antibodies. (Woodrow & Finn, 1966; Bowman *et al*, 1986). Thus it might be expected that the majority of NAIT-derived examples of anti-HPA-1a would preferentially react with $\alpha\text{v}\beta 3$.

The data presented here showed that the $\alpha\text{v}\beta 3$ -expressing cell line 'M21' was indeed better able to detect low levels of anti-HPA-1a than platelets (**Figure 5.13A**) whilst the related cell line 'M21-L11b' that expressed $\alpha\text{IIb}\beta 3$ gave similar reactivity to platelets (**Figure 5.13B**). These data therefore support our hypothesis that HPA-1a antibodies preferentially recognise $\alpha\text{v}\beta 3$, suggesting that reactivity against platelet $\alpha\text{IIb}\beta 3$ is only 'cross-reactivity' and thus the affinity of HPA-1a antibodies would be higher for $\alpha\text{v}\beta 3$ than $\alpha\text{IIb}\beta 3$. This is supported by the findings of Bakchoul, Peterson and Socher who observed high 'off-rates' of MAIPA-negative examples of anti-HPA-1a when using $\alpha\text{IIb}\beta 3$ as target in SPR assays (Socher *et al*, 2009; Bakchoul *et al*, 2011; Peterson, 2012).

The observed stronger reactivity of HPA-1a antibodies against integrin $\alpha\text{v}\beta 3$ is not only important in reinforcing our view that $\alpha\text{v}\beta 3$ may be the stimulating molecule in NAIT but also has other implications.

$\alpha\text{v}\beta 3$ is a key integrin in angiogenesis (Davis *et al*, 1993) and has been shown to be related to tumorigenic activity of tumour cells; cells lacking $\alpha\text{v}\beta 3$ (M21-L) exhibited reduced adhesion and proliferation compared to cells expressing $\alpha\text{v}\beta 3$ (M21) (Felding-Habermann *et al*, 1992; Felding-Habermann *et al*, 2002). $\alpha\text{v}\beta 3$ is also used by invading trophoblasts as an adhesion receptor and blocking of $\alpha\text{v}\beta 3$ by antibodies reduces their invasive capacity by 60 - 75% (Zhou *et al*, 1997; Merviel *et al*, 2001). Potentially therefore, blocking of $\alpha\text{v}\beta 3$ by maternal HPA-1a antibodies might also result in reduced invasion by the trophoblast. Reduced invasion has been shown to

reduce placental development and function and lead to IUGR (Brodsky & Christou, 2004). It would be important therefore if evidence of such effects of HPA-1a antibodies could be demonstrated.

In **Chapter 3**, we reported that HPA-1 antibodies were detected in 3/27 (11.1%) maternal samples referred for investigation of IUGR, thus providing some supporting evidence. More evidence was provided by Tiller who, in a study of 165 women with anti-HPA-1a, demonstrated an inverse linear relationship between the level of maternal anti-HPA-1a and the birth weight in male infants (Tiller *et al*, 2012). Thus there is mounting evidence for the role of $\alpha\text{v}\beta 3$ in NAIT.

Having shown that $\alpha\text{v}\beta 3$ was better able to detect low levels of anti-HPA-1a, we attempted to show a difference in fine epitope specificities of HPA-1a antibodies and a possible preference for $\alpha\text{v}\beta 3$ using two methods. Firstly we attempted to isolate the fine specificities of anti-HPA-1a using cross absorption and elution techniques. Although the use of eluates has been shown to be a sensitive method for detection of HPA-1a antibodies, in the experiments performed here it was not possible to demonstrate fine antibody specificities and both $\alpha\text{v}\beta 3$ and $\alpha\text{IIb}\beta 3$ were able to totally absorb all antibody, and testing of the eluates showed no differential reactivity (**Figure 5.16**) (Mueller-Eckhardt *et al*, 1982).

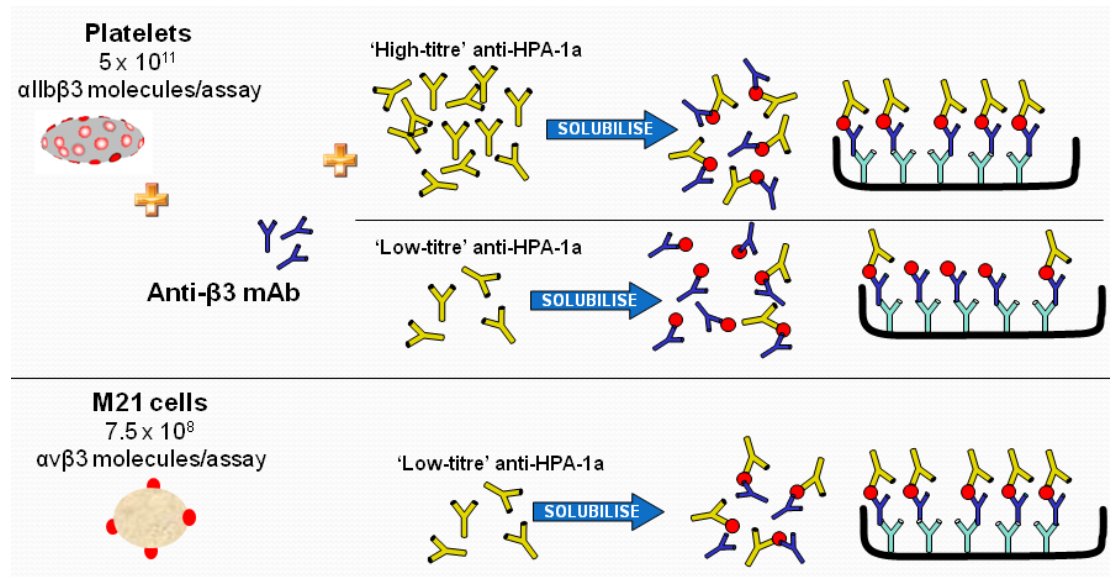
Secondly, we tested samples obtained from HPA-1b1b mothers in whom HPA-1a-mediated NAIT was suspected but where HPA-1a antibodies had not been detected. These experiments also failed to show examples of anti-HPA-1a that were reactive against $\alpha\text{v}\beta 3$ but not against $\alpha\text{IIb}\beta 3$. Clearly further work, using a much larger panel of samples is required to clarify the role of $\alpha\text{v}\beta 3$ as a target for detection of HPA-1a antibodies.

However, further proof of the different reactivity of the $\alpha\text{v}\beta 3$ expressing M21 cells was provided during the performance of quantitation assays. Here, titration of the NIBSC

high-potency anti-HPA-1a standard '03/152' clearly showed different curves when using $\alpha\text{v}\beta\text{3}$ compared to either platelet or M21-L11b $\alpha\text{IIb}\beta\text{3}$ (**Figure 5.22**) (Allen *et al*, 2005). Enumeration of the number of integrin molecules on these cells showed that two factors affected the ODs obtained.

The first factor was integrin number and density. Both M21 and M21-L11b cells were shown to have similar numbers of molecules present and at a similar density, both of which were significantly lower than on platelets (**Table 5.5**); this resulted in lower ODs against these cell types than against platelets when high levels of antibody were present. Thus integrin expression levels are important in determining strength of reactivity of HPA-1a antibodies as observed by Maslanka (Maslanka *et al*, 2012). We postulate that this is due to the dynamics of the MAIPA assay. At low levels of anti-HPA-1a some $\alpha\text{IIb}\beta\text{3}$ molecules on platelets will not have human alloantibody bound to them although they will still have bound capture mAb. When the platelet lysate is added to the wells of the microplate, only a proportion of the captured integrin molecules will therefore have human alloantibody attached. Using cells expressing lower integrin levels, all integrin molecules continue to be saturated with human alloantibody until eventually the same phenomenon occurs as with platelets; this hypothesis is shown graphically in **Figure 5.29**.

FIGURE 5.29. Cartoon showing effect of integrin number on MAIPA assay sensitivity.



We identified a second factor by using $\alpha v\beta 3$ and $\alpha\text{IIb}\beta 3$ in an otherwise identical membrane environment i.e. M21 and M21-LIIB cells since comparison of $\alpha v\beta 3$ on M21 cells and $\alpha\text{IIb}\beta 3$ on platelets potentially could have resulted in erroneous conclusions as integrins may behave differently in different environments; the use of the same cell line therefore removed this variable (Kieffer *et al*, 1991). We observed higher reactivity and different slopes of the titration curves against M21 cells compared to M21-LIIB cells suggesting that integrin conformation was important.

It might be argued that the difference was due to poor or incorrect expression of $\alpha\text{IIb}\beta 3$ in the M21-LIIB cells. However, it has been shown previously that these cells have complete post-translational processing and surface expression of $\alpha\text{IIb}\beta 3$ and the identical curves obtained with platelets and M21-LIIB cells at low levels of anti-HPA-1a also suggest that the molecule is expressed similarly between these cells (Cheresh & Spiro, 1987; Kieffer *et al*, 1991). It must therefore be concluded that HPA-1a antibodies recognise epitopes on $\alpha v\beta 3$ that are different to $\alpha\text{IIb}\beta 3$.

This observation has important repercussions since all studies on the relationship between maternal anti-HPA-1a levels and neonatal platelet count have used $\alpha\text{IIb}\beta 3$ as target rather than $\alpha\text{v}\beta 3$.

With respect to $\alpha\text{IIb}\beta 3$, in **Chapter 4** we showed that cation chelation affected assay sensitivity. In this chapter we show that cation chelation affects the measured level of antibodies. These findings are extremely important. Killie showed that the capture mAb used affected the measured level of anti-HPA-1a but did not provide an explanation for this (Killie *et al*, 2010). Our studies suggest that their findings were possibly due to the use of EDTA plasma samples.

Furthermore, not only does the change in integrin conformation induced by cation chelation affect the measured level of HPA-1a antibodies but also the change in conformation induced by different α -chains together with integrin number and density. Although we studied only a limited number of samples, it can be seen that in three cases (F0771, F1016 and F1071), the level falls on either side of the 3 i.u./mL threshold (determined by Killie to be the level at which severe thrombocytopenia was likely in the neonate) depending on which integrin was used ($\alpha\text{v}\beta 3$ or $\alpha\text{IIb}\beta 3$), and the number/density of that integrin (Killie *et al*, 2008).

Since integrin number affects the measured level of antibody it suggests that the use of platelets homo- or heterozygous for HPA-1a might produce different results; although this was not studied here it is an important factor that needs to be considered.

The finding that the measured levels of antibody depended on whether $\alpha\text{v}\beta 3$ or $\alpha\text{IIb}\beta 3$ was used was explored in more detail by studying the relationship between maternal anti-HPA-1a level and neonatal platelet count at birth when using the different integrins. The use of $\alpha\text{v}\beta 3$ (**Figure 5.27A**) did not improve the correlation between maternal antibody level versus neonatal platelet count over that observed

when using platelet $\alpha\text{IIb}\beta 3$ when using serum samples (**Figure 5.27**). However, the number of samples available was very small and all were collected post-delivery; the level of antibody in such samples has been shown not to correlate with neonatal platelet count (Killie *et al*, 2008).

Since this study had shown that the presence of EDTA had an adverse effect on measured antibody levels and some published studies used EDTA plasma when measuring antibody levels, the sensitivity of the MAIPA assay for predicting severe thrombocytopenia was investigated when using only serum samples and platelets as target. The assay showed a sensitivity of 76.9% when using 3 i.u./mL as a threshold. This was below the figure of 93% reported by Killie (Killie *et al*, 2008) who used the same threshold but similar to that of Bertrand (Bertrand *et al*, 2011) who used a revised threshold of 28 i.u./mL and observed a sensitivity of 82%. However, since our samples are all post-delivery samples it is perhaps surprising that such a high sensitivity was observed.

The data thus far suggest that $\alpha\text{v}\beta 3$ is more sensitive than $\alpha\text{IIb}\beta 3$ for the detection of low levels of anti-HPA-1a and that this is due to the different accessibility of the HPA-1a epitopes on $\alpha\text{v}\beta 3$. The recent development of recombinant fragments of $\beta 3$ that have been coupled to Luminex beads provided an opportunity to compare the effect of the absence of an α -chain with that of $\beta 3$ associated with different α -chains; this was of particular interest since the deletion of the β -A domain apparently increased sensitivity of detection of HPA-1a antibodies by 6% suggesting that there was masking of HPA-1a epitopes on the PSI domain by the β -A domain (Stafford *et al*, 2008; Chong *et al*, 2011).

The data obtained from testing a panel of anti-HPA-1a samples showed that, as in **Chapter 4** with the GTI ELISA assay, immobilised $\beta 3$ fragments on the Luminex beads were unaffected by the presence of EDTA whereas cellular $\alpha\text{IIb}\beta 3$ was

affected, reinforcing the conclusion that conformational changes in α IIb β 3 on platelets (and M21-LIIB cells) were responsible for loss of assay sensitivity. Excluding the data obtained with EDTA-anticoagulated samples therefore, comparison of the Luminex assay with that of MAIMA and MAIPA assays showed that no single assay was able to detect all examples of anti-HPA-1a (**Table 5.7**). However, the M21 MAIMA assay was again shown to be the most sensitive assay with a sensitivity of 94.1% versus 91.2% for all other assays (**Table 5.9**).

These data therefore suggest that it would be informative to undertake an antenatal screening programme in which samples were collected during pregnancy and screened for the presence of, and levels measured of, HPA-1a antibodies using β 3 in association with different α -chains and in the absence of an α -chain to determine if additional antibodies might be detected and/or a closer correlation between antibody level and neonatal platelet count might be observed.

Prior to such a study however, it would be useful to investigate whether increasing the number of integrin α v β 3 molecules expressed on M21 cells resulted in further increases in assay sensitivity or whether this simply would mirror the situation seen with platelets where the higher number of integrin molecules led to reduced assay sensitivity. Hayashi showed that expression of CD109 (and HPA-15 antigens) on K562 cells at levels 300 - 9,000 times that of platelets was technically possible and this resulted in strong reactivity in the MAIPA assay (Hayashi *et al*, 2010). Alternatively, the use of using other cell lines e.g. HUVECs could be explored; these cells express α v β 3 at >500,000 copies/cell (Byzova *et al*, 1998) and have been shown to express HPA-1a (Radtke *et al*, 1991).

It would also be informative to study the affinity of HPA-1a antibodies for α v β 3 and α IIb β 3 although this may be problematic since purifying and immobilising these integrins might induce conformational changes thus making affinity data less clear

and the method of purifying integrins has been shown to affect the results obtained (Bessos *et al*, 2008b).

In summary therefore, whilst the data presented in **Chapter 4** showed that the conformation of $\alpha\text{IIb}\beta 3$ was critically important for HPA-1a antibody detection when using the MAIPA assay, the work in this chapter shows that integrin conformation and density also affects the measured level of HPA-1a antibody.

This is an extremely important finding since two of the three large antenatal screening studies have used EDTA plasma samples when detecting and measuring levels of anti-HPA-1a antibodies using the MAIPA assay (Williamson *et al*, 1998; Bertrand *et al*, 2005; Killie *et al*, 2008). Whilst the studies of Williamson and Bertrand used HPA-1a homozygous platelets, the zygosity of the platelets in the study of Killie *et al* was not stated. Clearly, if different levels of antibody are measured in serum versus EDTA plasma samples and the use of heterozygous (HPA-1a1b) platelets compounds this effect then the interpretation of existing data regarding the relationship between the maternal HPA-1a antibody level and neonatal platelet count would change.

Importantly, larger studies are required to determine if a better correlation between antibody level and neonatal platelet count and/or outcome could be achieved by the use of $\beta 3$ in association with a different α -chain i.e. $\alpha\text{v}\beta 3$, since this is the molecule against which mounting evidence suggests is the primary immunising molecule in NAIT. If a better relationship were demonstrated it would overcome one of the failings of existing assays with respect to the WHO criteria for the introduction of an antenatal screening programme (Williamson, 1998; Murphy & Williamson, 2000; Murphy *et al*, 2002b). Further studies are also required on the role of HPA-1a antibodies and $\alpha\text{v}\beta 3$ in IUGR.

5.6 Summary of findings in this chapter

- The cell line used in this study as a model for syncytiotrophoblasts (M21) and its' companion line (M21-LIIB) were shown to express $\beta 3$ but at a lower level than on platelets. The level of reduction in expression was shown to be dependent on specificity of the mAb used.
 - Least reduction was observed with mAbs directed against αIIb (HIP8) and the $\beta 3$ PSI domain (SZ21 and 'Type I' HPA-1a mAbs).
 - The highest reduction in expression was observed with mAbs recognising epitopes involving, the hybrid domain (Y2/51 and 'Type II' anti-HPA-1a B2), and clones recognising the $\alpha\text{IIb}\beta 3$ complex (P2, PAB-1, PAB-6, 5B12).
- Using an immunofluorescence assay, it was possible to demonstrate reactivity of polyclonal HPA-1a antibodies against cell lines expressing $\alpha\text{v}\beta 3$.
- It is possible to modify the MAIPA assay so that melanoma cells expressing $\alpha\text{v}\beta 3$ or $\alpha\text{IIb}\beta 3$ may be used ('MAIMA' assay).
- Using this MAIMA assay in parallel with the standard MAIPA assay it was demonstrated that some examples of anti-HPA-1a react more strongly against cell lines expressing $\alpha\text{v}\beta 3$ than against platelets and others react less well. The reasons for this were identified as:
 - Anticoagulant used in test sample. EDTA plasma samples reacted less well against $\alpha\text{IIb}\beta 3$ on M21-LIIB cells and platelets than against $\alpha\text{v}\beta 3$ on M21 cells due to the sensitivity of $\alpha\text{IIb}\beta 3$ to cation chelation.
 - Integrin density. The M21 cell line expressed lower levels of integrin than platelets and thus the antibody-binding sites became saturated at

lower levels of antibody compared to platelets.

- The membrane environment surrounding the integrin, demonstrated by the different reactivity and susceptibility of $\alpha\text{IIb}\beta 3$ to cation chelation on platelets and M21-LIIb cells.
- When using serum samples, $\alpha\text{v}\beta 3$ on M21 cells was more sensitive than platelet $\alpha\text{IIb}\beta 3$ for the detection of low levels of anti-HPA-1a. This difference was not due to integrin density since $\alpha\text{IIb}\beta 3$ on M21-LIIb cells (present at similar density to $\alpha\text{v}\beta 3$ on M21 cells) was shown to be less sensitive than platelet $\alpha\text{IIb}\beta 3$ for the detection of anti-HPA-1a.
- Molecular modelling demonstrated differences in the key regions recognised by 'Type I' and 'Type II' HPA-1a antibodies between $\alpha\text{v}\beta 3$ and $\alpha\text{IIb}\beta 3$ suggesting that, as discussed above, some examples of anti-HPA-1a might preferentially react with $\alpha\text{v}\beta 3$.
- It was not possible to demonstrate fine specificities recognising $\alpha\text{v}\beta 3$ or $\alpha\text{IIb}\beta 3$ using absorption/elution experiments in the one example of polyclonal HPA-1a tested.
- The use of $\alpha\text{v}\beta 3$, and $\alpha\text{IIb}\beta 3$ in different membrane environments resulted in different measured levels of anti-HPA-1a.
- The correlation between measured anti-HPA-1a level and neonatal platelet count was similar using the various integrins/cell types.
- The M21 MAIMA assay is more sensitive than the platelet MAIPA and is equally sensitive to a next-generation recombinant protein assay ('Luminex').

5.7 Significance of these findings

These data clearly show the importance of integrin conformation and density when using the MAIPA assay to screen samples for the presence of HPA-1a antibodies and when assessing the amount of antibody present.

The presence of large numbers of $\beta 3$ integrin molecules e.g. on platelets, results in higher assay sensitivity when high levels of anti-HPA-1a are present but has the opposite effect when low levels of antibody are present and can result in false negative results. This is an important observation and needs to be taken into account when developing and standardising MAIPA assays.

The work in **Chapter 4** regarding EDTA-sensitivity of $\alpha\text{IIb}\beta 3$ was confirmed and extended. Not only did the disruption of $\alpha\text{IIb}\beta 3$ by EDTA result in variable detection of anti-HPA-1a but it also variably affected the measured levels of antibody; this varied widely between different examples of anti-HPA-1a when assaying serum versus EDTA plasma samples. In some cases there was no difference in measured level e.g. cases F0948 and F1071 (**Table 5.3**), whilst in others the difference was extreme e.g. cases F0947 and F0964. This is of fundamental importance in studies of the relationship between level of maternal anti-HPA-1a and neonatal platelet count.

More importantly, the measured level of anti-HPA-1a also varied depending on whether $\alpha\text{v}\beta 3$ or $\alpha\text{IIb}\beta 3$ was used as a target when using serum samples i.e. avoiding confounding effects of EDTA. In some cases the antibody levels were similar e.g. cases F0948 and F1071, whilst others showed higher values against $\alpha\text{v}\beta 3$ than $\alpha\text{IIb}\beta 3$ (cases F0975 and F1016) and a third group showed higher reactivity against $\alpha\text{IIb}\beta 3$ than against $\alpha\text{v}\beta 3$ (F0892, F1037). These data suggest that the relationship between the measured levels of anti-HPA-1a when using $\alpha\text{v}\beta 3$ and $\alpha\text{IIb}\beta 3$ and the neonatal platelet count need to be explored in larger studies.

Chapter 6.

Effect of binding of HPA-1a
antibodies on platelet
function and platelet
production

6.1 Introduction

Following tissue damage, platelets are attracted to, and provide the primary haemostatic plug for, the injury. A key receptor in the process of plug formation is $\alpha\text{IIb}\beta 3$ which plays a role in platelet aggregation, spreading of platelets across the tissue damage to form a primary barrier, and ultimately in clot retraction (Kasirer-Friede *et al*, 2007). Before this integrin can participate in these processes it must convert from the resting, inactive folded state that is unable to bind ligands to the activated, extended form with the headpiece in an open conformation that is capable of binding ligand (Xiao *et al*, 2004). This conversion process occurs as a result of 'inside-out' signalling transmitted across the platelet membrane and which is amplified through the PSI and hybrid domains resulting in an extended headpiece, separated 'legs' or tailpieces and swing-out of the hybrid and PSI domains (Xiao *et al*, 2004; Mould *et al*, 2005).

The hybrid domain is pivotal in this transformation. The prevention of swing-out of this domain (and of the PSI domain to which it is rigidly attached) prevents $\alpha\text{IIb}\beta 3$ from becoming activated (Takagi *et al*, 2003; Luo *et al*, 2004; Luo *et al*, 2007; Kamata *et al*, 2010). Conversely, the binding of mAbs to the PSI domain results in activation of $\alpha\text{IIb}\beta 3$ independently of inside-out signalling, possibly by a simple mechanical effect of levering apart the PSI and calf-1 domain of the α -chain, resulting in separation of the α and β 'knees' (Mould *et al*, 2005).

Binding of HPA-1a antibodies has been shown to block the binding of fibrinogen to cell lines expressing $\alpha\text{IIb}\beta 3$, probably by a simple steric hindrance effect since the lines used were incapable of 'inside-out' signalling and the HPA-1a epitopes are located near the fibrinogen binding site (Kroll *et al*, 2005a). Such steric effects were also suggested by Beadling (Beadling *et al*, 1995). Whether HPA-1a antibodies are able to exert inhibitory or activatory effects by preventing swing-out of, the PSI (and hybrid) domains is unknown. Such effects might be important in NAIT since inhibition

of platelet function might contribute to the bleeding effects observed in some cases, and activation of $\alpha\text{IIb}\beta 3$ might up-regulate recycling of integrin $\alpha\text{IIb}\beta 3$ to the internal pool thus rendering the platelets less adhesive (Schober *et al*, 2003).

Many methods have been used to study platelet function, although fewer have been used to study potential blocking effects of HPA-1a antibodies. Early studies using serotonin release and platelet aggregation demonstrated inhibitory effects of two examples of anti-HPA-1a obtained from patients with PTP and two from mothers of NAIT-affected children (van Leeuwen *et al*, 1984; Duncan & Rosse, 1986; Ryu *et al*, 1990). Subsequently, two groups specifically studied the interaction of fibrinogen with $\alpha\text{IIb}\beta 3$ and identified differences between PTP- and NAIT-derived examples of anti-HPA-1a. Using radio-labelled fibrinogen, Beadling reported that whilst six of seven of PTP-derived examples inhibited binding of this ligand to activated platelets, none of twelve NAIT-derived examples were able to do so (Beadling *et al*, 1995). These findings were confirmed by Kroll who used flow cytometry to study the binding of fibrinogen to HPA-1a positive and HPA-1a negative $\alpha\text{IIb}\beta 3$ expressed in a CHO cell line (Kroll *et al*, 2005a).

There are limitations to these studies. Aggregation assays do not necessarily mirror primary haemostatic effects that occur *in vivo* (Harrison, 2009), whilst the use of cell lines does not take into account trans-membrane signalling observed in platelets (Kroll *et al*, 2005a). In this study therefore, we investigated a novel application of the ROTEM® *delta* analyser to study the effects of HPA-1a antibodies on global haemostasis to potentially overcome the deficiencies described above. This assay uses the principles of rotational thrombo-elastometry to measure global haemostatic abilities of whole blood. It potentially has the ability to assess changes in platelet function induced by defects of $\alpha\text{IIb}\beta 3$ e.g. Glanzmann's thrombasthenia (Lang & von Depka, 2006) and blockade by HPA-1a antibodies although this has not been investigated to date.

We aimed to compare the effect of HPA-1a antibodies on platelet function measured using this assay with inhibitory effects observed by a more conventional (immunofluorescent) fibrinogen binding assay. We also investigate inhibitory effects using other markers of platelet activation, namely CD62P and PAC-1 epitope which have not previously studied in the context of HPA-1a antibodies.

In addition to potentially compromising platelet function, HPA-1a antibodies might also contribute to the severity of thrombocytopenia by inhibiting platelet production.

$\alpha\text{IIb}\beta 3$ can be detected on cultured megakaryocytes on days 5 - 6 (Rabellino *et al*, 1981; Vainchenker *et al*, 1982; Vinci *et al*, 1984; Chang *et al*, 2007). Adsorption of the maternal antibodies onto megakaryocytes might therefore result in low levels of circulating maternal anti-HPA-1a and/or negative screening assays. This is perhaps supported by the finding that, notwithstanding our findings in earlier chapters, in some cases of NAIT the level of maternal HPA-1a antibodies appears to be extremely low and yet the thrombocytopenia in the neonate is severe (Bertrand *et al*, 2006; Killie *et al*, 2008). Additionally there are many reports of clinically suspected NAIT where maternal antibodies cannot be detected (Mueller-Eckhardt *et al*, 1989; Dreyfus *et al*, 1997; Turner *et al*, 2005).

In vitro studies have shown that it is possible to inhibit platelet production using mAbs directed against $\alpha\text{IIb}\beta 3$ (Takahashi *et al*, 1999). However few studies have been performed using human antibodies.

Perdomo reported that quinine-induced antibodies were able to inhibit platelet production via their interaction with GPIb/IX whilst Greinacher was able to show that antibodies against the $\alpha\text{IIb}\beta 3$ antagonist eptifibatide were able to cause impairment of megakaryopoiesis (Greinacher *et al*, 2009; Perdomo *et al*, 2011).

Only one study has been published on the effect of HPA-1a antibodies on platelet

production and this was limited to three examples (Warwick *et al*, 1994).

In this chapter therefore, we also aimed to assess the inhibitory effects of the same panel of NAIT-derived HPA-1a antibodies used in the platelet function studies on megakaryopoiesis in order that we could determine the relationship between the level of maternal antibodies and their ability to inhibit platelet function and platelet production.

6.2 Methods

6.2.1 Preparation of purified IgG to be used in flow cytometric and ROTEM® *delta* assays

Samples were de-salted using Zeba-Spin columns and IgG prepared using Melon Gel spin purification kits as described (**Chapter 2.4.9**). One example of purified IgG was subjected to SDS-PAGE electrophoresis to determine purity and protein content relative to the original material (**Chapter 2.4.10**).

All IgG samples were assessed for anti-HPA-1a levels using a quantitative MAIPA as described previously (**Chapter 2.4.8**).

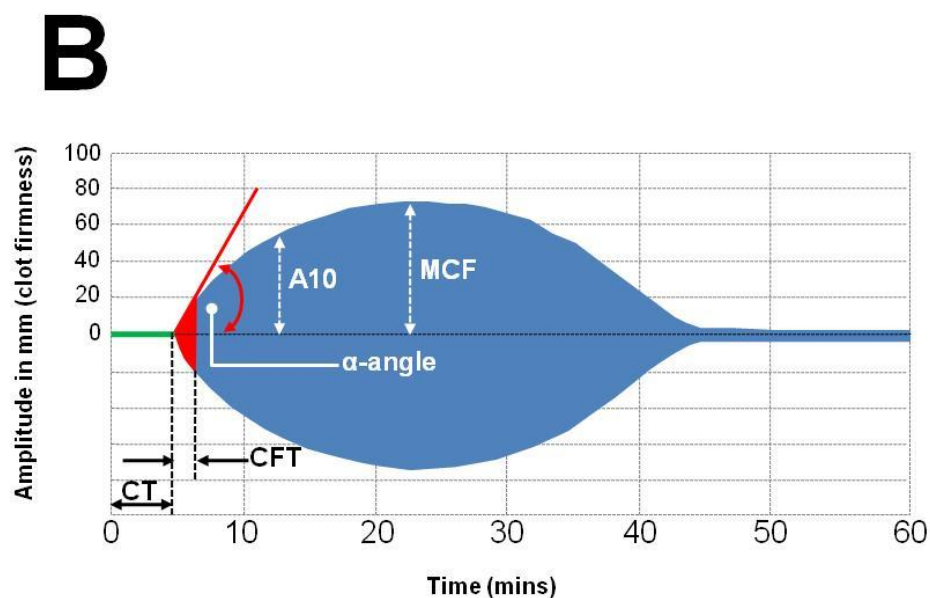
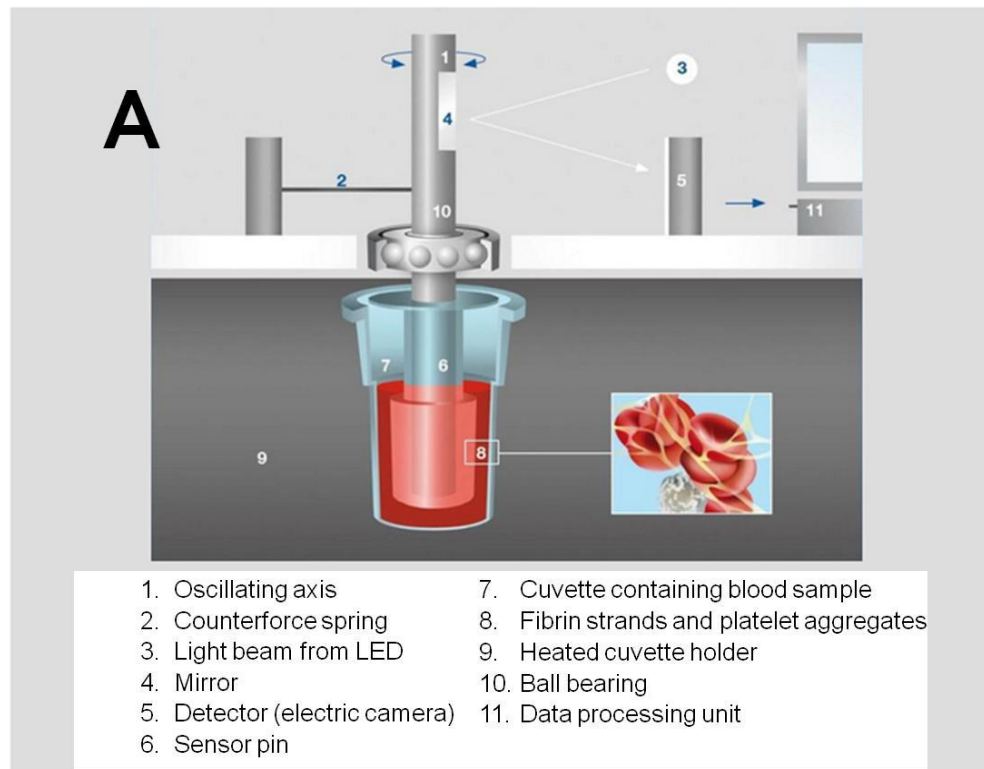
6.2.2 ROTEM® *delta* assay

The principle of the assay is shown in **Figure 6.1**.

FIGURE 6.1. Principle of the ROTEM assay. (A). The viscosity of whole blood is assessed by oscillation of a spindle (6) within a cuvette (7) (maintained at 37°C) containing whole blood. Light emitted from an LED (3) is reflected from a mirror (4) located on the spindle. Changes in reflected light are detected by a sensor (5) and analysed by the data processing unit (11). Coagulation is initiated by addition of activator reagents appropriate to the type of assay being performed and the increasing viscosity that occurs during coagulation results in decreasing movement of the spindle. (B) The degree of movement of the spindle is converted by software into a 'TEMogram' from which a large array of data on the kinetics of clot formation may be obtained, some of which are apparent in **Figure B**.

Following addition of the activator ('0 mins'), the time taken to commencement of clotting is recorded as the clotting time ('CT', green trace). The clot formation time ('CFT', red shaded area) is a measure of how rapidly the clot is forming and is the time taken for the clot to expand from 2 mm to 20 mm in amplitude. The α -angle (red line) is the angle between the middle axis of the profile and the 2 mm amplitude point and is also a measure of the kinetics of clot formation. The clot amplitude may be measured at various time points (e.g. 10 mins, 'A10') after clotting has started and the values indicate the firmness of the clot at those time points. The clot area is a measure of the size of the clot and can also be measured at different time points

('ARx'). The maximum clot firmness (MCF) is a measure of the maximum firmness of the clot and is dependent on the interaction of platelets with fibrinogen.



Images adapted from ROTEM®.

6.2.2.1 Preparation of test samples

Master solutions (total volume 75 μ L) of purified IgG containing anti-HPA-1a at a particular activity (i.u./mL) were prepared based on the required level of anti-HPA-1a activity (i.u./mL) in the final reaction volume to be used in the ROTEM assay. In some cases 75 μ L of undiluted sample was used, in others samples were diluted in TBS.

The master solution was added to 300 μ L fresh whole blood and then incubated at 37°C for 30 mins. All blood samples were maintained at 37°C prior to use and were used within 4 hrs of venesection including 'run-time' on the ROTEM® *delta* analyser.

6.2.2.2 Demonstration of function-blocking effects of HPA-1a antibodies in the ROTEM® *delta* assay

The 'EXTEM' ROTEM® assay was performed according to the manufacturers' instructions. This assay uses tissue factor to initiate the extrinsic coagulation pathway and whilst it does not measure platelet function *per se*, it does take account of the contribution of platelets to haemostasis

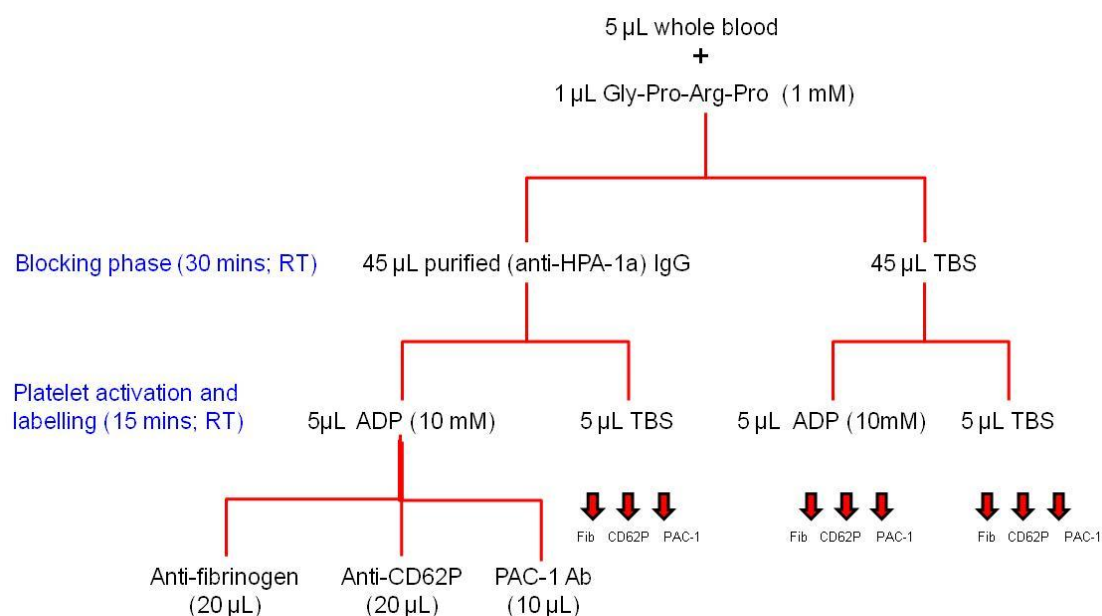
All reagents were allowed to warm to RT for at least 15 mins prior to use. New reaction pins were carefully placed on the ROTEM® *delta* probes and new reaction cuvettes seated firmly in the magnetic cuvette holders. Using the automated pipetting system, 20 μ L of 'r ex-TEM®' was aspirated from the reagent vial, an air bubble aspirated to separate the reagents, followed by 20 μ L Star-TEM® reagent. These reagents were dispensed carefully into the base of the reaction cuvette. 300 μ L blood sample, prepared as described above, was aspirated from the sample tubes, dispensed into the reaction cuvettes and mixed thoroughly with the pre-dispensed reagents. The reaction chamber was placed on the reaction pin immediately and the assay started. The reaction was allowed to proceed for approximately 60 mins.

6.2.3 Demonstration of function-blocking effects of HPA-1a antibodies by flow cytometry

To 5 μ L of donor whole blood was added 1 μ L Gly-Pro-Arg-Pro amide (final concentration 1 mM) to prevent fibrin polymerisation (Harfenist *et al*, 1982). Dilutions (in TBS /BSA) of purified IgG were prepared such that the final HPA-1a activity level in the assay (or protein level in negative controls and B2 anti-HPA-1a mAb) was the same as that used in the ROTEM® *delta* assay. 45 μ L diluted IgG was added to the blood samples and the samples incubated at 37°C for 30 mins.

To each tube was 5 μ L ADP (10 μ M final concentration) or 5 μ L TBS followed by 20 μ L FITC-conjugated anti-fibrinogen (diluted 1 in 5), 10 μ L FITC-conjugated PAC-1 antibody (neat) or 20 μ L FITC-conjugated anti-CD62P (neat). Samples were incubated at RT for 20 mins then 1 mL cold TBS/BSA added and the results analysed on a BD LSRII flow cytometer using FACSDiva software (Becton Dickinson). This method is summarised in **Figure 6.2**.

FIGURE 6.2 Method for assessing effect of HPA-1a antibodies on platelet activation.



Data was analysed as mean fluorescence intensity (MFI) and as percent positive cells. To assess percentage positive cells, a region encompassing approximately 2% of the cells within a relevant negative control was plotted then this region was applied to all other tests that related to that control.

6.2.4 Inhibition of megakaryocyte growth by HPA-1a antibodies

6.2.4.1 ABO grouping of cord blood

A donation of fresh cord blood was obtained from NHSBT and a 0.5 mL sample extracted on which an ABO grouping test was performed as follows:

The sample was washed twice in TBS then re-suspended to an approximate 5% suspension in TBS. 50 μ L monoclonal anti-A (clone BIRMA-1, Sanquin Diagnostics, Netherlands) and 50 μ L monoclonal anti-B (clone LB-2, ImuMed, Germany) were placed onto glass microscope slides. 50 μ L cell suspension was mixed thoroughly with the anti-A and anti-B and the slide rocked gently for 5 mins. The slides were then examined macroscopically for evidence of agglutination. Absence of agglutination confirmed that the unit was blood group 'O' and thus suitable for use.

6.2.4.2 Extraction of mononuclear cells (MNCs)

MNCs were isolated using density gradient separation. 15 mL volumes of Ficoll-Paque PLUS (density 1.077) were dispensed into 50 mL Falcon tubes. The cord blood was diluted 1 in 2 in MACS-BSA then layered carefully on to the surface of the Ficoll-Paque PLUS and the tubes centrifuged at 400 x *g* for 30 mins.

The MNC layer was removed carefully and dispensed into fresh 50 mL Falcon tubes. Approximately 45 mL MACS-BSA was added and the tubes centrifuged (400 x *g*, 10 mins).

The supernatant was removed, a further 45 mL MACS-BSA added and the tubes centrifuged (200 x *g*, 10 mins). The supernatant was removed and 10 μ L cell pellet

re-suspended in 190 μ L MACS-BSA buffer. A cell count was performed and the cells frozen in 10% DMSO in MACS-BSA buffer in aliquots of approximately 1×10^8 cells per vial.

6.2.4.3 Isolation of CD34+ cells and assessment of cell viability

CD34+ cells were isolated using a paramagnetic bead separation system.

One vial of cryopreserved MNCs was placed at 37°C until the ice had almost disappeared then the vial contents dispensed into 8 mL MACS-Rinse buffer. The cells were then pelleted by centrifugation (300 x *g*, 10 mins, 4°C). After removal of the supernatant the cells were re-suspended in 300 μ L MACS-Rinse buffer.

To the cell pellet was added 100 μ L FcR block (Miltenyi, Biotec, UK) then 100 μ L CD34 beads (Miltenyi Biotec, UK). The suspension was incubated at 4°C for 30 mins to allow the cells to adhere to the beads.

5 mL MACS-Rinse buffer was added and the cells pelleted (300 x *g*, 10 mins at 4°C).

The supernatant was removed and the cells re-suspended in a further 5 mL MACS-Rinse buffer.

A MACS-LS column (Miltenyi Biotec, UK) was prepared by rinsing the column with 5 mL MACS-Rinse buffer. The column was placed in MACS separator magnet, the cell suspension passed through a 30 μ M filter and then into the column.

The non-CD34+ cells were allowed to flow through the column and collected for enumeration and viability assessment.

The column was removed from the magnet and the CD34+ cells eluted from the column by passing 5 mL MACS-Rinse solution through the column.

A cell count and viability assessment was performed on the eluted cell suspension

using a Vi-CELL analyser (Beckman-Coulter, UK), the cells pelleted (300 x *g*, 4°C, 10 mins) and then re-suspended in 0.9 mL of Iscove's MDM.

6.2.4.4 Culture of megakaryocytes

Megakaryocytes were cultured using a MegaCult-C kit (STEMCELL technologies, UK) using a semi-solid medium as a support matrix.

To 1 mL aliquots of MegaCult-C medium was added 13.3 µL low-density lipoproteins to achieve a concentration in the final reaction mix of 40 µg/mL; 50 µL of the cell suspension was then added to the tubes.

To investigate the inhibitory effects of HPA-1a antibodies on megakaryocyte growth 195 µL purified IgG was added to the tubes. Two controls were similarly prepared; one tube to which 195 µL purified IgG from a group AB non-transfused male blood donor was added and another to which 195 µL of Iscove's medium was added.

600 µL of cold collagen solution was added to each tube, the contents mixed thoroughly then 750 µL dispensed immediately into each of two chambers of MegaCult slides. The final dilution of the anti-HPA-1a IgG was therefore approximately 1 in 9.5.

The slides were placed into a large Petri dish, a small, open Petri dish containing sterile distilled water, placed inside the large Petri dish to maintain humidity and the dishes incubated in a 37°C tissue culture incubator in 5% CO₂ in air.

6.2.4.5 Visualisation of megakaryocyte growth

All staining procedures were performed according to the manufacturers' instructions.

6.3 Results

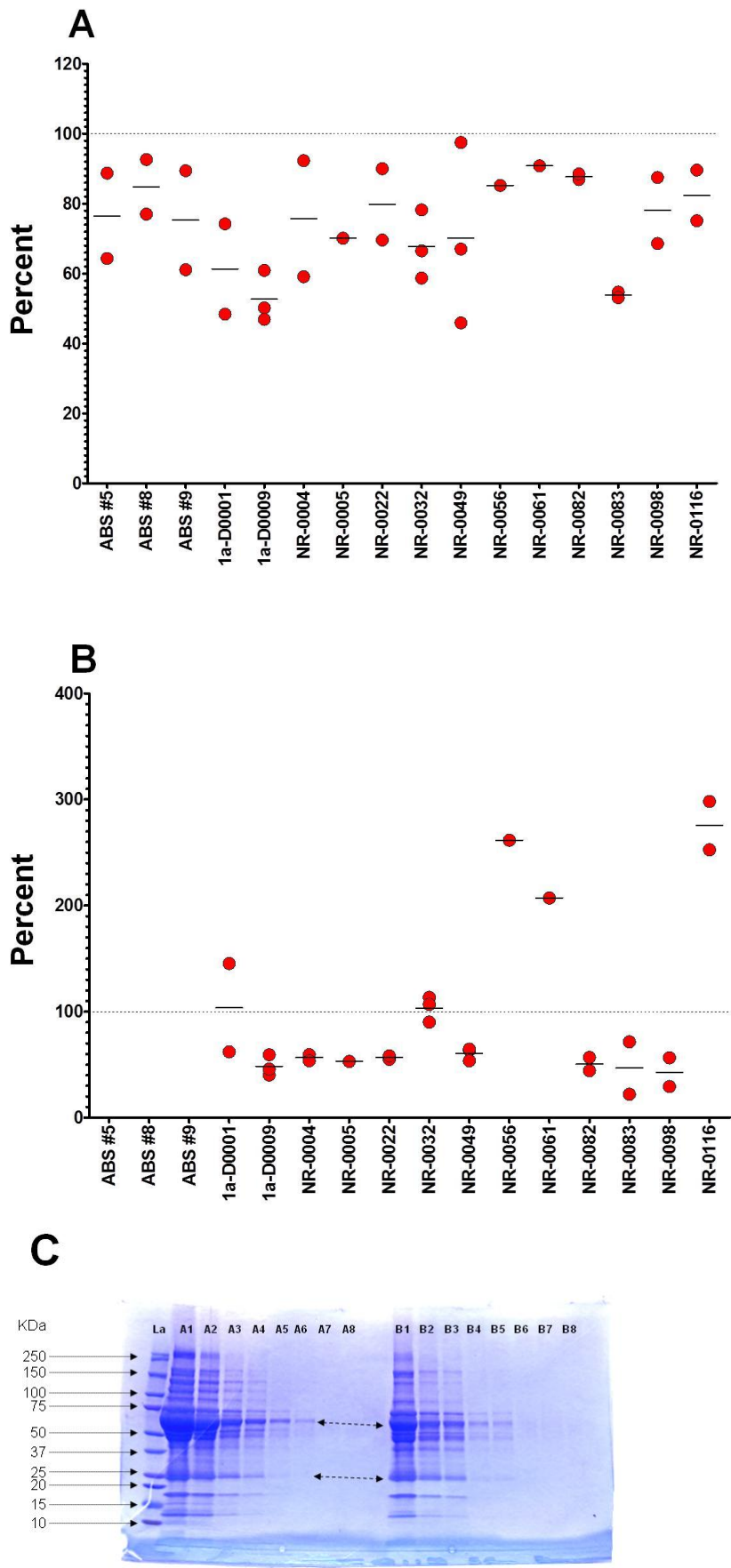
6.3.1 Purity of purified IgG

A total of 32 purifications on 16 examples of anti-HPA-1a were performed. The protein concentration of the purified IgG ranged from 45.9% to 97.5% (mean 72.8%) of that of the original material (**Figure 6.3A**).

Quantitation of HPA-1a antibody levels showed a mean of 93.2% antibody activity remained in the sample after purification (range 22.1 – 261.5%) (**Figure 6.3B**).

After completion of the inhibition studies, SDS-PAGE analysis of one example of purified IgG (chosen at random) showed that IgG was not the only component present but that IgG (as segregated heavy and light chains) remained in a much higher proportion compared to most other proteins (**Figure 6.3C**).

FIGURE 6.3. Protein concentration, anti-HPA-1a level and purity of purified IgG compared to starting material. (A) Protein concentration in purified IgG as percentage of protein concentration in original sample. The horizontal bar indicates the sample mean. (B) Anti-HPA-1a level in purified IgG as percentage of HPA-1a antibody level in original sample. The horizontal bar indicates the sample mean. (C) SDS-PAGE electrophoresis of original material and purified IgG from one sample (NR-0032) tested at a range of sample dilutions (indicated in key). The molecular weight of the bands is indicated beside the molecular weight ladder. The heavy (50 kD) and light (25 kD) chains of IgG are arrowed.



6.3.2 Demonstration of function-blocking effects of HPA-1a antibodies by flow cytometry

6.3.2.1 Binding of PAC-1 antibody

PAC-1 recognises an epitope near to the fibrinogen binding site that is only exposed upon activation and extension of $\alpha\text{IIb}\beta 3$ (Shattil *et al*, 1985; Shattil *et al*, 1987) and thus is potentially able to demonstrate inhibition of integrin extension by HPA-1a antibodies.

The binding of PAC-1 antibody to platelets following their exposure to the purified anti-HPA-1a or control IgG was assessed by indirect immunofluorescence using flow cytometry (**Table 6.1**).

TABLE 6.1. Effect of HPA-1a antibodies on PAC-1 binding. *The figures show the averaged MFI values from all experiments with ADP-activated platelets. Significance of results was assessed using the unpaired t-test (*P = <0.05; **P = <0.01; ***P = <0.0001). The purified anti-HPA-1a IgG data were analysed with respect to pooled data from the negative controls (purified IgG from the group AB serum non-transfused male blood donors at 5 mg/mL) (P-ABS). Significance of the B2 data was assessed with respect to the TBS control (P-TBS) Figures in brackets indicate only that only one assay was performed due to limited availability of test sample; statistical analysis could not be performed on these samples. Cells shaded red indicate a significant reduction in binding of PAC-1 antibody; cells shaded green indicate a significant increase in bound PAC-1 antibody compared to controls.*

Sample ID	iu/mL / *ng/mL	HPA-1a1a						HPA-1b1b					
		Mean MFI	SD	N	P-TBS	P-ABS		Mean MFI	SD	N	P-TBS	P-ABS	
TBS		1,475	252	11				1,645	545	4			
Neg controls 5, 8, 9		1,490	265	9	0.97			nt	na	0			
B2	50*	15	3	3	0.01*			2,232	1,069	2	0.61		
B2	10*	29	15	3	0.01*			1,660	668	2	0.99		
B2	5*	37	21	3	0.01*			1,482	611	2	0.87		
B2	1*	178	97	3	0.02*			1,414	535	2	0.81		
B2	0.1*	1,499	1,000	2	0.97			1,244	479	2	0.67		
B2	0.01*	1,499	955	2	0.97			1,100	307	2	0.55		
NR-0004	6.5	2,001	1,841	2		0.58		(2,059)	I.D.	1		I.D.	
NR-0005	5	714	240	3		0.14		nt	nt	0		nt	
NR-0022	9	202	116	3		0.009**		(169)	I.D.	1		I.D.	
NR-0032	50	41	18	2		0.04*		1,047	363	2		0.48	
NR-0049	1	549	220	3		0.08		963	426	2		0.41	
NR-0056	2	970	327	2		0.41		nt	nt	0		nt	
NR-0061	3	743	77	2		0.23		nt	nt	0		nt	
NR-0082	10	302	86	3		0.03*		2,653	13	2		0.08	
NR-0083	7.5	1,582	191	2		0.88		nt	nt	0		nt	
NR-0098	25	20	3	2		0.03*		1,582	102	2		0.88	
NR-0116	10	999	381	2		0.36		1,755	1,151	2		0.72	
1a-D0001	20	848	490	2		0.32		nt	nt	0		nt	
1a-D0009	50	135	68	3		0.02*		2,232	403	2		0.25	
1a-D0009	20	288	118	3		0.03*		2,692	1,042	2		0.12	
1a-D0009	5	1,537	280	3		0.93		nt	nt	0		nt	
1a-D0009	1	1,768	289	2		0.65		nt	nt	0		nt	

I.D. Insufficient data; nt: not tested; na: not applicable

The anti-HPA-1a mAb B2 exhibited dose-dependent blocking of PAC-1 antibody binding (or inhibition of activation of integrin $\alpha\text{IIb}\beta 3$) between 50 and 1 ng/mL.

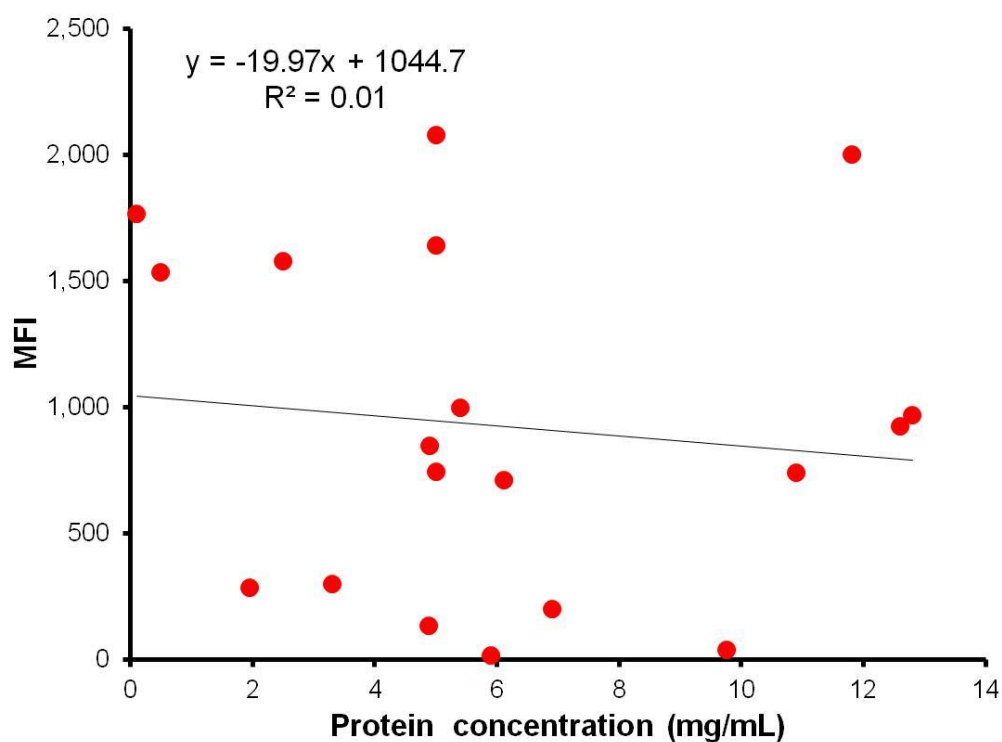
Three NAIT-derived examples of anti-HPA-1a (NR-0032, NR-0082 and NR-0098) resulted in reduced binding of PAC-1 that was HPA-1a specific. Samples NR-0032 and NR-0098 are paired EDTA plasma and serum samples respectively from the same venepuncture thus showing that the PAC-1 assay is reproducible.

One NAIT-derived example of anti-HPA-1a (NR-0022) reduced the binding of PAC-1 antibody to both HPA-1a1a and HPA-1b1b platelets although statistical analysis could not be performed since only one assay was performed using HPA-1b1b platelets.

The PTP-derived anti-HPA-1a D-0009 reduced binding of PAC-1 in a HPA-1a specific manner at concentrations of 50 and 20 i.u./mL.

In order to determine whether the blocking was related to protein concentration rather than level of anti-HPA-1a, the relationship between protein concentration and anti-HPA-1a level was explored (**Figure 6.4**).

FIGURE 6.4. Relationship between protein level and anti-HPA-1a level on blocking of binding of PAC-1 antibody. The bars show the averaged MFI values from all assays using ADP-activated platelets and the protein concentration of the purified IgG samples.



The blocking ability of the sample was not related to the protein level.

These data therefore suggest that PAC-1 binding is a sensitive, specific indicator of the inhibitory effects of HPA-1a antibodies on platelet function.

6.3.2.2 Binding of fibrinogen

An alternative indirect measure of the effect of HPA-1a antibodies on platelet function is inhibition of binding of fibrinogen by these antibodies (Joutsu-Korhonen *et al*, 2004; Kroll *et al*, 2005a).

The binding of fibrinogen to platelets following their exposure to the purified anti-HPA-1a or control IgG was assessed by indirect immunofluorescence using flow cytometry (**Table 6.2**).

TABLE 6.2. Effect of HPA-1a antibodies on fibrinogen binding. *The figures show the averaged MFI values from all experiments with ADP-activated platelets. Significance of results was assessed using the unpaired t-test (*P = <0.05; **P = <0.01; ***P = <0.0001). The purified anti-HPA-1a IgG data were analysed with respect to pooled data from the negative controls (purified IgG from the group AB serum non-transfused male blood donors at 5 mg/mL) (P-ABS). Significance of the B2 data was assessed with respect to the TBS control (P-TBS).*

Figures in brackets indicate only that only one assay was performed due to limited availability of test sample; statistical analysis could not be performed on these samples. Cells shaded red indicate a significant reduction in binding of fibrinogen; cells shaded green indicate a significant increase in bound fibrinogen compared to controls.

Sample ID	iu/mL / *ng/mL	HPA-1a1a					HPA-1b1b				
		Mean MFI	SD	N	P-TBS	P-ABS	Mean MFI	SD	N	P-TBS	P-ABS
TBS		5,578	960	11			9,437	1,197	4		
Neg controls 5, 8, 9		3,759	645	9	0.15		nt	na	0		
B2	50*	1,767	317	3	0.07		7,189	2,499	2	0.39	
B2	10*	1,560	148	3	0.06		7,231	2,304	2	0.39	
B2	5*	1,476	236	3	0.05		6,727	2,205	2	0.29	
B2	1*	1,498	223	3	0.05		6,464	1,764	2	0.23	
B2	0.1*	4,486	2,118	2	0.67		6,142	1,552	2	0.18	
B2	0.01*	13,300	4,747	2	0.02*		6,128	1,705	2	0.19	
NR-0004	6.5	1,781	378	2		0.20	(4,097)	I.D.	1		I.D.
NR-0005	5	4,289	1,982	3		0.74	nt	nt	0		nt
NR-0022	9	1,306	185	3		0.03*	(7,460)	I.D.	1		I.D.
NR-0032	50	1,853	406	2		0.22	4,399	1,573	2		0.69
NR-0049	1	1,913	335	3		0.14	5,149	950	2		0.37
NR-0056	2	2,452	563	2		0.39	nt	nt	0		nt
NR-0061	3	3,566	2,124	2		0.91	nt	nt	0		nt
NR-0082	10	10,250	1,121	3		0.0005***	7,076	300	2		0.046*
NR-0083	7.5	3,619	945	2		0.93	nt	nt	0		nt
NR-0098	25	949	42	2		0.08	7,883	1,206	2		0.02*
NR-0116	10	2,777	267	2		0.42	8,083	1,320	2		0.02*
1a-D0001	20	2,877	1,110	2		0.57	nt	nt	0		nt
1a-D0009	50	9,469	4,535	3		0.05	11,790	2,247	2		0.0009***
1a-D0009	20	1,830	269	3		0.13	11,390	329	2		0.0005***
1a-D0009	5	3,757	1,093	3		0.99	nt	nt	0		nt
1a-D0009	1	7,079	2,611	2		0.09	nt	nt	0		nt

I.D. Insufficient data; nt: not tested; na: not applicable

At concentrations between 50 and 1 ng/mL, the MFIs of B2 when tested against HPA-1a1a platelets were lower than those against HPA-1b1b platelets but the differences were not statistically significant.

Two samples (NR-0082 and B2 at 0.01 ng/mL) increased binding of fibrinogen to a statistically significant extent. The increase in fibrinogen binding with sample B2 (0.01 ng/mL) was HPA-1a specific since no increase in binding was observed with HPA-1b1b samples; the increase in fibrinogen binding with sample NR-0022 was not HPA-1a specific and was also increased with HPA-1b1b platelets.

One anti-HPA-1a sample (NR-0022) demonstrated a statistically significant inhibition of binding of fibrinogen. This appeared to be HPA-1a specific since no reduction in binding was observed with a HPA-1b1b sample but unfortunately insufficient purified IgG was available to permit replicate testing and thus statistical analysis.

Four additional samples (NR-0098, NR-0166, 1a-D0009 at 50 i.u./mL and 20 i.u./mL) induced significantly increased fibrinogen binding to HPA-1b1b samples.

These data therefore suggested that HPA-1a antibodies can inhibit or induce fibrinogen binding when using fibrinogen binding as a measure of platelet activation.

6.3.2.3 Binding of CD62P antibody

Finally, since CD62P expression is independent of integrin $\alpha\text{IIb}\beta 3$ activation and thus theoretically unaffected by the binding of HPA-1a antibodies to integrin $\alpha\text{IIb}\beta 3$, the expression of CD62P was used as a control to assess non-HPA-1a specific activation of platelets by the purified IgG samples (**Table 6.3**).

The binding of anti-CD62P to platelets following their exposure to the purified anti-HPA-1a or control IgG was assessed by indirect immunofluorescence using flow cytometry (**Table 6.3**).

TABLE 6.3. Effect of HPA-1a antibodies on CD62P. *The figures show the averaged MFI values from all experiments with ADP-activated platelets. Significance of results was assessed using the unpaired t-test (*P = <0.05; **P = <0.01; ***P = <0.0001). The purified anti-HPA-1a IgG data were analysed with respect to pooled data from the negative controls (purified IgG from the group AB serum non-transfused male blood donors at 5 mg/mL) (P-ABS). Significance of the B2 data was assessed with respect to the TBS control. Figures in brackets indicate only that only one assay was performed due to limited availability of test sample; statistical analysis could not be performed on these samples. Cells shaded red indicate a significant reduction in binding of CD62P antibody; cells shaded green indicate a significant increase in bound CD62P antibody compared to controls.*

Sample ID	iu/mL / *ng/mL	HPA-1a1a					HPA-1b1b				
		Mean MFI	SD	N	P-TBS	P-ABS	Mean MFI	SD	N	P-TBS	P-ABS
TBS		977	115	11			888	121	4		
Neg controls 5, 8, 9		427	50	9	0.0008***		nt	na	0		
B2	50*	710	92	3	0.23		893	241	2	0.98	
B2	10*	704	73	3	0.22		903	252	2	0.95	
B2	5*	681	71	3	0.19		865	241	2	0.93	
B2	1*	698	98	3	0.22		842	230	2	0.85	
B2	0.1*	775	2	2	0.45		864	216	2	0.92	
B2	0.01*	864	60	2	0.67		897	222	2	0.97	
NR-0004	6.5	251	45	2		0.14	(202)	I.D.	1		I.D.
NR-0005	5	319	48	3		0.25	nt	nt	0		nt
NR-0022	9	170	25	3		0.02*	(53)	I.D.	1		I.D.
NR-0032	50	216	4	2		0.08	270	92	2		0.20
NR-0049	1	184	35	3		0.02*	300	17	2		0.26
NR-0056	2	309	22	2		0.30	nt	nt	0		nt
NR-0061	3	308	13	2		0.29	nt	nt	0		nt
NR-0082	10	334	15	3		0.30	315	63	2		0.33
NR-0083	7.5	548	98	2		0.31	nt	nt	0		nt
NR-0098	25	184	4	2		0.049*	389	40	2		0.73
NR-0116	10	347	48	2		0.39	384	27	2		0.69
1a-D0001	20	680	139	2		0.07	nt	nt	0		nt
1a-D0009	50	449	183	3		0.87	1,404	648	2		0.008**
1a-D0009	20	518	202	3		0.53	516	128	2		0.47
1a-D0009	5	989	297	3		0.007**	nt	nt	0		nt
1a-D0009	1	1,349	293	2		0.0004***	nt	nt	0		nt

I.D. Insufficient data; nt: not tested; na: not applicable

Anti-HPA-1a mAb B2 was shown not to affect the binding of anti-CD62P antibody.

Samples NR-0049 and NR-0098 appeared to block CD62P expression/binding of anti-CD62P in a HPA-1a specific manner.

Sample NR-0022 blocked the binding of anti-CD62P against both HPA-1a1a and HPA-1b1b platelets.

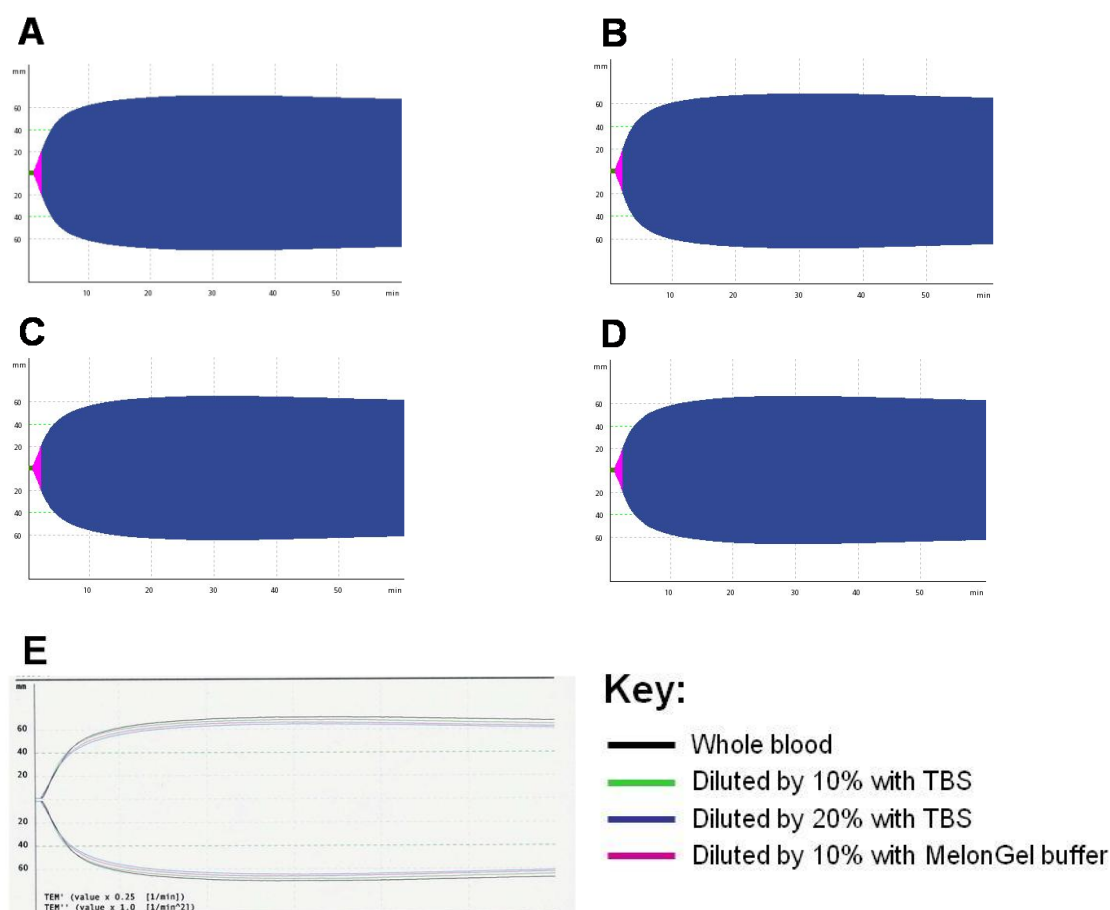
Sample 1a-D0009 increased binding of anti-CD62P at 50, 5 and 1 i.u./mL although this effect did not appear to be HPA-1a specific since the MFI when using HPA-1b1b platelets was increased with the 50 i.u./mL sample.

These data show that some purified IgG samples showed an inhibitory, and some an activatory, effect on platelets.

6.3.3 Demonstration of function-blocking effects of HPA-1a antibodies by modified ROTEM® *delta* assay

Since the proposed studies using the ROTEM assay would involve adding aliquots of IgG to normal donor blood thus diluting the sample, experiments were performed to study the effect of sample dilution on the ROTEM profile, and to determine the maximum dilution of donor sample by the test sample that still resulted in a normal ROTEM profile (**Figure 6.5**).

FIGURE 6.5. Effect of sample dilution on ROTEM profile. *Whole blood from donor D21 was tested undiluted in the ROTEM EXTEM assay (A). Blood from the same donor was diluted by 10% with TBS buffer (B), by 20% with TBS buffer (C) and by 10% with Melon Gel purification buffer to assess potential adverse impacts of the purification buffer used to prepare the IgG (D). (E) Overlay of figures A-D.*

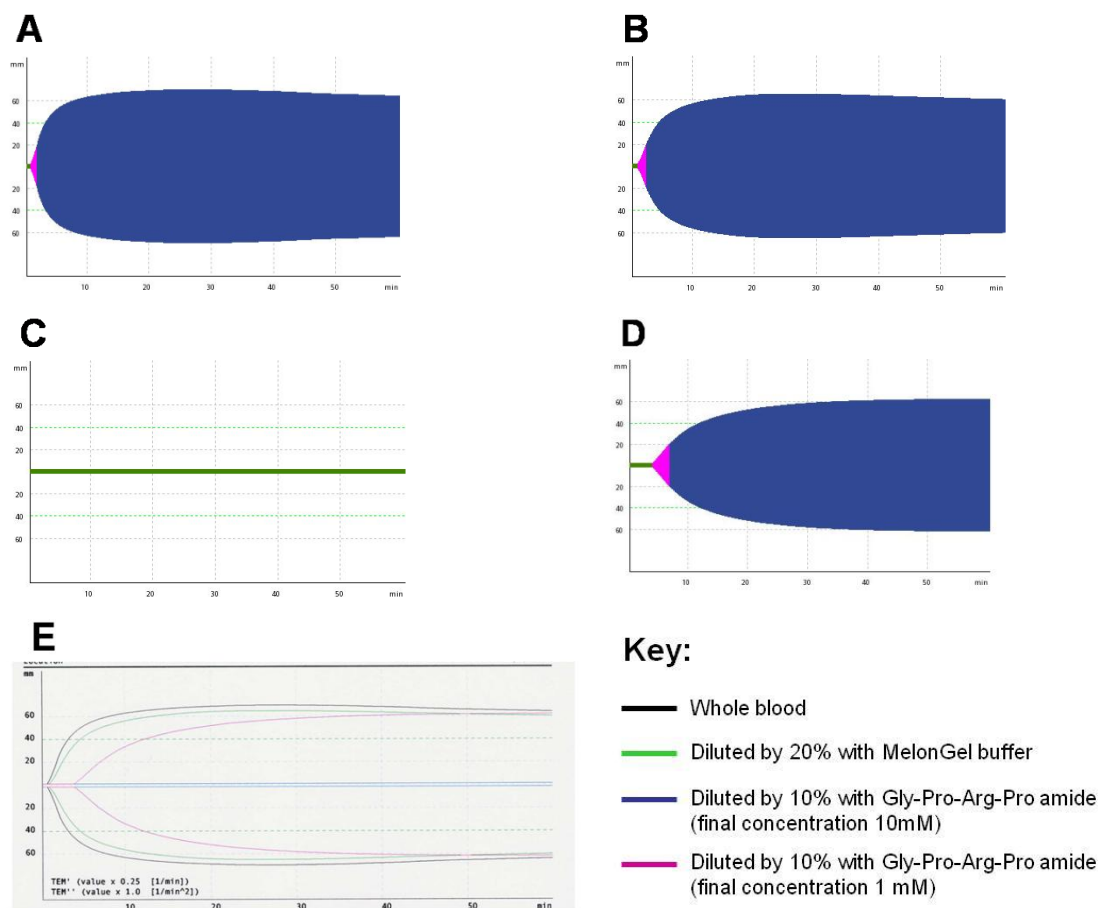


Diluting the donor blood sample by up to 20% with TBS appeared not to have a

deleterious effect on the ROTEM profile and this was selected as the extent by which all future blood samples would be diluted by test sample.

In order to show that an inhibitory effect could be demonstrated by ROTEM when using diluted blood, positive controls of blood diluted by 20% with Gly-Pro-Arg-Pro amide were evaluated (**Figure 6.6**).

FIGURE 6.6. Inhibition of fibrin polymerisation demonstrated by ROTEM. (A) Undiluted whole blood from donor D129. (B) Blood from same donor diluted 20% with Melon Gel buffer, diluted 20% with Gly-Pro-Arg-Pro amide (final concentration 10 mM) (C) and diluted 20% with Gly-Pro-Arg-Pro amide (final concentration 1 mM) (D). (E) Overlay of Figures 5.3 A-D.

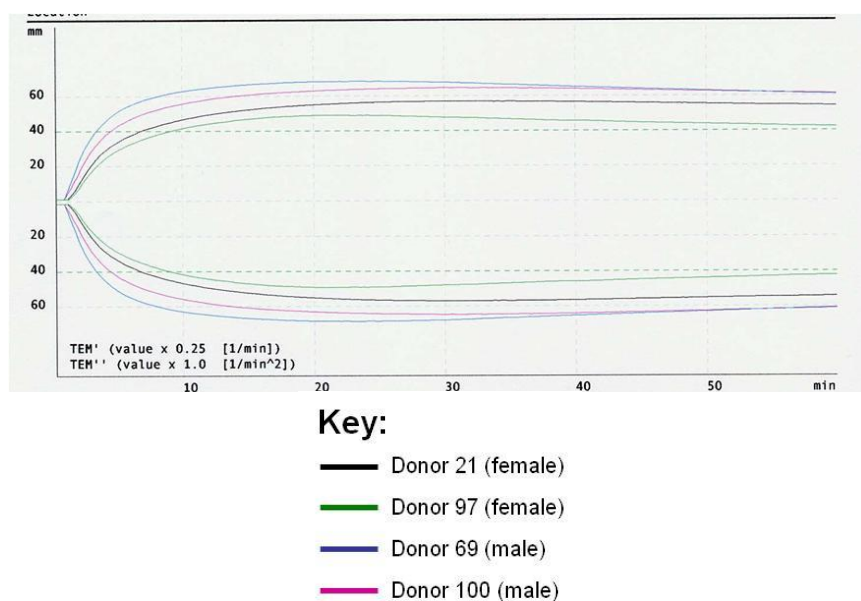


Despite the samples having been diluted by 20%, the ROTEM assay was able to show 'non-response' or 'partial response' profiles with Gly-Pro-Arg-Pro.

Thus, these initial controls showed that the ROTEM assay was able to produce normal profiles when using diluted blood samples, and abnormal profiles when Gly-Pro-Arg-Pro was present, suggesting that this assay was potentially suitable for evaluation of the effect of HPA-1a antibodies on haemostatic profiles.

The initial results with diluted samples from donors D21 and D129 appeared to show different ROTEM profiles. To determine whether this was an artefact arising from testing these samples in assays performed on different days, samples from four blood donors were tested simultaneously on the four available channels of the ROTEM device (**Figure 6.7**).

FIGURE 6.7. Inter-donor variation in ROTEM profiles when using diluted (20%) blood samples. Fresh blood samples from four blood donors were diluted by 20% with TBS and tested simultaneously in the ROTEM assay.



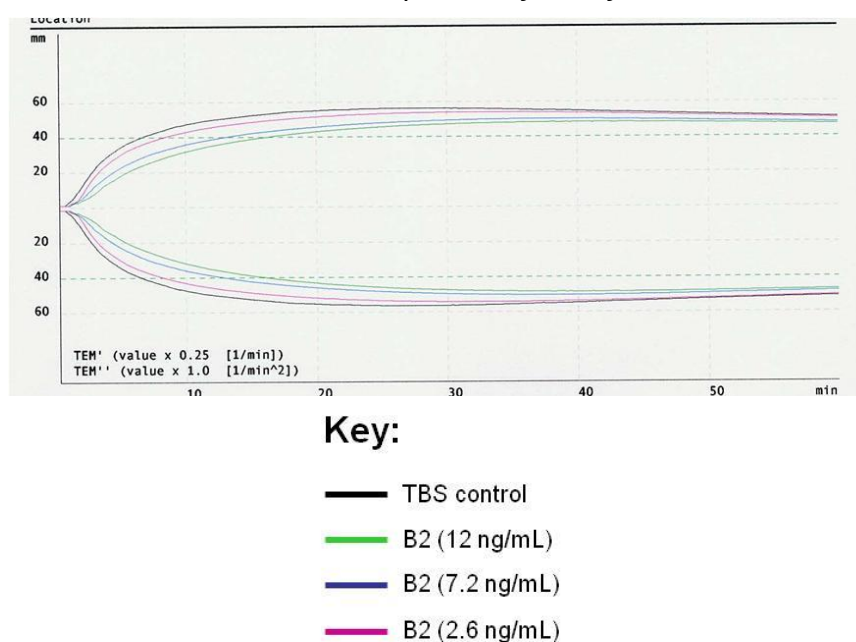
These data showed very different profiles. Since this would potentially result in a wide range of normal values that might mask small effects for any given test sample, it was decided that a reference control (whole blood diluted by 20% with TBS) would be included in each assay and test data analysed with respect to this control.

Having determined the potential suitability and the limitations of the assay, the sensitivity of the assay to inhibitory HPA-1a antibodies was explored.

The HPA-1a mAb 'B2' has been reported to block the binding of platelets to immobilised fibrinogen (Joutsu-Korhonen *et al*, 2004) and was thus a useful reagent to investigate function-blocking effects of HPA-1a antibodies in the ROTEM assay.

Aliquots of B2 were diluted to a range of concentrations and incubated with whole blood (20%:80% respectively) at 37°C for 30 mins after which the haemostatic function of the samples was evaluated. Typical results are shown in **Figure 6.8**.

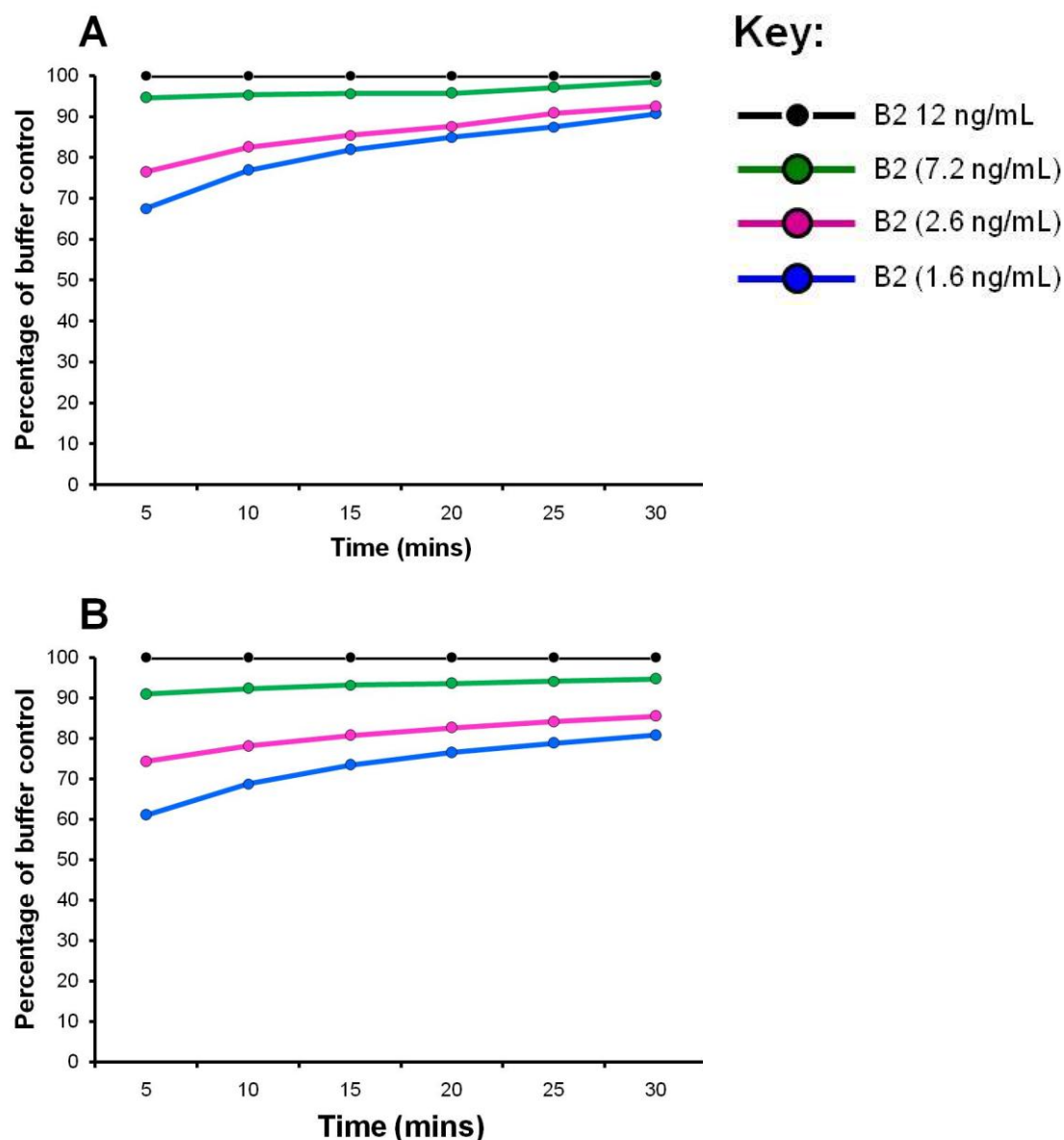
FIGURE 6.8. Demonstration of inhibitory effects of mAb B2 in ROTEM assay. Donor sample D113 was incubated with B2 anti-HPA-1a mAb at a range of concentrations then the sensitised samples analysed by ROTEM.



B2 was able to show a dose-dependent inhibitory effect on the ROTEM profile. Thus the modified ROTEM assay was shown to be suitable for analysis of inhibitory effects of mAbs.

The time course of the amplitude and area parameters was then studied to determine which time point provided maximum sensitivity (**Figure 6.9**).

FIGURE 6.9. Determination of optimum time point to obtain maximum assay sensitivity. (A) clot amplitude and (B) clot area.



Maximum sensitivity was obtained at 5 mins for both clot amplitude (A5) and clot area (AR5) parameters.

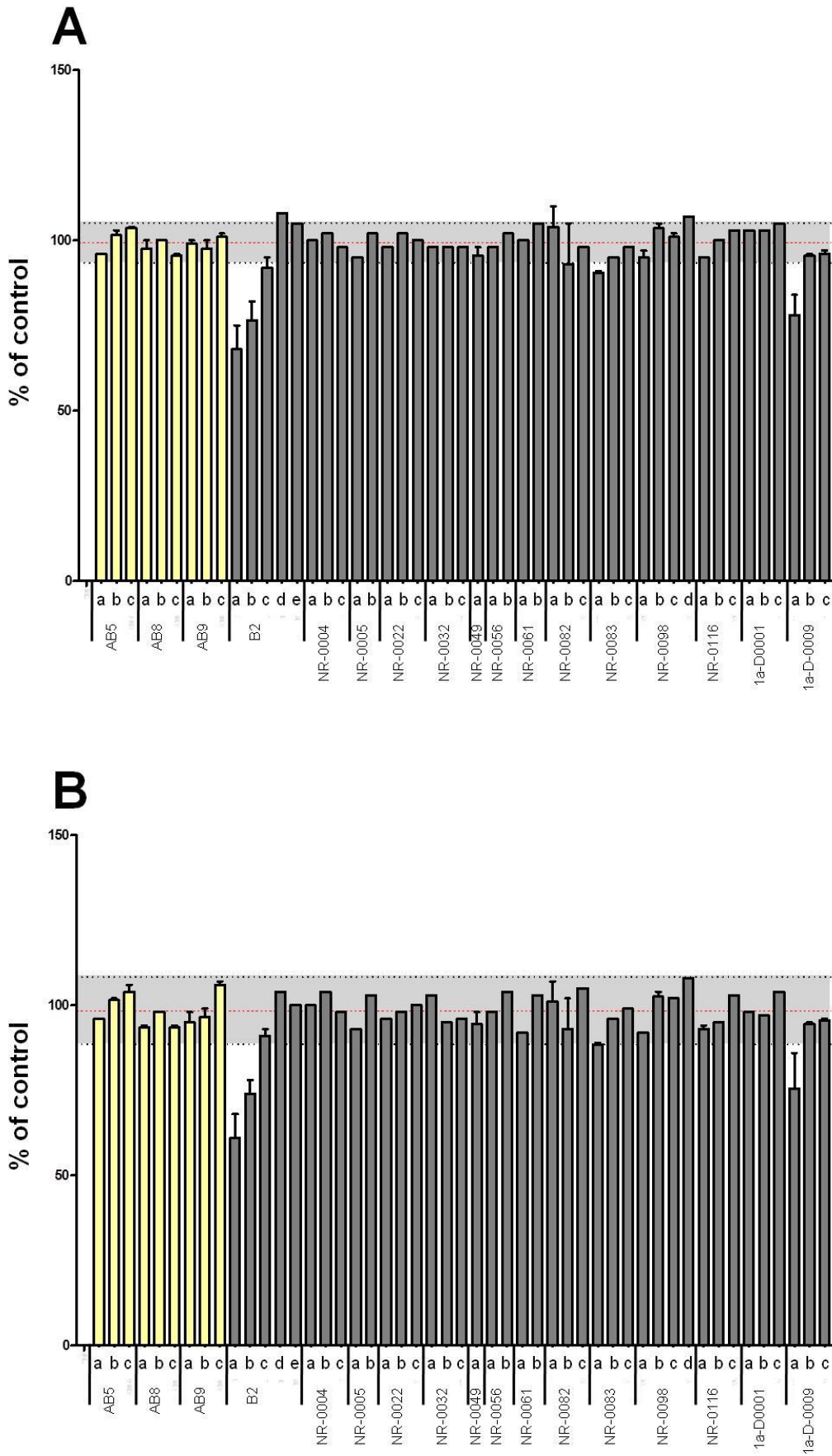
The effects of human polyclonal antibodies (detailed in **Table 6.4**) were then studied.

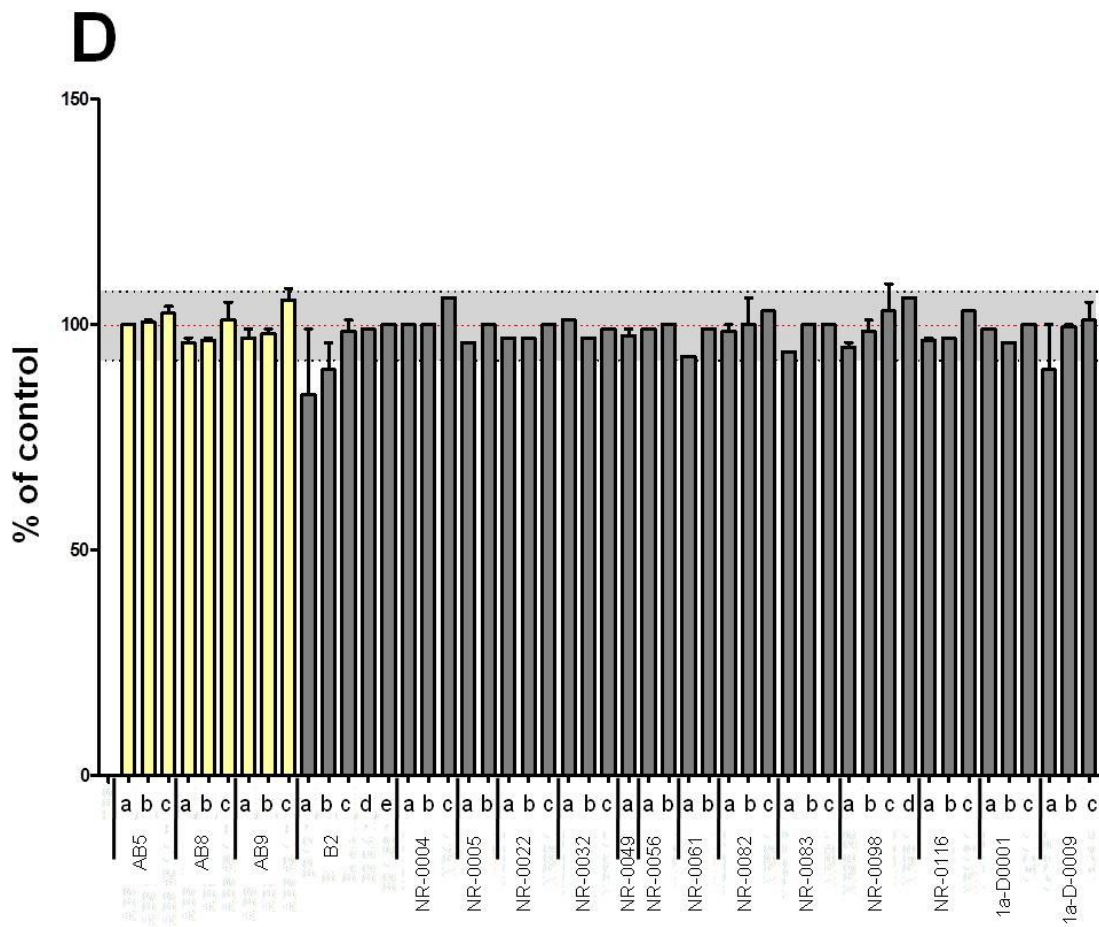
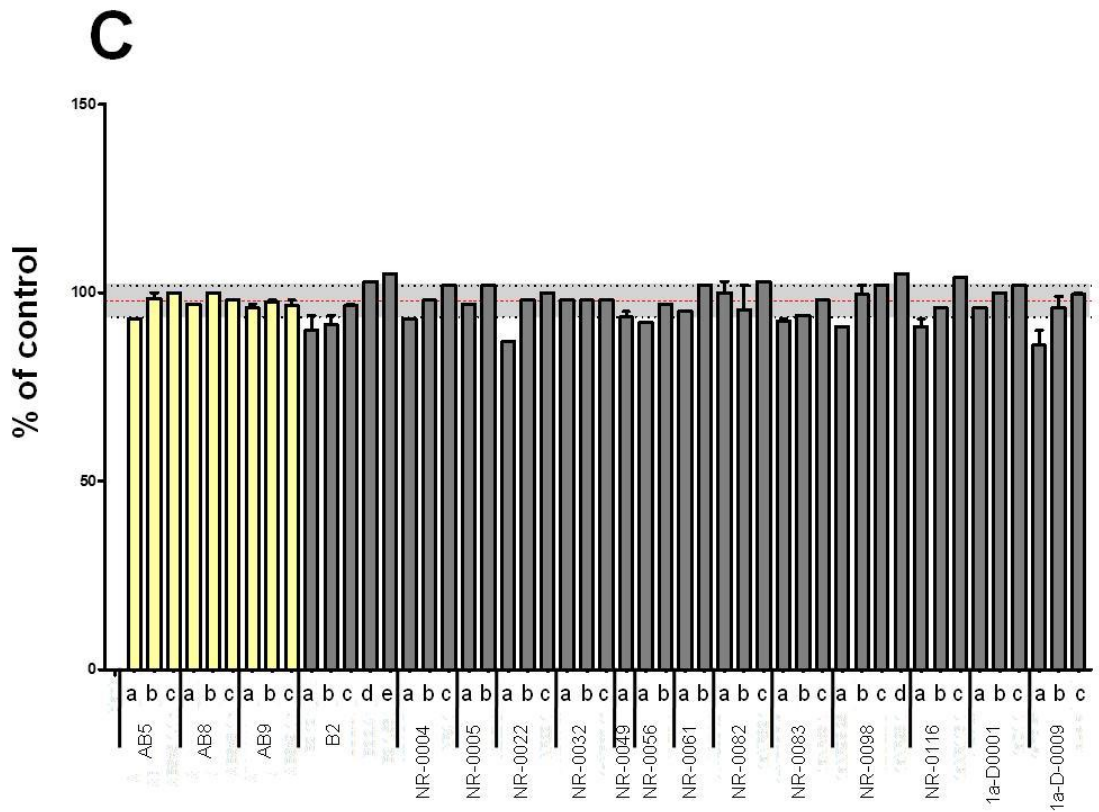
Purified IgG from the NAIT- and PTP-derived examples of anti-HPA-1a, were tested at a range of anti-HPA-1a levels (where the level of anti-HPA-1a was sufficiently high in the undiluted IgG preparation to permit dilution) in the ROTEM assay; 3 examples

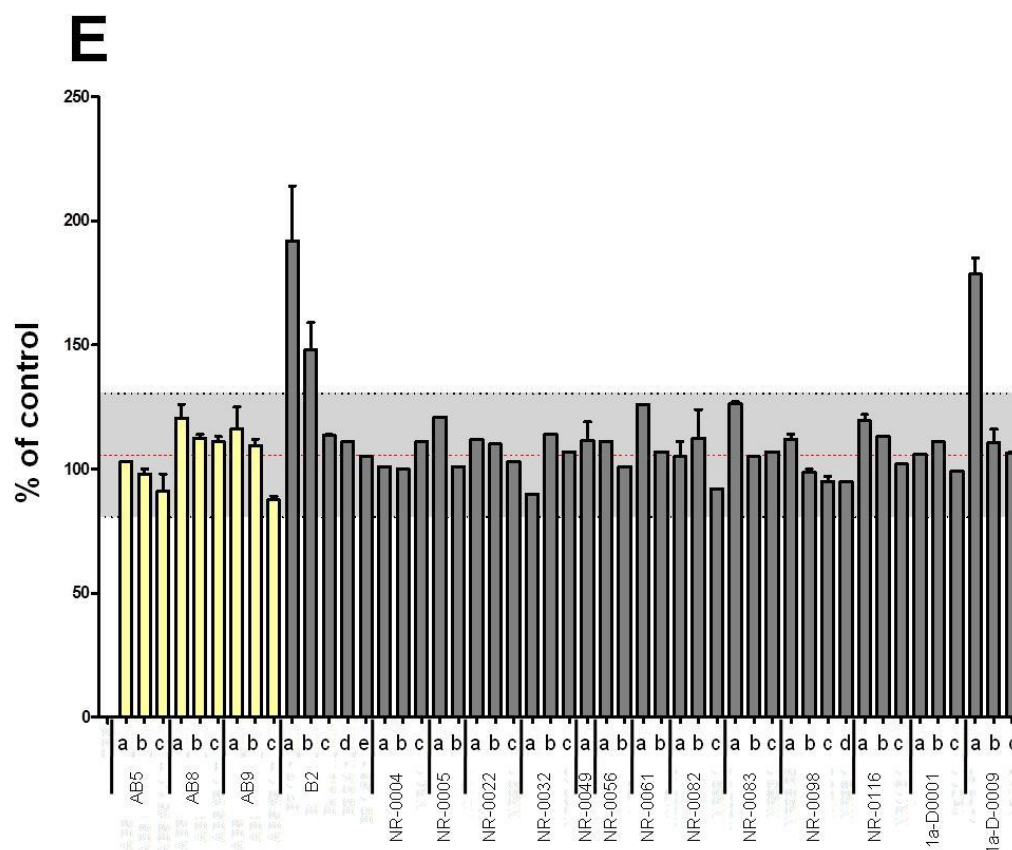
of purified IgG from blood group AB, non-transfused male blood donors were used as negative controls to determine normal ranges.

Data on various ROTEM® *delta* parameters that are dependent on the contribution of platelets to haemostasis, namely clot area, clot amplitude, α -angle, clot formation time and maximum clot firmness were analysed to determine the most informative parameters; these were analysed with respect to the buffer control within each assay (Figure 6.10).

FIGURE 6.10. Impact of pre-incubation of samples with purified IgG on ROTEM parameters of normal donor blood. *All results are shown as a percentage of the buffer control within the same assay. Test sample IDs are shown on the x axis; the letters a, b, c etc identify the concentration of anti-HPA-1a (i.u./mL) or protein (mg/mL), the decode of which is shown in **Table 6.4**. The negative controls (IgG from inert, group AB serum) are shaded yellow and test samples containing anti-HPA-1a in grey. The error bars show the SEM. (A) A5 (clot amplitude at 5 mins. (B) AR5 (clot area at 5 mins). (C) maximum clot firmness (MCF). (D) α -angle. (E) clot formation time (CFT). The dashed red line and the greyed area above and below this line indicate the mean value obtained with negative controls and ± 2 S.D respectively.*







Both the A5 and AR5 parameters showed that B2 had a significant inhibitory effect at concentrations of 12 and 7.2 ng/mL.

Anti-HPA-1a sample 1a-D0009 had an inhibitory effect at 50 i.u./mL as indicated by A5, AR5 and MCF parameters.

Anti-HPA-1a NR-0083 was inhibitory when assessed using the A5 parameter.

Anti-HPA-1a NR-0022 was inhibitory when assessed using the MCF parameter.

The α -angle was not able to demonstrate inhibitory effects of any of the samples.

The negative control IgG increased the mean CFT (dashed red line) to 105.5% (SD: 12.5%) of that of the buffer-only control.

B2 prolonged the CFT to >2 S.D. above the mean of the negative controls at concentrations of 12 and 7.2 ng/mL.

Anti-HPA-1a-D0009 significantly prolonged the CFT when added to the donor blood at a final level of 50 i.u./mL.

Thus, these data showed that the function-blocking effects of HPA-1a antibodies could successfully be demonstrated using this modified ROTEM assay using the parameters A5, AR5 and CFT.

TABLE 6.4. Samples tested in ROTEM assay.

Sample Number	Test ID	Sample type	HPA-1a antibody level (i.u./mL)	Protein concentration
AB5	a	NC	Not applicable	10 mg/mL
AB5	b	NC	Not applicable	4.4 mg/mL
AB5	c	NC	Not applicable	1.1 mg/mL
AB8	a	NC	Not applicable	10 mg/mL
AB8	b	NC	Not applicable	5 mg/mL
AB8	c	NC	Not applicable	1 mg/mL
AB9	a	NC	Not applicable	10 mg/mL
AB9	b	NC	Not applicable	5 mg/mL
AB9	c	NC	Not applicable	1 mg/mL
B2	a	1a mAb	0.6*	12 ng/mL
B2	b	1a mAb	0.36*	7.2 ng/mL
B2	c	1a mAb	0.13*	2.6 ng/mL
B2	d	1a mAb	0.12*	2.4 ng/mL
B2	e	1a mAb	0.08*	1.56 ng/mL
NR-0004	a	NAIT	6.5	10.6 mg/mL
NR-0004	b	NAIT	5	8.1 mg/mL
NR-0004	c	NAIT	1	1.6 mg/mL
NR-0005	a	NAIT	4.2	8.7 mg/mL
NR-0005	b	NAIT	1	2.1 mg/mL
NR-0022	a	NAIT	9	11.2 mg/mL
NR-0022	b	NAIT	5	6.2 mg/mL
NR-0022	c	NAIT	1	1.2 mg/mL
NR-0032	a	NAIT	50	6.9 mg/mL
NR-0032	b	NAIT	25	3.5 mg/mL
NR-0032	c	NAIT	10	1.4 mg/mL
NR-0049	a	NAIT	1	11.1 mg/mL
NR-0056	a	NAIT	1.6	9.5 mg/mL
NR-0056	b	NAIT	1	5.9 mg/mL
NR-0061	a	NAIT	3	9.6 mg/mL
NR-0061	b	NAIT	1	3.3 mg/mL
NR-0082	a	NAIT	10	5.3 mg/mL
NR-0082	b	NAIT	5	2.6 mg/mL
NR-0082	c	NAIT	1	0.5 mg/mL
NR-0083	a	NAIT	7.5	7.1 mg/mL
NR-0083	b	NAIT	5	4.8 mg/mL
NR-0083	c	NAIT	1	1.0 mg/mL
NR-0098	a	NAIT	25	5.3 mg/mL
NR-0098	b	NAIT	10	2.1 mg/mL
NR-0098	c	NAIT	5	1.1 mg/mL
NR-0098	d	NAIT	1	0.2 mg/mL
NR-0116	a	NAIT	10	8.7 mg/mL
NR-0116	b	NAIT	5	4.3 mg/mL
NR-0116	c	NAIT	1	0.9 mg/mL
1a-D0001	a	PTP	20	4.4 mg/mL
1a-D0001	b	PTP	5	1.1 mg/mL
1a-D0001	c	PTP	1	0.2 mg/mL
1a-D0009	a	PTP	50	5.0 mg/mL
1a-D0009	b	PTP	25	2.5 mg/mL
1a-D0009	c	PTP	5	0.5 mg/mL

** The activity of anti-HPA-1a 'B2' was assessed after this phase of the study was completed.*

NC: Negative control (group AB serum derived from non-transfused male blood donor); 1a mAb: anti-HPA-1a mAb; NAIT-derived anti-HPA-1a; PTP: anti-HPA-1a obtained from patients suffering an episode of post-transfusion purpura.

6.3.4 Comparison of all methods

The data obtained from the ROTEM assay was compared to the data obtained from the flow cytometry assays (**Table 6.5**).

TABLE 6.5. Comparison of ROTEM and flow cytometry data. *Statistical significance of flow cytometry data was determined by the unpaired t-test (*P = < 0.05; **P = < 0.01; ***P = < 0.001), and of ROTEM data by values falling outside of the mean $\pm$ 2 S.D. of the negative controls.*

Sample ID	iu/mL / *ng/mL	ROTEM (HPA-1a1a)			Flow cytometry (HPA-1a1a)			Flow Cytometry (HPA-1b1b)		
		A5	AR5	CFT	Fibrinogen	PAC-1	CD62P	Fibrinogen	PAC-1	CD62P
B2	50*	nt	nt	nt	0.07	0.01*	0.23	0.39	0.91	0.98
B2	12*	Yes	Yes	Yes	nt	nt	nt	nt	nt	nt
B2	10*	nt	nt	nt	0.06	0.01*	0.22	0.39	0.99	0.95
B2	7.2*	Yes	Yes	Yes	nt	nt	nt	nt	nt	nt
B2	5*	nt	nt	nt	0.05	0.01*	0.19	0.29	0.87	0.93
B2	2.6*	No	No	No	nt	nt	nt	nt	nt	nt
B2	1*	No	No	No	0.05	0.02*	0.22	0.23	0.81	0.85
B2	0.1*	No	No	No	0.67	0.97	0.45	0.18	0.67	0.92
B2	0.01*	No	No	No	0.02*	0.97	0.67	0.19	0.55	0.97
NR-0004	6.5	No	No	No	0.20	0.58	0.14	I.D.	I.D.	I.D.
NR-0005	5	No	No	No	0.74	0.14	0.25	nt	nt	nt
NR-0022	9	No	No	No	0.03*	0.009**	0.02*	I.D.	I.D.	I.D.
NR-0032	50	No	No	No	0.22	0.04*	0.08	0.69	0.48	0.20
NR-0049	1	No	No	No	0.14	0.08	0.02*	0.37	0.41	0.26
NR-0056	2	No	No	No	0.39	0.41	0.30	nt	nt	nt
NR-0061	3	No	No	No	0.91	0.23	0.29	nt	nt	nt
NR-0082	10	No	No	No	0.0005***	0.03*	0.30	0.046*	0.08	0.33
NR-0083	7.5	Yes	No	No	0.93	0.88	0.31	nt	nt	nt
NR-0098	25	No	No	No	0.08	0.03*	0.049*	0.02*	0.88	0.73
NR-0116	10	No	No	No	0.42	0.36	0.39	0.02*	0.72	0.69
1a-D0001	20	No	No	No	0.57	0.32	0.07	nt	nt	nt
1a-D0009	50	Yes	Yes	Yes	0.05	0.02*	0.87	0.0009***	0.25	0.008**
1a-D0009	20	No	No	No	0.13	0.03*	0.53	0.0005***	0.12	0.47
1a-D0009	5	No	No	No	0.99	0.93	0.007**	nt	nt	nt
1a-D0009	1	nt	nt	nt	0.09	0.65	0.0004***	nt	nt	nt

'Yes': sample had an inhibitory effect; 'No': sample did not exhibit inhibitory effect. nt: not tested; I.D. insufficient data.

These data showed that B2 was able to induce a dose-dependent effect of blocking of platelet function demonstrable both by the ROTEM® *delta* assay and binding of PAC-1 antibody; binding of the anti-fibrinogen antibody was reduced but this was not statistically significant. The ROTEM® *delta* assay had a sensitivity of approximately 7 ng/mL and the PAC-1 flow cytometry assay a sensitivity of 1 ng/mL. Subsequent quantitation of B2 showed that these figures equated to approximately 0.4 and 0.05 i.u./mL respectively.

The effects of polyclonal HPA-1a antibodies on platelet function assessed using flow cytometry assays varied according to the marker of platelet activation used; none of the markers correlated completely with the ROTEM data.

Of the NAIT-derived examples of anti-HPA-1a, 1/11 (9.1%) and 4/11 (36.4%) were able to inhibit the binding of anti-fibrinogen or PAC-1 antibody respectively. Three of these showed HPA-1a specific inhibition of PAC-1 binding whilst the remaining example was not tested against HPA-1b1b platelets in either the PAC-1 or fibrinogen binding assays due to lack of material. These data indicate either prevention of fibrinogen binding, prevention of activation of $\alpha\text{IIb}\beta\text{3}$ or steric hindrance of access to the target epitope by these labelled antibodies.

One of two examples of PTP-derived anti-HPA-1a blocked binding of PAC-1 and was inhibitory in the ROTEM® *delta* assay; the sensitivity of these two assays with this example was 20 and 50 i.u./mL respectively.

Three NAIT-derived examples of anti-HPA-1a (27.3%) prevented expression of CD62P. In two of these the blocking effect was HPA-1a specific and in the remaining example there was insufficient material to test against HPA-1b1b platelets.

Three examples (27.3%) of NAIT-derived anti-HPA-1a and one PTP-derived example induced increased binding of fibrinogen; this was not HPA-1a specific.

6.3.5 Megakaryocyte growth

The addition of anti-HPA-1a purified IgG to the culture tubes containing the CD34+ cells, growth medium and collagen prevented the collagen from gelling as expected. Upon completion of cell culture (day 11) and removal of the slide chamber supports, the collagen solution simply poured from the slide instead of remaining as a semi-solid matrix that could be stained and assessed for megakaryocyte growth. Staining of the slide confirmed that no cells were present.

A control culture using purified IgG from a non-transfused, male, group blood AB donor also resulted in non-gelling of the collagen.

A further control using Iscove's medium that was added to the collagen had the same effect.

These results showed that it was not possible to alter the ratios of the components of the Megacult-C kit as it resulted in non-gelling of the semi-solid matrix. Experiments to reduce the volume of purified IgG in order to try and prevent non-gelling were not performed since the samples were already being diluted 1 in 8. It was therefore thought that further dilution would result in the anti-HPA-1a activity being so low that any inhibitory effects would not be apparent.

6.4 Discussion and Conclusions

In this chapter we have shown that it is possible to demonstrate inhibitory effects of HPA-1a antibodies on global haemostasis in a simple point-of-care analyser, the ROTEM® *delta* analyser. This is a novel application of this device.

We also show that the use of the 'PAC-1' mAb, which binds only to activated $\alpha\text{IIb}\beta_3$, is a more sensitive marker for the inhibitory effects of HPA-1a antibodies than the more widely used fibrinogen binding assay.

The impact of mAb binding on platelet function has been well studied and a number of authors have shown that, depending on precise epitope specificity, it is possible to either inhibit or activate platelets using antibodies against $\alpha\text{IIb}\beta_3$ (Heinrich *et al*, 1985; Frelinger, III *et al*, 1991). mAbs against the domain of interest to this project i.e. the PSI domain including anti-HPA-1a 'B2' have been shown to affect ligand binding (Joutsu-Korhonen *et al*, 2004; Mould *et al*, 2005).

The inhibitory effects may be due to steric hindrance of the RGD binding site by the mAb, as reported for B' (Joutsu-Korhonen *et al*, 2004), or as a result of prevention of swing-out of the hybrid/PSI domain (Haas, 2008). Conversely, an induced swing-out of the PSI and hybrid domains by binding of mAb can result in integrin activation (Mould *et al*, 2005).

The potential inhibitory effects of HPA-1a antibodies are of relevance to NAIT since blocking of platelet function may contribute to the haemorrhagic effects observed in some cases. Early studies of polyclonal anti-HPA-1a antibodies suggested that, like B2, such antibodies impaired the binding of fibrinogen (van Leeuwen *et al*, 1984). Subsequently, it was shown that not all examples of anti-HPA-1a were able to block fibrinogen and whilst the majority of examples derived from patients suffering from PTP had a blocking effect, only a minority of NAIT-derived examples demonstrated were able to do so (Beadling *et al*, 1995; Kroll *et al*, 2005a).

These findings therefore suggested that impairment of platelet function by inhibition of fibrinogen binding was not a major contributor to the haemorrhagic symptoms of NAIT. However, as shown by Frelinger it is possible to alter platelet function as a result of antibody binding to epitopes not in proximity to the fibrinogen binding site (Frelinger, III *et al*, 1991).

Our findings regarding the inhibition of binding of fibrinogen agree with those of Beadling and Kroll and we found only one of 11 examples of NAIT-derived anti-HPA-1a to be inhibitory. However, our data show that inhibition of binding of the PAC-1 antibody, which binds near to the fibrinogen binding and competes with fibrinogen, is a more sensitive assay and we identified three additional examples of NAIT-derived anti-HPA-1a that exhibited specific inhibitory effects. Two of these samples were from the same mother and had been tested blind thus demonstrating that the PAC-1 assay is reproducible. We determined the sensitivity of the assay to be 1 ng/mL, approximately 3 orders of magnitude higher than the whole blood *in vitro* bleeding time and platelet aggregation assays, and four orders of magnitude higher than a fibrinogen binding assay used by Joutsii-Korhonen (Joutsii-Korhonen *et al*, 2004).

Studies with B2 showed that the PAC-1 binding assay is specific since no inhibition of binding of PAC-1 was observed when using HPA-1b1b platelets. Furthermore, the expected negative results when using anti-CD62P showed that B2 was specifically inhibiting activation of $\alpha\text{IIb}\beta 3$ (or was blocking the PAC-1/fibrinogen binding sites) (Joutsii-Korhonen *et al*, 2004).

Thus the PAC-1 binding assay is a sensitive, specific and reproducible assay for detecting inhibitory effects of HPA-1a antibodies. However, the mechanism by which PAC-1 is able to determine the functional effects of HPA-1a antibodies remains unclear. The PAC-1 epitope is close to the fibrinogen binding site and thus close to the HPA-1a epitope. The inhibitory effects therefore may, like inhibition of fibrinogen

binding, be simply due to steric hindrance of binding of PAC-1 antibody by the anti-HPA-1a antibodies. Alternatively, the binding of HPA-1a antibodies may prevent swing-out of the PSI/hybrid domain as discussed previously, thus preventing activation of $\alpha\text{IIb}\beta 3$ and exposure of the PAC-1 epitope. Further studies are required to clarify this mechanism.

Our results with anti-fibrinogen showed that not only was this assay less sensitive when using polyclonal examples of anti-HPA-1a, but also when using the mAb B2 and we were unable to demonstrate a significant inhibitory effect of this antibody. Furthermore, a number of samples appeared to induce fibrinogen binding to HPA-1a1a and/or HPA-1b1b platelets. Testing of the two samples from the same mother discussed above showed that the results were not reproducible and suggest that the activation effects were not HPA-1a specific. The most likely explanation is the presence of HLA class I antibodies as these are also known to affect platelet function, including induction of CD62P expression (Christie & Swinehart, 1992; Rubinstein *et al*, 1992; Christie *et al*, 1993). This would explain differences observed between donors since some donors might express particular HLA antigens whilst others lacked those antigens.

Thus, the use of these two markers of activation of $\alpha\text{IIb}\beta 3$ generated important data and suggested that the impact of HPA-1a antibodies on platelet function is greater than suggested by previous studies of fibrinogen binding; more studies are clearly required.

One limitation of the use of activation markers in flow cytometry assays is that they measure only one parameter in an *in vitro* assay that may not represent *in vivo* effects. We therefore investigated whether the ROTEM® *delta* assay, which measures global haemostasis and is therefore a closer parallel to *in vivo* conditions, might be able to demonstrate inhibitory effects of HPA-1a antibodies.

Using anti-HPA-1a B2, which we and others have shown to have inhibitory effects in flow cytometry assays, we successfully showed dose-dependent differences in the ROTEM® *delta* profiles. Analysis of data from the many parameters showed that the most informative parameters were amplitude of the clot at 5 mins (A5) (**Figure 6.9A**), the area of the clot at 5 mins (AR5) (**Figure 6.9B**), the maximum firmness of the clot (MCF) (**Figure 6.9C**) and the clot formation time (CFT) (**Figure 6.9E**). Using these parameters, the sensitivity of the assay was shown to be 5 ng/mL; thus this assay was also shown to be more sensitive than the assays used by Joutsu-Korhonen (Joutsu-Korhonen *et al*, 2004).

This modification of the ROTEM® *delta* assay to study the inhibitory effects of antibodies is a novel application of this assay. Whilst the ROTEM assay has been used to guide transfusion requirements for patients undergoing complex surgery or victims of major trauma (Afshari *et al*, 2011; Rourke *et al*, 2012) there are no published data on the effects of HPA-1a antibodies using this assay although Khurana used a similar assay, the thromboelastograph, to study the inhibitory effects of the anti- α IIb β 3 antibody 7E3 (Khurana *et al*, 1997).

Thus our data and that of Khurana clearly showed that inhibitory effects of mAbs could be demonstrated by thromboelastometry or elastography. However, our findings with the polyclonal anti-HPA-1a IgG samples differed from those obtained using analysis of platelet activation markers by flow cytometry. This may have been due to the technique by which the purified IgG was prepared since analysis of a representative IgG sample after the inhibition studies were complete showed the purity to be less than that suggested by the manufacturers of the purification system (**Figure 6.2C**). It is possible therefore that the discordant results were due to factors present in the 'purified IgG' other than IgG anti-HPA-1a.

Clearly, further work needs to be done on assessing the impact of HPA-1a antibodies

on platelet function but an improved method for producing purified IgG anti-HPA-1a needs to be developed. One such method would be the use of affinity columns (Protein A or Protein G). However, contaminating antibodies e.g. HLA class I would still be present and absorption against pooled, HPA-1b1b platelets would be required to remove these.

Alternatively, it might be possible to obtain purified IgG by elution of anti-HPA-1a from HPA-1a positive platelets or from immobilised $\alpha\text{IIb}\beta\text{3}$ followed by absorption against pooled, HPA-1b1b platelets. However, as shown in **Chapter 5**, the preparation of eluates was problematic and insufficient material may be available to allow testing to be performed. Additionally, the activity of the eluted antibodies may not reflect the activity observed in the original sample.

Concentration of the antibodies would allow assays to be performed at antibody levels the same as in the original samples and perhaps would further increase assay sensitivity. Furthermore, the addition of small, concentrated amounts of IgG in the ROTEM assay might allow use of normal ranges defined by the manufacturer.

In summary therefore, this study has shown that assessment of binding of PAC-1 antibody is a new, sensitive method for assessing inhibitory effects of HPA-1a antibodies. Additionally we have shown that it is possible to demonstrate that both monoclonal and polyclonal anti-HPA-1a antibodies are able to inhibit global haemostasis in the ROTEM® *delta* assay.

Unfortunately, the modification of the Megacult-C assay system used here to demonstrate possible inhibitory effects of HPA-1a antibodies on megakaryocyte growth resulted in complete assay failure due to the gel solution failing to become a semi-solid support matrix as expected. This was due to dilution of the matrix by the purified IgG (or negative control medium).

Further experiments in which less IgG solution was added to the medium were not performed since the IgG was already diluted approximately 1 in 8 in these initial experiments and further dilution would have compromised assay sensitivity.

For future studies a method of producing more concentrated IgG needs to be developed so that it would be possible to re-evaluate the Megacult-C system for its' abilities to demonstrate potential inhibitory effects of HPA-1a antibodies.

6.5 Limitations of the study

Studies of platelet function are reliant on blood that has been collected under conditions that minimise activation of platelets (Harrison, 2005). Since this study required a constant supply of blood samples that were homozygous for HPA-1a the only suitable source of samples was plateletpheresis donors that attend the Component Donation Unit of NHSBT (Oxford Centre). The ethical and Clinical Governance approvals granted to this study only permitted use of blood collected as part of the normal donation process and did not allow collection of fresh additional, carefully-handled samples. The samples therefore were initially collected into the plastic pouch attached the platelet collection pack used to collect samples for mandatory blood grouping and microbiology test and to reduce the risk of bacterial contamination of products (McDonald, 2011). From this pouch the samples were transferred into citrated blood tubes. There therefore was the possibility that the platelets may have been partially activated during the collection process.

The purified IgG used in this study was prepared using a rapid spin purification kit which uses negative selection of IgG rather positive selection such as in protein A or protein G affinity columns. The latter methods potentially select only a sub-population of IgG and require careful elution and control of pH when recovering the IgG and this might influence their effect on platelet function. However, the purity of the IgG obtained was not as high as anticipated and other (unidentified) contaminating proteins may have affected the assays; this was not determined until after completion of experiments.

It is possible that the prepared IgG contained not only anti-HPA-1a antibodies but also HLA class I antibodies. These are known to have activatory effects and may have confounded the results.

Only limited amounts of NAIT samples were available and in many cases these were

insufficient to allow replicate testing and/or testing against HPA-1b1b platelets.

The requirement to dilute the test samples decreased the sensitivity of the assay. Preparation of more concentrated material would have allowed testing of more samples with low levels of anti-HPA-1a and possibly increased sensitivity.

Dilution of the blood samples used in the ROTEM® *delta* analyser has not been validated by the manufacturer although dilution of the samples by up to 30% has been shown not to adversely affect the MCF parameter of the EXTEM assay (Weiss *et al*, 2010). Since inter-donor variation in the various ROTEM® *delta* values is known to occur (dependent on age, gender and platelet count) (Khurana *et al*, 1997; Theusinger *et al*, 2010) we used a novel way of data analysis. This was inclusion of a diluent control in each assay run and calculation of test results as a percentage of this. This method was validated as far as possible by demonstration of inhibitory responses with Gly-Pro-Arg-Pro and with the anti-HPA-1a mAb B2 but much larger studies are required to confirm that this gives the expected results.

6.6 Summary of findings in this chapter

- A novel adaptation of the ROTEM® *delta* assay was developed to assess inhibitory effects of platelet-reactive antibodies on global haemostasis.
- The modified ROTEM assay was demonstrated to be valid by showing the expected inhibitory responses of the fibrin polymerisation inhibitor peptide Gly-Pro-Arg-Pro and the known inter-donor variability in haemostatic response.
- Use of this assay showed that the anti-HPA-1a mAb B2 was able to exert an inhibitory effect on primary haemostasis.
- Parameters that were able to demonstrate inhibitory effects of B2 were A5, AR5, and CFT, although the results of these parameters were not concordant.
- Because of large variations in haemostatic response between blood donors, these effects were only demonstrable when determining the 'test' activity with respect to buffer control assayed in parallel.
- One of 11 NAIT-derived, and one of two PTP-derived examples of anti-HPA-1a were able to inhibit effects in the ROTEM® assay.
- Use of flow cytometry and a range of markers of platelet activation (PAC-1, fibrinogen binding and CD62P) showed that there was little correlation between markers or with ROTEM results. The most sensitive marker was PAC-1.
- Reduced PAC-1 binding was observed when using mAb B2, 4/11 NAIT-derived and 1/2 PTP-derived examples of anti-HPA-1a.

- Significant reduction in fibrinogen binding was observed with only one NAIT-derived anti-HPA-1a.
- Three NAIT-derived anti-HPA-1a samples resulted in reduced expression of CD62P.
- Two NAIT anti-HPA-1a samples induced increased fibrinogen binding; in one case this appeared to be HPA-1a specific and in the other the increase was non-HPA-1a specific.

6.7 Significance of the results

These data show that it is possible to assess the effect of platelet-reactive antibodies in an assay that uses a rapid IgG purification system and a point of care testing device, the ROTEM® *delta* analyser.

Samples containing anti-HPA-1a can result in apparent activation or inhibition of platelets, depending on which marker is used in flow cytometry assays.

Use of PAC-1 antibody demonstrated a higher incidence of inhibitory examples of NAIT-derived anti-HPA-1a than in previous studies.

Chapter 7

Discussion, conclusions
and future directions.

7.1 Discussion

7.1.1 Detection and quantitation of HPA-1a antibodies

Despite the development of a plethora of assays for the detection of platelet antibodies in response to specific problems with sensitivity, specificity and assay time, it is apparent that only a minority of these have been introduced into routine diagnostic use. More importantly, it is evident that the issue of assay sensitivity remains, and current assays not only fail to detect antibodies in up to 32% of clinically suspected cases of NAIT (Mueller-Eckhardt *et al*, 1989; Dreyfus *et al*, 1997; Turner *et al*, 2005; Ghevaert *et al*, 2007b; Bakchoul *et al*, 2011) but they also demonstrate large variations in antibody titre or quantitation in international assessments of laboratory performance (Allen *et al*, 2005; Sachs *et al*, 2012). Even when performed in a single laboratory the measured level of anti-HPA-1a correlates less than perfectly with neonatal outcome (Killie *et al*, 2008). These findings are of concern and are factors that have played a role in the decision not to implement antenatal screening for NAIT in the UK (Williamson, 1998; Murphy & Williamson, 2000; Murphy *et al*, 2002a). Clearly, an understanding of why current assays are unable to reliably detect and/or to measure levels of antibody is important to improve service provision and possibly to allow introduction of antenatal screening.

In this study, as part of a wider study of the incidence and outcomes of NAIT (Knight *et al*, 2011), we investigated whether non-detection of alloantibodies against HPAs is an issue in England. We also explored the hypothesis that the predominant reason for non-detection and variable measurement of anti-HPA-1a antibodies is use of $\beta 3$ integrin in a less than optimal conformation. Additionally we studied functional effects of HPA-1a antibodies that might contribute to the pathology of NAIT.

Our data show that in England, North Wales and Northern Ireland, clinically-reported NAIT occurs at a similar incidence (1 case per 8,080 births) to studies reported elsewhere and that this figure is considerably lower than that reported in antenatal

screening studies (Durand-Zaleski *et al*, 1996; Davoren *et al*, 2002; Tiller *et al*, 2009). However, unlike other studies we were unable to demonstrate a significant excess of mothers of HPA-1b1b (or any other) genotype amongst antibody-negative cases, suggesting that either the assays used by the national platelet immunology reference laboratory in England are extremely sensitive or that our study was underpowered to detect differences.

Our study, unsurprisingly, showed that NAIT was most frequently caused by HPA-1a antibodies. Taken together with our observation that the degree of thrombocytopenia and long-term outcome in previously undiagnosed neonates was more severe than in previously diagnosed and antenatally treated cases, these data confirm that an understanding and resolution of the problems of HPA-1a antibody detection and quantitation could potentially significantly impact on the burden of NAIT on both families and on health services.

Furthermore, by using the clinical information provided to subdivide referrals into a number of categories we made a number of observations and provided important new information on NAIT.

We found evidence that NAIT was under-recognised (or not investigated) in a significant number of cases; amongst the referrals that we categorised as 'possible history of NAIT in a previous pregnancy', there was evidence of previous children having symptoms indicative of NAIT in 5/19 cases (26.3%). In a study of the post-natal management of NAIT, only 20 - 40% of the expected number of cases were reported and the authors concluded that there was a need to improve the recognition of this disorder; our findings would support this and suggest that under-recognition may be one possible explanation for differences in incidence between prospective antenatal screening and clinical-reporting studies of NAIT (Murphy *et al*, 1999).

We also showed that HPA antibodies can be detected at a relatively high incidence in

mothers whose fetuses/infants we categorised as suffering from IUGR (11.1%, 3/27 cases) and 'cerebral abnormalities in the current fetus' (3.5%, 10/285 cases). These figures compare to an incidence of HPA antibodies amongst female blood donors of 0.05% suggesting that either HPA antibodies are important in these two clinical presentations of NAIT, or that antibody levels decline after pregnancy in most women (Dawkins *et al*, 1995). However, both Tiller and Mueller-Eckhardt provided some evidence to support our findings regarding IUGR, with a lower birth weight observed in infants born to mothers with HPA-1a antibodies than in those born to mothers without detectable HPA antibodies (Mueller-Eckhardt *et al*, 1989; Tiller *et al*, 2012).

Our findings regarding the incidence of HPA antibodies in cases presenting as hydrocephaly or ventriculomegaly are important since although there have been isolated reports of such presentations of NAIT our data provides a quantitative incidence figure amongst a relatively large group of referrals (Petersen *et al*, 1986; Montemagno *et al*, 1994; Murphy *et al*, 1996; Pova *et al*, 2007; Breeze *et al*, 2007).

Thus these data suggest that IUGR and cerebral abnormalities are potentially important indicators of NAIT but larger studies are required to confirm this.

This first phase of the study therefore has made a number of important observations and has confirmed the validity of studying HPA-1a antibodies since they are the predominant cause of NAIT in England.

In the main part of this study we investigated the role of integrin conformation on the detection of HPA-1a antibodies based on our hypothesis that differences in conformation of $\beta 3$ might be responsible for differences in assay sensitivity.

Our data show that *in vitro* activation of $\alpha \text{IIb}\beta 3$ is unlikely to be responsible for variation in assay sensitivity. However, we were able to show that two potential

sources of conformational changes of $\alpha\text{IIb}\beta\text{3}$, namely the use of EDTA plasma and the presence of EDTA in laboratory buffers both resulted in reduced assay sensitivity.

These effects were apparent in the two most widely used assays for platelet antibody detection in Europe, namely the Indirect PIFT and MAIPA assays. The conformational changes in $\alpha\text{IIb}\beta\text{3}$ caused by EDTA impacted on binding of the human alloantibody (PIFT and MAIPA) and capture mAbs used routinely in the MAIPA assay. The extent to which binding of capture mAb was affected depended on the epitope specificity but the majority of mAbs were affected. Of particular note was the almost complete loss of reactivity of clone P2, which is widely used in diagnostic laboratories and in a report on the NIBSC workshop exercises, out of a panel of 12 capture mAbs, P2 was the clone most associated with a failure to detect some examples of anti-HPA-1a, failing to detect on 11/17 occasions (Allen *et al*, 2007).

We also showed that the binding of clone Y2/51 to EDTA-treated platelets was significantly affected (approximately 40% loss of reactivity). This clone was associated with a failure to detect some examples of anti-HPA-1a (on 1/6 occasions) (Allen *et al*, 2007).

The reduced binding of capture mAbs was shown to significantly reduce the sensitivity of the MAIPA assay for the detection of NAIT-derived examples of anti-HPA-1a. This effect was apparent when comparing either EDTA plasma with serum (approximately 48% loss of sensitivity) or when using serum and comparing EDTA-treated versus control buffer-treated platelets (approximately 81% loss of sensitivity) (**Figure 4.5**). However, it might be argued that such findings are not relevant to diagnostic laboratories because of the assay conditions we used i.e. use of PRP. We therefore distributed serum and EDTA plasma samples obtained from a mother of a child that had been severely affected by NAIT and in whom we demonstrated the

presence of a potent anti-HPA-1a, to 39 participants in NIBSC workshop exercise 2011-B. The data returned from these participants showed that our findings were very relevant to diagnostic laboratories.

Two participants (5.1%) reported that the EDTA plasma sample did not contain platelet-specific antibodies whilst they reported the presence of anti-HPA-1a in the serum sample. One further laboratory did not detect the anti-HPA-1a in their MAIPA assay in the plasma sample but succeeded in detecting it in the serum sample. Thus 7.7% participants failed to detect this potent anti-HPA-1a in the MAIPA assay.

These results clearly demonstrate that the use of EDTA plasma samples can severely impact on the detection of anti-HPA-1a and substantiate our findings performed in a research environment.

Analysis of assay sensitivity (by comparing ODs) showed that in the MAIPA assay, the majority of participants (17/24, 70.8%) observed a reduction in assay sensitivity of >20% when using HPA-1a1a platelets and in 37.5% (9/24) the loss of sensitivity was severe (>50%). When using heterozygous, HPA-1a1b platelets the resulting loss of sensitivity was even more pronounced (mean loss 65.6%; range 0 - 96.6%) than when using HPA-1a1a platelets (mean loss 27.7%, range 0 - 85.1%). Thus, for the first time we were able to show that the testing of EDTA plasma samples in many routine diagnostic laboratories significantly impacted on the reactivity of HPA-1a antibodies, even strongly-reactive samples such as that used in this study.

These findings are extremely important since a number of groups have used EDTA plasma samples in their MAIPA assays when performing antenatal screening studies (Williamson *et al*, 1998; Killie *et al*, 2008).

Furthermore, our data show that not only does the use of EDTA plasma decrease the sensitivity of the MAIPA assay but it also affects the measured level of anti-HPA-1a.

However, the extent to which the level differs from the serum level varies from sample to sample (**Table 5.3**). This effect is greatest when low levels (<10 i.u./mL) of anti-HPA-1a are present (**Figure 5.21**) although more experiments are required to confirm this. This is also a potentially important finding since the study of Killie *et al* showed that the critical level of anti-HPA-1a at which severe thrombocytopenia was likely was 3 i.u./mL. Thus it is important that assays are able to reliably and reproducibly measure antibody levels below 10 i.u./mL (Killie *et al*, 2008).

Interestingly, the data from participants in the NIBSC workshop who used the ACE assays showed enhanced, rather than reduced, reactivity of EDTA plasma compared to the serum sample (serum mean OD 1.712, range 0.913 - 2.695; plasma mean OD 1.793, range 0.797 - 2.547, $P = 0.003$). Thus, immobilisation of $\alpha\text{IIb}\beta 3$ onto plastic prevents the EDTA-induced conformational change observed when the integrin is embedded in the platelet membrane. Bessos *et al*, in a comparative study of the MAIPA and an ELISA assay similar to that of the ACE assay, observed that the ELISA assay resulted in levels of anti-HPA-1a in plasma (presumed to be EDTA) samples that were on average 2.8 times those observed in the MAIPA assay although they noted, as found in our study that there was random variation in levels between samples in the two assays (Bessos *et al*, 2008a). Interestingly, they also noted that the largest variation was in the MAIPA assay performed on those samples containing anti-HPA-1a <1 i.u./mL. A similar effect was noted by Killie when comparing capture mAbs P2 and PAB-1 (both of which recognise epitopes that we have shown are extremely sensitive to EDTA disruption) with Y2/51 (Killie *et al*, 2010).

These data therefore suggest that immobilised $\alpha\text{IIb}\beta 3$ might be a more reliable target for the detection and quantitation of anti-HPA-1a (and perhaps other specificities). However, we and others have shown that the conformation of $\alpha\text{IIb}\beta 3$ in the ACE kits does not allow recognition by all examples of HPA antibodies, and Bessos showed

that there was no correlation between maternal anti-HPA-1a level measured by the ELISA assay and neonatal platelet count (Joiner *et al*, 1995; Kowalski *et al*, 1995a; Boehlen *et al*, 1998; Allen & Murphy, 1999; Bessos *et al*, 2008a). Thus a mechanism for immobilising $\alpha\text{IIb}\beta 3$ in a more natural conformation needs to be developed.

In **Chapter 5**, we studied the reactivity of $\alpha\text{IIb}\beta 3$ when expressed in the membrane of a melanoma cell line (M21-L1Ib) and showed that $\alpha\text{IIb}\beta 3$ in this environment was also sensitive to EDTA disruption. However, we observed some notable differences in reactivity of this integrin between the two membrane environments.

Firstly, there was less than perfect correlation with reactivity against platelets when using serum samples (**Figure 5.7B**), with platelets giving ODs 1.53 times those of M21-L1Ib cells.

Secondly, the level of anti-HPA-1a varied between serum and EDTA plasma samples.

Our studies and those of Felding-Habermann using mAbs clearly showed that M21-L1Ib cells express $\alpha\text{IIb}\beta 3$, although in our studies expression levels were much lower than on platelets and the precise level varied according to the clone used (Felding-Habermann *et al*, 1996; Felding-Habermann *et al*, 2002). Our data show that epitopes on the PSI domain seemed to be more accessible than those on other domains. Indeed, the anti-HPA-1a mAbs that are prototypical Type I (clones 23-15 and 19-7) and Type II (B2) also support these findings, suggesting that the orientation of the molecule was different in M21-L1Ib cells than in platelets. This might then explain the differences that we observed in the reactivity of the human alloantibodies which are predominantly Type I (Stafford *et al*, 2008).

However, an alternative explanation was provided by our studies of integrin density. We showed that M21-L1Ib cells expressed $\alpha\text{IIb}\beta 3$ at a density approximately 500

times less than that on platelets at around 4 per square micron compared to 2,000 per square micron on platelets (**Table 5.5**); these data are completely in agreement with those of Coller and confirm that the density of $\alpha\text{IIb}\beta 3$ integrin on platelets is one of the highest in cell biology (Coller & Shattil, 2008). We therefore postulate that on platelets the close proximity of $\alpha\text{IIb}\beta 3$ molecules (<20 nm apart) restricts some mAbs (i.e. those directed against the hybrid domain) and human polyclonal anti-HPA-1a antibodies from binding. The lower density of the molecule on M21-LIIB cells allows sufficient access to apparently increase the relative access of these epitopes compared to platelets and this then impacts on the strength of reactivity observed in the MAIMA assay. However, above a certain threshold of antibody all antigen binding sites on M21-LIIB cells become saturated and no increases in OD are observed. This, then, is an important finding and demonstrates that integrin density and total number of integrin molecules influence the sensitivity of the MAIPA/MAIMA assays.

In parallel with our investigations of $\alpha\text{IIb}\beta 3$ on M21-LIIB cells we explored our second hypothesis, shared by others, that the primary stimulating molecule in NAIT is placental syncytiotrophoblast $\alpha\text{v}\beta 3$ shed into the maternal circulation throughout pregnancy (Kumpel *et al*, 2008; Kumpel & Manoussaka, 2012). We used M21 cells, that express $\alpha\text{v}\beta 3$ and from which the M21-LIIB cells were derived, as a model for placental syncytiotrophoblasts (Felding-Habermann *et al*, 2002).

Our studies using these cells provided important new insights into the dynamics of the MAIPA assay as well as confirming other findings and suggesting further avenues for research.

Integrin $\alpha\text{v}\beta 3$ is known to be more resistant to EDTA (and protease) disruption than $\alpha\text{IIb}\beta 3$ (Fitzgerald *et al*, 1985; Vanderpuye *et al*, 1991). Our studies confirmed this in the context of detection of HPA-1a antibodies using serum versus EDTA plasma

samples (**Figure 5.12**) where we observed no difference in reactivity between these samples when using $\alpha\text{v}\beta 3$. Thus $\alpha\text{v}\beta 3$ appears to be a much more stable and more suitable molecule for the detection of anti-HPA-1a.

The question arises therefore as to whether HPA-1a antibodies preferentially recognise this integrin over $\alpha\text{IIb}\beta 3$.

We found two lines of evidence that potentially support this argument.

Despite the integrin number and density on M21 cells being very similar to that of M21-L11b cells (**Table 5.5**) there were notable differences in the reactivity of HPA-1a antibodies with these cells.

Firstly, the strength of reactivity of NAIT-derived anti-HPA-1a serum samples was higher against M21 cells than against M21-L11b cells (and platelets) when low levels of anti-HPA-1a were present (**Figure 5.13**).

Secondly, titration of the WHO anti-HPA-1a standard '03/152' showed significant differences in the reactivity of this reagent against $\alpha\text{v}\beta 3$ compared to that of $\alpha\text{IIb}\beta 3$ on M21-L11b cells and platelets (**Figure 5.22**). Whilst platelets showed the highest ODs between 100 and 50 i.u./mL, below this figure the M21 cells gave higher ODs. Based on integrin density and number of cells in each assay we calculated that there were almost 10^3 less molecules in the $\alpha\text{v}\beta 3$ MAIMA assay than in the platelet MAIPA and yet the strength of reactivity with $\alpha\text{v}\beta 3$ cells was higher. These results are intriguing and suggest that the HPA-1a epitopes on $\alpha\text{v}\beta 3$ are more accessible, are differently orientated or are preferentially recognised compared to HPA-1a epitopes on $\alpha\text{IIb}\beta 3$.

We therefore performed molecular modelling of both complexes and showed differences in the region of the HPA-1a epitopes (**Figure 5.14**) thus supporting our serological data. Unfortunately, we were unable to confirm differential recognition of

the epitopes on $\alpha\text{IIb}\beta 3$ and $\alpha\text{v}\beta 3$ by absorption/elution experiments although it should be noted that only one example of anti-HPA-1a was used and others may have shown different results.

One other possible explanation for the enhanced reactivity of low levels of antibody observed with M21 cells relates to the dynamics of the three main components of the MAIPA assay, the number of antigen binding sites, the number of capture mAb molecules and the number of alloantibody molecules. In **Figure 5.29** it can be seen that when low levels of alloantibody are present, their detection would be less than optimal when using cells with a high number of integrin molecules since only a proportion of these would have alloantibody bound to them; capture of the complexes onto the ELISA plate would therefore result in a weakened signal. Use of cells expressing low numbers of integrin molecules would still result in saturation of all molecules with alloantibody and thus maximal signal from the captured integrins.

However, this explanation would also apply to the M21-LIIb cells and as described earlier these cells did not demonstrate enhanced reactivity with the samples that were more reactive with M21 cells than platelets (**Figure 5.13**).

These findings therefore suggest that $\alpha\text{v}\beta 3$ is more sensitive than $\alpha\text{IIb}\beta 3$ for the detection of anti-HPA-1a. Unfortunately, testing of a number of samples from HPA-1b1b mothers in whom HPA-1a antibodies had not been detected by the NHSBT reference laboratory did not result in the expected detection of antibodies when using M21 cells (**Figures 5.23** and **5.24**). This may be because the assays used by that laboratory are now extremely sensitive; this hypothesis is perhaps supported by our findings in **Chapter 3** where we were unable to confirm an excess of antibody negative mothers of HPA-1b1b genotype.

Nonetheless, our observations that integrin conformation, density all appear to influence the ODs in the MAIPA assay remain valid.

We then studied the impact of these factors on the measured level of anti-HPA-1a.

Our data showed that this was also affected but the degree of variation varied between samples (**Table 5.3** and **Figure 5.20**). These data support our earlier findings but contrast with those of Bertrand who, in a report of a multi-centre study of anti-HPA-1a quantitation concluded that antibody levels in the supplied samples were similar despite variations in MAIPA protocol (Bertrand *et al*, 2012). However, close scrutiny of supplementary figure 3 in this report suggests that there were differences in antibody levels assessed by different protocols with some samples in some laboratories e.g. sample 4/laboratory A and samples 1 and 2/laboratory B.

Clearly then, in order to try and standardise antibody detection and quantitation it would be advantageous to use a target molecule that was consistent in conformation, number and density. The use of M21 cells expressing $\alpha\beta 3$ is one possible solution.

An alternative solution is to use immobilised integrin, although as described earlier, care needs to be taken that this is immobilised in the correct conformation. Recently, Stafford *et al* have produced a number of recombinant fragments of $\beta 3$, one of which appeared to be suitable for the detection of HPA-1a antibodies (Stafford *et al*, 2008). This fragment ($\Delta\beta A$ -Leu33), has been coupled to Luminex x-MAP beads and has successfully been shown to detect HPA-1a antibodies (Chong *et al*, 2011). We therefore compared the sensitivity of our M21 cell line (and M21-LIIB and platelets) with that of the Luminex technology in a collaborative project with Chong *et al*.

Using a panel of blinded samples we demonstrated that MAIPA or MAIMA assays using $\alpha IIb\beta 3$ were the least sensitive (platelet MAIPA 75.5 - 89.8%; M21-LIIB MAIMA 81.6 - 89.8% depending on capture mAb) (**Table 5.8**). Both M21 cell MAIMA and $\Delta\beta A$ -Leu33 –Luminex had high sensitivity (93.9 – 95.9% and 93.9% respectively).

However, this panel contained EDTA plasma examples of anti-HPA-1a and, as we

have shown, this adversely affects the $\alpha\text{IIb}\beta 3$ MAIPA. We therefore re-analysed the data having excluded such samples and all assays had a sensitivity of 91.2% except for the M21 MAIMA using clone VI-PL2 as capture mAb which had a sensitivity of 94.1% (**Table 5.9**).

These data clearly show that most examples of anti-HPA-1a are able to be detected using any of these assays. However, there was no correlation between the strength of reactivity observed in the Luminex assay with any of the cell types (**Figure 5.25**). Interestingly, it has recently been shown that some examples of anti-HPA-1a are able to recognise peptide sequences that bear no resemblance to sequences found in $\beta 3$ suggesting that a core of HPA-1a antibodies are able to be detected irrespective of integrin conformation (Thibaut *et al*, 2012). However, one major criticism of these comparative studies is that they all used alloantibodies detected using a particular method (usually MAIPA) and thus the panels of samples will exclude those that fail to react by that particular method. It would be informative therefore to prospectively screen a cohort of pregnant women with other assays or $\beta 3$ integrin in various conformations.

Although we did not perform any antibody quantitation studies using the Luminex assay we did investigate the relationship between the measured level of antibody when using different cell types and the neonatal platelet count on a limited number of samples. Unfortunately we could not demonstrate a strong relationship when using any of the cell types but this is perhaps not surprising since all of the samples available to us had been taken at or near to delivery and it is now known that a relationship can only be demonstrated in samples taken earlier in pregnancy (Killie *et al*, 2008; Bertrand *et al*, 2011).

7.1.2 Functional effects of HPA-1a antibodies

The pivotal role of platelets in primary haemostasis and in particular the role of $\alpha\text{IIb}\beta 3$

in many aspects of this process means that impairment of function by alteration of $\alpha\text{IIb}\beta 3$ function can potentially have severe haemorrhagic effects.

A number of studies have shown that HPA-1a antibodies are able to impair platelet function or the binding of ligand to $\alpha\text{IIb}\beta 3$ expressed in cell lines but that there are differences between NAIT-derived and PTP-derived examples, with only a minority of NAIT-derived exerting function-blocking effects (van Leeuwen *et al*, 1984; Duncan & Rosse, 1986; Ryu *et al*, 1990; Beadling *et al*, 1995; Kroll *et al*, 2005a).

These studies had a number of limitations since they studied either simply the interaction of $\alpha\text{IIb}\beta 3$ with fibrinogen or the aggregation of platelets in isolation from other haemostatic processes. In **Chapter 6** we investigated whether the use of the ROTEM® *delta* thromboelastograph, which provides a more broad assessment of haemostasis including the contribution of platelets, might provide new insights on the effect of HPA-1a antibodies.

We showed that modification of the assay to include the use of diluted whole blood samples still allowed the assay to function as expected. Importantly, we were able to show that the monoclonal anti-HPA-1a 'B2', which has previously been shown to inhibit the interaction of platelets with fibrinogen, was able to exhibit a dose-dependent inhibitory effect on the ROTEM® *delta* profile when using the parameters 'A5', 'AR5' and 'CFT' (Joutsu-Korhonen *et al*, 2004). These data therefore suggested that this assay potentially was a new mechanism by which inhibitory effects of HPA-1a antibodies might be assessed.

However, the use of PAC-1 as a marker for inhibition of $\alpha\text{IIb}\beta 3$ activation in a more conventional flow cytometry assay showed that the ROTEM® *delta* assay was not as sensitive for the detection of function-blocking effects of B2 (**Table 6.5**). The use of PAC-1 in this context has not been investigated before and so we also used inhibition of fibrinogen binding. Again, there were differences between this assay and both the

PAC-1 and ROTEM® *delta* assays.

Since the samples used in these experiments were purified IgG rather than purified anti-HPA-1a IgG there are a number of possible explanations for these findings.

Firstly, it is known that antibodies other than HPA-1a such as HLA class I are able to activate platelets; this would potentially result in opposing effects with any inhibitory HPA-1a antibodies that might be present, and the balance between these would determine the overall effect observed.

Secondly, one of the reasons for inhibition of platelet function is reported to be steric hindrance of binding of fibrinogen due to close proximity of the HPA-1a epitope and the fibrinogen binding site. It is possible that the differences observed between the two markers of activation of $\alpha\text{IIb}\beta\text{3}$ (fibrinogen and PAC-1) may be due to differences in epitopes recognised by the different HPA-1a antibodies, with some examples binding closer to the PAC-1 binding site than to the fibrinogen binding site and *vice versa*.

Lastly, we used IgG purified by a simple spin purification procedure rather than by a more complex affinity column process. Whilst the purification process successfully recovered almost all of the anti-HPA-1a IgG (mean recovery 93.3% of that present in the original sample), there was still a significant amount of protein present other than IgG (**Figure 6.3C**). We did not attempt to identify these additional proteins but it is possible that these may have affected the results in either the flow cytometry or ROTEM® *delta* assays.

We conclude therefore that the ROTEM® *delta* assay potentially is a suitable tool for the investigation of the inhibitory effects of HPA-1a antibodies (as evidenced by the results with 'B2') but further work is required using purified anti-HPA-1a IgG from NAIT-derived polyclonal examples of anti-HPA-1a. More importantly, the PAC-1

assay appears to be the most sensitive indicator of function-blocking effects of HPA-1a antibodies.

Lastly, we attempted to show that HPA-1a antibodies have an inhibitory effect on platelet production. Unfortunately, the commercial assay that we used proved not to be suitable to adaptation for inhibitory assays.

7.2 Conclusions

Our studies have shown that the sensitive detection and quantitation of HPA-1a antibodies are critically dependent on the conformation of the $\beta 3$ integrin on which this polymorphism is located. Whilst it appears that many examples of anti-HPA-1a can react with various conformations of $\beta 3$, some have more demanding epitope requirements and the sensitive detection of such antibodies can be challenging.

We showed that *in vitro* changes to the conformation of $\alpha \text{IIb}\beta 3$ through the use of the cation chelator EDTA can have a significant deleterious impact on assay sensitivity and these findings were validated in a number of laboratories that provide a service for the diagnosis of NAIT.

Our hypothesis that HPA-1a antibodies are produced as a result of exposure to integrin $\alpha \text{v}\beta 3$ and thus react primarily with this integrin rather than $\alpha \text{IIb}\beta 3$ was not completely proven in these studies but we did provide a number of pieces of supporting evidence. We showed that there were differences in the reactivity of HPA-1a antibodies with $\alpha \text{v}\beta 3$ and $\alpha \text{IIb}\beta 3$ within the same cellular environment i.e. M21 and M21-LIIB cells, with $\alpha \text{v}\beta 3$ resulting in more sensitive detection of some examples. Molecular models of these two proteins showed differences in the region of the HPA-1 polymorphism thus supporting these findings.

Additionally we provided indirect evidence in **Chapter 3** in our observations of 27.6% of referrals for investigation of suspected NAIT having come from primiparous mothers (who presumably had not been exposed to platelet $\alpha \text{IIb}\beta 3$) and of finding of HPA-1a antibodies in 11.1% of referrals for IUGR where it has been postulated that binding of HPA-1a antibodies to $\alpha \text{v}\beta 3$ may interfere with angiogenesis and reduce placental function (Tiller *et al*, 2012). However, further studies are still required on the interaction of HPA-1a antibodies with $\alpha \text{v}\beta 3$ and its' involvement in NAIT.

Finally, we showed that the measured level of HPA-1a antibodies depends on a number of factors including the conformation of $\beta 3$ which was affected by cation chelation (but to a variable extent between samples), by the α -chain with which it was associated and by the number and density of integrin molecules on the target cell. Thus, we provide a possible explanation for some differences between reported studies of maternal anti-HPA-1a antibody levels.

7.3 Future studies

Clearly, future studies on antibody detection and measurement of antibody levels should either use serum samples (and assay systems that do not contain EDTA or other cation chelators) or use incubation temperatures that do not lead to EDTA-induced conformational changes. Furthermore, the use of a target integrin molecule that has been immobilised into an appropriate conformation might have a positive impact on the inter-laboratory variation in antibody detection.

Since we were unable to conclusively show that HPA-1a antibodies preferentially recognise $\alpha v\beta 3$ but that there is increasing evidence that this is the case, it would be informative to study the affinity between HPA-1a antibodies and this integrin compared to that for $\alpha IIb\beta 3$ using SPR. This might present a challenge since, depending on how the assay is designed, purification of the integrins in order to immobilise them onto the SPR chips is likely to change their conformation, and purification of anti-HPA-1a IgG might only select sub-populations of antibodies. However, a recent development of SPR using intact cells might help to overcome one of these issues (Mizuguchi *et al*, 2012).

Our studies using the $\alpha v\beta 3$ MAIMA assay showed that even though there was approximately one order of magnitude less integrin molecules per cell and three orders of magnitude less molecules per assay compared to the platelet MAIPA assay the $\alpha v\beta 3$ assay was more sensitive. It would be informative therefore, to investigate

the use of cells transfected to express a varying number of molecules to better understand the complex inter-relationship between the components of the MAIPA/MAIMA assays.

References

- Ackroyd, J.F. (1949b) The cause of thrombocytopenia in sedormid purpura. *Clin.Sci.(Lond)*, **8**, 269-289.
- Ackroyd, J.F. (1949a) The mechanism of the reduction of clot retraction by sedormid in the blood of patients who have recovered from sedormid purpura. *Clin.Sci.(Lond)*, **8**, 235-627.
- Ackroyd, J.F. (1949c) The pathogenesis of thrombocytopenic purpura due to hypersensitivity to sedormid. *Clin.Sci.(Lond)*, **7**, 249-285.
- Ackroyd, J.F. (1951) The role of complement in sedormid purpura. *Clin.Sci.(Lond)*, **10**, 185-205.
- Adair, B.D., Xiong, J.P., Maddock, C., Goodman, S.L., Arnaout, M.A., & Yeager, M. (2005) Three-dimensional EM structure of the ectodomain of integrin $\alpha V\beta 3$ in a complex with fibronectin. *J.Cell Biol.*, **168**, 1109-1118.
- Adair, B.D. & Yeager, M. (2002) Three-dimensional model of the human platelet integrin $\alpha IIb\beta 3$ based on electron cryomicroscopy and x-ray crystallography. *Proc.Natl.Acad.Sci.U.S.A*, **99**, 14059-14064.
- Addison, W. (1842) 'On the colourless corpuscles and on the molecules and cyto blasts in the blood'. *Lond Med Gazette*, **30**, 144-146.
- Afshari, A., Wikkelsø, A., Brok, J., Møller, A.M., & Wetterslev, J. (2011) Thrombelastography (TEG) or thromboelastometry (ROTEM) to monitor haemotherapy versus usual care in patients with massive transfusion. *Cochrane.Database.Syst.Rev.*, CD007871.
- Ahlen, M.T., Husebekk, A., Killie, M.K., Kjeldsen-Kragh, J., Olsson, M.L., & Skogen, B. (2012) The development of severe neonatal alloimmune thrombocytopenia due to anti-HPA-1a antibodies is correlated to maternal ABO genotypes. *Clin.Dev.Immunol.*, **2012**, 156867.
- Allen, D., Ouwehand, W.H., De, H.M., Kekomaki, R., Kaplan, C., & Metcalfe, P. (2007) Interlaboratory variation in the detection of HPA-specific alloantibodies and in molecular HPA typing. *Vox Sang.*, **93**, 316-324.
- Allen, D., Rigsby, P., Bessos, H., Berry, J., Wilson, D., Ouwehand, W.H., Urbaniak, S., & Metcalfe, P. (2005) Collaborative study to establish the first international standard for quantitation of anti-HPA-1a. *Vox Sang.*, **89**, 100-104.
- Allen, D.L., Abrahamsson, S., Murphy, M.F., & Roberts, D.J. (2012) Human platelet antigen 1a epitopes are dependent on the cation-regulated conformation of integrin $\alpha IIb\beta 3$ (GPIIb/IIIa). *J.Immunol.Methods*, **375**, 166-175.
- Allen, D.L., Chapman, J., Phillips, P.K., & Ouwehand, W.H. (1994) Sensitivity of the platelet immunofluorescence test (PIFT) and the MAIPA assay for the detection of platelet-reactive alloantibodies: a report on two U.K. National Platelet Workshop exercises. *Transfus.Med.*, **4**, 157-164.
- Allen, D.L., Floyd, A.P., Billson, A.L., Bell, R.A.F., Davis, J.G., & Entwistle, C.C. (1992) The use of MAIPA (monoclonal antibody specific immobilisation of platelet antigens) is essential

- for the investigation of neonatal alloimmune thrombocytopenia (NAIT). *Transfus.Med.*, **2 (Supple 1)**, 47.
- Allen,D.L., Lucas,G.F., Murphy M.F., & Ouwehand,W.H. (2009) Platelet and neutrophil antigens. *Practical Transfusion Medicine* (ed. by Murphy M.F. & D. H. Pamphilon), pp. 44-59. Wiley-Blackwell, Chichester.
- Allen,D.L. & Murphy,M.F. (1999) Evaluation of an enzyme-linked immunosorbent assay kit (GTI PakPlus) for the detection of antibodies against human platelet antigens. *Transfus.Med.*, **9**, 384-386.
- Bain,B.J., Barnett,D., Linch,D., Matutes,E., & Reilly,J.T. (2002) Revised guideline on immunophenotyping in acute leukaemias and chronic lymphoproliferative disorders. *Clin.Lab Haematol.*, **24**, 1-13.
- Bajt,M.L. & Loftus,J.C. (1994) Mutation of a ligand binding domain of beta 3 integrin. Integral role of oxygenated residues in alpha IIb beta 3 (GPIIb-IIIa) receptor function. *J Biol.Chem.*, **269**, 20913-20919.
- Bakchoul,T., Kubiak,S., Krautwurst,A., Roderfeld,M., Siebert,H.C., Bein,G., Sachs,U.J., & Santoso,S. (2011) Low-avidity anti-HPA-1a alloantibodies are capable of antigen-positive platelet destruction in the NOD/SCID mouse model of alloimmune thrombocytopenia. *Transfusion*, **51**, 2455-2461.
- Balduini,C.L., Noris,P., Giorgiani,G., Martinetti,M., Klersy,C., Spedini,P., Belletti,S., Maccario,R., Gusberti,L., & Locatelli,F. (1999) Incompatibility for CD31 and human platelet antigens and acute graft-versus-host disease after bone marrow transplantation. *Br.J.Haematol.*, **106**, 723-729.
- Barron-Casella,E.A., Kickler,T.S., Rogers,O.C., & Casella,J.F. (1994) Expression and purification of functional recombinant epitopes for the platelet antigens, PIA1 and PIA2. *Blood*, **84**, 1157-1163.
- Barron-Casella,E.A., Nebbia,G., Rogers,O.C., King,K.E., Kickler,T.S., & Casella,J.F. (1999) Construction of a human platelet alloantigen-1a epitope(s) within murine glycoprotein IIIa: identification of residues critical to the conformation of the antibody binding site(s). *Blood*, **93**, 2959-2967.
- Beadling,W.V., Herman,J.H., Stuart,M.J., Keashen-Schnell,M., & Miller,J.L. (1995) Fetal bleeding in neonatal alloimmune thrombocytopenia mediated by anti-PIA1 is not associated with inhibition of fibrinogen binding to platelet GPIIb/IIIa. *Am.J.Clin.Pathol.*, **103**, 636-641.
- Berman,H.M., Westbrook,J., Feng,Z., Gilliland,G., Bhat,T.N., Weissig,H., Shindyalov,I.N., & Bourne,P.E. (2000) The Protein Data Bank. *Nucleic Acids Res.*, **28**, 235-242.
- Bertrand,G., Bakchoul,T., Javela,K., Kjaer Killie,M., Kaplan,C., & on behalf of The SSC of the ISTH (2012) Interlaboratory workshop on anti-HPA-1a alloantibody quantification with the mAb-specific immobilization of platelet antigen technique. *Journal of Thrombosis and Haemostasis*, **10**, 1172-1174.
- Bertrand,G., Drame,M., Martageix,C., & Kaplan,C. (2011) Prediction of the fetal status in noninvasive management of alloimmune thrombocytopenia. *Blood*, **117**, 3209-3213.
- Bertrand,G., Jallu,V., Gouet,M., Kjaer,K.M., Lambin,P., Husebekk,A., & Kaplan,C. (2005) Quantification of human platelet antigen-1a antibodies with the monoclonal antibody immobilization of platelet antigens procedure. *Transfusion*, **45**, 1319-1323.
- Bertrand,G., Martageix,C., Jallu,V., Vitry,F., & Kaplan,C. (2006) Predictive value of sequential maternal anti-HPA-1a antibody concentrations for the severity of fetal alloimmune

- thrombocytopenia. *J. Thromb. Haemost.*, **4**, 628-637.
- Bessos, H., Killie, M.K., Matviyenko, M., Husebekk, A., & Urbaniak, S.J. (2008a) Direct comparison between two quantitative assays in the measurement of maternal anti-HPA-1a antibody in neonatal alloimmune thrombocytopenia (NAIT). *Transfus. Apher. Sci.*, **39**, 221-227.
- Bessos, H., Matviyenko, M., Brown, P., Husebekk, A., Killie, M.K., Seghatchian, J., Santoso, S., & Urbaniak, S.J. (2008b) What's happening--probing of HPA-1a antigen-antibody interaction by surface plasmon resonance technology. *Transfus. Apher. Sci.*, **39**, 179-182.
- Bessos, H., Wilson, D.W., Metcalfe, P., Allen, D., & Urbaniak, S.J. (2005) Report on the 12th International Society of Blood Transfusion platelet immunology workshop. *Vox Sang.*, **89**, 105-113.
- Bizzozzero, G. (1882) Ueber einer neuen Formbestandtheil des Blutes und dessen Rolle bei der Thrombose und der Blutgerinnung. *Arch Path Anat Physiol.*, **90**, 261-332.
- Blue, R., Li, J., Steinberger, J., Murcia, M., Filizola, M., & Collier, B.S. (2010) Effects of limiting extension at the alphaIIb genu on ligand binding to integrin alphaIIb beta3. *J. Biol. Chem.*, **285**, 17604-17613.
- Blumberg, N., Masel, D., Mayer, T., Horan, P., & Heal, J. (1984) Removal of HLA-A, B antigens from platelets. *Blood*, **63**, 448-450.
- Bodary, S.C., Napier, M.A., & McLean, J.W. (1989) Expression of recombinant platelet glycoprotein IIb/IIIa results in a functional fibrinogen-binding complex. *J Biol. Chem.*, **264**, 18859-18862.
- Boehlen, F., Bulla, O., & de, M.P. (1998) Evaluation of a new antigen capture ELISA for detection and characterization of platelet alloantibodies. *Thromb. Res.*, **92**, 27-32.
- Boughton, B.J., Simpson, A.W., Bolt, C., Buchan, A., & McLeish, P. (1995) Platelet Membrane Glycoprotein IIb/IIIa has Sequence Homologies with Human Virus Proteins and Synthetic Viral Peptides Inhibit Anti-GPIIb/IIIa Antibodies in Autoimmune Thrombocytopenic Purpura. *Platelets.*, **6**, 75-82.
- Bournazos, S., Rennie, J., Hart, S.P., & Dransfield, I. (2008) Choice of anticoagulant critically affects measurement of circulating platelet-leukocyte complexes. *Arterioscler. Thromb. Vasc. Biol.*, **28**, e2-e3.
- Bowditch, R.D., TAni, P.H., Halloran, C.E., Frelinger, A.L., III, McMillan, R., & Ginsberg, M.H. (1992) Localization of a PIA1 epitope to the amino terminal 66 residues of platelet glycoprotein IIIa. *Blood*, **79**, 559-562.
- Bowman, J.M., Pollock, J.M., & Penston, L.E. (1986) Fetomaternal transplacental hemorrhage during pregnancy and after delivery. *Vox Sang.*, **51**, 117-121.
- Bray, P.F., Barsh, G., Rosa, J.P., Luo, X.Y., Magenis, E., & Shuman, M.A. (1988) Physical linkage of the genes for platelet membrane glycoproteins IIb and IIIa. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 8683-8687.
- Bray, P.F., Rosa, J.P., Johnston, G.I., Shiu, D.T., Cook, R.G., Lau, C., Kan, Y.W., McEver, R.P., & Shuman, M.A. (1987) Platelet glycoprotein IIb. Chromosomal localization and tissue expression. *J. Clin. Invest.*, **80**, 1812-1817.
- Breeze, A.C., Alexander, P.M., Murdoch, E.M., Missfelder-Lobos, H.H., Hackett, G.A., & Lees, C.C. (2007) Obstetric and neonatal outcomes in severe fetal ventriculomegaly. *Prenat. Diagn.*, **27**, 124-129.

- Brodsky,D. & Christou,H. (2004) Current concepts in intrauterine growth restriction. *J Intensive Care Med*, **19**, 307-319.
- Burrows,R.F. & Kelton,J.G. (1993) Fetal thrombocytopenia and its relation to maternal thrombocytopenia. *New.Eng.J.Med*, **329**, 1463-1466.
- Bussel,J.B., Zacharoulis,S., Kramer,K., McFarland,J.G., Pauliny,J., & Kaplan,C. (2005) Clinical and diagnostic comparison of neonatal alloimmune thrombocytopenia to non-immune cases of thrombocytopenia. *Pediatr.Blood Cancer*, **45**, 176-183.
- Byzova,T.V., Rabbani,R., D'Souza,S.E., & Plow,E.F. (1998) Role of integrin alpha(v)beta3 in vascular biology. *Thromb.Haemost.*, **80**, 726-734.
- Campbell,K., Rishi,K., Howkins,G., Gilby,D., Mushens,R., Ghevaert,C., Metcalfe,P., Ouwehand,W.H., & Lucas,G. (2007) A modified rapid monoclonal antibody-specific immobilization of platelet antigen assay for the detection of human platelet antigen (HPA) antibodies: a multicentre evaluation. *Vox Sang.*, **93**, 289-297.
- Carrell,N.A., Fitzgerald,L.A., Steiner,B., Erickson,H.P., & Phillips,D.R. (1985) Structure of human platelet membrane glycoproteins IIb and IIIa as determined by electron microscopy. *J Biol.Chem.*, **260**, 1743-1749.
- Cartwright,J.E., Fraser,R., Leslie,K., Wallace,A.E., & James,J.L. (2010) Remodelling at the maternal-fetal interface: relevance to human pregnancy disorders. *Reproduction.*, **140**, 803-813.
- Chae,H. & Park,H.H. (2009) EDTA inhibits the binding of clone 96.2C1, an anti-CD41a monoclonal antibody, to the platelets and addition of heparin and CaCl₂ to the antibody neutralizes the EDTA-induced inhibitory effect. *Korean J Hematol.*, **44**, 42-46.
- Chakravorty,S. & Roberts,I. (2012) How I manage neonatal thrombocytopenia. *Br.J Haematol.*, **156**, 155-162.
- Chang,Y., Bluteau,D., Debili,N., & Vainchenker,W. (2007) From hematopoietic stem cells to platelets. *J Thromb.Haemost.*, **5 Suppl 1**, 318-327.
- Cheresh,D.A. & Spiro,R.C. (1987) Biosynthetic and functional properties of an Arg-Gly-Asp-directed receptor involved in human melanoma cell attachment to vitronectin, fibrinogen, and von Willebrand factor. *J Biol.Chem.*, **262**, 17703-17711.
- Chong,W., Metcalfe,P., Mushens,R., Lucas,G., Ouwehand,W.H., & Navarrete,C.V. (2011) Detection of human platelet antigen-1a alloantibodies in cases of fetomaternal alloimmune thrombocytopenia using recombinant beta3 integrin fragments coupled to fluorescently labeled beads. *Transfusion*, **51**, 1261-1270.
- Christensen,R.D., Henry,E., Wiedmeier,S.E., Stoddard,R.A., Sola-Visner,M.C., Lambert,D.K., Kiehn,T.I., & Ainsworth,S. (2006) Thrombocytopenia among extremely low birth weight neonates: data from a multihospital healthcare system. *J.Perinatol.*, **26**, 348-353.
- Christie,D.J., Leja,D.N., Carlson,D.L., & Swinehart,C.D. (1993) Characterization of platelet activation induced by HLA antibodies associated with alloimmune thrombocytopenia. *J.Lab Clin.Med.*, **121**, 437-443.
- Christie,D.J. & Swinehart,C.D. (1992) Human platelet activating antibodies. *Semin.Thromb.Hemost.*, **18**, 186-192.
- Cleal,J.K. & Lewis,R.M. (2008) The mechanisms and regulation of placental amino acid transport to the human foetus. *J.Neuroendocrinol.*, **20**, 419-426.

- Clemetson, K.J. (2012) Platelets and primary haemostasis. *Thromb. Res.*, **129**, 220-224.
- Clofent-Sanchez, G., Lucas, S., Laroche-Traineau, J., Rispal, P., Pellegrin, J.L., Nurden, P., & Nurden, A. (1996) Autoantibodies and anti-mouse antibodies in thrombocytopenic patients as assessed by different MAIPA assays. *Br.J.Haematol.*, **95**, 153-160.
- Coller, B.S. & Shattil, S.J. (2008) The GPIIb/IIIa (integrin α IIb β 3) odyssey: a technology-driven saga of a receptor with twists, turns, and even a bend. *Blood*, **112**, 3011-3025.
- Collins, J. & Aster, R.H. (1987) Use of immobilized platelet membrane glycoproteins for the detection of platelet-specific alloantibodies in solid-phase ELISA. *Vox Sang.*, **53**, 157-161.
- Cooper, B. (2005) Osler's role in defining the third corpuscle, or "blood plates". *Proc. (Bayl. Univ Med Cent.)*, **18**, 376-378.
- Cramer, E.M., Savidge, G.F., Vainchenker, W., Berndt, M.C., Pidard, D., Caen, J.P., Masse, J.M., & Breton-Gorius, J. (1990) Alpha- granule pool of glycoprotein IIb-IIIa in normal and pathologic platelets and megakaryocytes. *Blood*, **75**, 1220-1227.
- Curtis, B.R., Fick, A., Lochowicz, A.J., McFarland, J.G., Ball, R.H., Peterson, J., & Aster, R.H. (2008) Neonatal alloimmune thrombocytopenia associated with maternal-fetal incompatibility for blood group B. *Transfusion*, **48**, 358-364.
- D'Andrea, G., Colaizzo, D., Vecchione, G., Grandone, E., Di, M.G., & Margaglione, M. (2002) Glanzmann's thrombasthenia: identification of 19 new mutations in 30 patients. *Thromb.Haemost.*, **87**, 1034-1042.
- Dankers, M.K., Roelen, D.L., Korfage, N., de, L.P., Witvliet, M., Sandkuijl, L., Doxiadis, I.I., & Claas, F.H. (2003) Differential immunogenicity of paternal HLA Class I antigens in pregnant women. *Hum.Immunol.*, **64**, 600-606.
- Davis, C.M., Danehower, S.C., Laurenza, A., & Molony, J.L. (1993) Identification of a role of the vitronectin receptor and protein kinase C in the induction of endothelial cell vascular formation. *J Cell Biochem.*, **51**, 206-218.
- Davoren, A., Curtis, B.R., Aster, R.H., & McFarland, J.G. (2004) Human platelet antigen-specific alloantibodies implicated in 1162 cases of neonatal alloimmune thrombocytopenia. *Transfusion*, **44**, 1220-1225.
- Davoren, A., McParland, P., Barnes, C.A., & Murphy, W.G. (2002) Neonatal alloimmune thrombocytopenia in the Irish population: a discrepancy between observed and expected cases. *J.Clin.Pathol.*, **55**, 289-292.
- Dawkins, B. (1993) Comparison of PIFT and MAIPA tests in the detection of anti-HPA-1a (PIA1) platelet antibodies. *Transf.Med.*, **3**, (Supple 1), 87.
- Dawkins, B. (1995) Monitoring anti-HPA-1a platelet antibody levels during pregnancy using the MAIPA test. *Vox Sang.*, **68**, 27-34.
- Dawkins, B., Franz, D., & Kalinowski, A. (1995) Anti-HLA antibodies are superior indicators for the presence of useful platelet alloantibodies. *Transf.Med.*, **5**, 79.
- de Moerloose, P., Boehlen, F., Extermann, P., & Hohfeld, P. (1998) Neonatal thrombocytopenia: incidence and characterization of maternal antiplatelet antibodies by MAIPA assay. *Br.J.Haematol.*, **100**, 735-740.
- Decary, F. (1988) Report on the Second Canadian Workshop on Platelet Serology. *Curr.Stud.Hematol.Blood Transfus.*, 1-12.

- Denis,C.V. & Wagner,D.D. (2007) Platelet adhesion receptors and their ligands in mouse models of thrombosis. *Arterioscler.Thromb.Vasc.Biol.*, **27**, 728-739.
- Desforages,M. & Sibley,C.P. (2010) Placental nutrient supply and fetal growth. *Int.J.Dev.Biol.*, **54**, 377-390.
- Dettke,M., Dreer,M., Hocker,P., & Panzer,S. (2001) Human platelet antigen-1a antibodies induce the release of the chemokine RANTES from human platelets. *Vox Sang.*, **81**, 199-203.
- Donné M (1842) Academy of Sciences, Paris: M. Donne on the Blood Globules. *Prov.Med.Surg.J.(1840.)*, **3**, 498-499.
- Doughty,R., James,V., & Magee,J. (1981) An enzyme linked immunosorbent assay for leucocyte and platelet antibodies. *J Immunol.Methods*, **47**, 161-169.
- Dreyfus,M., Kaplan,C., Verdy,E., Schlegel,N., Durand-Zaleski,I., & Tchernia,G. (1997) Frequency of immune thrombocytopenia in newborns: a prospective study. Immune Thrombocytopenia Working Group. *Blood*, **89**, 4402-4406.
- Drouin,A. & Cramer,E.M. (2002) Production of platelets. Platelets in thrombotic and non-thrombotic disorders; pathophysiology, pharmacology and therapeutics (ed. by P. Gresele, C. Page, V. Fuster, & J. Vermynen), pp. 25-40. Cambridge University Press, Cambridge.
- Duncan,J.R. & Rosse,W.F. (1986) Alloantibody-induced platelet serotonin release is blocked by antibody to the platelet PLA1 antigen. *Br.J.Haematol.*, **64**, 331-338.
- Duperray,A., Berthier,R., Chagnon,E., Ryckewaert,J.J., Ginsberg,M., Plow,E., & Marguerie,G. (1987) Biosynthesis and processing of platelet GPIIb-IIIa in human megakaryocytes. *J Cell Biol.*, **104**, 1665-1673.
- Durand-Zaleski,I., Schlegel,N., Blum-Boisgard,C., Uzan,S., Dreyfus,M., & Kaplan,C. (1996) Screening primiparous women and newborns for fetal/neonatal alloimmune thrombocytopenia: a prospective comparison of effectiveness and costs. Immune Thrombocytopenia Working Group. *Am.J.Perinatol.*, **13**, 423-431.
- Eng,E.T., Smagghe,B.J., Walz,T., & Springer,T.A. (2011) Intact alphaIIbbeta3 integrin is extended after activation as measured by solution X-ray scattering and electron microscopy. *J Biol.Chem.*, **286**, 35218-35226.
- Erlandsen,S.L., Greet,B.A., White,J., Leith,A., & Marko,M. (2001) High-resolution CryoFESEM of individual cell adhesion molecules (CAMs) in the glycocalyx of human platelets: detection of P-selectin (CD62P), GPI-IX complex (CD42A/CD42B alpha,B beta), and integrin GPIIbIIIa (CD41/CD61) by immunogold labeling and stereo imaging. *J.Histochem.Cytochem.*, **49**, 809-819.
- Felding-Habermann,B., Fransvea,E., O'Toole,T.E., Manzuk,L., Faha,B., & Hensler,M. (2002) Involvement of tumor cell integrin alpha v beta 3 in hematogenous metastasis of human melanoma cells. *Clin.Exp.Metastasis*, **19**, 427-436.
- Felding-Habermann,B., Habermann,R., Saldivar,E., & Ruggeri,Z.M. (1996) Role of beta3 integrins in melanoma cell adhesion to activated platelets under flow. *J.Biol.Chem.*, **271**, 5892-5900.
- Felding-Habermann,B., Mueller,B.M., Romerdahl,C.A., & Cheresh,D.A. (1992) Involvement of integrin alpha V gene expression in human melanoma tumorigenicity. *J Clin.Invest*, **89**, 2018-2022.
- Feuston,B.P., Culberson,J.C., & Hartman,G.D. (2003) Molecular model of the

- alpha(IIb)beta(3) integrin. *J.Med.Chem.*, **46**, 5316-5325.
- Fitzgerald,L.A., Charo,I.F., & Phillips,D.R. (1985) Human and bovine endothelial cells synthesize membrane proteins similar to human platelet glycoproteins IIb and IIIa. *J.Biol.Chem.*, **260**, 10893-10896.
- Fitzgerald,L.A. & Phillips,D.R. (1985) Calcium regulation of the platelet membrane glycoprotein IIb-IIIa complex. *J.Biol.Chem.*, **260**, 11366-11374.
- Fitzgerald,L.A., Poncz,M., Steiner,B., Rall,S.C., Jr., Bennett,J.S., & Phillips,D.R. (1987a) Comparison of cDNA-derived protein sequences of the human fibronectin and vitronectin receptor alpha-subunits and platelet glycoprotein IIb. *Biochemistry*, **26**, 8158-8165.
- Fitzgerald,L.A., Steiner,B., Rall,S.C., Jr., Lo,S.S., & Phillips,D.R. (1987b) Protein sequence of endothelial glycoprotein IIIa derived from a cDNA clone. Identity with platelet glycoprotein IIIa and similarity to "integrin". *J Biol.Chem.*, **262**, 3936-3939.
- Flug,F., Espinola,R., Liu,L.X., SinQuee,C., DaRosso,R., Nardi,M., & Karpatkin,S. (1991) A 13-mer peptide straddling the leucine33/proline33 polymorphism in glycoprotein IIIa does not define the PLA1 epitope. *Blood*, **77**, 1964-1969.
- Fontao-Wendel,R., Silva,L.C., Saviolo,C.B., Primavera,B., & Wendel,S. (2007) Incidence of transfusion-induced platelet-reactive antibodies evaluated by specific assays for the detection of human leucocyte antigen and human platelet antigen antibodies. *Vox Sang*, **93**, 241-249.
- Foxcroft,Z., Campbell,K., Merieux,Y., Urbaniak,S., Brierley,M., Rigal,D., Ouwehand,W.H., & Metcalfe,P. (2007) Report on the 13th International Society of Blood Transfusion Platelet Immunology Workshop. *Vox Sang.*, **93**, 300-305.
- Frelinger,A.L., III, Du,X.P., Plow,E.F., & Ginsberg,M.H. (1991) Monoclonal antibodies to ligand-occupied conformers of integrin alpha IIb beta 3 (glycoprotein IIb-IIIa) alter receptor affinity, specificity, and function. *J.Biol.Chem.*, **266**, 17106-17111.
- Fujiwara,K., Shimano,K., Tanaka,H., Sekine,M., Kashiwase,K., Uchikawa,M., Satake,M., & Nakajima,K. (2009) Application of bead array technology to simultaneous detection of human leucocyte antigen and human platelet antigen antibodies. *Vox Sang.*, **96**, 244-251.
- Furihata,K., Nugent,D.J., Bissonette,A., Aster,R.H., & Kunicki,T.J. (1987) On the association of the platelet-specific alloantigen, Pena, with glycoprotein IIIa. Evidence for heterogeneity of glycoprotein IIIa. *J Clin.Invest*, **80**, 1624-1630.
- Gavrilovskaya,I.N., Brown,E.J., Ginsberg,M.H., & Mackow,E.R. (1999) Cellular entry of hantaviruses which cause hemorrhagic fever with renal syndrome is mediated by beta3 integrins. *J Virol.*, **73**, 3951-3959.
- Gavrilovskaya,I.N., Shepley,M., Shaw,R., Ginsberg,M.H., & Mackow,E.R. (1998) beta3 Integrins mediate the cellular entry of hantaviruses that cause respiratory failure. *Proc.Natl.Acad.Sci.U.S.A*, **95**, 7074-7079.
- George,J.N., Caen,J.P., & Nurden,A.T. (1990) Glanzmann's thrombasthenia: the spectrum of clinical disease. *Blood*, **75**, 1383-1395.
- Ghevaert,C., Campbell,K., Stafford,P., Metcalfe,P., Casbard,A., Smith,G.A., Allen,D., Ranasinghe,E., Williamson,L.M., & Ouwehand,W.H. (2007a) HPA-1a antibody potency and bioactivity do not predict severity of fetomaternal alloimmune thrombocytopenia. *Transfusion*, **47**, 1296-1305.

- Ghevaert, C., Campbell, K., Walton, J., Smith, G.A., Allen, D., Williamson, L.M., Ouwehand, W.H., & Ransinghe, E. (2007b) Management and outcome of 200 cases of fetomaternal alloimmune thrombocytopenia. *Transfusion*, **47**, 901-910.
- Gibbins, J.M. (2004) Platelet adhesion signalling and the regulation of thrombus formation. *J. Cell Sci.*, **117**, 3415-3425.
- Gidwitz, S., Temple, B., & White, G.C. (2004) Mutations in and near the second calcium-binding domain of integrin α IIb affect the structure and function of integrin α IIb β 3. *Biochem. J.*, **379**, 449-459.
- Giles, C. (1981) The platelet count and mean platelet volume. *Br. J. Haematol.*, **48**, 31-37.
- Ginsberg, M.H., Lightsey, A., Kunicki, T.J., Kaufmann, A., Marguerie, G., & Plow, E.F. (1986) Divalent cation regulation of the surface orientation of platelet membrane glycoprotein IIb. Correlation with fibrinogen binding function and definition of a novel variant of Glanzmann's thrombasthenia. *J. Clin. Invest.*, **78**, 1103-1111.
- Goldberger, A., Kolodziej, M., Poncz, M., Bennett, J.S., & Newman, P.J. (1991) Effect of single amino acid substitutions on the formation of the PIA and Bak alloantigenic epitopes. *Blood*, **78**, 681-687.
- Gramatges, M.M., Fani, P., Nadeau, K., Pereira, S., & Jeng, M.R. (2009) Neonatal alloimmune thrombocytopenia and neutropenia associated with maternal human leukocyte antigen antibodies. *Pediatr. Blood Cancer*, **53**, 97-99.
- Greaves, M., Pickering, C., Porter, N.R., Magee, J.M., & Preston, F.E. (1983) Acquired Glanzmann's thrombasthenia. *Blood*, **61**, 209.
- Greinacher, A., Fuerll, B., Zinke, H., Mullejans, B., Kruger, W., Michetti, N., Motz, W., & Schwertz, H. (2009) Megakaryocyte impairment by eptifibatide-induced antibodies causes prolonged thrombocytopenia. *Blood*, **114**, 1250-1253.
- Gruel, Y., Boizard, B., Daffos, F., Forestier, F., Caen, J., & Wautier, J.L. (1986) Determination of platelet antigens and glycoproteins in the human fetus. *Blood*, **68**, 488-492.
- Gudino, M. & Miller, W.V. (1981) Application of the enzyme linked immunospecific assay (ELISA) for the detection of platelet antibodies. *Blood*, **57**, 32-37.
- Gulino, D., Ryckewaert, J.J., Andrieux, A., Rabiet, M.J., & Marguerie, G. (1990) Identification of a monoclonal antibody against platelet GPIIb that interacts with a calcium-binding site and induces aggregation. *J. Biol. Chem.*, **265**, 9575-9581.
- Gulliver, G. (1842) In: Elements of General and Minute Anatomy of Man and the Mammalia (ed. Gerber F). Added Notes and Appendix Comprising Researches on the Anatomy of the Blood, Chyle, lymph, thymous fluid, tubercle, etc etc. by George Gulliver. Hippolyte Bailliere, London.
- Haas, T.A. (2008) Allosteric regulation of α (IIb) β (3) by β (3) 95-105. *Thromb. Haemost.*, **99**, 701-710.
- Harfenist, E.J., Guccione, M.A., Packham, M.A., & Mustard, J.F. (1982) The use of the synthetic peptide, Gly-Pro-Arg-Pro, in the preparation of thrombin-degranulated rabbit platelets. *Blood*, **59**, 952-955.
- Harker, L.A. & Finch, C.A. (1969) Thrombokinetis in man. *J. Clin. Invest.*, **48**, 963-974.
- Harrington, W.J., Sprague, C.C., Minnich, V., Moore, C.V., Aulvin, R.C., & Dubach, R. (1953) Immunologic mechanisms in idiopathic and neonatal thrombocytopenic purpura.

Ann.Intern.Med., **38**, 433-469.

- Harris,L.K. & Aplin,J.D. (2007) Vascular remodeling and extracellular matrix breakdown in the uterine spiral arteries during pregnancy. *Reprod.Sci.*, **14**, 28-34.
- Harrison,P. (2005) Platelet function analysis. *Blood Rev.*, **19**, 111-123.
- Harrison,P. (2009) Assessment of platelet function in the laboratory. *Hamostaseologie.*, **29**, 25-31.
- Hartwig,J. & Italiano,J., Jr. (2003) The birth of the platelet. *J Thromb.Haemost.*, **1**, 1580-1586.
- Hayashi,T., Amakishi,E., Matsuyama,N., Yasui,K., Furuta,R.A., Hori,Y., Fukumori,Y., Tanaka,S., Curtis,B.R., Inoue,M., & Hirayama,F. (2010) Detection of antibodies against human platelet antigens 15a and 15b by using a cell line panel. *Br.J.Haematol.*, **151**, 402-404.
- Hayashi,T., Yasui,K., Matsuyama,N., Furuta,R.A., Hori,Y., Tanaka,S., Hirayama,F., Tani,Y., Shibata,H., & Inoue,M. (2009) Establishment of a novel method for detecting Nak antibodies by using a panel cell line. *Transfusion*, **49**, 390-392.
- Heemskerk,J.W., Bevers,E.M., & Lindhout,T. (2002) Platelet activation and blood coagulation. *Thromb.Haemost.*, **88**, 186-193.
- Heilmann,E., Hourdille,P., Pruvost,A., Paponneau,A., & Nurden,A.T. (1991) Thrombin-induced platelet aggregates have a dynamic structure. Time-dependent redistribution of glycoprotein IIb-IIIa complexes and secreted adhesive proteins. *Arterioscler.Thromb.*, **11**, 704-718.
- Heinrich,D., Scharf,T., Santoso,S., Clemetson,K.J., & Mueller-Eckhardt,C. (1985) Monoclonal antibodies against human platelet membrane glycoproteins IIb-IIIa. II. Different effects on platelet function. *Thromb.Res.*, **38**, 547-559.
- Helmerhorst,F.M., Bossers,B., de Bruin,H.G., Engelfriet,C.P., & von dem Borne,A.E. (1980) Detection of platelet antibodies: a comparison of three techniques. *Vox Sang*, **39**, 83-92.
- Helmerhorst,F.M., van Oss,C.J., Briynes,E.C.E., Engelfriet,C.P., & dem Borne,A.E.G.K. (1982) Elution of granulocyte and platelet antibodies. *Vox Sang.*, **43**, 196-204.
- Hewitt,J. & Burton,I.E. (1994) Incidence of autoantibodies to GPIIb/IIIa in chronic autoimmune thrombocytopenic purpura may be overestimated by the MAIPA. *Br.J Haematol.*, **86**, 418-420.
- Hod,E. & Schwartz,J. (2008) Platelet transfusion refractoriness. *Br.J.Haematol.*, **142**, 348-360.
- Holzhauser,S. & Zieger,B. (2011) Diagnosis and management of neonatal thrombocytopenia. *Semin.Fetal Neonatal Med.*, **16**, 305-310.
- Honda,S., Honda,Y., Bauer,B., Ruan,C., & Kunicki,T.J. (1995a) The impact of three-dimensional structure on the expression of PIA alloantigens on human integrin beta 3. *Blood*, **86**, 234-242.
- Honda,S., Tomiyama,Y., Pelletier,A.J., Annis,D., Honda,Y., Orzechowski,R., Ruggeri,Z., & Kunicki,T.J. (1995b) Topography of ligand-induced binding sites, including a novel cation-sensitive epitope (AP5) at the amino terminus, of the human integrin beta 3 subunit. *J.Biol.Chem.*, **270**, 11947-11954.

- Hook,E.B. & Regal,R.R. (1995) Capture-recapture methods in epidemiology: methods and limitations. *Epidemiol.Rev.*, **17**, 243-264.
- Hussain,M.A. & Siedlecki,C.A. (2004) The platelet integrin alpha(IIb) beta(3) imaged by atomic force microscopy on model surfaces. *Micron.*, **35**, 565-573.
- Jaegtvik,S., Husebekk,A., Aune,B., Oian,P., Dahl,L.B., & Skogen,B. (2000) Neonatal alloimmune thrombocytopenia due to anti-HPA 1a antibodies; the level of maternal antibodies predicts the severity of thrombocytopenia in the newborn. *BJOG.*, **107**, 691-694.
- Jallu,V., Bianchi,F., & Kaplan,C. (2005) Fetal-neonatal alloimmune thrombocytopenia and unexpected Glanzmann thrombasthenia carrier: report of two cases. *Transfusion*, **45**, 550-553.
- Joiner,K., Munizza,M., Keashen-Schnell,M.A., Nance,S., & Murphy,S. (1995) Role of GTI-PAK in platelet antibody detection; comparison of four methods. *Transf.*, **35**, 17S.
- Joiner,K., Nance,S., Keashen-Schnell,M., & Murphy,S. (1997) Incidence of human anti-mouse antibody (HAMA) in thrombocytopenic patients (TP). *Transfusion*, **37**, 95S.
- Joutsu-Korhonen,L., Preston,S., Smethurst,P.A., Ijsseldijk,M., Schaffner-Reckinger,E., Armour,K.L., Watkins,N.A., Clark,M.R., de Groot,P.G., Farndale,R.W., Ouwehand,W.H., & Williamson,L.M. (2004) The effect of recombinant IgG antibodies against the leucine-33 form of the platelet beta3 integrin (HPA-1a) on platelet function. *Thromb.Haemost.*, **91**, 743-754.
- Juji,T., Watanabe,Y., Ishikawa,Y., Fujiwara,K., Tonami,H., Tanaka,H., Satake,M., Akaza,T., Tadokoro,K., Kodera,Y., Sasazuki,T., Morishima,Y., & Takaku,F. (1999) Human platelet alloantigen (HPA)-5a/b mismatch decreases disease-free survival in unrelated bone marrow transplantation. *Tissue Antigens*, **54**, 229-234.
- Kamata,T., Handa,M., Ito,S., Sato,Y., Ohtani,T., Kawai,Y., Ikeda,Y., & Aiso,S. (2010) Structural requirements for activation in alphaIIb beta3 integrin. *J.Biol.Chem.*, **285**, 38428-38437.
- Kamphuis,M.M., Paridaans,N., Porcelijn,L., De,H.M., van der Schoot,C.E., Brand,A., Bonsel,G.J., & Oepkes,D. (2010) Screening in pregnancy for fetal or neonatal alloimmune thrombocytopenia: systematic review. *BJOG.*, **117**, 1335-1343.
- Kaplan,C., Daffos,F., Forestier,F., Cox,W.L., Lyon-Caen,D., Dupuy-Montbrun,M.C., & Salmon,C. (1988) Management of alloimmune thrombocytopenia: antenatal diagnosis and in utero transfusion of maternal platelets. *Blood*, **72**, 340-343.
- Kaplan,C., Freedman,J., Foxcroft,Z., Husebekk,A., Metcalfe,P., Muniz-Diaz,E., Ouwehand,W., Panzer,S., Rozman,P., & Skogen,B. (2007) Monoclonal platelet antigen capture assays (MAIPA) and reagents: a statement. *Vox Sang.*, **93**, 298-299.
- Kapur,K., Johnson,T., Beckmann,N.D., Sehmi,J., Tanaka,T., Kutalik,Z., Styrkarsdottir,U., Zhang,W., Marek,D., Gudbjartsson,D.F., Milaneschi,Y., Holm,H., DiIorio,A., Waterworth,D., Li,Y., Singleton,A.B., Bjornsdottir,U.S., Sigurdsson,G., Hernandez,D.G., Desilva,R., Elliott,P., Eyjolfsson,G.I., Guralnik,J.M., Scott,J., Thorsteinsdottir,U., Bandinelli,S., Chambers,J., Stefansson,K., Waeber,G., Ferrucci,L., Kooner,J.S., Mooser,V., Vollenweider,P., Beckmann,J.S., Bochud,M., & Bergmann,S. (2010) Genome-wide meta-analysis for serum calcium identifies significantly associated SNPs near the calcium-sensing receptor (CASR) gene. *PLoS.Genet.*, **6**, e1001035.
- Kasirer-Friede,A., Kahn,M.L., & Shattil,S.J. (2007) Platelet integrins and immunoreceptors. *Immunol.Rev.*, **218**, 247-264.

- Kaye,T. & Allen,D.L. (1994) Identification of platelet-reactive antibodies using an antigen-capture ELISA. *Transfus.Med.*, **4** (Supple 1), 49.
- Kekomaki,S., Kyllonen,L., Salmela,K., Koskimies,S., & Kekomaki,R. (2001) Platelet-specific alloantigens in cadaveric renal transplantation. A prospective study. Effect of HPA-5b mismatch in acute vascular rejection of renal allografts. *Tissue Antigens*, **57**, 154-157.
- Kekomaki,S., Salmela,K., Partanen,J., Koskimies,S., & Kekomaki,R. (1997) Alloimmunization against platelet antigens in renal transplant patients with acute vascular rejection. *Clin.Transplant.*, **11**, 19-24.
- Keowmaneechai,E. & McClements,D.J. (2002) Influence of EDTA and citrate on physicochemical properties of whey protein-stabilized oil-in-water emulsions containing CaCl₂. *J.Agric.Food Chem.*, **50**, 7145-7153.
- Khurana,S., Mattson,J.C., Westley,S., O'Neill,W.W., Timmis,G.C., & Safian,R.D. (1997) Monitoring platelet glycoprotein IIb/IIIa-fibrin interaction with tissue factor-activated thromboelastography. *J Lab Clin.Med*, **130**, 401-411.
- Kiefel,V. (1992) The MAIPA assay and its applications in immunohaematology. *Transfus.Med*, **2**, 181-188.
- Kiefel,V., Konig,C., Kroll,H., & Santoso,S. (2001) Platelet alloantibodies in transfused patients 36. *Transfusion*, **41**, 766-770.
- Kiefel,V., Santoso,S., Katzmann,B., & Mueller-Eckhardt,C. (1989) The Bra/Brb alloantigen system on human platelets. *Blood*, **73**, 2219-2223.
- Kiefel,V., Santoso,S., Katzmann,B., & Mueller-Eckhardt,C. (1988) A new platelet-specific alloantigen Bra. Report of 4 cases with neonatal alloimmune thrombocytopenia. *Vox Sang.*, **54**, 101-106.
- Kiefel,V., Santoso,S., Weisheit,M., & Mueller-Eckhardt,C. (1987) Monoclonal antibody--specific immobilization of platelet antigens (MAIPA): a new tool for the identification of platelet-reactive antibodies. *Blood*, **70**, 1722-1726.
- Kiefel,V., Schonberger-Richter,I., & Schilf,K. (2005) Anti-HPA-1a in a case of post-transfusion purpura: binding to antigen-negative platelets detected by adsorption/elution. *Transfus.Med.*, **15**, 243-247.
- Kieffer,N., Fitzgerald,L.A., Wolf,D., Cheresch,D.A., & Phillips,D.R. (1991) Adhesive properties of the beta 3 integrins: comparison of GP IIb-IIIa and the vitronectin receptor individually expressed in human melanoma cells. *J.Cell Biol.*, **113**, 451-461.
- Killie,M.K., Husebekk,A., Kjeldsen-Kragh,J., & Skogen,B. (2008) A prospective study of maternal anti-HPA 1a antibody level as a potential predictor of alloimmune thrombocytopenia in the newborn. *Haematologica*, **93**, 870-877.
- Killie,M.K., Salma,W., Bertelsen,E., Skogen,B., & Husebekk,A. (2010) Quantitative MAIPA: Comparison of different MAIPA protocols. *Transfus.Apher.Sci.*, **43**, 149-154.
- Kjeldsen-Kragh,J., Killie,M.K., Tomter,G., Golebiowska,E., Randen,I., Hauge,R., Aune,B., Oian,P., Dahl,L.B., Pirhonen,J., Lindeman,R., Husby,H., Haugen,G., Gronn,M., Skogen,B., & Husebekk,A. (2007) A screening and intervention program aimed to reduce mortality and serious morbidity associated with severe neonatal alloimmune thrombocytopenia. *Blood*, **110**, 833-839.
- Klee,G.G. (2000) Human anti-mouse antibodies. *Arch.Pathol.Lab Med.*, **124**, 921-923.

- Knight,M., Kurinczuk,J.J., Tuffnell,D., & Brocklehurst,P. (2005) The UK Obstetric Surveillance System for rare disorders of pregnancy. *BJOG.*, **112**, 263-265.
- Knight,M., Pierce,M., Allen,D., Kurinczuk,J.J., Spark,P., Roberts,D.J., & Murphy,M.F. (2011) The incidence and outcomes of fetomaternal alloimmune thrombocytopenia: a UK national study using three data sources. *Br.J.Haematol.*, **152**, 460-468.
- Knudsen,K.A., Smith,L., Smith,S., Karczewski,J., & Tuszynski,G.P. (1988) Role of IIb-IIIa-like glycoproteins in cell-substratum adhesion of human melanoma cells. *J Cell Physiol*, **136**, 471-478.
- Kowalski,M., Pierce,S., & Beck,M. (1995d) Comparison of three commercial platelet antibody screening test kits. *Blood*, **86**, 900a.
- Kowalski,M., Pierce,S., & Beck,M. (1995c) Comparison of three commercial platelet antibody screening test kits. *Blood*, **86**, 900a.
- Kowalski,M., Pierce,S., & Beck,M. (1995a) Comparison of three commercial platelet antibody screening test kits. *Blood*, **86**, 900a.
- Kowalski,M., Pierce,S., & Beck,M. (1995b) Comparison of three commercial platelet antibody screening test kits. *Blood*, **86**, 900a.
- Kroll,H., Penke,G., & Santoso,S. (2005a) Functional heterogeneity of alloantibodies against the human platelet antigen (HPA)-1a. *Thromb.Haemost.*, **94**, 1224-1229.
- Kroll,H., Yates,J., & Santoso,S. (2005b) Immunization against a low-frequency human platelet alloantigen in fetal alloimmune thrombocytopenia is not a single event: characterization by the combined use of reference DNA and novel allele-specific cell lines expressing recombinant antigens. *Transfusion*, **45**, 353-358.
- Kulkarni,S., Dopheide,S.M., Yap,C.L., Ravanat,C., Freund,M., Mangin,P., Heel,K.A., Street,A., Harper,I.S., Lanza,F., & Jackson,S.P. (2000) A revised model of platelet aggregation. *J.Clin.Invest*, **105**, 783-791.
- Kumpel,B.M. & Manoussaka,M.S. (2012) Placental immunology and maternal alloimmune responses. *Vox Sang.*, **102**, 2-12.
- Kumpel,B.M., Sibley,K., Jackson,D.J., White,G., & Soothill,P.W. (2008) Ultrastructural localization of glycoprotein IIIa (GPIIIa, beta3 integrin) on placental syncytiotrophoblast microvilli: implications for platelet alloimmunization during pregnancy. *Transfusion*, **48**, 2077-2086.
- Kunicki,T.J. & Aster,R.H. (1978) Deletion of the platelet-specific alloantigen P1A1 from platelets in Glanzmann's thrombasthenia. *J Clin.Invest*, **61**, 1225-1231.
- Kunicki,T.J. & Aster,R.H. (1979) Isolation and immunologic characterization of the human platelet alloantigen, P1A1. *Mol.Immunol.*, **16**, 353-360.
- Kunicki,T.J., Honda,S., Dawson,B., Honda,Y., Ruan,C., & Aster,R.H. (1995) The P1A alloantigen system is a sensitive indicator of the structural integrity of the amino-terminal domain of the human integrin beta 3 subunit. *Blood Cells Mol.Dis.*, **21**, 131-141.
- Kunicki,T.J., Pidard,D., Rosa,J.P., & Nurden,A.T. (1981) The formation of Ca⁺⁺-dependent complexes of platelet membrane glycoproteins IIb and IIIa in solution as determined by crossed immunoelectrophoresis. *Blood*, **58**, 268-278.
- Kunicki,T.J., Williams,S.A., Nugent,D.J., & Yeager,M. (2012) Mean platelet volume and

- integrin alleles correlate with levels of integrins alpha(IIb)beta(3) and alpha(2)beta(1) in acute coronary syndrome patients and normal subjects. *Arterioscler. Thromb. Vasc. Biol.*, **32**, 147-152.
- Kurata, Y., Oshida, M., Take, H., Furubayashi, T., Mizutani, H., Tomiyama, Y., Yonezawa, T., & Tarui, S. (1990) Acid treatment of platelets as a simple procedure for distinguishing platelet-specific antibodies from anti-HLA antibodies: comparison with chloroquine treatment. *Vox Sang.*, **59**, 106-111.
- Kurata, Y., Oshida, M., Take, H., Furubayashi, T., Nakao, H., Tomiyama, Y., Kanayama, Y., Nagao, N., Okubo, Y., Yonezawa, T., & . (1989) New approach to eliminate HLA class I antigens from platelet surface without cell damage: acid treatment at pH 3.0. *Vox Sang.*, **57**, 199-204.
- Landau, M. & Rosenberg, N. (2010) Molecular insight into human platelet antigens: structural and evolutionary conservation analyses offer new perspective to immunogenic disorders. *Transfusion*, **51**, 558-569.
- Lang, T. & von Depka, M. (2006) Possibilities and limitations of thromboelastometry/thromboelastography. *Hamostaseologie.*, **26**, S20-S29.
- Larson, M.K. & Watson, S.P. (2006) Regulation of proplatelet formation and platelet release by integrin alpha IIb beta3. *Blood*, **108**, 1509-1514.
- Leach, M.F. & Aubuchon, J.P. (2003) False reactivity in GTI Pak Plus ELISA kits due to the presence of anti-mouse antibody in patients' samples. *Immunohematology.*, **19**, 112-116.
- Lee, H., Nurden, A.T., Thomaidis, A., & Caen, J.P. (1981) Relationship between fibrinogen binding and the platelet glycoprotein deficiencies in Glanzmann's thrombasthenia type I and type II. *Br.J Haematol.*, **48**, 47-57.
- Leeksa, O.C., Giltay, J.C., Zandbergen-Spaargaren, J., Modderman, P.W., van Mourik, J.A., & von dem Borne, A.E. (1987) The platelet alloantigen Zwa or PIA1 is expressed by cultured endothelial cells. *Br.J.Haematol.*, **66**, 369-373.
- Leeksa, O.C., Zandbergen-Spaargaren, J., Giltay, J.C., & van Mourik, J.A. (1986) Cultured human endothelial cells synthesize a plasma membrane protein complex immunologically related to the platelet glycoprotein IIb/IIIa complex. *Blood*, **67**, 1176-1180.
- Levy-Toledano, S., Tobelem, G., Legrand, C., Bredoux, R., Degos, L., Nurden, A., & Caen, J.P. (1978) Acquired IgG antibody occurring in a thrombasthenic patient: its effect on human platelet function. *Blood*, **51**, 1065-1071.
- Li, C., Piran, S., Chen, P., Lang, S., Zarpellon, A., Jin, J.W., Zhu, G., Reheman, A., van der Wal, D.E., Simpson, E.K., Ni, R., Gross, P.L., Ware, J., Ruggeri, Z.M., Freedman, J., & Ni, H. (2011) The maternal immune response to fetal platelet GPIIb/IIIa causes frequent miscarriage in mice that can be prevented by intravenous IgG and anti-FcRn therapies. *J.Clin.Invest*, **121**, 4537-4547.
- Liu, L.X., Nardi, M.A., Casella, J.F., & Karparkin, S. (1996) Inhibition of binding of anti-PLA1 antibodies to platelets with monoclonal antibody LK-4. Evidence for multiple PLA1 receptor sites on platelet GPIIIa. *Blood*, **88**, 3601-3607.
- Loewenthal, R., Rosenberg, N., Kalt, R., Dardik, R., Landau, M., Yahalom, V., Avishai, O., Frenkel, O., Gazit, E., Steinberg, D.M., Lipitz, S., & Salomon, O. (2012) Compound heterozygosity of HLA-DRB3*01:01 and HLA-DRB4*01:01 as a potential predictor of fetal neonatal alloimmune thrombocytopenia. *Transfusion*, **In Press**.

- Lowe, R., Go, E., Tong, G., Voelcker, N., & Siuzdak, G. (2005) Monitoring EDTA and endogenous metabolite biomarkers from serum with mass spectrometry. *Spectroscopy*, **19**, 137-146.
- Lown, J.A.G., Dale, B.J., & Erber, W.N. (1993) Evaluation of the CD41/61 panel for routine application in the MAIPA assay. *Tissue Antigens*, **42**, 413.
- Lown, J.A.G. & Robinson, J.O. (1995) Acid treatment of platelets for HLA antigen removal inhibits expression of HPA-5 antigens. *Transf. Med*, **5**, 75.
- Lucas, G., Culliford, S., Green, F., Sidra, G., Calvert, A., Green, A., Harrison, P., Harvey, J., Allen, D., Smillie, D., Masurekar, A., Marks, D., Russell, N., & Massey, E. (2010) Recipient-derived HPA-1a antibodies: a cause of prolonged thrombocytopenia after unrelated donor stem cell transplantation. *Transfusion*, **50**, 334-339.
- Lucas, G.F. (1990) A survey of platelet serology in UK laboratories (1987): an assessment of the efficacy of using chloroquine-treated platelets to distinguish between platelet-specific and anti-HLA antibodies. The UK Platelet and Granulocyte Serology Working Group. *Clin. Lab Haematol.*, **12**, 185-200.
- Luo, B.H., Carman, C.V., & Springer, T.A. (2007) Structural basis of integrin regulation and signaling. *Annu. Rev. Immunol.*, **25**, 619-647.
- Luo, B.H., Strokovich, K., Walz, T., Springer, T.A., & Takagi, J. (2004) Allosteric beta1 integrin antibodies that stabilize the low affinity state by preventing the swing-out of the hybrid domain. *J Biol. Chem.*, **279**, 27466-27471.
- Ma, Y. & Wong, K. (2007) Reassociation and translocation of glycoprotein IIb-IIIa in EDTA-treated human platelets. *Platelets.*, **18**, 451-459.
- Ma, Y.Q., Qin, J., & Plow, E.F. (2007) Platelet integrin alpha(IIb)beta(3): activation mechanisms. *J Thromb. Haemost.*, **5**, 1345-1352.
- Maniecki, M.B., Etzerodt, A., Moestrup, S.K., Moller, H.J., & Graversen, J.H. (2011) Comparative assessment of the recognition of domain-specific CD163 monoclonal antibodies in human monocytes explains wide discrepancy in reported levels of cellular surface CD163 expression. *Immunobiology*, **216**, 882-890.
- Manoil, S., Curtis, B.R., & Aster, R.H. (1997) An autoantibody against platelet glycoprotein IIb/IIIa that recognises citrate but not EDTA platelets. *Transf.*, **37**, 94S.
- Mansour, W., Einav, Y., Hauschner, H., Koren, A., Seligsohn, U., & Rosenberg, N. (2011) An alphaIIb mutation in patients with Glanzmann thrombasthenia located in the N-terminus of blade 1 of the beta-propeller (Asn2Asp) disrupts a calcium binding site in blade 6. *J Thromb. Haemost.*, **9**, 192-200.
- Margadant, C., Monsuur, H.N., Norman, J.C., & Sonnenberg, A. (2011) Mechanisms of integrin activation and trafficking. *Curr. Opin. Cell Biol.*, **23**, 607-614.
- Maslanka, K., Guz, K., & Zupanska, B. (2003) Antenatal screening of unselected pregnant women for HPA-1a antigen, antibody and alloimmune thrombocytopenia. *Vox Sang*, **85**, 326-327.
- Maslanka, K., Michur, H., Guz, K., Wrobel, A., Uhrynowska, M., Misiak, A., Ejduk, A., Brojer, E., & Zupanska, B. (2012) The relevance of HPA-15 antigen expression for anti-HPA-15 antibody detection. *Int. J. Lab Hematol.*, **34**, 65-69.
- Masters, R. & Taaning, E. (1998) Three cases of platelet alloimmunisation associated with the presence of a novel platelet-specific antibody. *Vox Sang.*, **75**, 242-246.

- Matthys,V.S., Gorbunova,E.E., Gavrilovskaya,I.N., & Mackow,E.R. (2010) Andes virus recognition of human and Syrian hamster beta3 integrins is determined by an L33P substitution in the PSI domain. *J.Virol.*, **84**, 352-360.
- McDonald,C.P. (2011) Interventions Implemented to Reduce the Risk of Transmission of Bacteria by Transfusion in the English National Blood Service. *Transfus.Med Hemother.*, **38**, 255-258.
- McEver,R.P., Baenziger,N.L., & Majerus,P.W. (1980) Isolation and quantitation of the platelet membrane glycoprotein deficient in thrombasthenia using a monoclonal hybridoma antibody. *J.Clin.Invest*, **66**, 1311-1318.
- McMillan,R., Lopez-Dee,J., & Loftus,J.C. (2001) Autoantibodies to alpha(IIb)beta(3) in patients with chronic immune thrombocytopenic purpura bind primarily to epitopes on alpha(IIb). *Blood*, **97**, 2171-2172.
- McMillan,R., Mason,D., Tani,P., & Schmidt,G.M. (1982) Evaluation of platelet surface antigens: localization of the PIA1 alloantigen. *Br.J.Haematol.*, **51**, 297-304.
- McMillan,R., Tani,P., Millard,F., Berchtold,P., Renshaw,L., & Woods,V.L., Jr. (1987) Platelet-associated and plasma anti-glycoprotein autoantibodies in chronic ITP. *Blood*, **70**, 1040-1045.
- Menitove,J.E., Pereira,J., Hoffman,R., Anderson,T., Fried,W., & Aster,R.H. (1989) Cyclic thrombocytopenia of apparent autoimmune etiology. *Blood*, **73**, 1561-1569.
- Merviel,P., Challier,J.C., Carbillon,L., Foidart,J.M., & Uzan,S. (2001) The role of integrins in human embryo implantation. *Fetal Diagn. Ther.*, **16**, 364-371.
- Metcalfe,P., Watkins,N., Marsh,S.G.E., & Robinson,J. (2011) IPD - The Immuno Polymorphism Database. <http://www.ebi.ac.uk/ipd/hpa/table1.html>. (in press).
- Metcalfe,P., Allen,D., Chapman,J., & Ouwehand,W.H. (1997) Interlaboratory variation in the detection of clinically significant alloantibodies against human platelet alloantigens. *Br.J.Haematol.*, **97**, 204-207.
- Metcalfe,P. & Waters,W.H. (1993) HPA-1a typing by pcr amplification with sequence-specific primers (PCR-SSP). *Transf.Med.*, **3 (Supple 1)**, 82.
- Michelson,A.D. (1996) Flow cytometry: a clinical test of platelet function. *Blood*, **87**, 4925-4936.
- Mitchell,W.B., Li,J., Murcia,M., Valentin,N., Newman,P.J., & Coller,B.S. (2007) Mapping early conformational changes in alphaIIb and beta3 during biogenesis reveals a potential mechanism for alphaIIbbeta3 adopting its bent conformation. *Blood*, **109**, 3725-3732.
- Mizuguchi,T., Uchimura,H., Kataoka,H., Akaji,K., Kiso,Y., & Saito,K. (2012) Intact-cell-based surface plasmon resonance measurements for ligand affinity evaluation of a membrane receptor. *Anal.Biochem.*, **420**, 185-187.
- Montemagno,R., Soothill,P.W., Scarcelli,M., O'Brien,P., & Rodeck,C.H. (1994) Detection of alloimmune thrombocytopenia as cause of isolated hydrocephalus by fetal blood sampling. *Lancet*, **343**, 1300-1301.
- Morel-Kopp,M.-C. & Kaplan,C. (1993a) Importance of the choice of murine monoclonal antibody to detect and identify human anti-GPIIb/IX autoantibodies by the MAIPA procedure. *Tissue Antigens*, **42**, 421.
- Morel-Kopp,M.-C. & Kaplan,C. (1993b) Modification of the MAIPA technique to detect and

- identify anti-platelet GPIb/IX autoantibodies. *Thromb.Haem.*, **69**, 912.
- Morgenstern,E., Ruf,A., & Patscheke,H. (1992) Transport of anti-glycoprotein IIb/IIIa antibodies into the alpha-granules of unstimulated human blood platelets. *Thromb.Haem*, **67**, 121-125.
- Mould,A.P., Travis,M.A., Barton,S.J., Hamilton,J.A., Askari,J.A., Craig,S.E., Macdonald,P.R., Kammerer,R.A., Buckley,P.A., & Humphries,M.J. (2005) Evidence that monoclonal antibodies directed against the integrin beta subunit plexin/semaphorin/integrin domain stimulate function by inducing receptor extension. *J.Biol.Chem.*, **280**, 4238-4246.
- Mueller-Eckhardt,C., Kayser,W., Forster,C., Mueller-Eckhardt,G., & Ringenberg,C. (1982) Improved assay for detection of platelet-specific PIA1 antibodies in neonatal alloimmune thrombocytopenia. *Vox Sang.*, **43**, 76-81.
- Mueller-Eckhardt,C., Kiefel,V., Grubert,A., Kroll,H., Weisheit,M., Schmidt,S., Mueller-Eckhardt,G., & Santoso,S. (1989) 348 cases of suspected neonatal alloimmune thrombocytopenia. *Lancet*, **1**, 363-366.
- Mueller-Eckhardt,C., Mahn,I., Schulz,G., & Mueller-Eckhardt,G. (1978a) Detection of platelet autoantibodies by a radioactive anti-immunoglobulin test. *Vox Sang*, **35**, 357-365.
- Mueller-Eckhardt,C., Schulz,G., Sauer,K.H., Dienst,C., & Mahn,I. (1978b) Studies on the platelet radioactive anti-immunoglobulin test. *J.Immunol.Methods*, **19**, 1-11.
- Murcia,M., Jirouskova,M., Li,J., Coller,B.S., & Filizola,M. (2008) Functional and computational studies of the ligand-associated metal binding site of beta3 integrins. *Proteins*, **71**, 1779-1791.
- Murphy,M.F., Hambley,H., Nicolaides,K., & Waters,A.H. (1996) Severe fetomaternal alloimmune thrombocytopenia presenting with fetal hydrocephalus. *Prenat.Diagn.*, **16**, 1152-1155.
- Murphy,M.F., Verjee,S., & Greaves,M. (1999) Inadequacies in the postnatal management of fetomaternal alloimmune thrombocytopenia (FMAIT). *Br.J.Haematol.*, **105**, 123-126.
- Murphy,M.F. & Williamson,L.M. (2000) Antenatal screening for fetomaternal alloimmune thrombocytopenia: an evaluation using the criteria of the UK National Screening Committee. *Br.J.Haematol.*, **111**, 726-732.
- Murphy,M.F., Williamson,L.M., & Urbaniak,S.J. (2002a) Antenatal screening for fetomaternal alloimmune thrombocytopenia: should we be doing it? *Vox Sang.*, **83 Suppl 1**, 409-416.
- Murphy,M.F., Williamson,L.M., & Urbaniak,S.J. (2002b) Antenatal screening for fetomaternal alloimmune thrombocytopenia: should we be doing it? *Vox Sang.*, **83 Suppl 1**, 409-416.
- Murphy,M.F., Williamson,L.M., & Urbaniak,S.J. (2002c) Antenatal screening for fetomaternal alloimmune thrombocytopenia: should we be doing it? *Vox Sang.*, **83 Suppl 1**, 409-416.
- Murphy,M.F., Williamson,L.M., & Urbaniak,S.J. (2002d) Antenatal screening for fetomaternal alloimmune thrombocytopenia: should we be doing it? *Vox Sang.*, **83 Suppl 1**, 409-416.
- Newman,P.J., Derbes,R.S., & Aster,R.H. (1989) The human platelet alloantigens, PIA1 and PIA2, are associated with a leucine33/proline33 amino acid polymorphism in membrane glycoprotein IIIa, and are distinguishable by DNA typing. *J Clin.Invest*, **83**,

1778-1781.

- Newman,P.J., Martin,L.S., Knipp,M.A., & Kahn,R.A. (1985) Studies on the nature of the human platelet alloantigen, PIA1: localization to a 17,000-dalton polypeptide. *Mol.Immunol.*, **22**, 719-729.
- Nguyen,X.D., Dugrillon,A., Beck,C., Kerowgan,M., & Kluter,H. (2004) A novel method for simultaneous analysis of specific platelet antibodies: SASPA. *Br.J.Haematol.*, **127**, 552-560.
- Nguyen,X.D., Goebel,M., Schober,M., Kluter,H., & Panzer,S. (2010) The detection of platelet antibodies by simultaneous analysis of specific platelet antibodies and the monoclonal antibody-specific immobilization of platelet antigens: an interlaboratory comparison. *Transfusion*, **50**, 1429-1434.
- Nieminen,U. & Kekomaki,R. (1992) Quinidine-induced thrombocytopenic purpura: clinical presentation in relation to drug-dependent and drug-independent platelet antibodies. *B.J.Haem.*, **80**, 77-82.
- Nordhagen,R. & Flaathen,S.T. (1985) Chloroquine removal of HLA antigens from platelets for the platelet immunofluorescence test. *Vox Sang.*, **48**, 156-159.
- Noris,P., Simsek,S., Bruijne-Admiraal,L.G., Porcelijn,L., Huiskes,E., van der Vlist,G.J., van Leeuwen,E.F., van der Schoot,C.E., & dem Borne,A.E. (1995) Max(a), a new low-frequency platelet-specific antigen localized on glycoprotein IIb, is associated with neonatal alloimmune thrombocytopenia
156. *Blood*, **86**, 1019-1026.
- Nurden,A.T., Fiore,M., Nurden,P., & Pillois,X. (2011) Glanzmann thrombasthenia: a review of ITGA2B and ITGB3 defects with emphasis on variants, phenotypic variability, and mouse models. *Blood*, **118**, 5996-6005.
- Nurden,A.T., Rosa,J.P., Fournier,D., Legrand,C., Didry,D., Parquet,A., & Pidard,D. (1987) A variant of Glanzmann's thrombasthenia with abnormal glycoprotein IIb-IIIa complexes in the platelet membrane. *J Clin.Invest*, **79**, 962-969.
- Nurden,P. (1997) Bidirectional trafficking of membrane glycoproteins following platelet activation in suspension. *Thromb.Haemost.*, **78**, 1305-1315.
- Nurden,P., Poujol,C., Durrieu-Jais,C., Winckler,J., Combrie,R., Macchi,L., Bihour,C., Wagner,C., Jordan,R., & Nurden,A.T. (1999) Labeling of the internal pool of GP IIb-IIIa in platelets by c7E3 Fab fragments (abciximab): flow and endocytic mechanisms contribute to the transport. *Blood*, **93**, 1622-1633.
- O'Toole,T.E., Loftus,J.C., Plow,E.F., Glass,A.A., Harper,J.R., & Ginsberg,M.H. (1989) Efficient surface expression of platelet GPIIb-IIIa requires both subunits. *Blood*, **74**, 14-18.
- Palmeira,P., Quinello,C., Silveira-Lessa,A.L., Zago,C.A., & Carneiro-Sampaio,M. (2012) IgG placental transfer in healthy and pathological pregnancies. *Clin.Dev.Immunol.*, **2012**, 985646.
- Panzer,S. (2001a) Report on the Tenth International Platelet Genotyping and Serology Workshop on behalf of the International Society of Blood Transfusion. *Vox Sang.*, **80**, 72-78.
- Panzer,S. (2001b) Report on the Tenth International Platelet Genotyping and Serology Workshop on behalf of the International Society of Blood Transfusion. *Vox Sang.*, **80**, 72-78.

- Panzer,S., Kiefel,V., Bartram,C.R., Haas,O.A., Hinterberger,W., Mueller-Eckhardt,C., & Lechner,K. (1989) Immune thrombocytopenia more than a year after allogeneic marrow transplantation due to antibodies against donor platelets with anti-PIA1 specificity: evidence for a host-derived immune reaction. *Br.J.Haematol.*, **71**, 259-264.
- Pappalardo,P.A., Secord,A.R., Quitevis,P., Haimowitz,M.D., & Goldfinger,D. (2001) Platelet transfusion refractoriness associated with HPA-1a (PIA1) alloantibody without coexistent HLA antibodies successfully treated with antigen-negative platelet transfusions. *Transfusion*, **41**, 984-987.
- Parker,R.I., Rick,M.E., & Gralnick,H.R. (1984) A method to minimize platelet activation during platelet isolation. *Thromb.Res.*, **36**, 265-270.
- Pei,R., Lee,J., Chen,T., Rojo,S., & Terasaki,P.I. (1999) Flow cytometric detection of HLA antibodies using a spectrum of microbeads. *Hum.Immunol.*, **60**, 1293-1302.
- Pei,R., Lee,J.H., Shih,N.J., Chen,M., & Terasaki,P.I. (2003) Single human leukocyte antigen flow cytometry beads for accurate identification of human leukocyte antigen antibody specificities. *Transplantation*, **75**, 43-49.
- Perdomo,J., Yan,F., Ahmadi,Z., Jiang,X.M., Stocker,R., & Chong,B.H. (2011) Quinine-induced thrombocytopenia: drug-dependent GPIb/IX antibodies inhibit megakaryocyte and proplatelet production in vitro. *Blood*, **117**, 5975-5986.
- Petersen,S., Jansen,J., Taudorf,K., Thomsen,M., & Taaning,E. (1986) Alloimmune neonatal thrombocytopenia and hydrocephalus. Platelet antigen Zwa, ABO-antigens and HLA-antigens in mothers to infants with hydrocephalus. *Acta Paediatr.Scand.*, **75**, 86-88.
- Peterson,J.A. (2012) New approaches to detection of antibodies causing neonatal alloimmune thrombocytopenia (NAIT). *J Transf Med*, **5**, 58-59.
- Phillips,D.R., Teng,W., Arfsten,A., Nannizzi-Alaimo,L., White,M.M., Longhurst,C., Shattil,S.J., Randolph,A., Jakubowski,J.A., Jennings,L.K., & Scarborough,R.M. (1997) Effect of Ca²⁺ on GP IIb-IIIa interactions with integrilin: enhanced GP IIb-IIIa binding and inhibition of platelet aggregation by reductions in the concentration of ionized calcium in plasma anticoagulated with citrate. *Circulation*, **96**, 1488-1494.
- Pidard,D., Didry,D., Kunicki,T.J., & Nurden,A.T. (1986) Temperature-dependent effects of EDTA on the membrane glycoprotein IIb-IIIa complex and platelet aggregability. *Blood*, **67**, 604-611.
- Porcelijn,L., Huiskes,E., de Haas,M., Simsek,S., Overbeeke,M.A.M., & dem Borne,A.E.G.K. (1996) Antigen localisation with monoclonal antibodies. *Transf.Med.*, **6**, 196.
- Porcelijn,L., Huiskes,E., & dem Borne,A.E.G.K. (1994) 120ul MAIPA. *3rd European Conference on Platelet Immunobiology, Cambridge*.
- Porcelijn,L., van,B.W., Gratama,J.W., Van't,V.M., De,S.A., & Sintnicolaas,K. (2008) External quality assessment of platelet serology and human platelet antigen genotyping: a 10-year review. *Transfusion*, **48**, 1699-1706.
- Povoa,A.M., Ramalho,C., Machado,A.P., Matias,A., & Montenegro,N. (2007) Congenital posthemorrhagic hydrocephalus: a case of fetomaternal alloimmune thrombocytopenia. *Fetal Diagn. Ther.*, **22**, 321-324.
- Price,K.S., Chiroux,S., Goldman,M., & Shamy,A.A. (1999) Post-transfusion purpura 10 hours post-infusion of fresh frozen plasma containing anti-HPA-1a. *Blood*, **94 (Supple 1)**, 248a.

- Provasi,D., Murcia,M., Collier,B.S., & Filizola,M. (2009) Targeted molecular dynamics reveals overall common conformational changes upon hybrid domain swing-out in beta3 integrins. *Proteins*, **77**, 477-489.
- Rabellino,E.M., Levene,R.B., Leung,L.L., & Nachman,R.L. (1981) Human megakaryocytes. II. Expression of platelet proteins in early marrow megakaryocytes. *J Exp.Med.*, **154**, 88-100.
- Raborn,J., Wang,W., & Luo,B.H. (2011) Regulation of integrin alphaIIb beta3 ligand binding and signaling by the metal ion binding sites in the beta I domain. *Biochemistry*, **50**, 2084-2091.
- Radder,C.M., Beekhuizen,H., Kanhai,H.H., & Brand,A. (2004) Effect of maternal anti-HPA-1a antibodies and polyclonal IVIG on the activation status of vascular endothelial cells. *Clin.Exp.Immunol.*, **137**, 216-222.
- Radtke,F., Sinor,L.T., Stone,D.L., Farlow,I., Young,R., & Schram,R. (1991) A solid phase red cell adherence assay to detect anti-PIA1 antibodies utilising human umbilical vein endothelial cells. *Transf.*, **31 (Supple)**, 31S.
- Raymond,T., Gorbunova,E., Gavrilovskaya,I.N., & Mackow,E.R. (2005) Pathogenic hantaviruses bind plexin-semaphorin-integrin domains present at the apex of inactive, bent alphavbeta3 integrin conformers. *Proc.Natl.Acad.Sci.U.S.A*, **102**, 1163-1168.
- Regan,L., Braude,P.R., & Hill,D.P. (1991) A prospective study of the incidence, time of appearance and significance of anti-paternal lymphocytotoxic antibodies in human pregnancy. *Hum.Reprod.*, **6**, 294-298.
- Rivas,G.A., Usobiaga,P., & Gonzalez-Rodriguez,J. (1991) Calcium and temperature regulation of the stability of the human platelet integrin GPIIb/IIIa in solution: an analytical ultracentrifugation study. *Eur.Biophys.J.*, **20**, 287-292.
- Rivera,J., Lozano,M.L., Navarro-Nunez,L., & Vicente,V. (2009) Platelet receptors and signaling in the dynamics of thrombus formation. *Haematologica*, **94**, 700-711.
- Robb-Smith,A.H. (1967) Why the platelets were discovered. *Br.J.Haematol.*, **13**, 618-637.
- Rosa,J.P., Bray,P.F., Gayet,O., Johnston,G.I., Cook,R.G., Jackson,K.W., Shuman,M.A., & McEver,R.P. (1988) Cloning of glycoprotein IIIa cDNA from human erythroleukemia cells and localization of the gene to chromosome 17. *Blood*, **72**, 593-600.
- Rosa,J.P., Kieffer,N., Didry,D., Pidard,D., Kunicki,T.J., & Nurden,A.T. (1984) The human platelet membrane glycoprotein complex GP IIb-IIIa expresses antigenic sites not exposed on the dissociated glycoproteins. *Blood*, **64**, 1246-1253.
- Rosa,J.P. & McEver,R.P. (1989) Processing and assembly of the integrin, glycoprotein IIb-IIIa, in HEL cells. *J Biol.Chem.*, **264**, 12596-12603.
- Rourke,C., Curry,N., Khan,S., Taylor,R., Raza,I., Davenport,R., Stanworth,S., & Brohi,K. (2012) Fibrinogen levels during trauma hemorrhage, response to replacement therapy, and association with patient outcomes. *J Thromb.Haemost.*, **10**, 1342-1351.
- Rozman,P. (2002) Platelet antigens. The role of human platelet alloantigens (HPA) in blood transfusion and transplantation. *Transpl.Immunol.*, **10**, 165-181.
- Rubinstein,E., Urso,I., Boucheix,C., & Carroll,R.C. (1992) Platelet activation by cross-linking HLA class I molecules and Fc receptor. *Blood*, **79**, 2901-2908.
- Ryu,T., Davis,J.M., & Schwartz,K.A. (1990) Dose-dependent platelet stimulation and inhibition

- induced by anti-PIA1 IgG. *J.Lab Clin.Med*, **116**, 91-99.
- Sachs,U.J., Kiefel,V., Kroll,H., Bein,G., & Santoso,S. (2012) Report on the 15th International Society of Blood Transfusion platelet immunology workshop. *Vox Sang*.
- Sainio,S., Jarvenpaa,A.L., Renlund,M., Riikonen,S., Teramo,K., & Kekomaki,R. (2000) Thrombocytopenia in term infants: a population-based study. *Obstet.Gynecol.*, **95**, 441-446.
- Sainio,S., Javela,K., & Tuimala,J. (2012) Usefulness of maternal anti-HPA-1a antibody quantitation to predict severity of fetomaternal alloimmune thrombocytopenia. *J Transf Med*, **5**, 60-61.
- Santoso,S., Kiefel,V., & Mueller-Eckhardt,C. (1987) Redistribution of platelet glycoproteins induced by allo- and autoantibodies. *Thromb.Haemost.*, **58**, 866-871.
- Santoso,S., Kroll,H., Andrei-Selmer,C.L., Socher,I., Rankin,A., Kretzschmar,E., Watkins,N.A., & Ouwehand,W.H. (2006) A naturally occurring LeuVal mutation in beta3-integrin impairs the HPA-1a epitope: the third allele of HPA-1. *Transfusion*, **46**, 790-799.
- Santoso,S., Zimmermann,U., Neppert,J., & Mueller-Eckhardt,C. (1986) Receptor patching and capping of platelet membranes induced by monoclonal antibodies. *Blood*, **67**, 343-349.
- Schiffer,C.A. & Young,V. (1983) Detection of platelet antibodies using a micro-enzyme-linked immunosorbent assay (ELISA). *Blood*, **61**, 311-317.
- Schnaidt,M., Northoff,H., & Wernet,D. (1996) Frequency and specificity of platelet-specific alloantibodies in HLA-immunized haematologic-oncologic patients. *Transfus.Med.*, **6**, 111-114.
- Schober,J.M., Lam,S.C., & Wencel-Drake,J.D. (2003) Effect of cellular and receptor activation on the extent of integrin alphaIIb beta3 internalization. *J Thromb.Haemost.*, **1**, 2404-2410.
- Semana,G., Zazoun,T., Alizadeh,M., Morel-Kopp,M.C., Genetet,B., & Kaplan,C. (1996) Genetic susceptibility and anti-human platelet antigen 5b alloimmunization role of HLA class II and TAP genes. *Hum.Immunol.*, **46**, 114-119.
- Shattil,S.J., Cunningham,M., & Hoxie,J.A. (1987) Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. *Blood*, **70**, 307-315.
- Shattil,S.J., Hoxie,J.A., Cunningham,M., & Brass,L.F. (1985) Changes in the platelet membrane glycoprotein IIb/IIIa complex during platelet activation. *J Biol.Chem.*, **260**, 11107-11114.
- Shen,W.Z., Ding,Q.L., Jin,P.P., Wang,X.F., Jiang,Y.Z., Li,S.M., & Wang,H.L. (2009) A novel Pro126His beta propeller mutation in integrin alphaIIb causes Glanzmann thrombasthenia by impairing progression of pro-alphaIIb beta3 from endoplasmic reticulum to Golgi. *Blood Cells Mol.Dis.*, **42**, 44-50.
- Sheppard,R.L. & Henion,J. (1997a) Quantitative capillary electrophoresis/ion spray tandem mass spectrometry determination of EDTA in human plasma and urine. *Anal.Chem.*, **69**, 2901-2907.
- Sheppard,R.L. & Henion,J. (1997b) Determining EDTA in blood. *Anal.Chem.*, **69**, 477A-480A.
- Shibata,Y., Juji,T., Nishizawa,Y., Sakamoto,H., & Ozawa,N. (1981) Detection of platelet

- antibodies by a newly developed mixed agglutination with platelets. *Vox Sang.*, **41**, 25-31.
- Skacel,P.O., Stacey,T.E., Tidmarsh,C.E., & Contreras,M. (1989) Maternal alloimmunization to HLA, platelet and granulocyte-specific antigens during pregnancy: its influence on cord blood granulocyte and platelet counts. *Br.J Haematol.*, **71**, 119-123.
- Skouri,H., Bettaieb,A., Fromont,P., Elomri,H., Ennabli,S., & Bierling,P. (2005) Platelet and granulocyte alloimmunisation in multitransfused Tunisian patients. *Eur.J.Haematol.*, **75**, 248-251.
- Smith,C., Estavillo,D., Emsley,J., Bankston,L.A., Liddington,R.C., & Cruz,M.A. (2000) Mapping the collagen-binding site in the I domain of the glycoprotein Ia/IIa (integrin $\alpha(2)\beta(1)$). *J.Biol.Chem.*, **275**, 4205-4209.
- Smith,G.A., Rankin,A., Riddle,C., Cheetham-Wilkinson,C., Ranasinghe,E., Ouwehand,W.H., & Watkins,N.A. (2007) Severe fetomaternal alloimmune thrombocytopenia due to anti-human platelet antigen (HPA)-1a in a mother with a rare and silenced ITGB3*0101 (GPIIIa) allele. *Vox Sang.*, **93**, 325-330.
- Socher,I., Andrei-Selmer,C., Bein,G., Kroll,H., & Santoso,S. (2009) Low-avidity HPA-1a alloantibodies in severe neonatal alloimmune thrombocytopenia are detectable with surface plasmon resonance technology. *Transfusion*, **49**, 943-952.
- Sola-Visner,M., Saxonhouse,M.A., & Brown,R.E. (2008) Neonatal thrombocytopenia: what we do and don't know. *Early Hum.Dev.*, **84**, 499-506.
- Solenthaler,M., Krauss,J.K., Boehlen,F., Koller,R., Hug,M., & Lammle,B. (1999) Fatal fresh frozen plasma infusion containing HPA-1a alloantibodies. *Br.J.Haematol.*, **106**, 258-259.
- Soulier,J.P., Patereau,C., & Drouet,J. (1975) Platelet indirect radioactive Coombs test. Its utilisation for Pla1 grouping. *Vox Sang.*, **29**, 253-268.
- Spencer,J.A. & Burrows,R.F. (2001) Feto-maternal alloimmune thrombocytopenia: a literature review and statistical analysis. *Aust.N.Z.J.Obstet.Gynaecol.*, **41**, 45-55.
- Staatz,W.D., Peters,K.J., & Santoro,S.A. (1990) Divalent cation-dependent structure in the platelet membrane glycoprotein Ia-IIa (VLA-2) complex. *Biochem.Biophys.Res.Comm.*, **168**, 107-114.
- Stafford,P., Ghevaert,C., Campbell,K., Proulx,C., Smith,G., Williamson,L.M., Ranasinghe,E., Watkins,N.A., Huntington,J.A., & Ouwehand,W.H. (2008) Immunologic and structural analysis of eight novel domain-deletion beta3 integrin peptides designed for detection of HPA-1 antibodies. *J.Thromb.Haemost.*, **6**, 366-375.
- Stanworth,S.J., Hackett,G.A., & Williamson,L.M. (2001) Fetomaternal alloimmune thrombocytopenia presenting antenatally as hydrops fetalis. *Prenat.Diagn.*, **21**, 423-424.
- Starcevic,M., Tomicic,M., Malenica,M., & Zah-Matakovic,V. (2010) Neonatal alloimmune thrombocytopenia caused by anti-HLA-A24 alloantibodies. *Acta Paediatr.*, **99**, 630-632.
- Stefanini,M., Plitman,G.I., Dameshek,W., Chatterjea,J.B., & Mednicoff,I.B. (1953) Studies on platelets. XI. Antigenicity of platelets and evidence of platelet groups and types in man. *J Lab Clin.Med.*, **42**, 723-738.
- Sutherland,D.R., Yeo,E., Ryan,A., Mills,G.B., Bailey,D., & Baker,M.A. (1991) Identification of a cell-surface antigen associated with activated T lymphoblasts and activated

- platelets. *Blood*, **77**, 84-93.
- Switala-Jelen,K., Dabrowska,K., Opolski,A., Lipinska,L., Nowaczyk,M., & Gorski,A. (2004) The biological functions of beta3 integrins. *Folia Biol.(Praha)*, **50**, 143-152.
- Taaning,E. (1985) Microplate enzyme immuno-assay for detection of platelet antibodies. *Tissue Antigens*, **25**, 19-27.
- Taaning,E. & Skibsted,L. (1990) The frequency of platelet alloantibodies in pregnant women and the occurrence and management of neonatal alloimmune thrombocytopenic purpura. *Obstet.Gynae.Survey*, **45**, 521-525.
- Taaning,E. & Svejgaard,A. (1994) Post-transfusion purpura: a survey of 12 Danish cases with special reference to immunoglobulin G subclasses of the platelet antibodies. *Transfus.Med.*, **4**, 1-8.
- Taaning,E. & Tonnesen,F. (1999) Pan-reactive platelet antibodies in post-transfusion purpura. *Vox Sang.*, **76**, 120-123.
- Takada,Y. & Hemler,M.E. (1989) The primary structure of the VLA-2/collagen receptor alpha 2 subunit (platelet GPIa): homology to other integrins and the presence of a possible collagen-binding domain. *J Cell Biol.*, **109**, 397-407.
- Takagi,J., Strokovich,K., Springer,T.A., & Walz,T. (2003) Structure of integrin alpha5beta1 in complex with fibronectin. *EMBO J*, **22**, 4607-4615.
- Takahashi,R., Sekine,N., & Nakatake,T. (1999) Influence of monoclonal antiplatelet glycoprotein antibodies on in vitro human megakaryocyte colony formation and proplatelet formation. *Blood*, **93**, 1951-1958.
- Tamerius,J.D., Curd,J.G., Tani,P., & McMillan,R. (1983) An enzyme-linked immunosorbent assay for platelet compatibility testing. *Blood*, **62**, 744-749.
- Tanaka,T., Umesaki,N., Nishio,J., Maeda,K., Kawamura,T., Araki,N., & Ogita,S. (2000) Neonatal thrombocytopenia induced by maternal anti-HLA antibodies: a potential side effect of allogenic leukocyte immunization for unexplained recurrent aborters. *J.Reprod.Immunol.*, **46**, 51-57.
- Theusinger,O.M., Nurnberg,J., Asmis,L.M., Seifert,B., & Spahn,D.R. (2010) Rotation thromboelastometry (ROTEM) stability and reproducibility over time. *Eur.J.Cardiothorac.Surg.*, **37**, 677-683.
- Thibaut,J., Merieux,Y., Rigal,D., & Gillet,G. (2012) A novel assay for the detection of anti-human platelet antigen antibodies (HPA-1a) based on the peptide aptamer technology. *Haematologica*, **97**, 696-704.
- Thornton,M.A., Poncz,M., Korostishevsky,M., Yakobson,E., Usher,S., Seligsohn,U., & Peretz,H. (1999) The human platelet alphaIIb gene is not closely linked to its integrin partner beta3. *Blood*, **94**, 2039-2047.
- Thude,H., Schorner,U., Helfricht,C., Loth,M., Maak,B., & Barz,D. (2006) Neonatal alloimmune thrombocytopenia caused by human leucocyte antigen-B27 antibody. *Transfus.Med.*, **16**, 143-149.
- Tiller,H., Killie,M., Skogen,B., Oian,P., & Husebekk,A. (2009) Neonatal alloimmune thrombocytopenia in Norway: poor detection rate with nonscreening versus a general screening programme. *BJOG.*, **116**, 594-598.
- Tiller,H., Killie,M.K., Husebekk,A., Skogen,B., Ni,H., Kjeldsen-Kragh,J., & Oian,P. (2012)

- Platelet antibodies and fetal growth: maternal antibodies against fetal platelet antigen 1a are strongly associated with reduced birthweight in boys. *Acta Obstet. Gynecol. Scand.*, **91**, 79-86.
- Tomiyama, Y., Kunicki, T.J., Zipf, T.F., Ford, S.B., & Aster, R.H. (1992) Response of human platelets to activating monoclonal antibodies: importance of Fc gamma RII (CD32) phenotype and level of expression. *Blood*, **80**, 2261-2268.
- Tsuno, N.H., Matsuhahi, M., Hasegawa, K., Tamura, S., Kawabata, M., Kaneko, J., Aoki, T., Sugawara, Y., Kokudo, N., & Takahashi, K. (2012) HPA-4 incompatibility may cause rejection reactins in solid organ transplantation. *J Transf Med*, **5**, 79.
- Turner, M.L., Bessos, H., Fagge, T., Harkness, M., Rentoul, F., Seymour, J., Wilson, D., Gray, I., Ahya, R., Cairns, J., & Urbaniak, S. (2005) Prospective epidemiologic study of the outcome and cost-effectiveness of antenatal screening to detect neonatal alloimmune thrombocytopenia due to anti-HPA-1a. *Transfusion*, **45**, 1945-1956.
- Uhrynowska, M. & Zupanska, B. (1996) Platelet-specific antibodies in transfused patients. *Eur. J. Haematol.*, **56**, 248-251.
- Vainchenker, W., Deschamps, J.F., Bastin, J.M., Guichard, J., Titeux, M., Breton-Gorius, J., & McMichael, A.J. (1982) Two monoclonal antiplatelet antibodies as markers of human megakaryocyte maturation: immunofluorescent staining and platelet peroxidase detection in megakaryocyte colonies and in in vivo cells from normal and leukemic patients. *Blood*, **59**, 514-521.
- Valentin, N., Vergracht, A., Bignon, J.D., Cheneau, M.L., Blanchard, D., Kaplan, C., Reznikoff-Etievant, M.F., & Muller, J.Y. (1990) HLA-DRw52a is involved in alloimmunization against PL-A1 antigen. *Hum. Immunol.*, **27**, 73-79.
- Valentin, N., Visentin, G.P., & Newman, P.J. (1995) Involvement of the cysteine-rich domain of glycoprotein IIIa in the expression of the human platelet alloantigen, PIA1: evidence for heterogeneity in the humoral response. *Blood*, **85**, 3028-3033.
- van Boxtel, C.J. & Engelfriet, C.P. (1973) Immunofluorescence Microphotomerry for the Detection of Platelet Antibodies. II. Sensitivity of the method compared to that of conventional serological methods. *Scandinavian Journal of Immunology*, **2**, 531-540.
- van Boxtel, C.J., Engelfriet, C.P., & Feltkamp, T.E.W. (1973) Immunofluorescence Microphotometry for the Detection of Platelet Antibodies. I. Standardization of the Method. *Scandinavian Journal of Immunology*, **2**, 217-230.
- van der Weerdt, C.M., Veenhoven-Vonriesz, L.E., Nijenhuis, L.E., & Van, L.J. (1963) The Zw blood group system in platelets. *Vox Sang.*, **8**, 513-530.
- van Gils, J.M., Stutterheim, J., van Duijn, T.J., Zwaginga, J.J., Porcelijn, L., De, H.M., & Hordijk, P.L. (2009) HPA-1a alloantibodies reduce endothelial cell spreading and monolayer integrity. *Mol. Immunol.*, **46**, 406-415.
- van Leeuwen, E.F., Leeksa, O.C., van Mourik, J.A., Engelfriet, C.P., & dem Borne, A.E. (1984) Effect of the binding of anti-Zwa antibodies on platelet function. *Vox Sang.*, **47**, 280-289.
- Van Loghem, J.J.J., Dorfmeijer, H., Van Hart, M., & Schreuder, F. (1959) Serological and genetical studies on a platelet antigen (Zw). *Vox Sang.*, **4**, 161-169.
- Vanderpuye, O.A., Labarrere, C.A., & McIntyre, J.A. (1991) A vitronectin-receptor-related molecule in human placental brush border membranes. *Biochem. J.*, **280 (Pt 1)**, 9-17.
- Vinci, G., Tabilio, A., Deschamps, J.F., Van, H.D., Henri, A., Guichard, J., Tetteroo, P.,

- Lansdorp,P.M., Hercend,T., Vainchenker,W., & . (1984) Immunological study of in vitro maturation of human megakaryocytes. *Br.J Haematol.*, **56**, 589-605.
- von dem Borne,A.E. & Decary,F. (1990) ICSH/ISBT Working Party on platelet serology. Nomenclature of platelet-specific antigens. *Vox Sang.*, **58**, 176.
- von dem Borne,A.E., van Leeuwen,E.F., von Riesz,L.E., van Bortel,C.J., & Engelfriet,C.P. (1981) Neonatal alloimmune thrombocytopenia: detection and characterization of the responsible antibodies by the platelet immunofluorescence test. *Blood*, **57**, 649-656.
- von dem Borne,A.E., Verheugt,F.W., Oosterhof,F., von,R.E., de la Riviere,A.B., & Engelfriet,C.P. (1978) A simple immunofluorescence test for the detection of platelet antibodies. *Br.J.Haematol.*, **39**, 195-207.
- Wagner,C.L., Mascelli,M.A., Neblock,D.S., Weisman,H.F., Coller,B.S., & Jordan,R.E. (1996) Analysis of GPIIb/IIIa receptor number by quantification of 7E3 binding to human platelets. *Blood*, **88**, 907-914.
- Warwick,R.M., Vaughan,J., Murray,N., Lubenko,A., & Roberts,I. (1994) In vitro culture of colony forming unit-megakaryocyte (CFU-MK) in fetal alloimmune thrombocytopenia. *Br.J.Haematol.*, **88**, 874-877.
- Watkins,N.A., Schaffner-Reckinger,E., Allen,D.L., Howkins,G.J., Brons,N.H., Smith,G.A., Metcalfe,P., Murphy,M.F., Kieffer,N., & Ouwehand,W.H. (2002a) HPA-1a phenotype-genotype discrepancy reveals a naturally occurring Arg93Gln substitution in the platelet beta 3 integrin that disrupts the HPA-1a epitope. *Blood*, **99**, 1833-1839.
- Watkins,N.A., Smethurst,P.A., Allen,D., Smith,G.A., & Ouwehand,W.H. (2002b) Platelet alphaIIb beta3 recombinant autoantibodies from the B-cell repertoire of a post-transfusion purpura patient. *Br.J.Haematol.*, **116**, 677-685.
- Weisel,J.W., Nagaswami,C., Vilaire,G., & Bennett,J.S. (1992) Examination of the platelet membrane glycoprotein IIb-IIIa complex and its interaction with fibrinogen and other ligands by electron microscopy. *J Biol.Chem.*, **267**, 16637-16643.
- Weiss,G., Lison,S., Spannagl,M., & Heindl,B. (2010) Expressiveness of global coagulation parameters in dilutional coagulopathy. *Br.J Anaesth.*, **105**, 429-436.
- Wencel-Drake,J.D. (1990) Plasma membrane gpIIb-IIIa: evidence for a cycling receptor pool. *Am.J.Pathol.*, **136**, 61-70.
- Wencel-Drake,J.D., Frelinger,A.L., Dieter,M.G., & Lam,S.C.T. (1993) Arg-Gly-Asp-dependent occupancy of GPIIb/IIIa by apyrase: evidence for internalisation and cycling of a platelet integrin. *Blood*, **81**, 62-69.
- Wencel-Drake,J.D., Plow,E.F., Kunicki,T.J., Woods,V.L., Keller,D.M., & Ginsberg,M.H. (1986a) Localization of internal pools of membrane glycoproteins involved in platelet adhesive responses. *Am.J Pathol.*, **124**, 324-334.
- Wencel-Drake,J.D., Plow,E.F., Kunicki,T.J., Woods,V.L., Keller,D.M., & Ginsberg,M.H. (1986b) Localization of internal pools of membrane glycoproteins involved in platelet adhesive responses. *Am.J Pathol.*, **124**, 324-334.
- Wencel-Drake,J.D., Plow,E.F., Kunicki,T.J., Woods,V.L., Keller,D.M., & Ginsberg,M.H. (1986c) Localization of internal pools of membrane glycoproteins involved in platelet adhesive responses. *Am.J Pathol.*, **124**, 324-334.
- West,K.A., Anderson,D.R., McAlister,V.C., Hewlett,T.J., Belitsky,P., Smith,J.W., & Kelton,J.G. (1999) Alloimmune thrombocytopenia after organ transplantation. *N.Engl.J.Med.*, **341**, 1504-1507.

- Westman,P., Hashemi-Tavoularis,S., Blanchette,V., Kekomaki,S., Laes,M., Porcelijn,L., & Kekomaki,R. (1997) Maternal DRB1*1501, DQA1*0102, DQB1*0602 haplotype in fetomaternal alloimmunization against human platelet alloantigen HPA-6b (GPIIIa-Gln489). *Tissue Antigens*, **50**, 113-118.
- White,J.G. & Escolar,G. (2000) EDTA-induced changes in platelet structure and function: adhesion and spreading. *Platelets*, **11**, 56-61.
- Wilcox,D.A., Wautier,J.L., Pidard,D., & Newman,P.J. (1994) A single amino acid substitution flanking the fourth calcium binding domain of alpha IIb prevents maturation of the alpha IIb beta 3 integrin complex. *J Biol.Chem.*, **269**, 4450-4457.
- Williamson,L.M. (1998) Screening programmes for foetomaternal alloimmune thrombocytopenia. *Vox Sang.*, **74 Suppl 2**, 385-389.
- Williamson,L.M., Hackett,G., Rennie,J., Palmer,C.R., Maciver,C., Hadfield,R., Hughes,D., Jobson,S., & Ouwehand,W.H. (1998) The natural history of fetomaternal alloimmunization to the platelet-specific antigen HPA-1a (PIA1, Zwa) as determined by antenatal screening. *Blood*, **92**, 2280-2287.
- Wilson,J.M. & Jungner,Y.G. (1968) [Principles and practice of mass screening for disease]. *Bol.Oficina Sanit.Panam.*, **65**, 281-393.
- Woodrow,J.C. & Finn,R. (1966) Transplacental haemorrhage. *Br.J Haematol.*, **12**, 297-309.
- Woods,V.L., Jr., Oh,E.H., Mason,D., & McMillan,R. (1984) Autoantibodies against the platelet glycoprotein IIb/IIIa complex in patients with chronic ITP. *Blood*, **63**, 368-375.
- Wu,G.G., Kaplan,C., Curtis,B.R., & Pearson,H.A. (2010) Report on the 14th International Society of Blood Transfusion Platelet Immunology Workshop. *Vox Sang*, **99**, 375-381.
- Xiao,T., Takagi,J., Collier,B.S., Wang,J.H., & Springer,T.A. (2004) Structural basis for allostery in integrins and binding to fibrinogen-mimetic therapeutics. *Nature*, **432**, 59-67.
- Xiong,J.P., Mahalingham,B., Alonso,J.L., Borrelli,L.A., Rui,X., Anand,S., Hyman,B.T., Rysiok,T., Muller-Pompalla,D., Goodman,S.L., & Arnaout,M.A. (2009) Crystal structure of the complete integrin alphaVbeta3 ectodomain plus an alpha/beta transmembrane fragment. *J Cell Biol.*, **186**, 589-600.
- Xiong,J.P., Stehle,T., Diefenbach,B., Zhang,R., Dunker,R., Scott,D.L., Joachimiak,A., Goodman,S.L., & Arnaout,M.A. (2001) Crystal structure of the extracellular segment of integrin alpha Vbeta3. *Science*, **294**, 339-345.
- Xiong,J.P., Stehle,T., Zhang,R., Joachimiak,A., Frech,M., Goodman,S.L., & Arnaout,M.A. (2002a) Crystal structure of the extracellular segment of integrin alpha Vbeta3 in complex with an Arg-Gly-Asp ligand. *Science*, **296**, 151-155.
- Xiong,J.P., Stehle,T., Zhang,R., Joachimiak,A., Frech,M., Goodman,S.L., & Arnaout,M.A. (2002b) Crystal structure of the extracellular segment of integrin alpha Vbeta3 in complex with an Arg-Gly-Asp ligand. *Science*, **296**, 151-155.
- Zhang,W., Nardi,M.A., Borkowsky,W., Li,Z., & Karparkin,S. (2009) Role of molecular mimicry of hepatitis C virus protein with platelet GPIIIa in hepatitis C-related immunologic thrombocytopenia. *Blood*, **113**, 4086-4093.
- Zhou,Y., Fisher,S.J., Janatpour,M., Genbacev,O., Dejana,E., Wheelock,M., & Damsky,C.H. (1997) Human cytotrophoblasts adopt a vascular phenotype as they differentiate. A strategy for successful endovascular invasion? *J.Clin.Invest*, **99**, 2139-2151.

- Zhu,J., Luo,B.H., Xiao,T., Zhang,C., Nishida,N., & Springer,T.A. (2008) Structure of a complete integrin ectodomain in a physiologic resting state and activation and deactivation by applied forces. *Mol.Cell*, **32**, 849-861.
- Zucker,M.B. & Grant,R.A. (1978) Nonreversible loss of platelet aggregability induced by calcium deprivation. *Blood*, **52**, 505-513.

Appendix 1. List of Abbreviations

Abbreviation	Full Text
aa	Amino acid
ACE	Antigen capture ELISA
ADP	Adenosine diphosphate
AITP	Autoimmune thrombocytopenia
ATCC	American type culture collection
βTD	Beta tail domain
BPSU	British Paediatric Surveillance Unit
BSA	Bovine serum albumin
CD	Cluster of differentiation
CDB	Cell dissociation buffer
CPD	Citrate phosphate dextrose
CHO	Chinese hamster ovary
CI	Confidence interval
CT	Clotting time
CFT	Clot formation time
CFU-MK	Colony-forming unit, megakaryocytic
CTG	Cardiotachograph
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EBI	European bioinformatics institute
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMBL	European molecular biology laboratory
EMEM	Eagle's minimal essential medium
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FMAIT	Feto-maternal alloimmune thrombocytopenia
GI	Gastro-intestinal
GP	Glycoprotein
H&I	Histocompatibility and Immunogenetics
HBsAg	Hepatitis B surface antigen
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HITS	Histocompatibility and Immunogenetics Typing System

Appendix 1 (cont'd...)

HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HPA	Human platelet antigen
HUVEC	Human umbilical vein endothelial cell
ICH	Intracranial haemorrhage
IUD	Intrauterine death
ITP	Idiopathic thrombocytopenic purpura
i.u.	International units per mL
IUD	Intra-uterine death
IUGR	Intra-uterine growth restriction
IVIG	Intravenous immunoglobulin
kD	kiloDalton
LED	Light emitting diode
mAb	Monoclonal antibody
MACE	Modified antigen capture ELISA
MAIMA	Monoclonal antibody specific immobilisation of melanoma cell antigens
MAIPA	Monoclonal antibody specific immobilisation of platelet antigens
MCF	Maximum clot firmness
MFI	Mean fluorescence intensity
mL	Millilitre
MNC	Mononuclear cell
NAIT	Neonatal alloimmune thrombocytopenia
NHSBT	National Health Service Blood and Transplant
NIBSC	National Institute for Biological Standards and Control
na	Not applicable
NK	Not known
NND	Neonatal death
NPEU	National Perinatal Epidemiology Unit
N.S.	Not significant
OD	Optical density
OPD	Ortho-phenylene diamine
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDB	Protein data bank
PE	Phycoerythrin
PIFT	Platelet immunofluorescence test
PMT	Photo-multiplier tube

Appendix 1 (cont'd...)

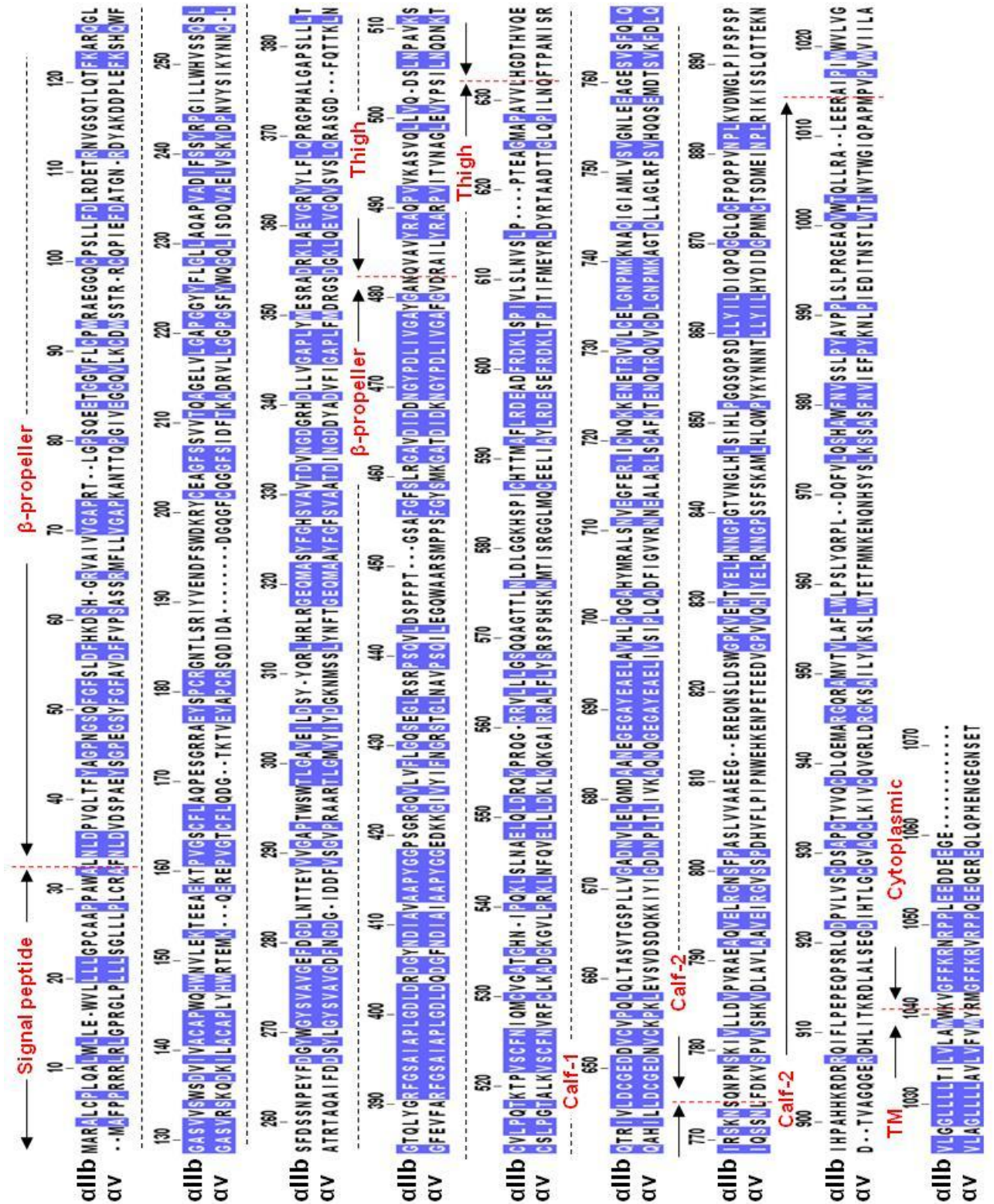
PRP	Platelet-rich plasma
PSI	Plexin semiphosphorin integrin
PTP	Post-transfusion purpura
RPT	Refractoriness to platelet transfusion
RT	Room temperature
RTA	Road traffic accident
SASPA	Simultaneous analysis of platelet specific antibodies
SCCS	Surface connected canicular system
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SPR	Surface plasmon resonance
TBS	Tris buffered saline
TOP	Termination of pregnancy
UK	United Kingdom
UKOSS	United Kingdom Obstetric Surveillance System
VLA	Very late antigen
vWF	Von Willebrand Factor
WHO	World Health Organisation

Appendix 2.

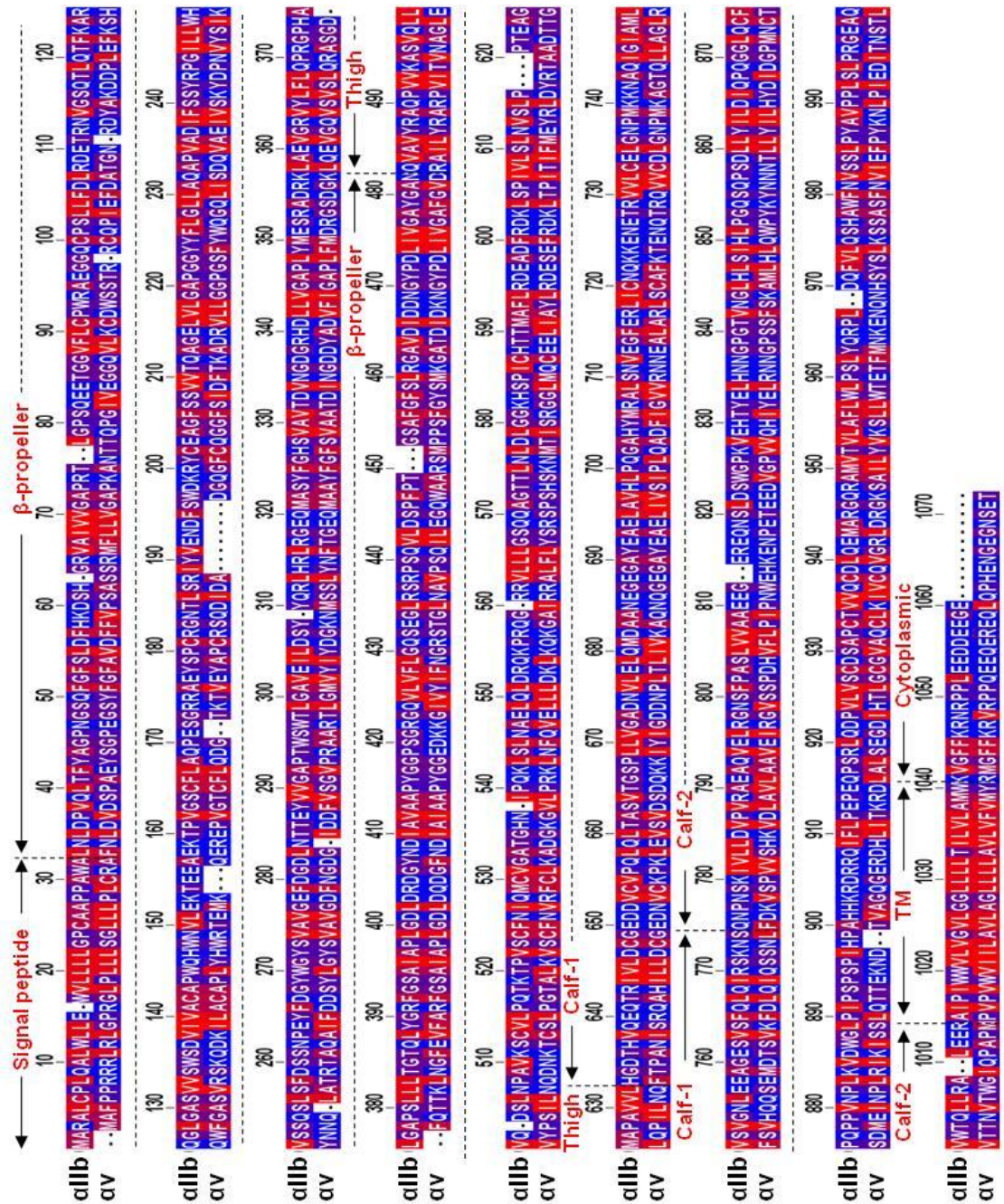
Protein sequences for $\alpha\text{IIb}\beta 3$ (protein P08514) and $\alpha\text{v}\beta 3$ (protein P06756) were retrieved from the UniProt protein knowledgebase (UniProtKB) then exported in FASTA format. The files were imported into, and aligned using, Jalview Version 2.7 (Dundee, UK) and amino acids coloured according to identity (**Appendix 2A**) or hydrophobicity (**Appendix 2B**).

Domains are indicated by red text.

Appendix 2A. Comparison of amino acid sequences of αIIb and αV showing sequence identity



Appendix 2B. Comparison of amino acid sequences of α IIb and α v showing hydrophobicity



Appendix 3. Samples used in study

3.1 Samples obtained from blood donors

Sample ID	Donor ID	Material
AB3	Unknown	Serum
AB5	Unknown	Serum
AB HE	Unknown	Serum
1a D-0003	Donor 3	CP
1a D-0004	Donor 4	CP
1a D-0005	Donor 5	RP
1a D-0006	Donor 5	Serum
1a D-0008	Donor 2	CP
1a D-0010	Donor 7	Serum
1a D-0011	Donor 2	Serum
1a D-0012	Donor 8	RP
1a D-0013	Donor 9	CP
1a D-0015	Donor 4	CP
1a D-0016	Donor 10	CP
1a D-0017	Donor 11	CP
1a D-0018	Donor 12	CP
1a D-0024	Donor 15	CP

3.2 Samples obtained from patients having had an episode of PTP

Sample ID	ID	Material
1a D-0001	Unknown	RP
1a D-0009	Unknown	RP

3.3 Samples from mothers of infants suspected of having NAIT

Sample ID	Mother ID	Material	Parity	Sample taken gestation (weeks)	New NAIT case?
NR-0001	F1072	EDTA	G1	T	Yes
NR-0002	F1071	EDTA	G1	T	Yes
NR-0003	F1021	EDTA	G1	T	Yes
NR-0004	F1037	Serum	G2	T	Yes
NR-0005	F1048	Serum	G2	T	Yes
NR-0021	F0709	EDTA	G3	T	Yes
NR-0022	F0709	Serum	G3	T	Yes
NR-0023	F0652	EDTA	G2	NK	No
NR-0031	F0768	EDTA	G4	15	Yes
NR-0032	F0892	EDTA	G1	T	Yes
NR-0039	F0771	EDTA	G1	NK	Yes
NR-0040	F0761	EDTA	G4	NK	Yes

Appendix 3.3 (cont'd...)

Sample ID	Mother ID	Material	Parity	Sample taken gestation (weeks)	New NAIT case?
NR-0046	F0788	EDTA	G2	T	Yes
NR-0049	F0771	Serum	G1	T	Yes
NR-0056	F1071	Serum	G1	T	Yes
NR-0058	F0737	EDTA	G1	NK	Yes
NR-0061	F1016	Serum	G1	T	Yes
NR-0064	F0734	Serum	G5	NK	Yes
NR-0073	F0315	EDTA	G2	NK	NK
NR-0075	F0964	EDTA	G4	T	Yes
NR-0077	F0992	Serum	G1	T	Yes
NR-0078	F0788	Serum	G3	26	No
NR-0082	F0315	Serum	G2	NK	NK
NR-0083	F0964	Serum	G4	T	Yes
NR-0084	F1016	EDTA	G1	T	Yes
NR-0090	F0992	EDTA	G1	T	Yes
NR-0098	F0892	Serum	G1	T	Yes
NR-0099	F0975	EDTA	G3	T	Yes
NR-0102	F0672	Serum	G3	NK	No
NR-0103	F1048	EDTA	G2	T	Yes
NR-0104	F1037	EDTA	G2	NK	Yes
NR-0110	F1001	Serum	G2	T	Yes
NR-0116	F0975	Serum	G3	T	Yes
NR-0125	F0876	EDTA	G4	NK	Yes
NR-0128	F0251	Serum	G3	NK	NK
NR-0137	F0160	RP	NK	NK	NK
NR-0145	F0138	RP	NK	NK	NK
NR-0149	F0132	RP	NK	NK	NK
NR-0150	F0113	RP	NK	NK	NK
NR-0151	F0006	RP	NK	PT	No
NR-0152	F0006	RP	NK	PT	No
NR-0153	F0006	RP	NK	PT	No
NR-0154	F0111	RP	NK	NK	NK
NR-0156	F0612	EDTA	G3	PT	No
NR-0157	F0506	EDTA	G1	NK	Yes
NR-0201	F0327	Serum	G1	NK	NK
NR-0204	F0272	Serum	G1	Term	Yes

CP: citrated plasma; EDTA: EDTA plasma; RP: re-calcified citrated plasma; T: Term; PT: pre-term; NK: not known; G: Gravida

Appendix 4. Data collection from laboratories participating in NIBSC international quality assurance Exercise 2011-B.

4A. Worksheet distributed to participants to collect raw data.

Use this form to report raw data for each sample e.g. O.D. or MFI

Please adapt this form for your own use - e.g. add more rows/columns for more donors/techniques if you need them

Sample 1		Technique 1	Technique 2	Technique 3	Technique 4	(add more techniques if required by Inserting Columns)
Donor	HPA Genotype	IbIIIIa DO	Test: Units:	Test: Units:	Test: Units:	
1						
2						
3						
4						
5						
6						

(add more donors if required by Inserting Rows)

Sample 2		Technique 1	Technique 2	Technique 3	Technique 4	(add more techniques if required by Inserting Columns)
Donor	HPA Genotype	IbIIIIa Units:	IaIIa Units:	IbIX Units:	CD109 Units:	
1						
2						
3						
4						
5						
6						

(add more donors if required by Inserting Rows)

Sample 3		Technique 1	Technique 2	Technique 3	Technique 4	(add more techniques if required by Inserting Columns)
Donor	HPA Genotype	IbIIIIa Units:	IaIIa Units:	IbIX Units:	CD109 Units:	
1						
2						
3						
4						
5						
6						

(add more donors if required by Inserting Rows)

Sample 4		Technique 1	Technique 2	Technique 3	Technique 4	(add more techniques if required by Inserting Columns)
Donor	HPA Genotype	IbIIIIa Units:	IaIIa Units:	IbIX Units:	CD109 Units:	
1						
2						
3						
4						
5						
6						

(add more donors if required by Inserting Rows)

Appendix 4. Data collection from laboratories participating in NIBSC international quality assurance Exercise 2011-B

4B. MAIPA questionnaire distributed to participants.

Workshop 2011-B MAIPA questionnaire

Laboratory name:			
Laboratory number:			
Platelets			
1. Which anticoagulant was the blood collected into?			
If 'Other', please specify			
2. How were the platelets prepared from the sample e.g. differential centrifugation, gel filtration etc?			
3. Prior to use, were the platelets: frozen, stored in suspension at 4°C or used freshly prepared			
If 'Other', please specify			
4. If stored at 4°C, what solution were the platelets suspended in?			
5. What solution were the platelets washed in prior to use?			
6. What solution were the platelets suspended in prior to use?			
7. At what concentration?		x 10 ³ /μL	
First incubation (test sample)			
8. What volume of platelet suspension was used per reaction?			μL
9. Were the platelets pelleted and the supernatant removed before adding the test sample or were they used in suspension?			
10. What volume of test sample was used?			μL
11. How long were the platelets incubated with the test sample?			mins
12. At what temperature were the platelets incubated with the test sample?			°C
13. What solution was used to wash the platelets after completion of the first incubation?			
14. What was the pH of this wash solution?			
15. How many washes did the platelets have in this solution?			
16. After washing, were the platelets re-suspended in a solution?			
17. If yes, what was this solution?			
Second incubation [monoclonal antibody (mAb)]			
18. Name of clone:	Clone 1		
	Clone 2		
19. Please indicate the supplier of the mAb	Clone 1		
	Clone 2		
20. At what dilution was this used?	Clone 1		
	Clone 2		
21. What solution was used to dilute the mAb?			
22. What was the final concentration of the mAb?	Clone 1		μg/mL
	Clone 2		μg/mL
23. What volume of mAb was added to the platelets?	Clone 1		μL
	Clone 2		μL
24. How long was the second incubation?			mins
25. At what temperature was the second incubation performed?			°C
26. What solution was used to wash the platelets after the 2 nd incubation?			
27. What was the pH of this wash solution?			
28. How many washes did the platelets have in this solution?			
Any other comments?			

Appendix 5. Details of MAIPA assay used by participants in NIBSC workshop exercise 2011-B. *Question numbers relate to the numbers on the questionnaire circulate to participants (Appendix 4). Cells shaded red indicate presence of EDTA.*

Question		Lab ID		
		75	A	A
1	Anticoagulant	ACD or EDTA	EDTA	EDTA
2	Platelet extraction	Centrifugation	Centrifugation	Centrifugation
3	Fresh/frozen	Stored at 4°C	Frozen	Frozen
4	Storage solution	PBS/EDTA	Not stored	Not stored
5	Washed in	PBS/EDTA	PBS/EDTA	PBS/EDTA
6	Suspended in	PBS/BSA	PBS/EDTA	PBS/EDTA
7	Plt concentration (x10 ³ /μL)	1000	100	100
8	Volume of platelets	30 μL	100 μL	100 μL
	Total No. Platelets	3 x 10 ⁶	1 x 10 ⁷	1 x 10 ⁷
9	Pellet or Suspension	Suspension	Pellet	Pellet
10	Test sample	40 μL	25 μL	25 μL
	Sample dilution	1 in 1.75	Neat	Neat
Primary incubation (test sample)				
11	Time	30-60 mins	30 mins	30 mins
12	Temp	37°C	37°C	37°C
13	Wash solution	PBS	TBS/BSA	TBS/BSA
14	pH of wash solution	7.4	7.4	7.4
15	Number of washes	2	2	2
16	After wash: pellet or suspension	Suspension	Suspension	Suspension
17	Which solution	PBS/BSA	TBS/BSA	TBS/BSA
Secondary incubation (capture mAb)				
18	Clone	PAB-1	PAB-1	PAB-6
20	Dilution	Neat	1 in 10	1 in 10
21	Diluent for mAb	na	TBS/BSA	TBS/BSA
22	Final mAb conc	26 μg/mL	1.2 μg/mL	1.2 μg/mL
23	Volume of mAb	10 μL	40 μL	40 μL
24	Time	30 mins	30 mins	30 mins
25	Temp	37°C	37°C	37°C
26	Wash solution	PBS	TBS/BSA	TBS/BSA
27	pH of wash solution	7.4	7.4	7.4
28	Number of washes	2	3	3

Appendix 5 (cont'd...)

Question	Lab Number	Lab ID		
		B	17	16
1	Anticoagulant	Citrate	EDTA	ACD
2	Platelet extraction	Centrifugation	Centrifugation	Centrifugation
3	Fresh/frozen	Fresh	Stored at 4°C	Frozen (then 4°C)
4	Storage solution	Not stored	EDTA(10mM) / BSA / PBS	PBS/EDTA
5	Washed in	TBS/BSA/EDTA	EDTA(10mM) / BSA / PBS	PBS/EDTA
6	Suspended in	TBS/BSA	EDTA(10mM) / BSA / PBS	PBS/EDTA
7	Plt concentration (x10 ³ /μL)	200	100	205
8	Volume plts	50 μL	100 μL	100 μL / 30 μL
	Total # Plts	1 x 10 ⁷	1 x 10 ⁷	2.05 x 10 ⁷
9	Pellet or Suspension	Suspension	Suspension	Pellet
10	Test sample	25 μL	25 μL	100 μL
	Sample dilution	1 in 3	1 in 4	Neat
Primary incubation (test sample)				
11	Time	40 mins	30 mins	30 mins
12	Temp	37°C	37°C	37°C
13	Wash solution	TBS/BSA/EDTA	TBS	PBS/BSA
14	pH of wash solution	7.4	7.4	7.0
15	Number of washes	3	3	3
16	After wash: pellet or suspension	Pellet	Pellet	Pellet
17	Which solution	na	na	na
Secondary incubation (capture mAb)				
18	Clone	PAB-6	PAB-1 + PAB-6	Y2/51
20	Dilution	1 in 10	1 in 50	1 in 15
21	Diluent for mAb	TBS/BSA	TBS	PBS/EDTA
22	Final mAb conc	2.7 μg/mL	25 μg/mL	Unknown
23	Volume of mAb	40 μL	40 μL	10 μL
24	Time	40 mins	30 mins	30 mins
25	Temp	RT	37°C	37°C
26	Wash solution	TBS/BSA/EDTA	IGEPAL/Tween	PBS/EDTA
27	pH of wash solution	7.4	Unknown	7.0
28	Number of washes	3	9	2

Appendix 5 (cont'd...)

Question	Lab Number	Lab ID		
		70	66	46
1	Anticoagulant	EDTA	CPD	Citrate
2	Platelet extraction	Centrifugation	Centrifugation	Centrifugation
3	Fresh/frozen	Frozen	Stored at 4°C	Stored at 4°C
4	Storage solution	NS	NaCl + NaN ₃	0.9% NaCl + 1% NaN ₃
5	Washed in	PBS/BSA/EDTA	Not washed	EDTA (3mM) / NaCl
6	Suspended in	NaCl/BSA	PBS/BSA	PBS/BSA
7	Plt concentration (x10 ³ /μL)	100	1000	1000
8	Volume plts	500 μL	40 μL	40 μL
	Total # Plts	5 x 10 ⁷	4 x 10 ⁷	4 x 10 ⁷
9	Pellet or Suspension	Pellet	Suspension	Pellet
10	Test sample	100 μL	40 μL	50 μL
	Sample dilution	Neat	1 in 2	Neat
Primary incubation (test sample)				
11	Time	30 mins	60 mins	30 mins
12	Temp	37°C	37°C	37°C
13	Wash solution	NaCl/BSA	NaCl	NaCl
14	pH of wash solution	7.4	6 to 7	7.4
15	Number of washes	1	2	3
16	After wash: pellet or suspension	Pellet	NS	Suspension
17	Which solution	na	x	PBS/BSA
Secondary incubation (capture mAb)				
18	Clone	Y2/51	SZ21	P2
20	Dilution	Neat	Neat	1 in 100
21	Diluent for mAb	na	na	PBS/BSA
22	Final mAb conc	20 μg/mL	200 μg/mL	10 μg/mL
23	Volume of mAb	50 μL	6 μL	20 μL
24	Time	30 mins	*	30 mins
25	Temp	37°C	*	37°C
26	Wash solution	NaCl/BSA	*	NaCl
27	pH of wash solution	7.4	*	7.4
28	Number of washes	2	*	3

Appendix 5 (cont'd...)

Question	Lab Number	Lab ID		
		56	60	66
1	Anticoagulant	EDTA	EDTA	CPD
2	Platelet extraction	Centrifugation	Centrifugation	Centrifugation
3	Fresh/frozen	Fresh	Frozen	Stored at 4°C
4	Storage solution	PBS/EDTA/NaN ₃	NS	NaCl/NaN ₃
5	Washed in	PBS/EDTA	PBS/EDTA	Not washed
6	Suspended in	PBS/EDTA	PBS/EDTA	PBS/BSA
7	Plt concentration (x10 ³ /μL)	750	250	1000
8	Volume plts	100 μL	50 μL	40 μL
	Total # Plts	7.5 x 10 ⁷	1.25 x 10 ⁷	4 x 10 ⁷
9	Pellet or Suspension	Pellet	Pellet	Suspension
10	Test sample	50 μL	50 μL	40 μL
	Sample dilution	Neat	Neat	1 in 2
Primary incubation (test sample)				
11	Time	45 mins	35 mins	60 mins
12	Temp	37°C	37°C	37°C
13	Wash solution	PBS/EDTA	PBS/EDTA	NaCl
14	pH of wash solution	6.9	6.0	6 to 7
15	Number of washes	2	3	2
16	After wash: pellet or suspension	Pellet	Pellet	NS
17	Which solution	na	na	
Secondary incubation (capture mAb)				
18	Clone	P2	P2	P2
20	Dilution	1 in 40	1 in 40	Neat
21	Diluent for mAb	PBS/EDTA/BSA	PBS/BSA	na
22	Final mAb conc	5 μg/mL	5 μg/mL	200 μg/mL
23	Volume of mAb	50 μL	40 μL	6 μL
24	Time	45 mins	35 mins	*
25	Temp	37°C	37°C	*
26	Wash solution	PBS/EDTA	PBS/BSA	*
27	pH of wash solution	6.9	6.0	*
28	Number of washes	3	3	*

Appendix 5 (cont'd...)

Question	Lab Number	Lab ID		
		75	36	43
1	Anticoagulant	ACD or EDTA	ACD	ACD
2	Platelet extraction	Centrifugation	Centrifugation	Centrifugation
3	Fresh/frozen	Stored at 4°C	Stored at 4°C	Frozen
4	Storage solution	PBS/EDTA	NaCl + NaN ₃	PBS/EDTA/BSA
5	Washed in	PBS/EDTA	NaCl + NaN ₃	PBS/EDTA/BSA
6	Suspended in	PBS/BSA	PBS/BSA	PBS/EDTA/BSA
7	Plt concentration (x10 ³ /μL)	1000	100	5
8	Volume plts	30 μL	100 μL	3 μL
	Total # Plts	3 x 10 ⁶	1 x 10 ⁷	1.5 x 10 ⁴
9	Pellet or Suspension	Suspension	Pellet	Suspension
10	Test sample	40 μL	50 μL	120 μL
	Sample dilution	1 in 1.75	Neat	(Neat)
Primary incubation (test sample)				
11	Time	30-60 mins	30 mins	30 mins
12	Temp	37°C	37°C	37°C
13	Wash solution	PBS	NaCl	NaCl
14	pH of wash solution	7.4	5.65	7.0
15	Number of washes	2	3	3
16	After wash: pellet or suspension	Suspension	Pellet	Pellet
17	Which solution	PBS/BSA	na	na
Secondary incubation (capture mAb)				
18	Clone	P2	6C9	6C9
20	Dilution	Neat	1 in 10	1 in 40
21	Diluent for mAb	na	PBS/BSA	PBS/EDTA/BSA
22	Final mAb conc	200 μg/mL	20 μg/mL	5 μg/mL
23	Volume of mAb	10 μL	40 μL	40 μL
24	Time	30 mins	30 mins	*
25	Temp	37°C	37°C	*
26	Wash solution	PBS	0.9% NaCl	*
27	pH of wash solution	7.4	5.65	*
28	Number of washes	2	3	*

Appendix 5 (cont'd...)

Question	Lab Number	Lab ID		
		69	63	B
1	Anticoagulant	Citrate	EDTA	Citrate
2	Platelet extraction	Centrifugation	Centrifugation	Centrifugation
3	Fresh/frozen	Stored at 4°C	Stored at 4°C	Fresh
4	Storage solution	NaCl + NaN ₃	NaCl + NaN ₃	Not stored
5	Washed in	PBS/FCS	PBS/EDTA	TBS/BSA/EDTA
6	Suspended in	PBS/FCS	PBS/BSA	TBS/BSA
7	Plt concentration (x10 ³ /μL)	350	200	200
8	Volume plts	50 μL	100 μL	50 μL
	Total # Plts	1.75 x 10 ⁷	1 x 10 ⁷	1 x 10 ⁷
9	Pellet or Suspension	Suspension	Suspension	Suspension
10	Test sample	50 μL	50 μL	25 μL
	Sample dilution	1 in 2	1 in 3	1 in 3
Primary incubation (test sample)				
11	Time	45 mins	30 mins	40 mins
12	Temp	37°C	37°C	37°C
13	Wash solution	PBS/FCS	NaCl	TBS/BSA/EDTA
14	pH of wash solution	7.0	7.1 to 7.3	7.4
15	Number of washes	1	2	3
16	After wash: pellet or suspension	Pellet	Suspension	Pellet
17	Which solution	na	PBS/BSA	na
Secondary incubation (capture mAb)				
18	Clone	6C9	6B9	VI-PL2
20	Dilution	1 in 10	NS	1 in 50
21	Diluent for mAb	PBS/FCS	PBS	TBS/BSA
22	Final mAb conc	20 μg/mL	4 μg/mL	10 μg/mL
23	Volume of mAb	40 μL	40 μL	40 μL
24	Time	45 mins	30 mins	40 mins
25	Temp	37°C	37°C	RT
26	Wash solution	PBS/FCS	0.9% NaCl	TBS/BSA/EDTA
27	pH of wash solution	7.0	7.1 to 7.3	7.4
28	Number of washes	3	3	3

NS: not stated; na: not applicable; * participant used original MAIPA method in which the test sample and capture mAb are simultaneously incubated with the platelets

