PREDICTING THE SEVERITY OF NEONATAL ALLOIMMUNE THROMBOCYTOPENIA THROUGH RISK STRATIFCATION BY ASSESSING THE IgG SUBCLASSES INVOLVED

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Thesis Overview

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Abbreviations

A260	Absorption at wavelength 260
ADCC	Antibody-Dependent Cell-mediated Cytoxicity
AIT	Autoimmune thrombocytopenia also called idiopathic thrombocytopenia
BSA	Bovine Serum Albumin
CA	California
CD	Cluster of Differentiation
cDNA	Complementary Deoxy-ribonucleic Acid
CT	cytotrophoblasts
DAB	diaminobenzidine tetrahydrochloride
dH_2O	Distilled water
DMSO	Di-Methyl Sulfoxide
DNA	Deoxy-ribonucleic Acid
dNTP	Deoxy-nucleoside triphosphate
ECM	Extra-Cellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced Green Fluorescent Protein
ELISA	Enzyme Linked Immunosorbent Assay
FcR	Fc Receptors
FcRn	Neonatal Fc Receptor
FMH	Fetal-maternal haemorrhage
GP	Glycoprotein
GPVI	Glycoprotein number VI
HCPC	The Health and Care Professions Council
H&I	Histocompatibility and Immunogenetics
HEK	Human Embryonic Kidney
HFDN	Haemolytic disease of the fetus and newborn
HIP	HPA-screening in pregnancy
HLA	Human Leucocyte Antigen
HPA	Human Platelet Antigen
HRP	Horseradish Peroxidase
HSST	Higher Specialist Scientific Training
IBGRL	International Blood Group Reference Library
ICH	Intra Cranial Haemorrhage
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IVIg	Intravenous immunoglobulin

KB	Kilo Byte
LB	Luria-Bertani
MA	Massachusetts
MAIPA	Monoclonal antibody immobilisation of platelet glycoprotein assay
MFI	Mean/Median Fluorescent Intensity
MHC	Major histocompatibility complex
MWCO	Molecular Weight Cut Off
NAIT	Neonatal alloimmune thrombocytopenia
NCBI	National Centre for Biotechnology Information
NHS	National Health Service
NHS BT	National Health Service Blood and Transplant
NK	Natural Killer
PBS	Phosphate Buffered Saline
PBS-TBN	Phosphate Buffered Saline BSA Tween 20 sodium azide
PCR	Polymerase Chain Reaction
PDPU	Protein and Development Production Unit
pEGFP-N3	Plasmid Green Fluorescent Protein Number 3
PIFT	Platelet immunofluorescence test
PROFNAIT	Clinical trial
PTP	Post-transfusion purpura
RE	Restriction Endonuclease
rGPVI	Recombinant Glycoprotein Number 6
RPE	red phycoerythrin
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
SPR	Surface Plasmon Resonance
ST	Synciotiotrophoblasts
TRALI	Transfusion Acute Lung Injury
UK	United Kingdom
USA	United States of America
UV	Ultraviolet (light)
vWF	von Willebrand factor
WHO	World Health Organisation

Abstract

Neonatal alloimmune thrombocytopenia (NAIT) is a potentially fatal condition that affects the fetus or newborn. Maternally derived IgG alloantibodies directed against paternally inherited specific platelet antigens cross the placenta and target fetal platelets. The most severe cases result in death due to intra-cranial haemorrhage (ICH) caused mostly by antibodies to human anti-platelet antigen (HPA)-1a (also known as glycoprotein IIb/IIIa or integrin αIIbβ3). Previous studies have tried, unsuccessfully, to predict which HPA-1a-positive mothers may be at risk of ICH and fetal death, focussing on antibody titre and /or IgG subclass. More recent evidence has implicated antibodies to integrin αvβ3, which shares a subunit with HPA-1a and is required for angiogenesis. Therefore, ICH could be due to vascular pathology caused by HPA-1a antibodies that cross-react with αvβ3.

This study aimed to develop an assay that could detect antibodies to αvβ3 and to test if these were present in NAIT cases which had ICH. The second aim was to use Luminex™ technology, as previous studies used less sensitive methods, to investigate whether IgG subclass could be linked to NAIT severity.

Human embryonic kidney (HEK) cells were transfected with expression vectors encoding the heterodimers αvβ3 or αIIbβ3. Protein expression was validated using monoclonal antibodies and known HPA positive sera by flow cytometry. Transfected cell strains were incubated with serum samples from NAIT referral cases testing positive for HPA-1a antibodies and were divided into ICH and non-ICH cohorts. Analysis using flow cytometry revealed that samples from both ICH and non-ICH when tested undiluted reacted with both HEK strains. A possible prozone effect was assessed by testing neat and 4-fold-diluted samples from the two cohorts but no such effect was observed. In order to detect αvβ3-specific antibodies, the αIIbβ3 antibody was absorbed out of the patient sera using a β3-coated resin. After absorption, three ICH samples were tested with αvβ3 transfected cells but did not have detectable antibody. The two cohorts (ICH v non-ICH) were tested at doubling dilutions from neat to 1/64 using the two different strains but no differences were observed between them.

Luminex™ technology using an in-house multiplex bead assay with recombinant HPA-1a, HPA-1b and GPVI proteins was used to detect IgG antibodies and their subclass. Cut-offs were established using One Lambda's normalised background ratio plus 3SDs from 22 female apheresis donors. Fifteen samples from the ICH cohort were analysed with 23 non-ICH, 7 post-transfusion purpura (PTP) and 15 platelet refractory patients. Differences were observed between non-ICH and platelet refractory samples for total IgG ($p=0.027$) and IgG1 (p-0.011), and with ICH, non-ICH, PTP versus platelet refractory for IgG2 subclass ($p <0.0001$). This did not demonstrate any markers to be able to predict ICH.

In conclusion, this study did not demonstrate an association with αvβ3 HPA-1a specific antibody, HPA-1a titre or HPA-1a IgG subclass with ICH in the English population of confirmed NAIT cases.

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Declaration

No portion of the work referred to in the thesis has been submitted in support of an application or qualification of this or any other university or other institute of learning.

Copyright Statement

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About the Author

I completed a pharmacology degree at the University of Bath in 2000 which included a year in a research laboratory. After graduation, I started training as a Clinical Scientist in Histocompatibility and Immunogenetics (H&I) field at the Welsh Blood Service.

After obtaining the BSHI Diploma in 2003, I worked in the Molecular Genetics department in H&I, overseeing the development of the 'in-house HLA typing kits. I also was responsible for validating and introducing the 'in-house' sequenced-based typing methodology. I became a registered Clinical Scientist with the Healthcare Protection Agency (HCPC) 2006.

In 2009, I completed a Master of Science degree in Transfusion and Transplantation at the University of Bristol. I achieved Part 1 of the Royal College of Pathologists certificate. Following this, I worked on a number of projects for the Welsh Bone Marrow Donor Registry such as the tender for HLA-typing by sequenced-based for new donors and for 'out-sourcing' HLA-typing.

Throughout my time as a BSHI qualified Clinical Scientist with the Welsh Blood Service, I was part of the on-call team for deceased donor typing and as a flow cytometry cross-matcher.

In April 2015, I moved to NHS Blood and Transplant at Filton to become the H&I Laboratory Operations Manager. This involved learning about Serology for Platelet and Granulocyte Immunology. My responsibilities include reviewing neonatal alloimmune thrombocytopenia investigations (NAIT) cases, and for reviewing the local HLA platelet refractory patients.

In September 2015, I started the Higher Specialist Scientific Training (HSST) programme. Since then, I have achieved all milestones expected of a trainee on this programme, including passing the Part 2 examination of the Royal College of Pathologists in 2020.

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Chapter 1: Introduction

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1.1 Platelets and haemostasis

Platelets play a major role within haemostasis and thrombosis; they are small anuclear cells (1-2 microns) with a discoid shape which circulate within blood vessels and activate clot formation when there is vessel injury (Li *et al*., 2012). Platelets are formed from their precursor cells the megakaryocytes, within the bone marrow. After red blood cells, they are the most abundant cells within the body and have a lifespan of 5-7 days (Li *et al*., 2012; Holinstat, 2017).

Platelets adhere to vessel walls at the site of vessel injury which causes primary haemostasis (Gale, 2011; Vadasz *et al*., 2015). During vessel injury the subendothelium becomes exposed, displaying proteins such as collagen which receptors on platelets recognise and bind (adhesion) (Vadasz *et al*., 2015). Binding of the platelet receptors to their newly exposed ligands such as von Willebrand factor (vWF) causes their activation which results in conformational changes to the platelet's structure and receptor expression (Austin, 2017). Once the platelets have become activated this aids in recruiting further platelets as they adhere to each other when activated and this amplifies the response to attract further platelets to aggregate (Austin, 2017).

The secondary wave of haemostasis is the activation of the coagulation pathway which produces thrombin and finally fibrin to halt bleeding (Li *et al*., 2012; Vadasz *et al*., 2015). Figure 1.1 illustrates the primary and secondary waves of haemostasis and platelet involvement in this process. When platelets aggregate at sites which do not have injury this results in conditions such as strokes and myocardial infarctions (Vadasz *et al*., 2015).

The platelet receptors that contribute to haemostasis are the glycoproteins (GP) and integrins (Ramstrom, Rainby & Lindahl, 2002). Integrins have an α and β subunit which are non-covalently linked; in platelets there are five α units and two β subunits which can combine to form receptors (Kasirer-Friede, Kahn & Shattil, 2007). The most notable platelet integrin is αIIbβ3, formed from glycoproteins GPIIb (αIIb) and GPIIIa (β3), which has the highest density and binds to multiple ligands (Gale, 2011). The GPs often combine with other GPs to form platelet receptors.

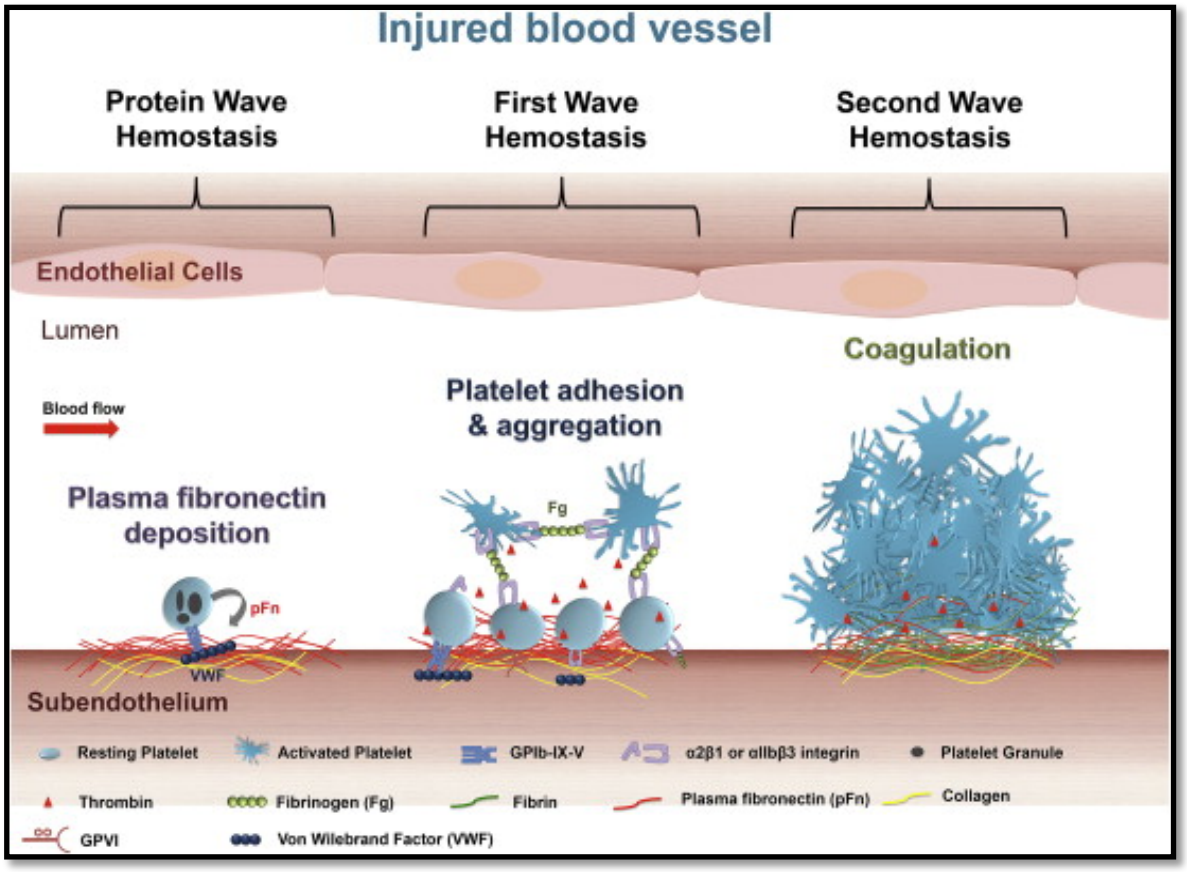

Figure 1.1 Schematic representation of the role of platelets in haemostasis. This diagram shows the first wave of haemostasis where vessel injury exposes collagen and endothelial proteins which the platelet receptors can bind to. This causes the platelets to change shape and start to adhere to the endothelial wall and aggregate. This leads into the second wave of haemostasis which is the coagulation pathway, resulting in a haemostatic plug. Taken from Vadasz *et al*., (2015).

The receptor primarily responsible for platelet adhesion in high shear rates is GPIb-IX-V binding to vWF (Ni & Freedman, 2003; Andrews & Berndt, 2004). αIIbβ3 integrin binds to fibrinogen or vWF, which is responsible for platelet adhesion in low shear rates. Binding to fibrinogen results in a stable adhesion as this ligation causes a conformational change in the receptors to increase the affinity (Austin, 2017). Table 1.1 lists the main GPs involved in haemostasis.

Glycoprotein	Expression	Function	Ligands
Ia	Monocytes T and B cells NK Cells Platelets	Platelet adhesion during low flow rates	Collagen
Ib	Platelets	Complexes with GPIX and to help in platelet adhesion and tethering in high flow rates Role in platelet turnover	von Willebrand factor Complement receptor of macrophages
IIb	Platelets	Form a complex with GPIIIa to become αIIbβ3 integrin Complex central in clot formation	Fibrinogen von Willebrand factor Fibronectin Vitronectin
IIIa	Platelets Trophoblasts Endothelial cells	Form a complex with GPIIb to become αIIbβ3 integrin – mediates platelet aggregation Complex central in clot formation Forms a complex with αv as well	Fibrinogen von Willebrand factor Fibronectin Vitronectin
CD109	Platelets Activated T cells Endothelial cells Progenitor bone marrow cells	Precise function unknown	Collagen

Table 1.1. Examples of glycoproteins, with their function and expression. The main glycoproteins (GP) of platelets with their expression, ligands and function; showing which human platelet antigens are located on which glycoprotein. Taken from Scuh *et al*., (2002) and Hayashi & Hirayama (2015).

1.1.1 Human Platelet Antigens (HPA)

Changes to GP structure, due to single nucleotide polymorphisms (SNPs), can expose distinctive epitopes (Curtis, 2015). These structural differences on the GPs give rise to the platelets own antigen system called the Human Platelet Antigen (HPA) (Sachs, 2013). HPAs are expressed on a number of different GPs; Figure 1.2 is a diagrammatic representation of where the different HPAs are located on each of the GPs listed in Table 1.2 (Curtis, 2015).

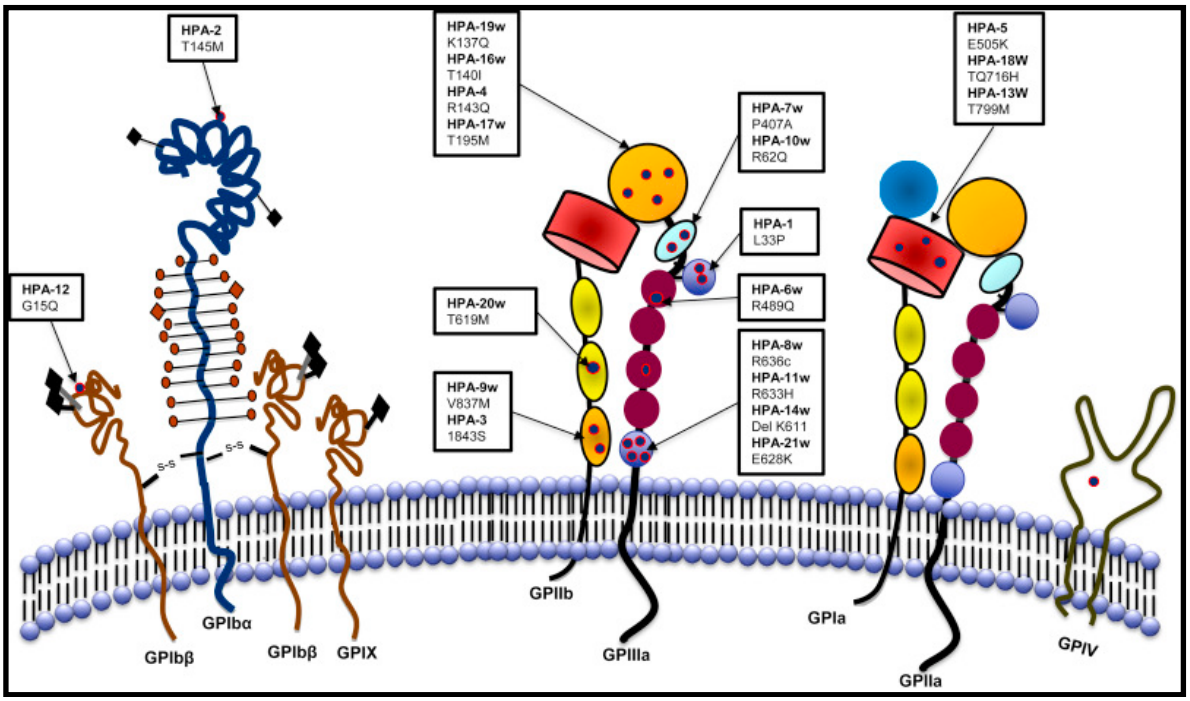

Figure 1.2. Schematic representation of the different glycoproteins and the human platelet antigens location. Taken from Zdravic *et al*., (2016) shows the glycoproteins GPs that are important in auto and alloimmune thrombocytopenias, including the positions of the human platelet antigens (HPA) and their position on GPs.

The HPA system has its own nomenclature system. Each different epitope region on the GPs is given a number such as HPA-1 (located on GPIIIa) and HPA-2 (located on GPIb). In each of these antigens there are further SNPs which give rise to further epitopes and these are called 'a' or 'b' (rarely 'c'). Hence, they are called HPA-1a or HPA-1b; the wild-type antigen is present at a higher frequency in the population and designated 'a', e.g., HPA-1a. The mutated antigen is referred to as 'b', e.g., HPA-1b and is less frequent (Curtis, 2015). There are currently 41 platelet antigens (including antigen sites and their SNPs) which have been shown to cause both allo and auto-immune responses (Porceliign, Huiskes & Haas, 2020). HPA inheritance is through autosomal co-dominance (Bussel & Primiani, 2008). The genotype frequency of these antigens depends on ethnicity; most studies are from a Caucasoid background, but there are published frequencies of other groups (Table 1.2, Curtis & McFarland, 2013).

The majority of the named HPAs are located on the αIIbβ3 integrin (GPIIb/GPIIIa complex), with a high proportion on the β3 part of the integrin (Curtis & McFarland, 2013). The most common HPA involved in an alloimmune response is HPA-1a (Curtis & McFarland, 2013). Platelets also express human leukocyte antigens (HLA) and the ABH antigen system; these in addition with HPA are the main causes of any alloimmune response directed to the

platelets (Hayashi & Hirayama, 2015). However, the majority of the antibody response in autoimmune thrombocytopenia conditions is directed to GPIIbIIIa followed by GPIbα (Li *et al.*, 2012).

	Allele Frequency (Percentages)			
HPA Antigens	**Caucasoid**	**African**	**Asian**	**Glycoprotein**
1a1a	72	90	100	GPIIIa
1a1b	26	10	0	
1b1b	2	Not reported	Not reported	
2a2a	85	71	95	GPIbα
2a2b	14	29	5	
2b2b	1	Not reported	Not reported	
3a3a	37	68	59.5	GPIIb
3a3b	48	32	40.5	
3b3b	15	Not reported	Not reported	
4a4a	>99.9	100	99.5	GPIIIa
4a4b	<0.1	0	0.5	
4b4b	<0.1	Not reported	Not reported	
5a5a	88	82	98.6	GPIa
5a5b	20	18	0.4	
5b5b	1	Not reported	Not reported	CD109
15a15a	35	65	53	
15a15b	42	35	47	
15b15b	23	Not reported	Not reported	

Table 1.2. The main human platelet antigens and their location on their glycoproteins. This table shows the main human platelet antigens and their location on the glycoproteins. Antigen frequencies (percentages) of three populations are shown for the different homozygous and heterozygous combinations. Taken and adapted from Curtis & McFarland (2013).

1.2. Clinical Indications related to thrombocytopenia

1.2.1 Introduction

A normal circulating platelet count in a healthy adult is 150-400x10^9/L and conditions with a reduced number of circulating platelets cause bleeding, potentially leading to haemorrhages (Vadasz *et al*., 2015). Thrombocytopenia is a term used to describe platelet disorders where the number of circulating platelets is below 150x10^9/L (Li *et al*., 2012). The three over-arching conditions that can cause thrombocytopenia are immune, genetic and malignancy related (Li *et al*., 2012; Vadasz *et al*., 2015). Malignancy disorders indicated in thrombocytopenia are blood disorders such as chronic lymphocytic leukaemias, lymphomas and solid phase cancers such as breast and ovarian cancer (Li *et al*., 2012). Genetic disorders range from mutations which may decrease GP expression to lack of platelet production (Freson *et al*., 2007). Immune mediated thrombocytopenia can be divided into autoimmune or alloimmune.

1.2.2 Immune thrombocytopenia

1.2.2.1 Autoimmune thrombocytopenia (AIT)

AIT is a bleeding disorder that is caused by an autoantibody binding to platelets to cause their destruction (Vadasz *et al.*, 2015). More recently these autoantibodies have been shown to decrease platelet production by suppressing megakaryocyte development as well as causing platelet destruction (Li *et al*., 2012). AIT has its own clinical characteristics and is reported to affect 1 to 2.5/10,000 people (Li *et al*., 2012; Vadasz *et al*., 2015). This condition can be divided into primary causes where there are no additional causes of thrombocytopenia; and secondary causes which include drug-induced and infection associated thrombocytopenia (Vadasz *et al*., 2015). People who are suffering from infections such as *cytomegalovirus, Helicobacter pylori* and *Epstein-Barr* virus have been shown to have thrombocytopenia. This has been thought to be due to molecular mimicry whereby the infectious agent's structure is similar to that of GPs and these antibodies cross-react with platelets to cause their destruction (Li *et al*., 2012).

Primary AIT, also called idiopathic thrombocytopenia, is diagnosed when the platelet count is below 100x10^9L. The exact pathway is unknown, although one proposed mechanism is that autoantibodies opsonise to the person's own GPs, which facilitates platelet clearance

by Fcγ-receptors on macrophages of the reticuloendothelial system (Li *et al*., 2012; McKenzie *et al*., 2013). These antibodies have been shown to suppress platelet production by inducing megakaryocyte apoptosis (Li *et al*., 2012; McKenzie *et al*., 2013; Vadasz *et al*., 2015). The majority of the autoantibodies (70%) are directed to GPIIb/IIIa, whilst others are directed to GPIbα (Li *et al*., 2012).

1.2.2.2 Alloimmune thrombocytopenia

Alloimmune thrombocytopenia is the production of alloantibodies following exposure to platelets from another person which have a different HPA expression. These alloantibodies bind to the 'foreign' platelets and cause their destruction (Vadasz *et al*., 2015). This exposure to alloantigens is commonly through blood transfusions and pregnancy. The former leads to transfusion refractoriness, which is defined as a failure to increase the patient's platelet count, following two consecutive transfusions, due to HLA-, ABO- or HPA-specific antibodies, and is treated by supplying units that the person does not have antibodies to (Stanworth *et al*., 2015). Pregnancy alloantibodies can lead to a condition which affects fetuses and newborns called neonatal alloimmune thrombocytopenia (NAIT) (Sachs, 2020). NAIT will be discussed in more detail in Section 1.3. A third condition is called post-transfusion purpura (PTP), which occurs after a transfusion with an incompatible unit, resulting in a severe drop in the platelet count. This is caused by the patient having an existing HPA antibody (from pregnancy or transfusion) which destroys the transfused platelets, but by unknown mechanisms also destroys their own platelets and any platelets transfused which are negative for the antibody (Li *et al*., 2012).

1.3. Neonatal alloimmune thrombocytopenia (NAIT)

NAIT is a potentially fatal condition which affects both the fetus and newborn. The reported incidence of NAIT is a between 1 in 1,000 to 2000 pregnancies, this range is due to the differences in screening programmes and clinical diagnosis (see Section 1.3.2) (Arnold, Smith & Kelton, 2008). NAIT is under-reported as a platelet count is not routinely taken at birth and, in some cases, there is a maternal history of miscarriage which has not been investigated (Vadasz *et al*., 2015; Tiller *et al*., 2017). Maternally derived IgG alloantibodies directed against specific platelet antigens cross the placenta via the fetal Fc receptor (FcRn) and target the fetal platelets to cause thrombocytopenia (Sachs, 2020). These

alloantibodies arise by an incompatibility between the maternal and paternal platelet antigen, with the fetus inheriting a paternal antigen which is bound by the maternal antibody (Sachs, 2013; Zdravic *et al*., 2016).

Thrombocytopenia in a newborn can be the result of conditions unrelated to NAIT, such as premature birth, infections and pre-eclampsia in the mother (Resch *et al*., 2018). The differential diagnosis in NAIT must take other conditions into consideration to explain the thrombocytopenia. Severe NAIT is classified as a platelet count $<50x10^9$/L and the main concern is that the lack of platelets can lead to intracranial haemorrhage (ICH) (de Vos *et al.,* 2020).

NAIT can be caused by any platelet incompatibilities between the paternal and maternal phenotype. However, studies have shown that 80% of cases in the Caucasoid population are due to HPA-1a antibodies (Sachs, 2020); when the mother is HPA-1b1b positive and is sensitised by an HPA-1a+ partner. Around 2% of the Caucasoid population is homozygous for HPA-1b1b and around 10% produce antibodies (de Vos *et al.,* 2020). In a Caucasoid population, HPA-5b antibodies account for 10-15% of NAIT cases, the remaining cases are mostly due to HPA-3a and HPA-15a antibodies or novel/private antibodies (Peterson *et al*., 2013, de Vos *et al.,* 2020).

1.3.1 Indication

During pregnancy NAIT is suspected if there is evidence of ventriculomegaly or ICH during the twenty-week ultrasound scan (Martillotti *et al*., 2017). ICH has been reported to occur prior to 20 weeks' gestation, with most cases presenting during the second trimester (Vadasz *et al*., 2015). The majority of primary NAIT cases are diagnosed following birth, if a baby presents with blood spots on the skin such as petechiae (small) or purpura (large) (Wienzek-Lischka *et al*., 2017). ICH may occur *in utero*, during birth or post-delivery and can cause neurological damage leading to mental and physical disabilities such as blindness and cerebral palsy (Kamphius & Oepkes, 2011). Death occurs in 5% of cases and this can occur *in utero* or post-delivery (Yougbare, Zdravic & Ni, 2018). Studies have shown that ICH occurs in 1:10,000 of births affected by NAIT (Tiller *et al*., 2020).

Some have hypothesised that NAIT due to HPA-2 antibodies (GPIα) would present in a non-classical way and cause miscarriages which would never get reported (Zdravic *et al*., 2016; Kaplan, Ni & Freedman, 2013). From murine studies it has been suggested that HPA-2 antibodies would bind to the placenta and cause miscarriage due to a placental thrombus rather than a reduction in fetal platelets (Kaplan, Ni & Freedman, 2013). The other hypothesis is that these antibodies cause such a mild thrombocytopenia they are under reported as there are no symptoms.

1.3.2 Possible routes of sensitisation

1.3.2.1 Pre-pregnancy

β3 is also present in sperm and saliva (Bussel & Primiani, 2008), which could induce maternal sensitisation prior to pregnancy. This is not the accepted route of sensitisation as an affected second pregnancy is more severe than a first affected pregnancy, indicating a primary response during gestation and not prior to this (Bussel & Primiani, 2008). This is further supported by the likelihood of the second fetus having a haemorrhage being 79% if the pregnancy is untreated and the likelihood of NAIT is 100% (based on the paternal HPA genotype) (Tiller *et al*., 2017; Winkelhorst *et al*., 2017).

1.3.2.2 During pregnancy

There are conflicting reports on the routes and timing of maternal sensitisation and how the immune privilege usually afforded to the fetus by the placenta is broken-down (Zdravic *et al*., 2016). This is confounded by screening studies during pregnancy for NAIT. Some countries have screening programmes whereas other countries, such as the UK, use clinical diagnosis during and post-delivery (Knight *et al.,* 2011; Tiller *et al*., 2017). The difference in reported incidence is likely due to some countries using screening studies whilst others using clinical symptoms and there are cases of NAIT without clinical symptoms (Knight *et al.,* 2011; Tiller *et al*., 2017). NAIT is reported to occur in around 50% of first pregnancies but screening programmes put this figure around 25% in mothers who are homozygous for HPA-1b (Bussel & Primiani, 2008; Brojer *et al*., 2016).

Some hypothesise that fetal platelets cross the placenta into the maternal circulation triggering an immune response if there are HPA incompatibilities (Bussel & Primiani, 2008),

whilst others argue that the amount of fetal platelets crossing the placenta into the maternal circulation is not enough to mount an alloresponse sufficient to cause NAIT (Kumpel *et al*., 2008; Espinoza *et al*., 2013). Fetal platelet antigens are expressed by the 16th gestational week and fetuses will have a normal platelet count from the 18th gestational week (Tiller *et al*., 2017). This route of sensitisation does not seem plausible as the effects in NAIT are observed in first pregnancies by 20 weeks' gestation and the timeline would not fit with a primary immune response. More research in fetal platelets crossing the placenta is required to show if this is a route of sensitisation.

GPIIIa forms the β3 subunit of the αIIbβ3 integrin but in other cells, it can combine with αv to form the αvβ3 integrin (Zdravic *et al*., 2016, Sachs, 2020). This integrin is expressed by villous syncytiotrophoblast (ST) in the placenta and the maternal immune response is triggered by αvβ3 to produce antibodies that cross the placenta in mothers who are HPA-1b1b (Espinoza *et al*., 2013). There have been reports that the shedding of ST during the pregnancy could immunise the mother to make β3 antibodies (Kumpel, 2012). This route of sensitisation could explain how first pregnancies are affected but does not explain how only how some HPA-1b1b mothers are affected and but not all. The additional mechanisms which trigger this antibody response has not been elucidated.

1.3.2.3 At delivery

Screening studies indicate the route of sensitisation is at birth in 75% of cases following a fetal-maternal haemorrhage (FMH) at birth where the fetal platelets can pass into the maternal circulation (Kjeldsen-Kragh & Bengtsson, 2020). This exposes the maternal immune system to platelets and an alloimmune response is initiated, during subsequent pregnancies, when maternal circulation is re-exposed and this causes an amnestic antibody response and may explain why subsequent pregnancies are more severely affected (Brojer *et al*., 2016).

Once sensitisation within NAIT is properly elucidated, this may aid in developing specific treatments to try and prevent the development of HPA specific antibodies. Recently a clinical trial called PROFNAIT has been established to obtain maternal sera post-birth which contain HPA-1a antibodies, and this was licensed in 2019 as NAITgam (Tiller *et al*., 2017, Kjeldsen-Kragh & Bengtsson, 2020). These HPA-1a antibodies will be purified from multiple

sera and made into a preparation which can be injected into women during pregnancy or post-delivery; whereby the injected anti-HPA-1a sequester all the maternal HPA-1a antibodies, prior to memory B cells from forming. After injecting this anti-HPA-1a preparation, this will hopefully be used as a prophylaxis post-birth to NAIT affected mothers to decrease the severity of NAIT in subsequent pregnancies (Brojer *et al*., 2016). This treatment would be akin to the established anti-D treatment for haemolytic disease of the fetus and newborn (HFDN).

1.3.3 Pathogenesis of NAIT

The route of sensitisation may not have been elucidated but formation of the antibodies has been established. The maternal immune system recognises the platelet antigens from the fetus as foreign and the maternal antigen presenting cells process and present these peptides to CD4+ T helper cells (Zdravic *et al*., 2016). The helper cells proliferate and produce cytokines which causes B cells to differentiate into plasma cells and produce specific HPA antibodies; the cytokines can promote class switching and somatic hypermutation (Zdravic *et al*., 2016). The maternal HPA antibodies cross the placenta (see Section 1.5) and bind to the fetal platelets. Antibody binding results in opsonisation through the Fc receptor on macrophages and phagocytosis and the platelets are cleared in the reticulo-endothelial system of the spleen (Zdravic *et al*., 2016).

Studies suggests that HPA-1a antibody production is associated with the major histocompatibility complex (MHC) human leucocyte antigen (HLA), DRB3*01:01 allele (Kumpel, 2012; Zdravic *et al*., 2016; Wienzek-Lischka *et al*., 2017). Screening studies showed that 90% of HPA-1a antibody-positive mothers were DRB3*01:01; which is present in 28% of the general population (Kjeldsen-Krogh *et al*., 2007; Tiller *et al*., 2017). The difference between HPA-1a and HPA-1b is the result of a single amino acid substitution; HPA-1a has a leucine at position 33 that is substituted to proline in HPA-1b (Proulx *et al*., 1994). Studies have shown that DRB3*01:01 binds to the leucine at this position but not the proline; this DRB3*01:01/proline combination is presented to CD4+ T cells. The DRB3*01:01 association is not routinely screened for during NAIT referrals in all laboratories; as previous studies have shown that this has a positive predictive value of 35%, with a negative predictive value of 99.6% (Kamphuis & Oepkes, 2011). But newer

studies have shown that if the mother has two copies of the DRB3*01:01 gene, there is a dose dependent effect which causes a low platelet count in the fetus (Kjeldsen-Kragh *et al.,* 2019). Only a small population, around 10%, of HPA-1b1b women make HPA-1a antibodies and it has been reported that these women may be primed through viral or bacterial infections during pregnancy which somehow causes the immune system to make HPA antibodies (Vadasz *et al*., 2015; Tiller *et al*., 2017).

It is not known how ICH occurs in NAIT, though recent research links NAIT severity and ICH to subtle differences in the antibodies produced. Studies have shown that HPA-1a antibodies can bind to the vitronectin receptor ($\alpha v\beta 3$ integrin) on endothelial cells (Santoso *et al*., 2016). $\alpha v\beta 3$ has been shown to have a role in capillary angiogenesis throughout the body including the brain and placenta (Yougbare, Zdravic & Ni, 2018). Therefore, an antibody binding to $\alpha v\beta 3$ would block vitronectin from binding and prevent angiogenesis. This, rather than the low platelet count, may be the cause of the ICH. This hypothesis could explain why newborns with a platelet count over $50x10^9$/L present with ICH (Yougbare *et al*., 2015, de Vos *et al.,* 2020).

Studies have reported that there could be potentially three different antibodies involved in NAIT. Such as one antibody specific to β3, which would react to platelets and the endothelial cells and then antibodies which are specific to compound epitopes on each integrin such as αvβ3 and αIIbβ3, which would either be specific to endothelial cells or platelets (Santoso *et al*., 2016).

Antibodies which react to HPA-1a part of the β3 give rise to two distinct types of antibodies which may cause different biological effects. Type I which is when the antibody binds close to the plexin/semaphoring/integrin domain or Type II which bind close to the epidermal growth factor domain (Porcelign, Huiskes & de Haas, 2020). These differences in antibody binding sites may explain why 'weak' HPA-1a antibodies may result in severe thrombocytopenia, whereas antibodies with a high antibody titre may not result in a severe platelet count. This may be due to plexin/semaphoring/integrin domain antibody binding antibodies being less severe on decreasing fetal platelet counts. Type II antibodies may cause ICH through 'blocking' the function of the αIIbβ3 and its function of binding fibrinogen in the second wave of haemostasis (Sachs, 2020).

An antibody that is specific for αvβ3 could support sensitisation through ST exposure during the pregnancy. These αvβ3 antibodies could explain a few observations that have been reported in NAIT children such as low birth weights; if αvβ3 antibodies bind to the placenta this could impact placental angiogenesis and hence reduce the nutrients supplied to the fetus causing the low birth weight or potentially miscarriages (Yougbare, Zdravic & Ni, 2018).

1.3.4 Treatment

NAIT treatment guidelines have only recently been published which offers guidance for post-natal treatment of newborns and treatment of at-risk pregnancies (Lieberman *et al.,* 2019). If NAIT is diagnosed during the pregnancy the standard treatment is to administer intravenous immunoglobulin (IVIg) which is produced from a pool of blood donors which reduces IgG antibodies in the circulation by unknown mechanisms (Bussel & Primiani, 2008). This treatment is given weekly and there is evidence that this decreases the incidence of ICH and some studies have reported an increase in neonatal platelet count (Esponiza *et al*., 2013; Tiller *et al*., 2017). A second line treatment, in addition to IVIg during pregnancy, is to administer steroids as this aids the actions of IVIg (Tiller *et al.,* 2017). Historically intra-uterine platelet transfusions were given throughout pregnancy, but this increases the risk of intrauterine death and now IVIg is given as standard from 20 weeks or when an ICH has occurred (Espinoza *et al*., 2013).

Following birth, the standard treatment is to transfuse a compatible neonatal platelet unit to the newborn, in some cases this can be with the addition of IVIg (Tiller *et al*., 2017). NAIT normally resolves within 21 days and treatment is withdrawn when the newborns platelet count reaches normal ranges.

1.4. Antibodies in NAIT

1.4.1 Antibodies

The initial immune response produces IgM antibodies, which have high avidity but are not truly specific to antigens (Kumpel & Manoussaka, 2011). Repeat exposure to the antigen produces IgG antibodies. As stated in Section 1.3.3, the processed HPA peptides are

presented to CD4+ T cells which causes antibody production (Zdravic *et al*., 2016). The pregnant endometrium is reported to be a mildly inflammatory site and is therefore primed to initiate an immune response (Kumpel & Manoussaka, 2011).

Criteria	**IgG1**		**IgG2**		**IgG3**		**IgG4**	
Molecular Mass (kD)	146		146		170		146	
Mean audit serum level (g/L)	6.98		3.8		0.51		0.56	
Half-Life (days)	21		21		7/~21[1]		21	
Placental transfer	++++		++		++/++++[1]		+++	
Antibody response to proteins	++		+/-		++		++[2]	
Complement activation C1q binding	++		+		+++		-	
For receptors								
FcγRI	+++[3]	65[4]	-	-	++++	61	+	34
$FcγRIIa_{H131}$	+++	5.2	++	0.45	++++	0.89	++	0.17
$FcγRIIa_{R131}$	+++	3.5	+	0.10	++++	0.91	++	0.21
FcγRIIb/c	+	0.12	-	0.02	++	0.17	+	0.20
$FcγRIIIa_{F158}$	++	21.2	-	0.03	++++	7.7	-	0.20
$FcγRIIIa_{V158}$	+++	2.0	+	0.07	++++	9.8	++	0.25
FcγRIIIb	+++	0.2	-	-	++++	1.1	-	-
FcRn (at pH<6.5)	+++		+++		++/+++[1]		+++	

Table 1.3 Properties of human IgG subclasses. Taken from Vidarsson *et al.,* (2014).

As described in Table 1.3, antibody structure falls into two distinct parts - the Fab portion which is where antigen recognition takes place and the Fc portion, which is where receptors (FcR) on the effector cells bind to initiate the downstream effects (Shroeder & Cavacini, 2010). IgG has four different Fc subclasses, IgG1, IgG2, IgG3 and IgG4, which were named in order of the amounts found in serum. Membrane bound proteins have been shown to induce IgG1 together with some IgG3, IgG2 can be produced, and a mature response is IgG4 (Vidarsson, Deckers & Rispens, 2014). Therefore, the immune response is adaptive as the

[1] Depends on allotype
[2] After repeated encounters with protein antigens ofterallergens
[3] Multivalent binding to transfected celled. Adapted from Bruhns *et al.,* (2008)
[4] Association constant (x 10^6 M^{-1}) for monovalent binding (Bruhns *et al*., 2008)

subclasses change and Fab region which undergoes somatic hypermutation to make the binding more specific with increased affinity (Kumpel & Manoussaka, 2011).

The effector function that results from a bound antibody depends upon the subclass. There are two main effector functions. The first is antibody-dependent cell-mediated cytoxicity (ADCC) which results in engulfment of the antigen by effector cells such as monocytes, macrophages, and natural killer (NK) cells (Shroeder & Cavacini, 2010). The second is the initiation of the complement cascade by C1q binding, which results in cell death. IgG1 and IgG3 are efficient activators of the complement pathway due to the affinity of the FcγR for all three kinds of receptor (I, II and III) (Shroeder & Cavacini, 2010). IgG2 and IgG4 antibody binding leads to the ADCC pathway, though, - IgG2 but not IgG4 can weakly bind C1q.

1.4.2 Antibodies and platelet destruction in NAIT

There have been very limited studies on the different IgG subclasses involved in NAIT and no final conclusions made to whether a certain subclass could be indicated (Sachs, 2020). The main IgG subclasses thought to be involved in NAIT are IgG1 and IgG3, as these are predominantly produced against immunisation from proteins (Kumpel & Manoussaka, 2011). One study indicated that IgG3 is present with IgG1 but could not be correlated to platelet counts (Proulx *et al*. 1994).

Antibody IgG subclass investigations which have been performed in kidney rejection cases, have shown that if the sensitising route is through pregnancy (akin to NAIT) and transplantation, the serum concentration order is IgG1>IgG3>IgG2>IgG4, which supports the concept noted above (Lowe *et al*., 2013). Using sensitive techniques such as surface plasmon resonance (SPR), Khovanova *et al*., (2015) showed that IgG4 was a potential biomarker for antibody mediated rejection and kidney graft loss. Therefore, a study is required to investigate whether the differences in IgG subclasses can be used to predict more severe outcomes in NAIT such as ICH, thereby identifying a potential biomarker to identify the most ‘at risk’ for ICH. These fetuses could then be treated during pregnancy.

Recent studies have investigated antibody post-translational modifications, such as the addition/removal of sugars (glycosylation), which affects FcRn binding (Kapur *et al*., 2014;

Wilcox, Holder & Jones, 2017). During pregnancy, galactosylation and sialylation of IgG antibodies is increased, which may lead to preferential transport across the placenta in comparison to non-modified antibodies (Wilcox, Holder & Jones, 2017). In NAIT, increased glycosylation of IgG1 has been reported accompanied by decreased fucosylation. The latter modification enhances affinity for FcγRIII affinity, thereby promoting platelet phagocytosis once the antibody had passed into the fetal circulation and supporting the theory that platelet destruction is mediated by ADCC (Kapur *et al*., 2014; Zdravic *et al*., 2016).

The use of developing technologies such as SPR has shown that low avidity antibodies can cause NAIT as there are clinical symptoms without detectable antibodies (Curtis, 2015). Low avidity antibodies can give a false negative result by standard techniques (see Section 1.7.2) (Delbos *et al*., 2016). However, studies have shown that sera found to be HPA-1a antibody-negative by monoclonal antibody immobilisation of platelet glycoprotein assay (MAIPA) can be positive if analysed by SPR. SPR measures antibody association with the antigen, even if this is quickly disassociated from the antigen, which is not detected by MAIPA (Curtis, 2015). This technology is not routinely used for HPA antibody detection and is used as a research tool because of the complexity of the methodology.

1.5. Antibody transfer across the placenta

1.5.1 Introduction to the placenta

The placenta is formed from fetal cells and its primary role is to provide nutrients to the fetus, enabling development (Sadovsky & Jansson, 2015). This is a temporary organ which is only present during pregnancy and is a histological barrier separating the fetal and maternal circulation (Wilcox, Holder & Jones, 2017). Following egg fertilisation, a ball of cells is formed called the blastocyst, the outer cells are trophoderm cells; these cells come into contact with maternal cells lining the uterus to initiate implantation and placental development, which eventually consists of functional units called chorionic villi comprised of an epithelial bi-layer formed of the synciotiotrophoblast (ST – mentioned in Section 1.3.2) which is the outer-most layer of the placenta (Kumpel & Manoussaka, 2011; Sadovsky & Jansson, 2015) and the underlying cytotrophoblasts (CT) surrounding a core made up of stromal cells and extra-cellular matrix (ECM) and placental blood vessels (Figure 1.3) (Kumpel & Manoussaka, 2011; Sadovsky & Jansson, 2015).

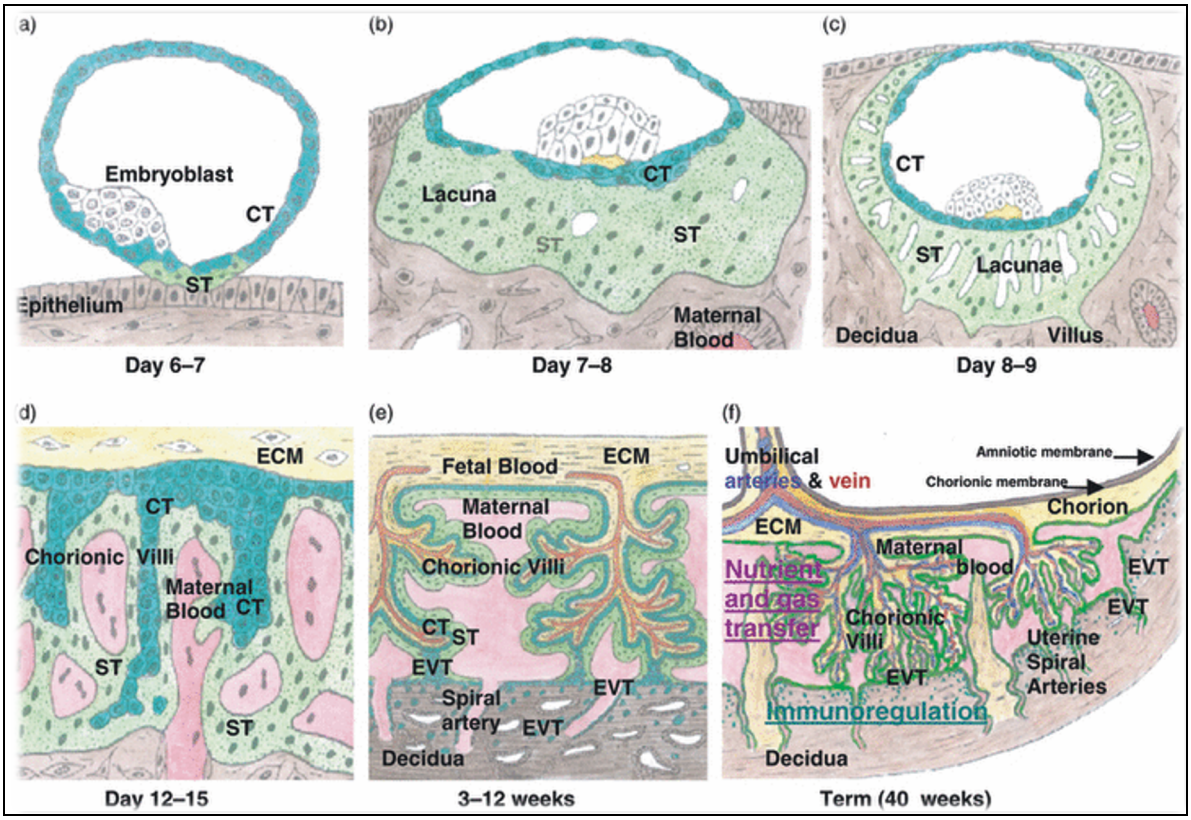

Figure 1.3. Schematic representation of placenta development. Taken from Kumpel & Manoussaka (2011) shows how the development of the placenta from the embryoblast (a) embryoblast stage which is within days of fertilisation to a mature chorionic villus at 40 weeks' gestation(f). The chorionic villus which are main units of the placenta start forming after day 12 (d) and by around a month's gestation (e) fetal circulation forms and starts transferring from the villus space from the mother's system (Kumpel & Manoussaka (2011).

Figure 1.3 shows that maternal blood can circulate through the intervillous space and come in direct contact with the STs (Sadovsky & Jansson, 2015; Wilcox, Holder & Jones, 2017). The interface between ST cells and the maternal circulation is essential for the fetus to receive the nutrients required for growth and development (Kane & Acquah, 2009). The majority of nutrient transfer across the placenta is through diffusion along chemical gradients; some transfer is dependent upon transport proteins such as channels and carrier-mediated active transport, and by endocytosis/exocytosis (Kane & Acquah, 2009).

1.5.2 Transfer of antibodies across the placenta

The transfer of maternal antibodies across the placenta is essential in providing immunity to the fetus/newborn in early life (Wilcox, Holder & Jones, 2017). Women are actively immunised during pregnancy to pass on specific immunity to certain diseases which will protect the newborn (Wilcox, Holder & Jones, 2017). In some circumstances, such as NAIT, the maternal antibodies transferred across the placenta are detrimental to the fetus/newborn, but how do antibodies cross the placenta?

IgG is the only class of antibody to be transported across the placenta. The transmission is mediated by the FcRn which is expressed on the ST of the placenta (Chucri *et al*., 2010). Models have shown that circulating IgG is taken up through endocytosis to bind with membrane bound FcRn within an endosome in the ST (Simister, 2003). Binding of IgG to the FcRn is dependent on an acidic pH environment, and the low pH in the transcytotic vesicle prevents the antibody degradation (Wilcox, Holder & Jones, 2017). The endosome is transported to the inner membrane and IgG released into the villous stroma; this area has a neutral pH which causes the dissociation of the IgG and the FcRn and the IgG is released into the fetal circulation (Kane & Acquah, 2009; Wilcox, Holder & Jones, 2017). The FcRn is homologous to HLA-Class I and is associated with β2-microglobulin; two FcRn molecules bind one IgG molecule (Kane & Acquah, 2009).

Placental antibody transmission has been studied using cordocentesis techniques and comparing the antibody levels found in the cord and the maternal circulation (Simister, 2003; Wilcox, Holder & Jones, 2017). IgG transmission commences around 12-13 weeks' gestation, and the concentration increases throughout pregnancy (Simister, 2003; Wilcox, Holder & Jones, 2017). In the second trimester there is an exponential increase in total IgG with gestational age (van den Berg *et al*., 2011). The reason is unclear, but this could be explained by more FcRn becoming available. By the third trimester the total amount of IgG can be twice the concentration registered in the second trimester (van den Berg *et al*., 2011).

The different IgG subclasses mentioned in Section 1.4.1 have been shown to be transported across the placenta with decreased affinity of IgG1> IgG4> IgG3 and IgG2 (Sadovsky & Jansson, 2015; Wilcox, Holder & Jones, 2017). Different IgG subclasses also differ in their maternal levels during gestation and the amount found in the fetal system. During gestation the individual IgG subclasses increase at different rates; IgG1 is three times the level of IgG2 by 32 weeks, whereas IgG4 levels in the newborn are higher than in the maternal system (Kane & Acquah, 2009).

1.5.3 Antibody transfer in NAIT

IgG1 is reported to be the predominant antibody found in NAIT in combination with IgG3 (Kumpel & Manoussaka, 2011; Brojer *et al*., 2016). As IgG1 has the highest affinity to FcRn and therefore most likely to be transferred across the placenta this would support the suggestion that this subclass is involved in NAIT; IgG3 is passed less efficiently through the placenta (Einarsdottir *et al*., 2014).

As stated previously (Section 1.4.2) the addition of sugars such as galactose and the reduction of fructose to the antibodies increases the affinity to FcRn and therefore placental transport. These antibodies have been shown to be important in causing ICH in NAIT (Kapur *et al*., 2014). Specific glycans in IgG3 can increase its affinity to FcRn and enable this subclass to pass efficiently through the placenta (Einarsdottir *et al*., 2014). One key site for FcRn binding is at histidine at position 435 (H435) in IgG which is present in IgG1, IgG2 and IgG4. The majority of IgG3 antibodies have arginine at 435; a subclass called G3m has the H435 and can efficiently cross the placenta (Einarsdottir *et al*., 2014). This could explain why IgG3 has been implicated in NAIT in addition to IgG1.

The IgG HPA-1a antibodies in NAIT, cross the placenta into the fetus and can cause ICH. The timing of ICH occurring in the fetus varies; there have been case studies in which ICH has been reported in the first trimester (Giovgrandi *et al*., 1990). One study found that the majority of ICH occurred by the end of the second trimester, when fetal IgG antibody levels can be 50% of those of the maternal system (van den Berg *et al*., 2011; Tiller *et al*., 2013). Other studies have stated that ICH occurs within the third trimester (Spencer & Burrows, 2001; Brojer *et al*., 2016). Tiller *et al*., (2013) concluded that there is a difference reported in the timing of ICH, but this could be due to the numbers of cases included in the studies. A recent systematic review looking at maternal HPA-1a antibody levels did not include levels and ICH (Kjaer *et al*., 2019). In this systematic review, they found only two studies which looked at antibody titre and ICH, but they concluded levels could not be used to predict severity (Sachs, 2020).

1.6. Predicting NAIT Severity

1.6.1 Introduction

For NAIT, the ideal scenario would be to develop an accurate laboratory test which can predict if a fetus or newborn is at risk of ICH and death. Studies have previously tried to link antibody titre to platelet count at birth as an indicator of severity and the risk of ICH. Investigators have focussed on antibody concentration and glycosylation and, to a more limited extent, the different IgG subclasses (Sachs, 2020). So far, no consensus on how to predict NAIT severity has been reached. The only reliable predictor is that if a previous pregnancy has been affected, then subsequent affected pregnancies will be more severe if left untreated throughout gestation (Espinoza *et al*., 2013). The risk of having NAIT in a second pregnancy is 100% (based on the paternal HPA genotype) and the risk of a second pregnancy having ICH is 79%, if the pregnancy is untreated (Tiller *et al*., 2017; Winkelhorst *et al*., 2017). The major problem that has been highlighted is that low HPA antibody titres can cause severe NAIT, and high antibody titres may not cause NAIT, which relates to the avidity of the antibody (Peterson *et al*., 2013). Therefore, obstetric history is still the most robust method of predicting NAIT severity (Constantinescu, Zamfirecu & Vladareanu, 2012). There is also no correlation between platelet counts and the occurrence of ICH, which is why αvβ3 antibodies are now thought to cause ICH (de Vos *et al*., 2020).

1.6.2 Antibody concentrations

There have been numerous studies analysing maternal IgG antibody titre throughout gestation and at birth to use this to try and predict NAIT severity (Proulx *et al*., 1994; Jaegtvik *et al*., 2000; Ghevaert *et al*., 2007; Bessos *et al*., 2008a; Killie *et al*., 2008; Bertrand *et al*., 2011). The majority analyse HPA-1a antibody concentration as the predominant cause of NAIT. There are reports that antibody to HPA-5b does not cause as severe form of NAIT due to the low number of copies on the platelets (Porcelign, Huiskes & Haas, 2020).

The published data is confusing and conflicting, including prospective studies which link antibody titre to severity as well as retrospective ones that are unable to make this link (Proulx *et al*., 1994; Jaegtvik *et al*., 2000; Ghevaert *et al*., 2007; Bessos *et al*., 2008a; Killie *et al*., 2008; Bertrand *et al*., 2011). The studies use different methodologies, including

commercial Enzyme Linked Immunosorbent Assay (ELISA) and the well-publicised MAIPA assay, and conflicting results are apparent in a direct comparison (Bessos *et al*., 2008a). Killie *et al*., (2008) reported that samples taken at gestational weeks 22 and 34 were associated with severe NAIT if the antibody titre was over 3.0IU/ml. Whilst Bertrand *et al*., (2011) found that an antibody titre of 28IU/ml prior to 28 weeks' gestation correlated with severe NAIT. Jaegtvik *et al*. (2000) reported HPA-1a antibody titres over 7.4IU/ml were required for severe NAIT to occur. These reports are generally from first pregnancies but there is uncertainty as to whether miscarriages/abortions had been discounted. In women who have had multiple NAIT newborns, antibody titres decrease with successive pregnancies and so titre measurement may not be reliable (Killie *et al*., 2008).

Other studies using ELISA did not find an association between maternal antibody concentrations and NAIT, which may be due to the ELISA technique not being as sensitive as MAIPA (Proulx *et al*., 1994, Bessos *et al*., 2008a). Ghevaert *et al*., (2007) found in a retrospective study with 133 samples using MAIPA that there was no link to antibody concentration and NAIT severity.

A recent systematic review by Kjaer *et al.,* (2019) found that antibody levels should be tested by quantitative MAIPA to allow for comparisons. In this review they found four prospective and eight retrospective studies. All studies measured concentration at different times but more of the retrospective studies assessed samples taken at birth. Antibody titre cut-offs were different and the platelet count for classifying severe NAIT was different with a range of 20-50x10^9/L (Kjaer *et al.,* 2019). This is an example of why the literature is confusing and why antibody titre is not included in risk stratification in many countries.

There are instances where antibodies cannot be detected during pregnancy but is detectable post-natally. This may be due to the fact that during pregnancy, the antibodies may be low avidity or bound to the placenta or is flowing into the fetal circulation and is therefore below the level of detection in maternal blood.

In summary, there is no threshold antibody titre that can reliably predict NAIT severity. The differences in the antibody titres reported are probably due to study size, i.e., small studies

may show a different significant antibody titre than larger studies which include more variance; some are prospective screening studies which have analysed thousands of samples (Killie *et al*., 2008), whilst others include data from less than hundred samples (Bertrand *et al*., 2011).

1.6.3 Antibody subclasses

There have been limited studies to assess if the level of a certain IgG subclass can predict NAIT severity. Mawas *et al*., (1997) found that they could not predict NAIT with total IgG or with IgG1, IgG2 and IgG4. They did show a significant association between NAIT severity and IgG3 (Mawas *et al*., 1997), but this has not been investigated further (Sachs, 2020). Proulx *et al*., (1994) did not show any association between the different IgG subclasses and NAIT severity, as they did not link to clinical data (Sachs, 2020). Newer studies have not analysed whether the relative levels of antibody subclasses change over gestation or through subsequent pregnancies (Sachs, 2020). Recent studies have only analysed IgG1 in attempts to detect low avidity antibodies which might cause ICH, but this has not been investigated with other subclasses (Curtis, 2015; Delbos *et al*., 2016).

1.7 Current methodologies within the laboratory to support HPA antibody detection

The standard laboratory tests used to identify NAIT are HPA genotyping and antibody screening/identification. Each uses a range of in-house techniques and commercially available kits (Sachs, 2013).

1.7.1 Genotyping

HPA genotyping of the mother, father and fetus/newborn is required to identify if there is a genetic HPA difference between the maternal and paternal immune systems. Platelet immunology laboratories investigating potential NAIT cases will generally use a polymerase chain reaction (PCR) technique to test for at least HPA-1, 2, 3, 4, 5 and 15 incompatibilities (Sachs, 2013). The NHS Blood and Transplant (NHS BT) approach is to perform sequence-based typing as this aids in identifying novel mutations. The paternal genotype is used to assess risks for subsequent pregnancies. New techniques have recently been developed to

use free fetal DNA which is found in the maternal blood and the fetus is genotyped to determine genotype (Mammasse *et al*., 2020).

1.7.2 Antibody Detection

Three antibody detection methods are utilised for diagnosing an alloimmune platelet antibody.

1.7.2.1 Monoclonal antibody immobilisation of platelet glycoprotein assay (MAIPA)

Kiefel *et al*., (1987) first described the MAIPA. According to Zdravic *et al*., (2016) and Tiller *et al*., (2017), this is the standard test for identifying platelet-specific glycoprotein antibodies. The MAIPA (Figure 1.4) is an 'in-house' enzyme-linked immunosorbent assay (ELISA). MAIPA is time-consuming as it can take up to two days to perform, though with optimisation it can be reduced to one day (Campbell *et al*., 2007). MAIPA has the advantage of being reliable, sensitive, and quantitative (if required) and can use different monoclonal antibodies to increase detection by binding to different sites, in case the original monoclonal was 'blocking' the maternal antibody from binding and being detected (Campbell *et al*., 2007). This test can be used to perform a cross-match using paternal platelets and maternal serum; binding of antibodies in the maternal sera to the paternal platelets is indicative of NAIT. This is a definitive prognosis of NAIT and can be used if a private epitope is suspected. A private epitope arises when the has been a change the sequence which gives rise to a new amino acid and thus an epitope, HPA-1a or HPA-1b is an example; these epitopes can be found anywhere on the glycoprotein. False negative results are obtained if the monoclonal antibodies used for detection are 'blocking' maternal antibodies from binding to the specific GPs (Socher *et al*., 2009; Porcelijn *et al*., 2014). However, it requires multiple wash steps and low antibodies (weak binding) can be washed away which can give a false negative result (Zdravic *et al*., 2016).

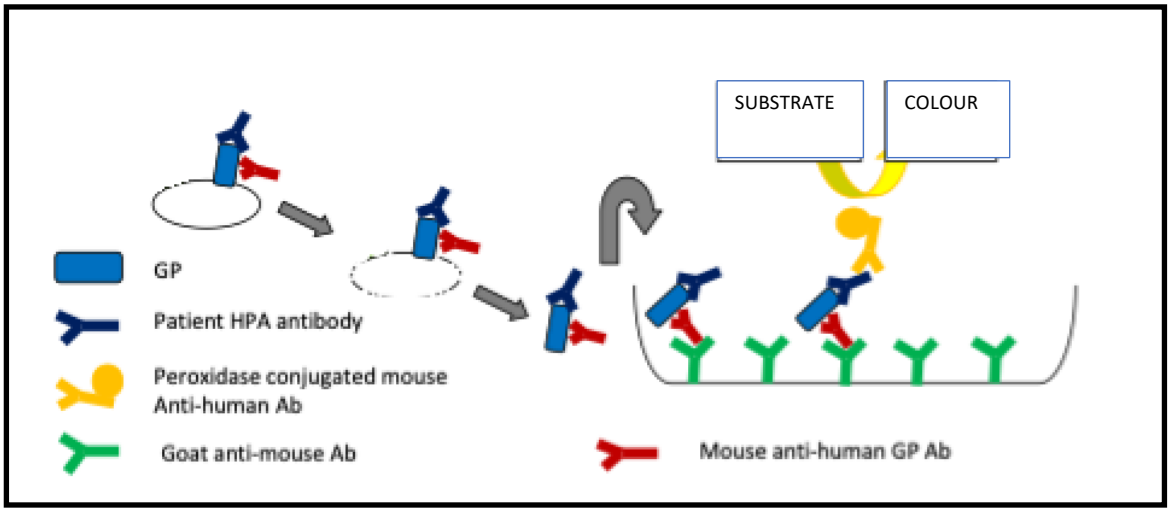

Figure 1.4. Schematic of the MAIPA assay and the steps involved. Known HPA typed panel cells are incubated with patient serum. If an antibody to the GP (glycoprotein) is present in the patient serum, it will bind. After this first incubation a mouse anti-human GP antibody is also added, which binds to the GP at a different position from the patient antibody. The platelet is solubilised, so fragments are left of bound GP-patient HPA antibody and Mouse anti-human GP antibody. The mix is added to goat anti-mouse antibody coated wells. This binds the fragment and with the addition of peroxidise conjugated mouse anti-human antibody and a substrate which cause a colour change if antibody present. This is read at a specific optical density. The commercial ELISA does not go through the solubilisation step and the well contains GPs attached for the antibody to bind but is a similar assay at the well stage. Based on Kiefel *et al*., (1987).

1.7.2.2 Platelet immunofluorescence test (PIFT)

This is a screening assay using fresh frozen platelets with known HPA genotypes. Patient sera are incubated with the platelets followed by the addition of anti-human red phycoerythrin (RPE) conjugated secondary antibody and analysed on a flow cytometer. This has the advantage of using whole cells expressing the platelet antigen system and can sometimes detect antibodies not found in the MAIPA, such as HPA-3a antibodies (Porcelijn, Huiskes & Haas, 2020). The disadvantage of this assay is that it detects HLA antibodies as well, if cognate to the antigens expressed on the platelets, which would hinder HPA antibody detection.

1.7.2.3 Bead-based assay

Bead-based assays, such as those employed by the Luminex™ technology, have been used within the transplant field for the last two decades (Lachmann *et al*., 2013). Solubilised HPA molecules are attached to a microbead that has a unique combination of fluorescent dyes which are identified upon passing through the Luminex™ analyser (Figure 1.5). This technology has revolutionised Human Leucocyte Antigen (HLA) antibody identification

assays in solid organ transplant. HLA antibody screening has proved to be more sensitive than immunoassay methodology (Lachmann *et al*., 2013).

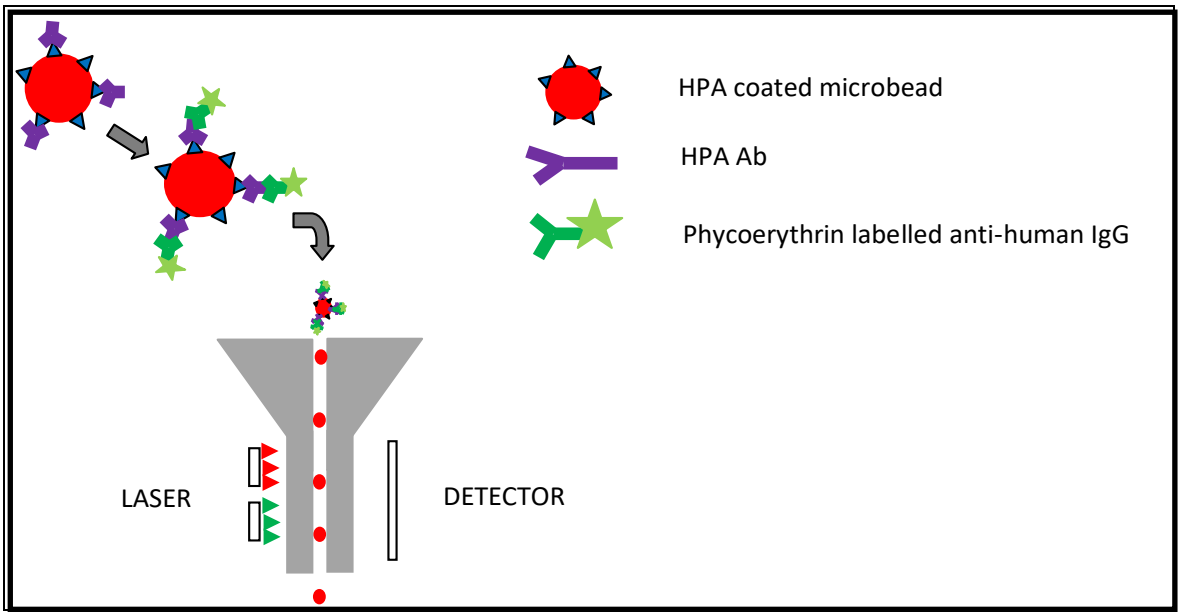

Figure 1.5. Schematic representation of the Luminex™ bead-based assay. Inside the microbead there is a unique dye blend that allows it to be identified when passed through the laser. If a patient has HPA antibodies present in the serum this will bind to the HPA molecule on the microbead. The antibody binding can be detected by a phycoerythrin labelled anti-human IgG. One laser identifies the bead, and the other laser detects bound antibody.

Porcelijn *et al*., (2014) studied a commercial HPA bead-based assay in comparison with the MAIPA. The results are promising but there have been instances where the results have not been concordant with the Luminex™ assay as this method has not detected multiple HPA specificities resulting from transfusion reactions (Porcelijn *et al*., 2014). Other laboratories around the world are developing their own bead-based assay to detect HLA/HPA antibodies, which are superior to MAIPA in detecting some HPA antibodies (Chong *et al*., 2010; Metzner *et al.*, 2017; Tao *et al.*, 2019). The advantages are that it is not a complex process, takes only three hours, and a small amount of serum/plasma is used in comparison to the MAIPA. This assay has been shown to detect HPA-1a antibodies in NAIT cases which have been negative by MAIPA (Stanton *et al*., 2018).

This assay is ideal for modifying to be able to fully quantify the HPA-1a antibody concentration and to assess each of the IgG subclasses. This would replicate the study by Lowe *et al*., (2013) (Section 1.4.2) by using the same principles of HLA IgG subclass detection applied to the HPA-1a antibody scenario. This methodology has the potential to

use ‘in-house’ technology which has previously been published for HPA-1a detection (Chong *et al*., 2010) with a cost benefit over commercial kits.

1.8. Research areas identified

Santoso *et al*., (2016) proposed that antibodies to the αvβ3 heterodimer cause ICH and other complications such as low birth weight, but only a limited number of papers support this proposal (Yougbare *et al*., 2015). Therefore, studies to investigate whether the effects of these antibodies can be distinguished from those recognising the αIIbβ3 heterodimer are required. Testing for antibodies which are specific for αvβ3 heterodimer would be a valuable diagnostic tool. Especially as the literature indicates that αvβ3 antibodies in maternal serum could indicate that the fetus or newborn is at risk from ICH (Santoso *et al*., 2016).

As discussed in Section 1.6.2 no antibody concentration is able to reliably predict NAIT severity. Studies proposing various different antibody concentrations that can predict NAIT severity are over five years old and have not measured antibody concentration using newer technologies such as Luminex™. The published results vary depending on methodology, sample size and prospective versus retrospective design.

There are limited studies which have investigated IgG subclass, both over twenty years ago (Poulx *et al*., 1994; Mawas *et al*., 1997). Even though much of the literature states that IgG1 and IgG3 are important in NAIT, there has not been any clear published research comparing these IgG subclasses. Furthermore, studies on post-translocation modifications by sugars have focussed only on antibody subclass IgG1 only (Kapur *et al*., 2014). These studies have not been performed on new technologies such as Luminex™.

Consequently, information on antibody concentration, subclass and heterodimers is required for improving knowledge and understanding of pathogenic antibodies in NAIT.

1.9 Aims and Objectives

Hypothesis 1: The presence of αvβ3 antibodies in NAIT maternal samples are linked to ICH in the fetus or newborn.

The initial aim will be to establish methodology to detect αvβ3 antibodies using flow cytometry. This will be divided into 3 objectives.

Objective 1: Clone αv and combine with β3 to form a αvβ3 integrin that can be expressed on HEK293 cells.

Objective 2: Establish an absorption method to remove αIIbβ3 antibodies from samples.

Objective 3: Test and compare the results from the flow cytometry investigations between the ICH and non-ICH cohort.

Hypothesis 2: A high HPA-1a IgG titre causes severe NAIT.

The second aim is to establish an antibody titre that can be used to predict NAIT severity.

Objective 4: To test if 'prozone' is indicated in ICH samples.

Objective 5: To identify the most suitable range of dilutions that could be used to identify HPA-1a antibody titrations.

Hypothesis 3: That there is a difference in the IgG subclasses during gestation that can be used to predict NAIT severity.

The third aim is to validate a method that can detect all the IgG subclasses for HPA-1a antibodies.

Objective 6: To test four different cohorts who have previously tested positive for HPA-1a antibodies and analyse if there are differences in IgG subclass.

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Chapter 2: Materials & Methods

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2.1 Test Sera

Database searches were performed using the laboratory information management system for samples which were HPA-1a positive by MAIPA and were part of a NAIT investigation. These samples were cross-referenced with a 'NAIT' database to inform whether the fetus or newborn had suffered from ICH. Two cohorts were selected one with ICH and the other with non-ICH. The searches were performed between 2010-2018. Sera chosen for flow cytometer and absorptions experiments were selected from the above cases on sample volume.

All samples from both cohorts were tested using the recombinant HPA-1a Luminex™ assay (Section 2.17). For the Luminex™ assay the cohorts were extended to include post-transfusion purpura and platelet refractory patients who had been referred to the laboratory between 2010-2018 and possessed an HPA-1a antibody detected by MAIPA.

2.2 DNA constructs

A cDNA clone encoding αv (NCBI ref sequence NM_002210.4) was purchased from Sino Biological (Philadelphia, USA) and reconstituted as instructed. This cDNA was used to transform XL1-Blue competent cells (Agilent, California, USA) as per manufacturer's instructions. Briefly, cells were thawed on ice and incubated with the αv clone and a DNA control vector on ice for 30 minutes. Tubes were heat pulsed for 45 seconds at 42^{o}C and put back on ice for two minutes; 225µl of recovery medium (Lucigen, Wisconsin USA) was added to each tube and incubated on a shaker at 37^{o}C for one hour. The cells were plated onto Luria-Bertani (LB; Sigma, Poole UK) ampicillin agar plates at varying volumes and incubated overnight at 37^{o}C. Single clones were picked from the plates and added to 5ml LB ampicillin liquid culture media and incubated overnight at 37^{o}C on a shaker.

Stocks of cDNA were made from the bacteria using Wizard® *Plus* SV Minipreps DNA purification system kit (Promega, Wisconsin USA). Briefly, the cells were pelleted by centrifugation at 10,000x g for five minutes and the supernatant discarded. The cells were resuspended in 250µl of resuspension solution, vortexed and transferred to a 1.5ml microcentrifuge tube, 250µl of cell lysis solution was added, mixed by inversion followed by 10µl of alkaline phosphatase and incubated for five minutes at room temperature.

Neutralisation solution (350 µl) was added prior to centrifugation at 14,000x g for 10 minutes. The supernatant was decanted into a spin column and centrifuged for one minute. The fall through was discarded prior to adding 750µl of wash solution and centrifuging again for one minute. The spin column was washed again using 250µL of wash solution and centrifuged for two minutes. The spin column was transferred to a clean microcentrifuge tube and the DNA eluted using nuclease free water. The DNA concentration was measured by absorbance at A260 on a DeNovix DS-11+ Spectrophotometer (Delaware USA)

2.3 Addition of EcoR1/BamH1 restriction sites

Primers were designed around the αv open reading frames, which incorporated EcoR1 and BamH1 restriction sites (Table 2.1) and amplified using the polymerase chain reaction (PCR) reaction using sequence specific primers (Sigma-Aldrich, Darmstadt, Germany) with Phusion HF Taq (ThermoFisher, Massachusetts, USA) and dNTPs (Sigma, Poole, UK). Reactions were set up as detailed in Table 2.2 and thermal cycler (Nexus Eppondorf) conditions detailed in Table 2.3.

Primers	**Sequence**
5’	ATA TGAATTCATGGCTTTTCCGCCGCGG
3’	ATAGGATCCAGTTTCTGAAGTTTCCTTCACC

Table 2.1. The sequences of the 5’ and 3’ primers used to insert the restriction enzyme sites of EcoR1 (yellow) and BamH1 (green) sites.

Reagent	**Amount added in µL (a)**	**Amount added in µL (b)**
dH2O	28	26.5
DNA (50ng/µL)	5	5
5xPhusion buffer	10.0	10.0
10µM 5’ primer	2.5	2.5
10µM 3’ primer	2.5	2.5
10mM dNTPs	1.0	1.0
Phusion HF Taq	0.5	0.5
DMSO (100%)	N/A	1.5

Table 2.2. Reagents and amounts used for each PCR reaction. Columns ‘a’ and ‘b’ are the volumes used with and without the addition of DMSO to aid the denaturation of high GC rich nucleotide sequence.

Due to the high GC base content of the product, dimethyl sulfoxide (DMSO; Sigma, Poole UK) was added to a final concentration of 3% to aid with denaturation. The PCR annealing temperature was set at both 58°C and 61°C with an elongation time of one minute 45 seconds due to the expected size of the product (Table 2.3).

Temperature (°C)	**Time**	**Temperature (°C)**	**Time**
98	30 sec	98	30 sec
98 58 72	10 sec 30 sec 30 cycles 1min 45 sec	98 61 72	10 sec 30 sec 30 cycles 1min 45 sec
72	10 min	72	10 min
4	Infinity	4	infinity

Table 2.3. Thermal cycler conditions with the differing annealing temperatures.

2.4 Visualisation of PCR amplified DNA

PCR products were electrophoresed on a 1% agarose gel (VWR, Pennsylvania USA) at 200V for 30 minutes (Bio-Rad, California USA). The gel and the running buffer were prepared using Tris-acetate-EDTA (Sigma, Poole UK) and DNA loaded using loading buffer (New England BioLabs, Massachusetts USA) and stained with SybrSafe (Invitrogen, California USA). The PCR products were visualised on a BioRad Gel Doc EZ Imager (California USA).

2.5 DNA Isolation from Gel Electrophoresis

DNA was isolated from the agarose gel by excising the expected size product (~3Kb) from the gel under UV light. The DNA was extracted using Wizard®*SV* gel and PCR clean-up system kit as per manufacturer's instructions (Promega, Wisconsin USA). Briefly, 10µL membrane binding solution was added to each 10mg of excised gel and incubated at 55°C (one to five minutes) and vortexed until the gel dissolved. The mix was transferred to a micro-column and incubated at room temperature for one minute. The column was centrifuged at 16,000x g for one minute and the flow through discarded. The column was washed with 700µL of membrane wash solution, centrifuged and followed by a second wash of 500µL membrane wash solution. After a five-minute centrifugation step, the DNA was eluted with 30µL of nuclease-free water.

2.6 Digestions and ligation of insert into vector

Double restriction enzyme digests were set up for the αv PCR product with added EcoR1 and BamH1 sites and the vector pEGFP-N3 (Invitrogen, California USA). One microgram of both the insert and the vector were digested with 20 units each of EcoR1-HF and BamH1-HF, 5µL of 10x CutSmart® 10x restriction enzyme buffer (New England BioLabs, Massachusetts USA) and made up to 50µL with dH_2O. Control reactions were set up for each restriction enzyme with the vector as a separate reaction. The reactions were incubated for one hour at 37°C, the enzymes were denatured at 65°C for five minutes, placed on ice and 10µL of DNA loading buffer was added to each tube. Gel electrophoresis was performed as in Section 2.4. The αv and the pEGFP-N3 digested products were cut from the gel and the DNA extracted as per Section 2.2.

The NEBioCalulator (https://nebiocalculator.neb.com/#!/ligation) was used to calculate the ratio of 3:1 insert DNA and vector DNA and amounts of DNA to be mixed. For the ligation reaction, 4µL each of insert (100ng) and vector DNA (50ng) was pipetted into a reaction mix containing 2µL of ligase buffer, 1µL of T4 DNA ligase (New England BioLabs, Massachusetts USA) and dH_2O to a final volume of 20µL. This mixture was placed in a thermal cycler for six hours at 16.0°C followed by 10 minutes at 65.0°C to inactivate the ligase. The products from this reaction were transformed into XL1-Blue competent cells as per procedure in Section 2.2 but was grown on Kanamycin (50µg/mL) LB plates. Colonies were picked for Minipreps as described previously (Section 2.2). Restriction enzyme digestion was performed on the Minipreps DNA and visualised on an agarose gel. Clones which exhibited two bands on the gel, suggesting that the insert was present, were sent for confirmatory sequencing. Figure 2.1 shows the steps taken in the process of ligating αv into the pEGFP-N3 vector.

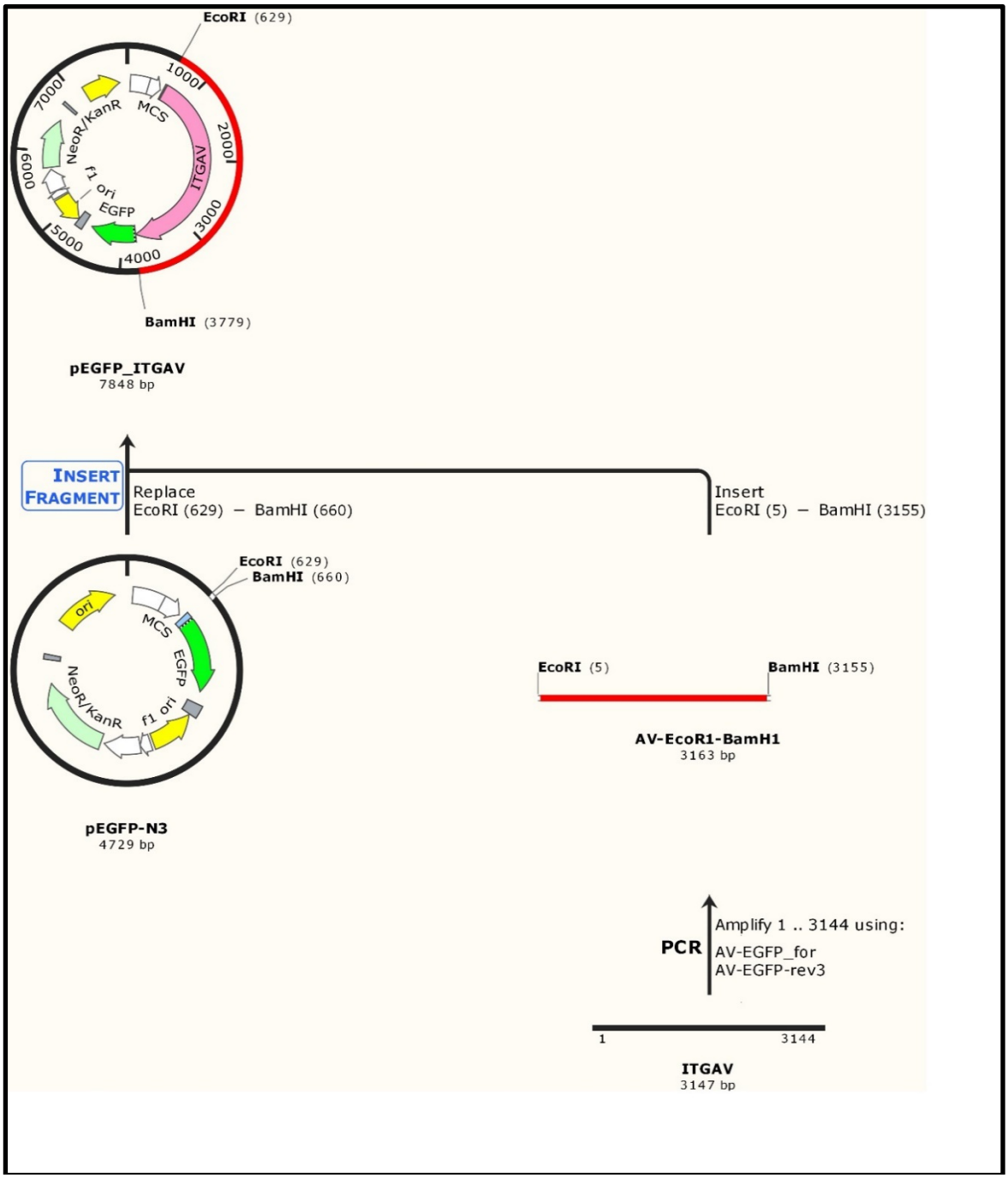

Figure 2.1. The steps taken in the cloning process starting with the αv (ITGAV) clone and the first PCR reaction adding the restriction sites of EcoR1 and BamH1. The digestion of αv and pEGFP-N3 with the restriction enzymes and the ligation together forms a plasmid which incorporates the DNA of interest.

2.7 Confirmatory Sequencing

Sequencing was performed by a third-party department on a 3500xl genetic analyser (ThermoFisher, Massachusetts USA), using Big Dye Terminator v3.1 chemistry (ThermoFisher, Massachusetts USA). Seven sequencing reactions were used with each of the primers listed in Table 2.4. Sequences were analysed using SnapGene software

(SnapGene® from GSL Biotech; available at snapgene.com) and checked against the αv sequencing information supplied by Sino Biological.

Primer name	Sequence 5'-3'
EGFP SeqFor	GGTAGGCGTGTACGGTGGGAGG
AV-EGFP-SeqF1	CATTGTACCATTGGAGAACTG
AV-EGFP-SeqF2	GACATTAATGGAGATGATTATGC
AV-EGFP-SeqF3	CACTGCCTGGAACAGCTCTC
AV-EGFP-SeqF4	GGTGCCTACGAAGCTGAGC
AV-EGFP-SeqF5	TGTATATCCTTCATTATGATATTG
EGFP SeqRev	GGACCAGCTCGACCTGCCGCTGC

Table 2.4. Primer information used to confirm the αv sequence.

2.8 Site directed mutagenesis of β3 to make HPA-1b version

QuikChange Lightning kit (Agilent, California, USA) was used to perform a mutagenesis reaction that changed nucleotide 176 (from the initiator methionine) from a T>C using the primer below; red indicating the relevant nucleotide change:

5' ATGAGGCCCTGCCTCCGGGCTCACCTCGCTG 3'

The manufacturer's instructions were followed for the site directed mutagenesis and all components were included (except primer) in the kit. Table 2.5 details the reaction constituents and Table 2.6 details the thermal cycler conditions. Following the mutagenesis reaction, 1µL of DpnI was added to the reaction to digest the original DNA template and incubated at 37°C for 5 minutes.

Reagent	Amount added in µL
dH_2O	18
DNA (100ng/µl)	1
QuikChange reaction buffer	2.5
QuiK solution	0.5
Primer 10µM	1
dNTPs	1
QuikChange enzyme blend	1

Table 2.5. The constituents of the mutagenesis reaction to change the nucleotide base of the original product expressing HPA-1a to HPA-1b.

Temperature (°C)	Time
95	2 min
95 55 65	20 sec 30 sec 30 cycles 3 min 54 sec
65	5 min
4	Infinity

Table 2.6. The cycling conditions for the site directed mutagenesis reaction. The elongation time of 3 min 54 sec is calculated as 30sec per kb of the plasmid.

From the mutagenesis reaction, 1.5µL of DNA was taken and transformed into XL1-Blue competent cells and plated on LB plates as previously described. Overnight colonies were picked for DNA Minipreps as described in Section 2.2 and was sent for confirmatory sequencing. Confirmed HPA-1b DNA was transformed into XL1-Blue competent cells for DNA Midipreps to ensure enough DNA was available for transfection experiments (Section 2.10). This Section was kindly performed by the scientists at PDPU when initial attempts failed.

2.9 DNA extraction using QIAfilter plasmid midi and maxi kits

DNA was extracted from transformed cells grown overnight in LB liquid media using the QIAfilter plasmid midi kit (Qiagen, Hilden Germany) according to manufacturer's instructions. Briefly, the harvested culture was centrifuged at 4,000x g for 20 minutes at 4°C, the pellet was re-suspended in 4ml of resuspending buffer, followed by 4ml of cell lysis buffer, inverted 6 times prior to incubating for five minutes at room temperature. Four millilitres of chilled neutralising buffer were added and inverted six times. The lysate is poured into the QIAfilter cartridge and incubated at room temperature for 10 minutes during which equilibration buffer is added to a Qiagen tip and allowed to empty. The plunger is fitted to the barrel of the QIAfilter cartridge, and the lysate transferred into the Qiagen tip. The tip is then washed with wash buffer and the DNA eluted with 5ml of elution buffer. The DNA is precipitated by adding 3.5ml of isopropanol and centrifuging at 17,000x g for 30 minutes at 4°C. The supernatant is discarded and 2ml of 70% ethanol is added and centrifuged at 17,000x g for 10 minutes. The DNA is dissolved in TE buffer for use in transfection experiments.

2.10 Cell culture and transfection

HEK293WT (Health Protection Agency, Salisbury UK) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, Poole UK) supplemented with L-glycine (2mmol/L (Sigma, Poole UK) and 10% (vol/vol) fetal calf serum (FCS; HyClone, ThermoFisher Scientific, Massachusetts USA). For transfection experiments, the cells were gently washed with Dulbecco's PBS (Sigma, Poole UK) prior to the addition of 3ml Trypsin-ethylenediaminetetraacetic acid (EDTA; Sigma Poole UK) and incubated at 37^{o}C for two minutes. The trypsin-EDTA was neutralised with culture medium and cells pipetted into sterile tubes and centrifuged at 168x g for three minutes. The supernatant was discarded, and pellet was resuspended in 10ml of medium. The cells were counted using Nucleocounter NC-200 and Vial 1-cassette (Chemometec, Allerod Denmark) and adjusted to 6x10^{6} cells/ml. One millilitre of this suspension was added to 15ml of media for each 55cm^{2} tissue culture petri dish prepared for each planned transfection. Each transfection was set up in duplicate and the cells incubated at 37^{o}C in a CO_2 (%) incubator for 24 hours to allow the cells to obtain >80% confluency.

Transfections were performed using the transfection reagent TransIT-2020 (Mirus Bio, Wisconsin USA) following the reagent protocol for a 55cm^{2} petri dish. Briefly, for each transfection mix, 7.5µg DNA was added to serum-free DMEM to a final volume of 1500µl. If DNA is co-transfected, then 15 µg DNA, consisting of 7.5µg β3 DNA and 7.5 µg of αv or αIIb DNA is added to the serum-free DMEM. The αIIb DNA (in pEGFP-N3 plasmid) and β3 (in pcDNA3.1+zeo plasmid) were donated from the Protein and Development Production Unit (PDPU) at NHS Blood and Transplant. TransIT-2020 (45µl) is added to the mix and incubated for 30 minutes at room temperature prior to adding to each petri dish. These petri dishes were returned to the incubator and left for 48 hours.

2.11 Flow cytometry methodology for transfected proteins

Transfected and untransfected cells were gently dislodged from each petri dish with a scraper, pipetted into a universal tube and centrifuged at 168x g for three minutes. The cells were washed three times using 10ml of PBS containing 0.5% (w/v) bovine serum albumin (BSA; Sigma, Poole UK). The count was adjusted to 7x10^{6}cells/ml and 30µl of the cell suspension was incubated with 30µl patient sera or diluted monoclonal antibody (as

per manufacturer's instructions) in V bottom 96-well plates at 37°C on a plate shaker for 30 minutes.

The plates were washed three times with PBS/0.5% BSA using 150µl/well for the first wash and centrifuged at 550x g for two minutes, followed by 250µl/well of PBS/0.5%BSA for the subsequent washes. The cells were then incubated for 30 minutes with 100µl RPE-conjugated secondary antibody, which were either goat anti-human IgG or rabbit anti-mouse IgG (diluted 1/50 in PBS/0.5%BSA). Each well was washed as above and transferred to a flow-cytometry tube to a total volume of 450µl in PBS/0.5%BSA for analysis on a Navios flow cytometer with supplied CXP software (Beckman Coulter, High Wycombe, UK).

2.12 Control sera and antibodies

Control sera specific to HPA-1a and HPA-1b were identified from previously tested patient samples. The HLA positive control serum is a mixture of HLA Class I and HLA Class II antibody positive patient sera which are positive with all beads on the One Lambda LSM12 screening assay. The various negative controls for all assays (called AB+number) are from non-transfused AB red cell blood donors.

The murine anti-human monoclonal antibodies used to validate the transfected cells are displayed in Table 2.7.

Antibody	Clone	Manufacturer
Anti-integrin GPIIb/IIIa (αIIbβ3)	PAB1	Gift from PDPU
Anti-integrin GPIIb/IIIa (αIIbβ3)	PAB6	Gift from PDPU
Anti-integrin αvβ3	LM609	Merck Millipore
Anti-integrin β3 (CD61)	SAP	Merck Millipore
Anti- human CD41 (αIIb)	PMC6/248	BioRad
Anti- integrin αv	P2W7	Abcam Cambridge UK

Table 2.7. The murine monoclonal antibodies which were used to validate the transfected cell lines. These included antibodies to the whole structure such as clones PAB1/6 and LM609 and to parts of the structure such as CD41 and CD61.

Secondary antibodies used in the flow cytometer assays were goat anti-human IgG conjugated to red phycoerythrin (RPE) purchased from Jackson ImmunoResearch (Philadelphia USA) and rabbit anti-mouse IgG conjugated to RPE supplied from DAKO (Glostrup Denmark).

2.13 Biotinylation of β3 protein for attachment to streptavidin beads

Purified recombinant β3 protein was biotinylated using EZ-Link NHS-PEG4-Biotin (Thermo Scientific, Illinois USA) according to the manufacturer's instructions. Briefly, 5.4mg of recombinant β3 protein was washed and buffer exchanged into PBS using Vivaspin 6; 10 molecular weight cut-off (MWCO) centrifugal filter devices (Sartorius, Epsom, UK) to a final concentration of 1mg/ml. A 20-fold molar excess of biotin to protein was calculated for the protein. The calculation used was from the reagent pack insert.

The biotin was prepared directly before use by the addition of 170µl dH_2O to provide a 20mM stock solution. The calculated volume of 20mM biotin was added to the 1ml reaction tube containing the protein and incubated on a roller mixer for one hour at room temperature. Non-reactive biotin was removed by washing with 2 x 4ml PBS using a Vivaspin 6; 10 MWCO centrifugal filter device as described above.

Streptavidin agarose resin beads (Thermo Scientific, Illinois USA) were mixed well and 1ml removed to give 0.5ml of packed beads. 6.03mg/ml biotinylated β3 protein was mixed with the beads and incubated on a roller mixer for 30 minutes at room temperature, washed twice in PBS and resuspended in PBS/0.1% sodium azide and stored at 4^{o}C until required.
For serum absorption, 250µL of packed β3 bound beads were washed three times with PBS and centrifuged at 2,500x g for two minutes prior to incubating with 180µL of test sera at room temperature on a roller for 30 minutes. The beads were then centrifuged, and the serum removed and placed in a fresh tube. The beads were washed three times with PBS. To regenerate the beads, 250µL of 0.1M glycine-HCl (Sigma, Poole UK) pH3.0 was added and centrifuged at 2,500x g for two minutes, the supernatant was removed and added to a tube containing 12µL of 1M tris-HCl pH8 (Sigma, Poole UK). This process was repeated for a total of five times and each time was called an eluate numbered one to five. The whole process was repeated twice more on each sample so that each sample had three rounds of absorptions. The beads were washed three times with PBS before the next absorption.

Each of the five eluates were kept from each absorption round and had the protein concentration measured at A280 on a DeNovix DS11+ Spectrophotometer. A 20µL sample from the eluates was taken from each of the tubes for Western Blotting, the remaining

eluates from each round of absorptions were pooled and buffer exchanged using Vivaspin 6; 50 MWCO centrifugal filter device (Sartorius, Epsom, UK).

2.14 Western Blotting

20µL of eluate (Section 2.13) and 5µL of protein gel loading buffer (Coomassie blue staining dye 0.5µg/well) were incubated at 100^{o}C for two minutes. Commercial gels were used and assembled in a gel cassette (Bio-Rad Mini-PROTEAN®, USA) and the samples loaded into the appropriate wells. A Prosieve colour (red/blue) kilodaltons (kDa) protein marker (Lonza, 50550) was run on each gel. The gels were electrophoresed using a tris-glycine-SDS running buffer at 100 volts for 75 minutes. The gels were transferred onto a membrane using a Bio-Rad Trans Blot Turbo transfer/blotting system (USA) for seven minutes. The PVDF membranes were immersed in blocking buffer containing 80ml of PBS-0.2% (w/v) Tween 20 (Sigma, UK) and 5% (w/v) skim-milk powder (Oxoid™, UK) and incubated for one hour at room temperature on a rocker mixer. The membranes were washed with PBS-0.2% Tween 20 and HRP-conjugated anti-human IgG antibody diluted 1/1000 in 10ml of blocking buffer was added to the membrane and incubated at room temperature for 1 hour on a rocker mixer. The membranes were washed three times in PBS-0.2% Tween 20, five minutes per wash. One diaminobenzidine tetrahydrochloride (DAB) tablet (10mg; Sigma, Poole UK) was dissolved in 30ml PBS-0.2% Tween 20 and 30µL of hydrogen peroxide (Sigma, Poole UK) was added immediately before use. The DAB substrate was poured into the tray containing the membranes. Once developed the membranes were washed with water to stop the reaction, dried and photographed.

2.15 Platelet immunofluorescence test

The PIFT was performed to analyse whether there were HPA-1a antibodies in the absorbed sera in a method similar to Section 2.11. Fresh frozen platelets at a concentration of 2.5x10^{6} cells were dispensed into a U shaped 96 well plate and 25µl of control sera or test sera and incubated at 37^{o}C for 30 minutes. The samples were washed four times with 200µl of EDTA/1%BSA and centrifuging at 668x g for three minutes between each wash. Samples were incubated for 30 minutes with a 1/50 dilution of goat anti-human IgG conjugated with RPE (25µL/well, Section 2.12), each well was washed as above and resuspended in 200µl of

EDTA/1%BSA and analysed on a CytoFLEX flow cytometer with supplied software (Beckman Coulter, California USA).

2.16 Preparation of Luminex™ Beads with Recombinant Proteins

Recombinant proteins for rHPA-1a, rHPA-1b and rGPVI were kindly supplied by the H&I Colindale Research Department and were produced as described by Chong *et al.,* (2011). These proteins had previously been biotinylated at 2mg/mL using the methodology described in Chong *et al.,* (2011) and stored at -80^{o}C. Three different xMAP LumAvidin colour coded bead regions (Luminex Corporation, Texas USA) were prepared as per the manufacturer's instructions. Each 1ml bead set contains 2.5x10^{6} beads. Briefly, beads were centrifuged for 2 minutes at 10,000x g and sonicated for 3 minutes, followed by vortexing gently. The beads were pelleted for two minutes at 8,000x g, resuspended in 1ml of PBS containing 1% (w/v) BSA and divided into eight vials, so that each vial contained 125µl (3.125x10^{5} beads). PBS/1% BSA was added to each vial, so that the final volume was 1.25ml. For each biotinylated protein, 2.5µg was added to eight vials containing beads of a specific bead region. These were incubated on a roller mixer in the dark for 90 minutes and then pelleted for two minutes at 8,000x g and washed three times in 1mL of PBS containing 0.1%(w/v) BSA; 0.02% (w/v) Tween 20; 0.05% (w/v) sodium azide (PBS-TBN). Each set of 8 vials from an individual bead region were combined into one vial and resuspended in 600µL of PBS-TBN to give ~4,000 beads/1µL and stored separately at 4^{o}C until required.

2.17 Luminex™ antibody testing

Prior to use the beads were sonicated for three minutes and vortexed for 20 seconds. The beads were multiplexed together by mixing 1µL (~4000 beads) of each bead different colour coded bead region with 7µL of PBS-TBN buffer per 10µL reaction volume. Patient sera/plasma were centrifuged at 10,000x g for five minutes and diluted 1:4 with PBS-TBN.

Twenty microliters of diluted sera/plasma were added to 10µl of the bead multiplex mix in a 96 well filter plate and incubated in the dark on a shaker for 30 minutes at room temperature. Each patient sample was tested in five wells for total IgG and each IgG subclass. Following incubation, the plate was washed 1x100µl PBS-TBN and a further 4 washes with 200µL of PBS-TBN using a vacuum manifold. One hundred microliters of PE-

conjugated anti-human IgG diluted 1 in 100 (see Table 2.8 for details of antibodies) was added to each reaction well and incubated for 30 minutes using the same conditions as the previous incubation and wash steps. Beads were resuspended in 80µL of PBS-TBN and placed on a shaker for two minutes prior to being analysed on a Fluoroanalyser (Luminex 200™, Luminex Corporation, Texas USA). The median fluorescent intensity (MFI) was measured from 100 events per bead region and used for analysis.

Antibody	**Clone**	**Manufacturer**
Goat anti-human IgG-PE	F(ab)2	One Lambda (California USA)
Mouse anti-human IgG1	4E3	SevernBiotech (Oxfordshire UK)
Mouse anti-human IgG2	31-7-4	SevernBiotech (Oxfordshire UK)
Mouse anti-human IgG3	HP6050	SevernBiotech (Oxfordshire UK)
Mouse anti-human IgG4	HP6025	SevernBiotech (Oxfordshire UK)

Table 2.8. List of antibodies used in the Luminex™ experiments.

2.18 Statistical analysis of results

For all flow cytometer tests, the mean fluorescent intensities (MFI) for each antibody to αvβ3 and αIIbβ3 were used to calculate a ratio. Six AB negative sera's (non-transfused blood donors) MFIs mean were calculated plus four standard deviations (SD) to establish a positive cut-off. Each test samples MFI was divided into the cut-off value to produce a ratio. A ratio over one was classed as positive. Scatter graphs from these experiments were generated using Prism software (version 5 or 9) and when comparing two cohorts Mann-Whitney U was performed using the same software. If more than one cohort or variable was present these were compared by Kruskal-Wallis using Prism software. Column diagrams were generated using Microsoft Excel (2016).

For all Luminex™ tests, the positive cut-off was established using twenty-two female apheresis donors returning from pregnancy. The median fluorescent intensity (MFI) was used from these samples and the mean plus three SDs established as the cut-off. Prism software was used to generate scatter plots and to perform Kruskal-Wallis analysis.

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Chapter 3: Results Chapter 1

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3.1 Introduction

Santoso *et al.,* (2016) hypothesised that three different antibodies may potentially be involved in NAIT which have differences in their integrin recognition properties. One antibody is specific to an epitope located on the heterodimer αIIbβ3 expressed on platelets and another is specific for αvβ3 expressed on endothelial cells, the other reacts with both (β3 antibodies) (Santoso *et al*., 2016). They studied the antibodies in NAIT affected children and found that for those who had suffered ICH, αvβ3 antibodies were present in addition to β3 specific antibodies; whilst β3 but not αvβ3 antibodies were present in the 'no ICH' cohort.

Currently there are no commercial assays available to detect αvβ3 antibodies; previous studies have used endothelial cells, derived from umbilical veins, with both an HPA-1a1a and HPA-1b1b phenotype. These were used as a research tool to test for antibodies that were specific to the αvβ3 antigen. The aim of this study was to produce an αvβ3 antigen that was not reliant on endothelial umbilical vein cell isolation which could be expressed stably in an alternative cell line. Within the laboratory there are established cell lines that have been utilised to express recombinant antigens, which are used to detect clinically relevant antibodies. The decision was made to use the same cells for this study (Wozniak *et al.,* 2011), since the system has been validated for clinical diagnostic use, the aim was to use the same expression vector and cloning techniques to express the αvβ3 integrin.

Standard cloning techniques were used for obtaining the αv DNA plasmids. Previously the PDPU department had developed plasmids for αIIb and the HPA-1a version of β3. All available plasmids were used to form the integrins of both αIIbβ3 and αvβ3 of the HPA-1a phenotype. The αIIbβ3 integrin was tested as this would be useful in validating as a screening methodology which is specific to this antigen. To demonstrate that the αvβ3 antibodies were specific to the HPA-1a epitope a recombinant β3 polypeptide was produced, that possessed the HPA-1b epitope (single nucleotide polymorphism of leucine to proline at position 33 in ITB3 gene).

This chapter details the process of establishing an αv expression vector and the generation of αvβ3 and αIIbβ3 integrins by HEK293WT cells that can be used to detect antibodies present in the serum from NAIT patients. This involved optimising and validating the system. The proteins once transiently expressed were subsequently validated using a combination of monoclonal antibodies to the polypeptides (e.g., αv, αIIb and β3) or the integrin dimers (e.g., αvβ3 and αIIbβ3), as well as human sera with known HPA antibody specificities. The research laboratory where this study was based, had previously published cloning and transient transfection work to aid the confirmation and classification of a new HPA antigen. Therefore, the cloning of αv into the expression vector and all transfections were based on previously published methods (Poles *et al.*, 2019). The cloning described in this chapter only relates to αv and β3 HPA-1b plasmids. The αIIb and β3 HPA-1a plasmids were kindly donated from the Protein Development and Production Unit (PDPU).

3.2 Cloning

The first stage in the cloning of αv was to design PCR primers to amplify the sequence from the commercial pGEM-T DNA plasmid and incorporate restriction endonuclease (RE) sites for ligating into mammalian expression vector pEGFP-N3 (see section 3.4 Snapgene map). The restriction endonucleases (RE), BamH1 and EcoR1 were selected to ligate into the multiple cloning sites of the pEGFP-N3 vector as use of these would avoid cleaving the αv sequence. The construct was analysed using SnapGene software (SnapGene® from GSL Biotech; available at snapgene.com) which aids in helping visualise each step of the cloning process such as PCR primer amplification and ligation with the RE to ensure that the αv sequence would be cloned in frame.

DNA from the purchased αv plasmid was transformed into E. coli XL1 chemically competent cells to make stock DNA. This DNA preparation was used for PCR amplifications to add the RE sites. Agarose gel electrophoresis showed multiple weak bands in the initial PCR reaction (not shown) indicating either the thermal cycling conditions were not optimal or poor primer design. Consequently, the primers and the thermal cycling conditions were reviewed.

The elongation time of 30 seconds in the cycling program was too short for the amplification of a 3KB product suggesting one reason for weak amplification; the time was extended to one minute 45 seconds and in addition, annealing temperatures of 58°C and 61°C were trialled. On review of the primer sequence, it was noted that there was a high G/C base content, suggesting that the addition of dimethyl sulfoxide (DMSO) may improve the PCR reaction.

Figure 3.1 shows the agarose gel electrophoresis image for PCR optimisation. Experiments which included DMSO in the reaction mix resulted in the appropriate size product band (~3KB) and less non-specific amplifications; little differences were seen between either the annealing temperatures of 58°C or 61°C when reviewing the gel image. The expected ~3KB product from reactions that included DMSO in the mix, were excised from the agarose gel and the DNA purified using Promega Wizard® SV Gel and PCR Clean-up system.

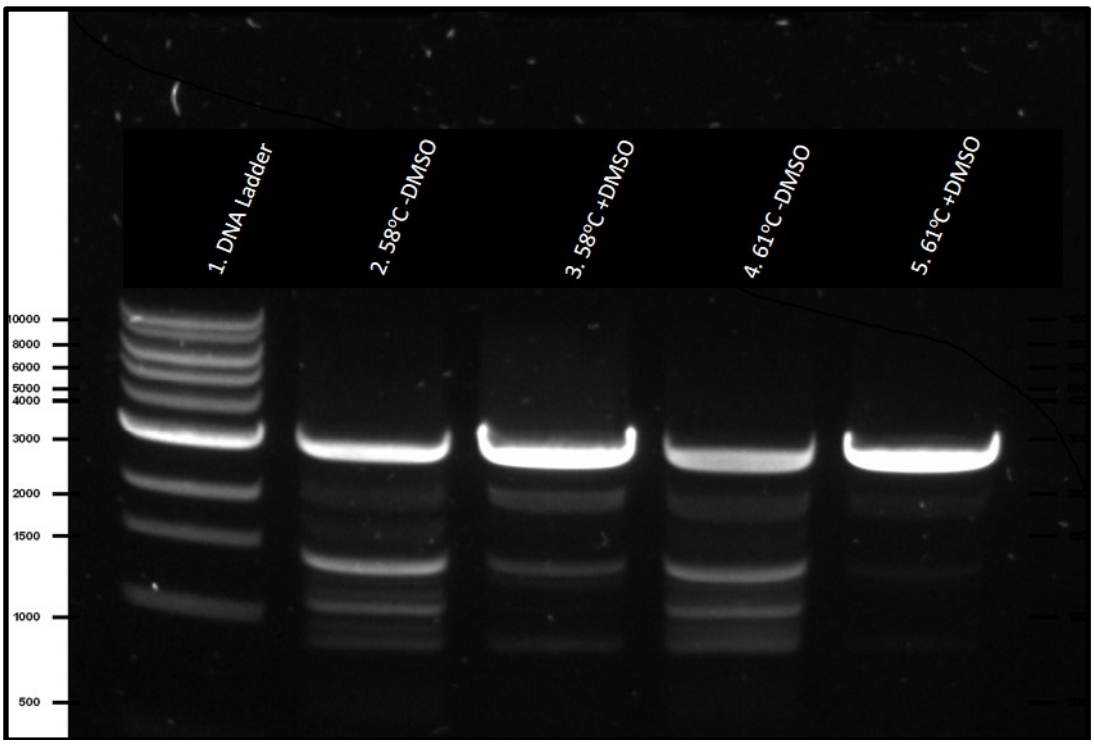

Figure 3.1. Agarose gel electrophoresis image for PCR optimisation. Lane 1 1kb ladder (New England Biolabs); Lanes 2 and 3 58°C annealing temperature in the absence of DMSO (lane 2) and presence (lane 3). Lanes 4 and 5 61°C annealing temperature in the absence and with DMSO (lane 4) and presence (lane 5). The expected size of the product is 3KB. As can be seen lanes 3 and 5 had less non-specific amplification and a strong specific band. These bands were excised, and the DNA extracted.

3.3 Double RE digestion of αv and pEGFP-N3

The plasmid pEGFP-N3 had previously been used in the laboratory for cloning the αIIb polypeptide and for other antibody detection systems (Poles *et al*., 2019). This plasmid enables easy detection of transfected cells using the FL1 detector of a flow cytometer analyser as the green fluorescent protein expressed with the protein of interest can be used to select the transfected cells and to 'gate' around these.

Gel purified DNA from the αv optimised reaction and the vector pEGFP-N3 were digested with both BamH1 and EcoR1 according to manufacturer's instructions. Controls included: undigested plasmid and DNA incubated with BamH1 only and EcoR1 only. The results from these reactions were visualised using agarose gel electrophoresis. Following digestion, αv and pEGFP-N3 DNA were purified using the Promega Wizard® SV Gel and PCR Clean-up system.

3.4 Ligation of αv and pEGFP-N3 and choosing clones

The purified vector and insert DNA from Section 3.3 were ligated using a ratio of 1:3 vector to insert and transformed into E. coli XL1 chemically competent cells. Individual colonies were picked from LB-kanamycin agar plates, inoculated into 5ml LB kanamycin medium and grown overnight in a 37^{o}C incubator. Plasmid DNA was extracted using Promega Wizard® Plus SV Mini-preps Purification System. DNA from six different clones were digested with BamH1 and EcoR1 RE and were visualised by agarose gel electrophoresis (Figure 3.2). Only three of the clones, one, five and six had the appropriate size inserts and these were sent for confirmatory DNA sequencing. All other clones were discarded. Figure 3.3 is a schematic of the ligation of αv and the pEGFP-N3 plasmid.

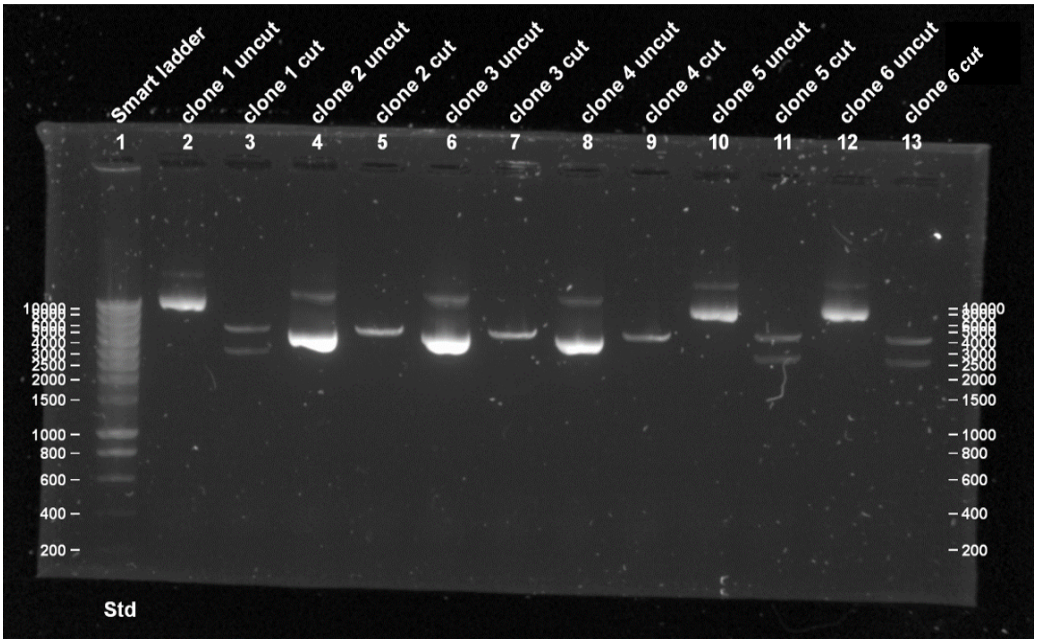

Figure 3.2. Gel image of plasmid DNA extracted from each of the clones following incubation with (cut) and without (uncut) the restriction enzymes BamH1 and EcoR1. Lane 1: SmartLadder MW marker (200-10,000kb). The digested plasmids, which show two bands, are classed as successful and were sequenced to confirm the αv had the correct sequence. These were clones 1, 5 and 6 which are visualised in lanes 3, 11 and 13. All other clones were discarded.

3.5 Sequencing results

The sequencing results were analysed using SnapGene software (SnapGene® from GSL Biotech; available at snapgene.com) and matched the bought clones sequence (data not shown). It was noted that each clone had the expected silent mutations cysteine to threonine (for both) at positions 87 C/T and 2028 C/T as described in the accompanying datasheet. Therefore, these three clones were propagated for transfection experiments. As these were silent mutations it was not thought that they would affect antibody binding, which was later proved with the αvβ3 monoclonal binding.

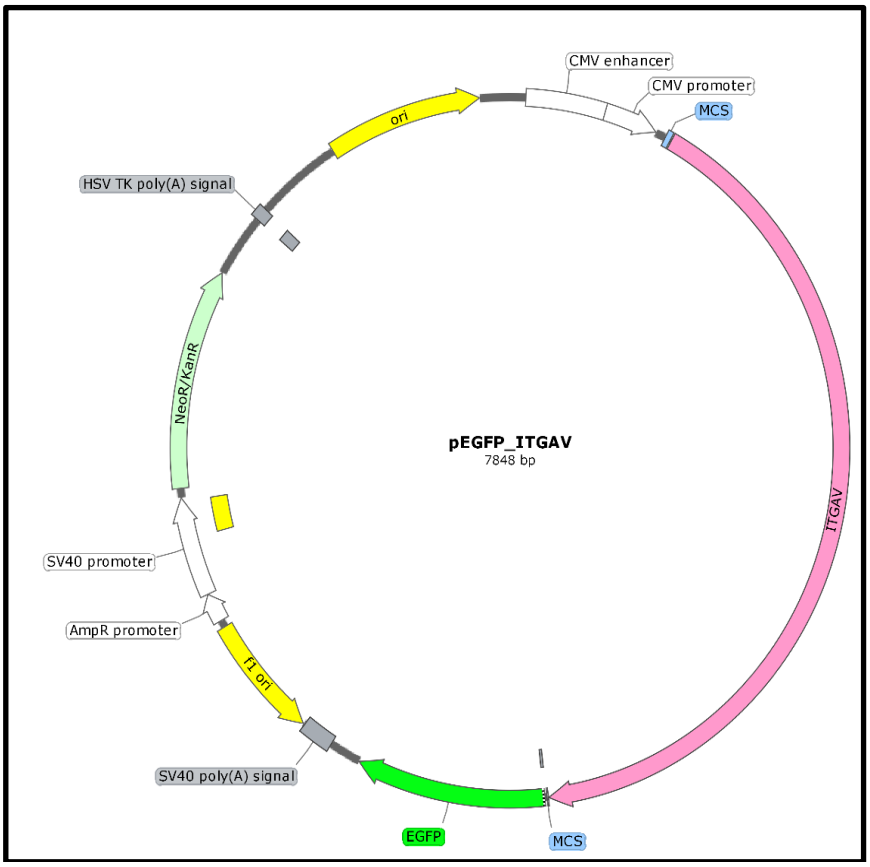

Figure 3.3. Schematic of the incorporated αv (ITGAV) within the pEGFP plasmid.

3.6 Transformed and digest results

The ligated αv clones number 1, 5 and 6 were transformed into *E.coli* XL1 chemically competent cells to prepare enough DNA for transfection experiments. Only two cultures grew (clone 5 chosen) and DNA was extracted using the QIAfilter Plasmid Midi kit. As a check that the DNA with the clone was inserted into the vector after the transformation, a double digest was performed using BamH1 and EcoR1 as two products would be seen (Figure 3.4).

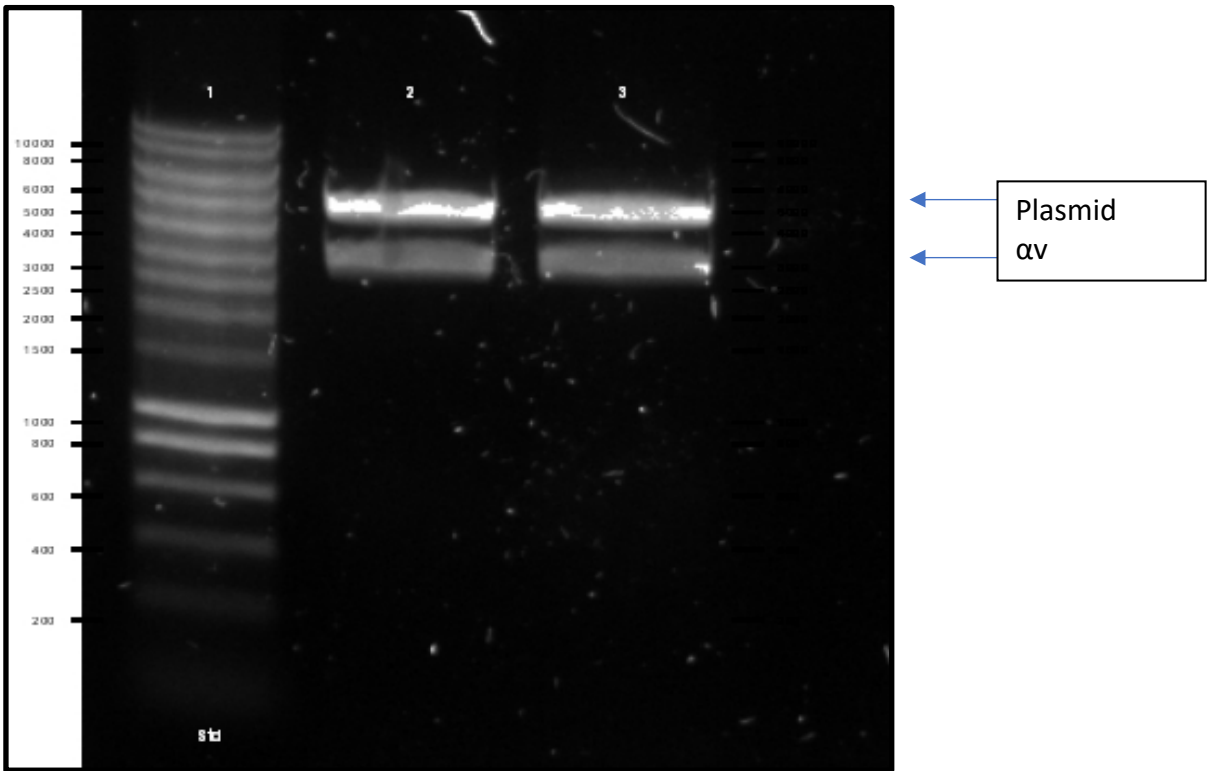

Figure 3.4. Gel electrophoresis image of DNA double digestion with BamH1 and EcoR1 showing the expected two bands (around 3KB and 5KB). Lane 1: SmartLadder MW marker; Lanes 2 and 3 show digested plasmid DNA. The lower band is the αv insert and the higher band is the plasmid.

3.7 Initial transfection results

HEK293WT cells were co-transfected with DNA coding for αIIb and β3 or αv and β3 in separate flasks, using the protocol published by Poles *et al.,* (2019). As a control each of the sub-units αIIb, β3 and αv were transfected singularly into HEK293WT cells in separate flasks. Early experiments tested native HEK293WT cells and transformed HEK293 cells with monoclonal antibodies directed against each separate alpha (αv and αIIb) and beta (β3) polypeptides and both integrins (αvβ3 and αIIbβ3) – see Figure 3.5.

Flow cytometry was used for the detection of the expressed proteins on the transiently transfected cells as described in Poles *et al*., (2019). Briefly, cells expressing EGFP (therefore αv and αIIb, and αvβ3 and αIIbβ3) are detected in the FL1 channel on the flow cytometer. RPE-conjugated monoclonal antibodies (e.g., goat anti-human IgG and goat anti-mouse IgG) directed against the expressed proteins including the β3 polypeptide are detected in the FL2 channel. The analysis is performed on unpermeabilised cells which are expressing the whole integrins (Figure 3.5).

Untransfected HEK293WT cells express low or undetectable levels of both αvβ3 and αIIbβ3 integrins. The results (Figure 3.5) indicate that the correct proteins have been transiently expressed, and there was no unexpected monoclonal antibody binding, as each monoclonal antibody reacted with its cognate antigen. Therefore, as anticipated, the αvβ3 expressed protein reacted only with the monoclonal antibodies against αv, β3 (CD61) and αvβ3, and the antibodies specific to αIIbβ3 (PAB1 and PAB6) did not bind. Conversely, for the expressed αIIbβ3 protein the only monoclonal antibodies which bound were ones that were specific to β3 (CD61) and αIIb (CD41) and the αIIbβ3 (PAB1 and PAB6) complex, but not αv and αvβ3 heterodimer (Section 2.12 details monoclonal antibodies and clones). Figure 3.6 displays the flow cytometer outputs from the experiments with a monoclonal which is negative to each cell and positive (e.g., αvβ3 cell with PAB1 -negative and with αvβ3 - positive).

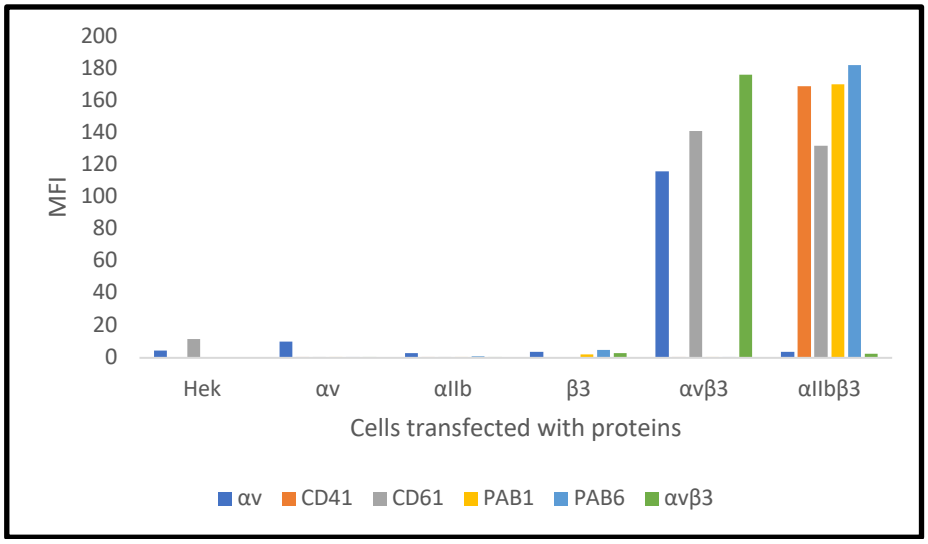

Figure 3.5. Reactions of monoclonal antibodies with cells. The X axis shows the cells with their transfected proteins. The Y axis shows the mean fluorescent intensity (MFI) of the bound monoclonal antibodies, each represented as a different colour bar. Each monoclonal bound to its expected antigens and cross-reactivity is negligible. The different coloured bars relate to the specific monoclonal antibodies used tested against each transfected cells. The specificities are CD41 (αIIb), CD61 (β3), PAB1 (αIIbβ3), PAB6 (αIIbβ3), αv and αvβ3 (see Section 2.12 for antibody clones). Please note that these results arise from a single assay and were not tested on multiple occasions.

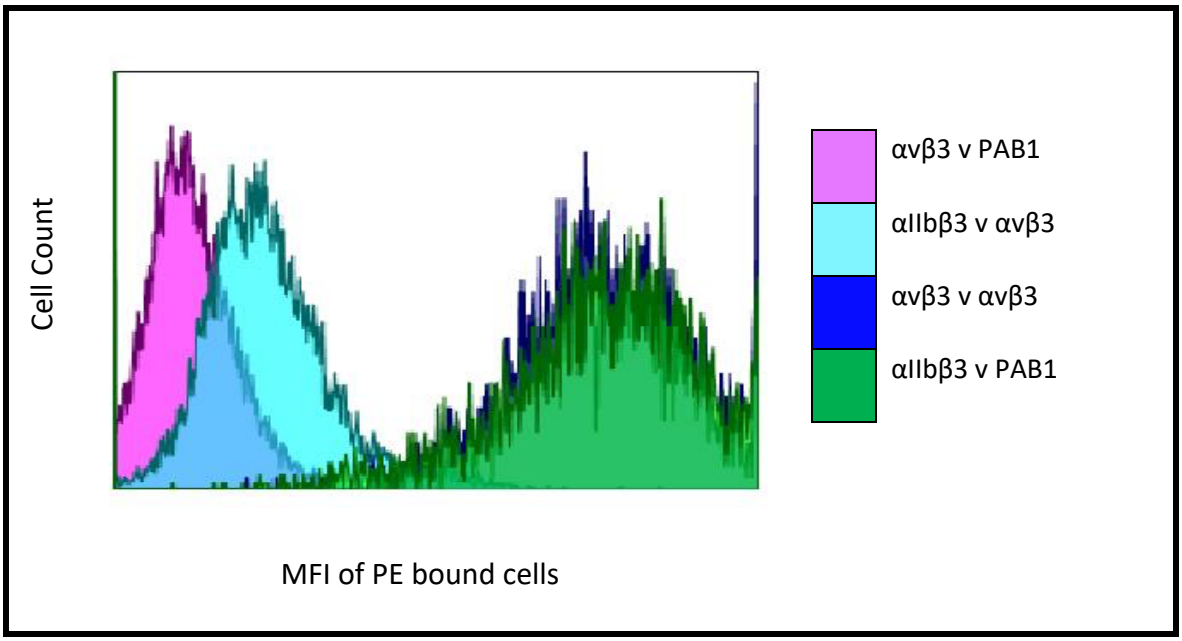

Figure 3.6. Schematic version of the histograms from the flow cytometer of the αvβ3 or αIIbβ3 transfected cells reacting with a positive or negative monoclonal antibody. The X axis shows the mean fluorescent intensity (MFI) from FL2 of the bound anti-mouse antibodies which have been conjugated with phycoerythrin (RPE). The Y axis shows the cell count. On the left is the negative reactions displayed and when the rabbit anti-mouse IgG PE is bound there is a shift to the right.

Only the combined α and β chains are expressed on the cell surface which is evidenced from the monoclonal antibodies binding (Figure 3.5). The alpha chain with the EGFP can be incorporated within the cell and still be detected by the flow cytometer by channel FL1 and is used to indicate how much of the whole cell population has this polypeptide incorporated and this is expressed as a percentage (Figure 3.7). The percentage indicates transfection efficiency and the higher the percentage indicates how many cells have incorporated the polypeptide. An initial experiment showed that more alpha chain polypeptide was incorporated into the cell than when combined as a dimer and expressed on the cell surface (Figure 3.7). As can be seen from Figure 3.7, αIIb polypeptide had the highest transfection efficiency followed by the αv, and αIIbβ3. The αvβ3 expression showed less than 20% transfection efficiency when tested with the αvβ3 antibody. This highlighted the need for further experiments to improve transfection efficiency by investigating DNA transfection ratios and the length of time of cell culture.

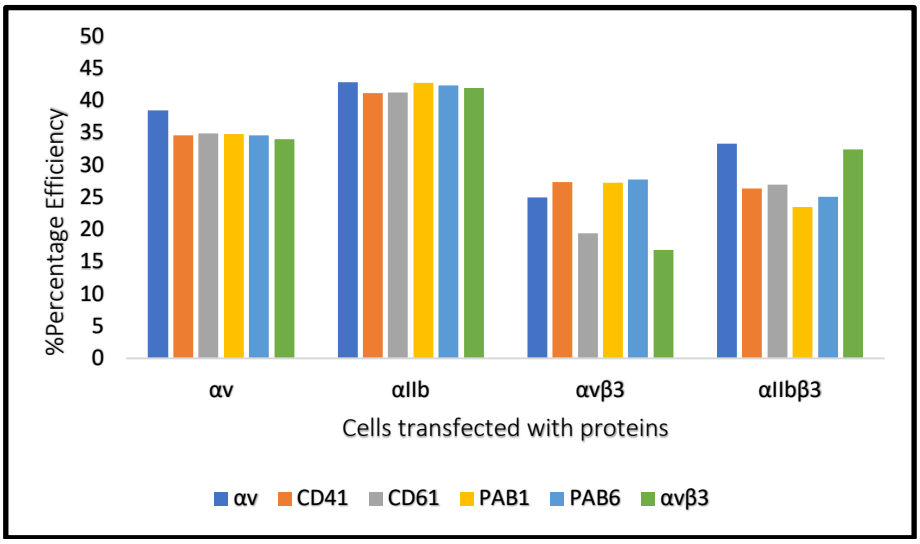

Figure 3.7. A measurement of transfection efficiency. This graph displays the transfection efficiencies which show the incorporated EGFP and not surface expression for the individual subunits and the complexes and does not indicate monoclonal antibody binding. This is based on the number of cells expressing the EGFP proteins which is detected by the FL1 channel of a flow cytometer. Each of the subunit or complexes were tested in separate wells with each of the monoclonal antibodies and this data is shown in Figure 3.5. The αVβ3 integrin shows less efficient transfection of HEK293 cells than the αIIbβ3 complex. β3 is not shown as does not contain the EGFP. The different coloured bars relate to the specific monoclonal antibodies used tested against each transfected cells. The specificities are CD41 (αIIb), CD61 (β3), PAB1 (αIIbβ3), PAB6 (αIIbβ3), αv and αvβ3 (see Section 2.12).

3.8 Transfection changing ratios

From the literature review, it was noted that αv is inherently expressed in HEK293WT cells (Ling *et al.*, 2002). This could potentially interfere with the expression of the cloned DNA as inherent αv may bind to the transfected β3 protein. Therefore, not all the transfected αv will form the dimer with the β3 polypeptide chain and subsequently expressed on the cell surface but the inherent αv will also form a dimer with the transfected β3 and move to the cell surface but not be detected due to the lack of EGFP.

The next experiments were aimed at improving the transfection efficiency of the αv plasmid, by varying the ratio of αv to β3 DNA. The ratios tested were 1:1, 2:1 and 3:1. These experiments were performed only once (Figure 3.8) as no significant difference was observed between the level of proteins expressed by the transfected cells when tested with the monoclonal antibodies and known positive and negative human polyclonal sera. The PI 1 positive control, an HPA-1a antibody that is well established in the laboratory but unexpectedly this did not show a positive shift with the MFI by the flow cytometer to the cells expressing αIIbβ3 and αvβ3. On investigation, this serum had been diluted previously and was not used at its correct dilution for this test. The known HPA-1a antibody serum

(single patient) had the highest binding to the αvβ3 protein with the 1:1 ratio of the αv and β3 DNA.

What was noticeable, was a decrease in the transfection efficiency when the αvβ3 monoclonal and HPA-1a antibodies bound to the αvβ3 expressing cells, antibody binding may result in cell loss expressing the proteins prior to analysis by flow cytometry. From these results it was decided to keep the DNA ratio of 1:1 for both αv and β3 DNA as suggested in the transfection reagent (Mirus Bio TransIT®-2020) manufacturer's instructions, and to use 15µg of DNA in total.

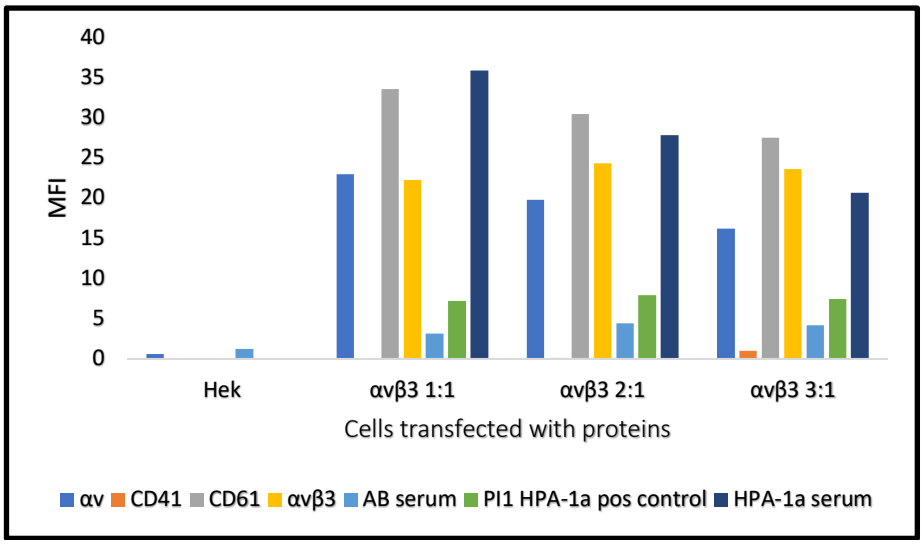

Figure 3.8. A measurement of MFI changing the αv ratios. The X axis shows the cells with their transfected expression vectors and the differing ratios of the αv DNA compared to the β3 DNA. The Y axis shows the MFI for FL1 and FL2 obtained for the monoclonal antibodies and human sera. Each bar indicates the monoclonal antibodies (CD41 (αIIb), CD61 (β3), PAB1 (αIIbβ3), αv, αvβ3), AB serum is a negative control, PI 1 is a polyclonal human HPA-1a positive control and a previously screened patient HPA-1a antibody positive serum.

The transfection efficiency was analysed from these results (Figure 3.9). There did not seem any difference in protein expression when analysing the different sera and monoclonal antibodies to the different ratios of DNA used in the transfection experiments for αv compared to the β3. Therefore, future experiments would look at extending the transfection reagent incubation timings.

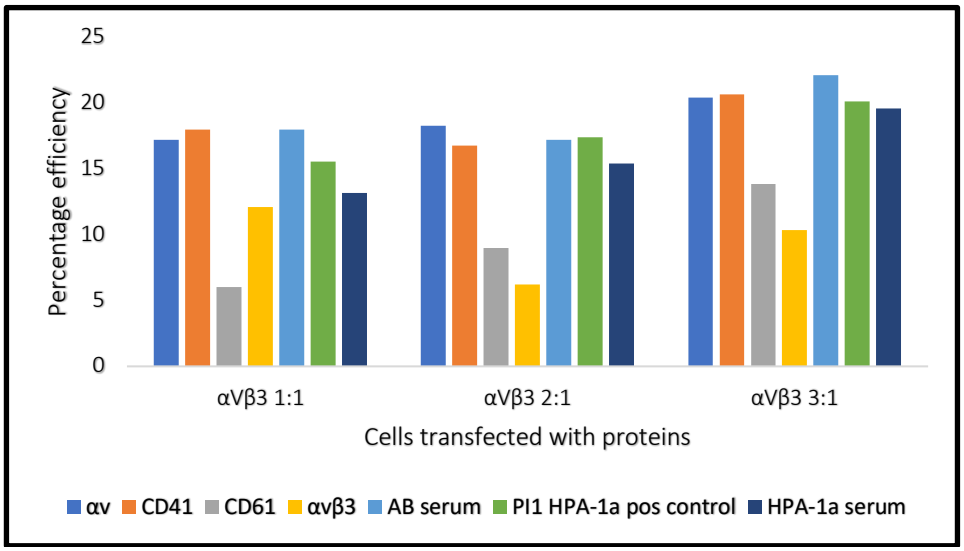

Figure 3.9. A measurement of transfection efficiency whilst changing αv ratio. This investigates the transfection efficiencies for the different transfected ratios of αv DNA to β3 of 1:1, 2:1 and 3:1. This is based on the number of cells expressing the EGFP proteins which is detected by the FL1 channel of a flow cytometer and not an indication of antibody binding. The different coloured bars relate to the specific monoclonal antibodies used tested against each transfected cell. The specificities are CD41 (αIIb), CD61 (β3), PAB1 (αIIbβ3), PAB6 (αIIbβ3), αv and αvβ3 (see Section 2.12).

3.9 Transfections timings

In the study by Poles *et al*., (2019), transfected cells were cultured for 24 hours before harvesting. It was decided to compare the transfection efficiencies after culturing the cells for 24, 40 and 48 hours with the transfection agent to try and increase protein expression. This was exposing the cells to plasmid of both α/β subunits and treating with transfection reagent for these defined periods. Each of the cells expressing proteins were tested with each monoclonal antibody to the individual units and the complexes (Figure 3.10). When comparing αvβ3 over three time points using the amount of monoclonal bound as an indicator, there was equal reactivity at 40 and 48 hours, this was similar for αIIbβ3. When measuring the transfection efficiency by using the EGFP and FL1 gate, this supported the use of 40 and 48 hours' incubations with the plasmid and reagent (Figure 3.11), 48 hours was used for all subsequent experiments.

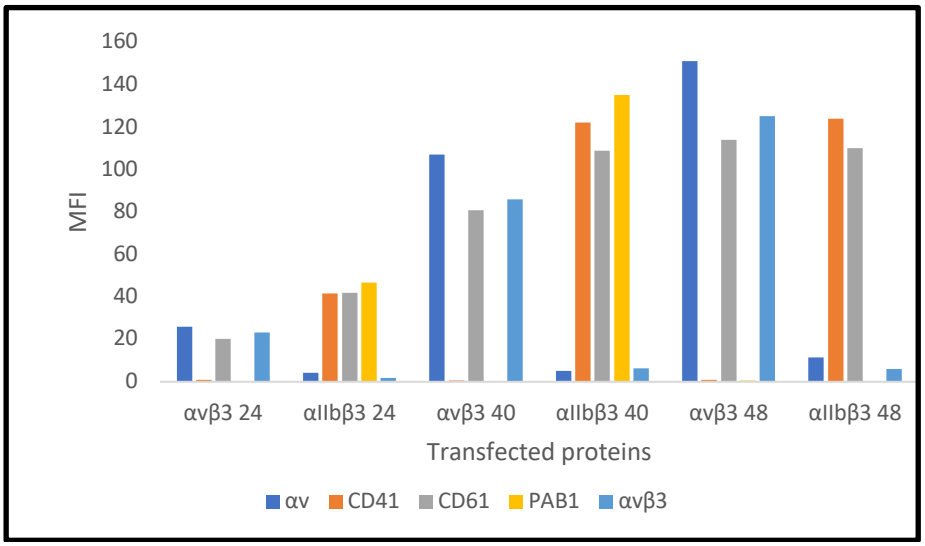

Figure 3.10. Reactions of monoclonal antibodies with cells over different time periods. The X axis is displaying the different integrins transfected and the various transfection timings at 24, 40 and 48 hours. Each bar indicates the monoclonal antibodies (CD41 (αIIb), CD61 (β3), PAB1 (αIIbβ3), αv, αvβ3). There was increased mean fluorescence (Y axis) with the cognate monoclonal binding to the integrin with increasing the transfection timings. Due to laboratory error αIIbβ3 expressing cells with the PAB1 monoclonal was discarded.

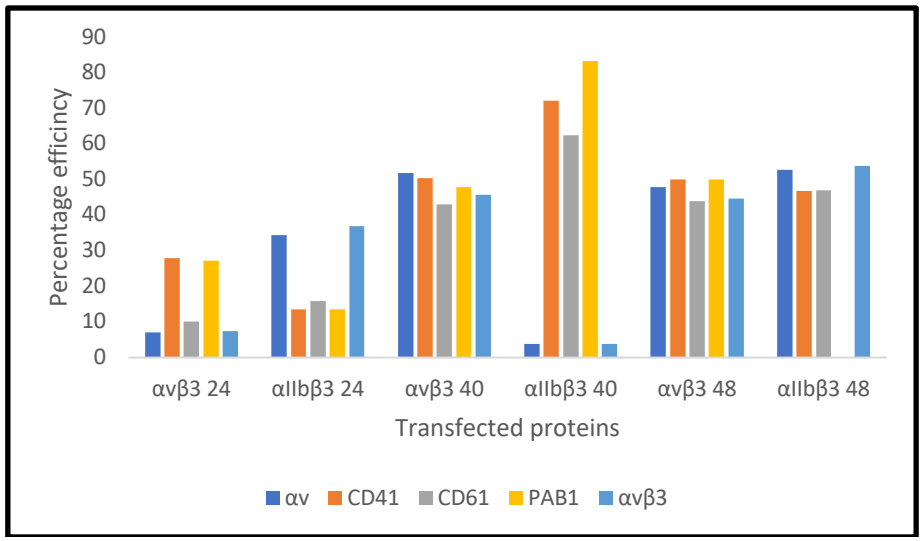

Figure 3.11. A measurement of transfection efficiency over different time periods. Assessing the transfection efficiency by increasing the incubations of the transfection timings from 24 hours to 40 and 48 hours. This was performed by using the FL1 channel and the EGFP to count the number of cells There is an increased transfection efficiency with extending incubations from 24 hours to 48 hours. At 48 hours there was little difference between the αvβ3 and the αIIbβ3 integrin. Due to laboratory error αIIbβ3 expressing cells v the αIIβ3 complex monoclonal was discarded.

3.10 Testing with human polyclonal sera

The majority of the previous experiments were performed using commercially available monoclonal antibodies (see Section 2.12 for details). It was important to test whether human polyclonal serum containing previously identified HPA-1a antibodies reacted with the transfected proteins. HPA-1a positive patient serum reacted with both the αvβ3 and

the αIIbβ3 integrins by producing a mean fluorescence shift in the RPE channel (Figure 3.12). As expected, negative AB serum and HPA-1b patient serum did not bind to the cells and had a negative result. This showed that the expressed proteins were reactive with polyclonal sera and not just monoclonal antibodies.

At this stage it also became apparent that the HPA-1a sera always reacted with both αvβ3 and αIIbβ3, suggesting a need to differentiate between antibodies to the two complexes to properly identify αvβ3 antibodies.

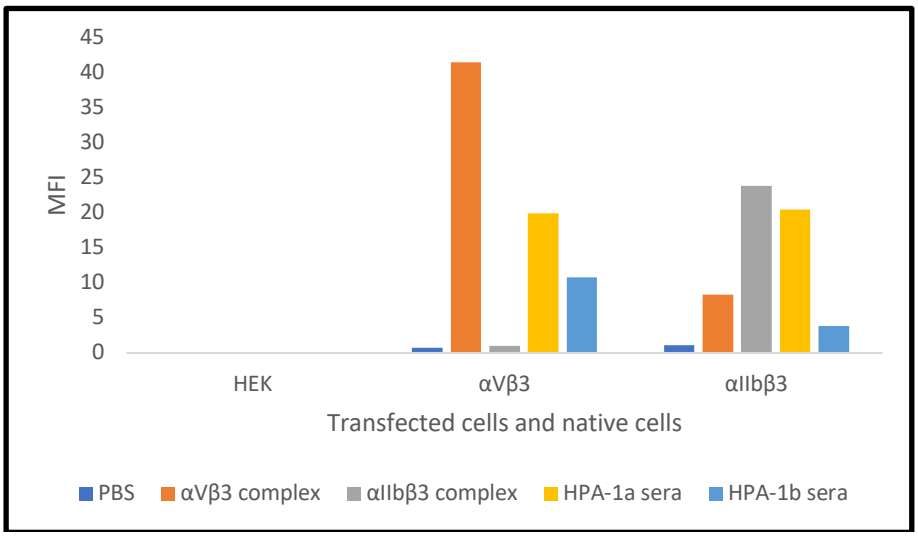

Figure 3.12. Comparison of monoclonal and polyclonal antibodies for HPA-1a and HPA-1b expressing cells. This shows the results of testing untransfected HEK293WT and the co-transfected proteins of both αvβ3 and αIIbβ3 proteins. These were incubated with a range of samples such as PBS, monoclonal' s specific to each complex and HPA-1a and HPA-1b serum. The results show that HPA-1a serum bind to both αvβ3 and αIIbβ3 expressing cells but the HPA-1b serum did not bind to these cells. HEK293 cells did not react with any incubation with monoclonal or polyclonal serum.

3.11 Testing specificity by binding to HPA-1b

An option was to design an HPA-1b β3 form of both proteins to see if this would be an easier way to differentiate from both antibodies. The theory was that the HPA-1a antibodies would not bind to the HPA-1b versions of the αIIbβ3 proteins but hopefully would bind to the HPA-1b versions of αvβ3 proteins if it recognised the structure as a whole conformation and not just the HPA-1b part of the β3.

Using Agilent Quik Change™ Multi Lightning Kit, a plasmid was constructed which contained the HPA-1b β3 version. This kit enables site directed mutagenesis of the HPA-1a β3 form in the pcDNA 3.1+zeo vector to the HPA-1b form of the β3 vector by performing a PCR

reaction and transforming into *E.coli* competent cells to obtain enough DNA (Section 3.6). Sequencing of this product confirmed the HPA-1b version (data not shown).

The results showed that the expressed proteins on the transfected cells reacted to their cognate monoclonal/polyclonal antibodies. For example, the HPA-1a polyclonal serum reacted only with αvβ3 and αIIbβ3 HPA-1a versions of the cells and vice versa for HPA-1b cells and polyclonal serum. The monoclonal antibodies direct to each cells expressing αvβ3 and αIIbβ3 reacted to the HPA-1a and HPA-1b versions of the cells co-transfected with αv or αIIb. This is shown in Figure 3.13. The αvβ3 monoclonal against the αvβ3 HPA-1a transfected cells had an experiment error in the flow analyser and no data was collected. In this experiment an HLA human polyclonal positive control was used to see if HLA antibodies in sera bound to the transfected HEK293 cells which this experiment has shown HLA antibodies do not interfere in this assay. This is important as it has been shown that HLA antigens are expressed on HEK293WT cells previously, which could cause false positive results for αvβ3 and αIIbβ3 transfected cells (Wozniak *et al*., 2011).

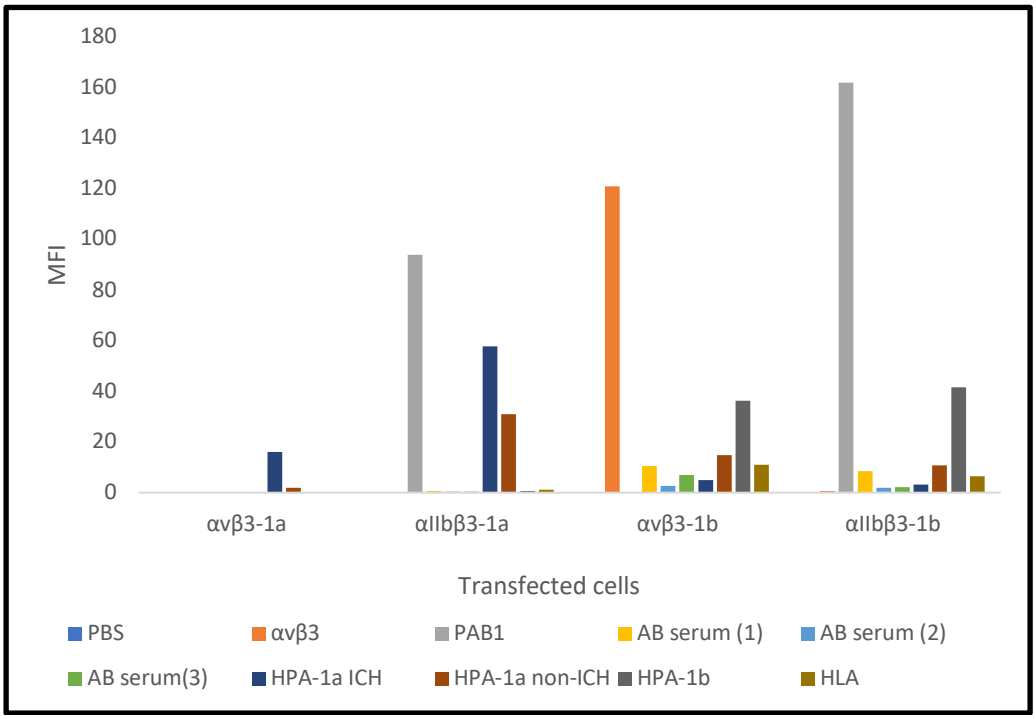

Figure 3.13. Testing of monoclonal and polyclonal antibodies for HPA-1a and HPA-1b expressing cells. Phosphate buffered saline (PBS) and a range of monoclonal antibodies (CD41 (αIIb), CD61 (β3), PAB1 (αIIbβ3), αv, αvβ3)/polyclonal antibodies (human sera) were incubated with either HPA-1a or HPA-1b versions of the αvβ3 and αIIbβ3 transfected cell lines. A secondary antibody (RPE) was added which allows detection through the FL2 channel (if an antibody is bound) of a flow cytometer and the output was expressed as MFI. Three different AB sera were used as the negative control to establish any background binding in the detection system. The polyclonal sera included known NAIT mothers with/without and with a fetus/newborn intracranial haemorrhage (ICH) or platelet refractory patients to HLA or HPA proteins.

Figure 3.13 shows that the HPA-1b versions of the transfected cells had a higher background binding of the AB serum which was not seen in the HPA-1a forms. The monoclonal antibodies had a higher MFI value with the HPA-1b forms than the HPA-1a forms of the proteins. In laboratory experiments with flow cytometry there are often several different AB sera (sera obtained from blood group AB red cell blood donors) used to work out the mean MFI shift and from here standard deviations (SDs). Polyclonal negative sera are used to give an indication of background binding of sera and bound conjugated antibody which can be used to determine positive cut-off levels Within the laboratory the cut-offs used for flow cytometry assays are the mean of the negative controls plus 4 SDs, everything above this is classed as positive. Also, our laboratory uses ratios to compare assays which are performed over multiple days. Every assay performed, had the negative mean plus 4 SDs calculated, and the test samples MFI divided into this to give the ratio. A ratio of 1 is equivocal and over 1 is classed as positive. The raw MFI data for Figure 3.13 was calculated into ratios and is represented in Figure 3.14 and in Table 3.1.

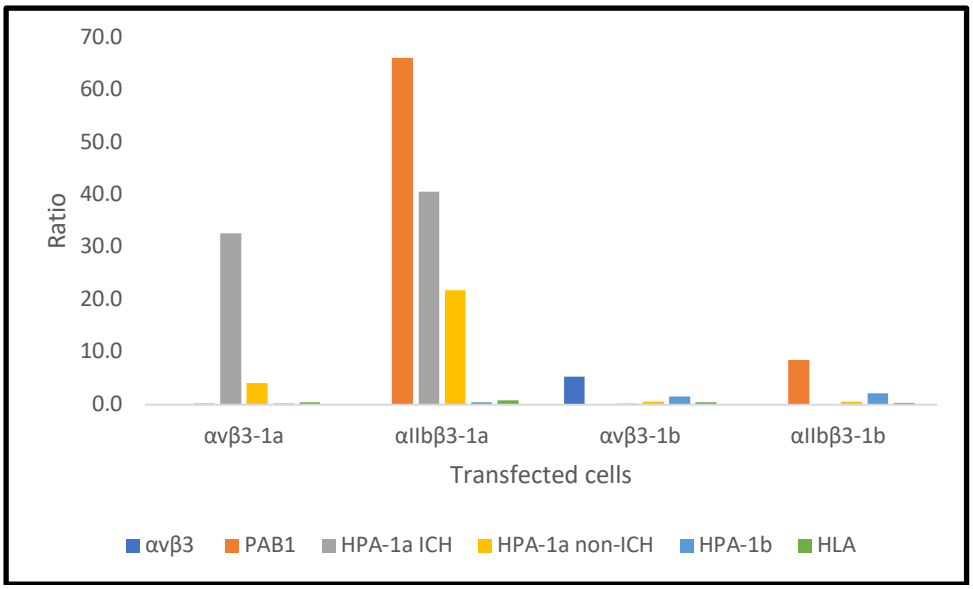

Figure 3.14. Results of testing of monoclonal and polyclonal antibodies for HPA-1a and HPA-1b expressing cells displayed as ratios. The data here are corrected to take into account the background MFI from AB negative sera and applying a cut-off of the mean of the AB sera plus 4SDs. The data is expressed as ratios and everything over 1 is deemed positive. An example of the calculations is for αvβ3-1a has a mean+4Ds of 0.49 (MFI) and for the HPA-1a ICH sample has an MFI of 16.1, 16.1/0.49 gives a ratio of 32.65 which is displayed above. Using a ratio allows comparisons between the different transfected cells and their proteins. The αvβ3 monoclonal tested with the αvβ3 HPA-1a expressing cells had a laboratory error and data was not collected. Shown is the specific monoclonal antibodies and human polyclonal sera reacting with the expected transfected cultured cells of both the β3 variants of HPA-1a and HPA-1b version.

	Transfected Cultured Cells			
Monoclonal/Polyclonal Serum	αvβ3	αIIbβ3	αVβ3-1b	αIIbβ3-1b
αvβ3 complex	NA	0.1	5.4	0.0
αIIbβ3 complex (PAB1)	0.2	66.1	0.0	8.5
HPA-1a ICH	32.7	40.6	0.2	0.2
HPA-1a non-ICH	4.1	21.9	0.7	0.6
HPA-1b	0.2	0.5	1.6	2.2
HLA	0.4	0.9	0.5	0.3

Table 3.1. Calculated ratios of the experiments. The data is expressed as ratios and everything over 1 is deemed positive. An example of the calculations is for αvβ3-1a has a mean+4SDs of 0.49 (MFI) and for the HPA-1a ICH sample has an MFI of 16.1, 16.1/0.49 gives a ratio of 32.65 which is displayed above. Using a ratio allows comparisons between the different transfected cells and their proteins. The αvβ3 monoclonal tested with the αvβ3 HPA-1a expressing cells had a laboratory error and data was not collected. The positive values are highlighted in yellow. Shown is the specific monoclonal antibodies and human polyclonal sera reacting with the expected transfected cultured cells of both the β3 variants of HPA-1a and HPA-1b version.

In the study of Santoso *et al.,* (2016), they stated that in NAIT the αvβ3 antibody they detected was a compound antibody. Therefore, in this scenario the antibody will be detected by cells expressing the HPA-1a version of the β3 (discussed further in Section 4.7). Thus, αvβ3 antibodies will not be detected by cells bearing the HPA-1b form of the β3.

3.12 Summary

This chapter describes the approach taken to produce an αvβ3 protein which could be used to detect antibodies to αvβ3. Standard cloning techniques that are well established within the laboratory were used to produce an expression vector containing the sequence for the αv polypeptide that could be combined with the previously cloned β3. As a control the previously validated αIIbβ3 integrin was used to test for HPA-1a antibodies (Poles *et al*., 2019).

During the αv cloning process there was optimisation of the initial PCR reaction to add on the RE sites for BamH1 and EcoR1 by extending the annealing time and adding DMSO to improve the reaction. The αIIbβ3 specific monoclonal antibodies only bound to their cognate antigens and not to the αvβ3 proteins.

The initial transfection experiments were performed using the protocol published in Poles *et al*., (2019), which showed that the cells co-transfected with αIIbβ3 and αvβ3 integrins only bound the expected monoclonal antibodies. The monoclonal antibodies which were

specific to one part of the α or β chain only bound to cause a positive result when the whole integrin was expressed, as only the dimers are expressed on the cell surface (Section 3.7).

The initial experiments indicated that the transfection efficiency would have to be improved to increase the number of expressed proteins on the cells for the patient sera testing. Optimising the transfection and the culture time became the focus of the next few experiments. A 1:1 ratio of αv and β3 in cotransfection gave results similar to those obtained at increased ratios. Extending the cell culture following transfection to 48 hours consistently resulted in over 40% transfection efficiency. Therefore, these parameters were used for all future experiments.

The early optimisation experiments showed that known human polyclonal HPA-1a positive sera caused positive reactions with both αvβ3 and αIIbβ3 expressing cells. This indicated that there was a problem of differentiating between αIIbβ3 and αvβ3 antibodies as both of the cells expressing αvβ3 and αIIbβ3 would always be positive. One option was to develop a β3-HPA-1b version of both αvβ3 and αIIbβ3 expressing cells. This was prior to understanding the definition of a compound antibody, which in this instance meant the serum must be HPA-1a antibody positive with the addition of a second epitope which recognised the αvβ3 antibody (Kjeldsen-Kragh & Bengtsson, 2020). This gave a further objective in Section 4.1 in establishing a methodology to absorb out the αIIbβ3 antibody to try and detect the αvβ3 antibody.

Further validation was undertaken using the HPA-1a and HPA-1b version the αIIbβ3 and αvβ3 expressing cells which showed that HPA-1a and HPA-1b specific antibodies could be detected. The last experiments calculated ratios of the mean of three different AB serum plus 4SDs, which is already established in the laboratory. Once the AB negative serum was taken into account and adjusted for the MFI shift the antibody results were clearly specific for their intended cognate αIIbβ3 and αvβ3 integrins.

Time limitations affected the work as each experiment took a week to perform and only low number of samples could be analysed at once given the total number of HEK293 cells. Therefore, each experiment was only conducted once, which means that experimental variability could not be estimated.

In summary, αIIbβ3 and αvβ3 integrins have been transiently transfected into HEK293WT and a system has been validated and optimised which can detect HPA-1a and HPA-1b antibodies to the αIIbβ3 and αvβ3 integrins. There is the potential to use these as a screening tool to detect HPA-1a and HPA-1b antibodies independent of HLA antibodies, but the sensitivity of the assays requires determining against the current PIFT method.

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Chapter 4: Results Chapter 2

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4.1 Introduction

This chapter will investigate whether NAIT severity can be predicted by testing maternal samples to identify if there is a biomarker such as αvβ3 antibodies (HPA-1a version) or αIIbβ3 antibody titre which can potentially be linked to ICH. There have been limited published studies on the ability of laboratory tests to predict which NAIT cases will result with ICH in a fetus/newborn (recently reviewed by Sachs, 2020). Various studies have investigated maternal IgG HPA-1a αIIbβ3 antibody titres to establish if there is a level which causes ICH and if a particular IgG subclass is linked to ICH (Sachs, 2020). More recently, a study by Santoso *et al.,* (2016) associated the presence of αvβ3 antibodies with ICH. All NAIT ICH cases were positive for both αvβ3 and αIIbβ3 antibodies, whereas the NAIT cases without ICH possessed only αIIbβ3 antibodies. The antibody must be HPA-1a positive with the β3 version expressing a leucine at position 33 (ITGB3 gene) and not the proline which is found on the β3 which is the HPA-1b version.

The αvβ3 integrin (vitronectin receptor) has been shown to be involved in capillary angiogenesis throughout the body, including the brain (Yougbare *et al.,* 2015). Potentially, αvβ3 antibodies could inhibit angiogenesis and explain why some NAIT cases present with ICH. It has been suggested that an HPA-1a compound epitope involving both the αv and β3 subunits might bind specific NAIT-implicated antibodies resulting in ICH (Santoso *et al.,* 2016).

The primary aim of the work described in this chapter was to investigate if there are αvβ3 antibodies detected in the ICH patient cohort and not present in the non-ICH cohort. If this hypothesis is proven in a retrospective study, a prospective application may aid the prediction of ICH in a NAIT fetus/newborn. In addition, testing was performed to investigate whether there are differences in HPA-1a antibody titre between the two cohorts that could also help to predict ICH. These experiments were performed using the recombinant αvβ3 and αIIbβ3 developed and validated in Chapter 3, using the same experimental approach of incubating cells and serum followed by a fluorescently conjugated anti-human IgG secondary antibody for detection by a flow cytometer. An additional study was included to investigate if the transfected HEK293 cells expressed HLA

and thus could bind HLA antibodies which could cause a positive reaction which may be falsely attributed to αIIbβ3 or αvβ3 antibodies.

The objectives were:

- To investigate whether HLA antibodies can bind to HEK293 cells. This was performed by reacting cells engineered to transiently express αvβ3 or αIIbβ3 with a range of sera containing HLA antibodies from previously pregnant female platelet donors.

- To assess whether there was a difference between the ICH and non-ICH patient cohorts when their sera were tested against HEK293 cells expressing recombinant αvβ3 or αIIbβ3 proteins of both HPA-1a and HPA-1b versions.

- To perform absorption studies using an HPA-1a recombinant β3 integrin polypeptide coupled to streptavidin beads to remove HPA-1a antibodies and leaving antibodies reactive to the HPA-1a + αvβ3 complex only.

- To determine if there was a difference in HPA-1a antibody titre between the two cohorts. This was achieved by testing both expressed protein complexes against dilutions of the sera to establish an antibody titre.

4.2 Evidence that samples containing HLA antibodies do not bind to HEK293 cells transiently expressing αvβ3 and αIIbβ3 integrins.

HLA antibodies are formed during pregnancy and after transfusion and transplantation. A high number of NAIT referrals have HLA antibodies present (30% of multiparous women have HLA antibodies), therefore there is a requirement to distinguish between HLA and HPA antibodies (Petermann *et al.,* 2018). HEK293WT express HLA antigens and this experiment was to ensure that HLA antibodies present in some sera do not cause interference when testing against the transiently transfected HEK293 cells expressing the recombinant αvβ3 and αIIbβ3 integrins (Wozniak *et al*., 2011).

Of 18 female apheresis donors (returning to the donor panel following pregnancy), nine samples were HLA antibody positive and nine were HLA antibody negative. From the results shown in Figure 4.1, sera which contained HLA antibodies did not significantly bind to HEK293. Only one sample bound to cells expressing the αvβ3 and αIIbβ3 integrins, indicating the presence of HPA-1a antibodies. This specificity was confirmed by a standard laboratory identification method using MAIPA (Section 1.7.3). There was no significant difference in cell binding between the HLA antibody positive group and the non-HLA

antibody group (Mann-Whitney U test). There was no difference in antibody binding between the HEK293 cells expressing αvβ3 (p=0.29) and αIIbβ3 (p=0.18). The HLA positive samples were screened by One Lambda's single antigen identification kits for use on the Luminex™ 200 Fluoroanalyzer. One sample had an HLA antibody which was cognate to the HEK293 HLA-type, with a median fluorescent intensity (MFI) of over 9,000, a positive reaction would be expected to occur in the flow experiments. A positive reaction may not have occurred as protein expression may vary with tissue culture over-time.

HEK293 cells have an HLA-type which contains some high frequency antigens such as HLA-A*02, A*03; B*07, -. The HEK293 cells expressing these HLA antigens could potentially interfere with the assay and cause positive results due to HLA antibodies and not to the transfected proteins. There are techniques available to reduce the HLA expression such as an incubation with trypsin prior to harvesting. These cells were exposed to trypsin on each culture passage which may have reduced the HLA expression.

To summarise, these recombinant cells lines have the potential to be used as a screening tool for antibodies directed against the expressed proteins, HLA antibodies do not interfere in this assay. This confers confidence that the positive reactions obtained in the following patient testing are due to antibodies to antigens carried on the HPA-1a versions of αvβ3 and αIIbβ3 integrins.

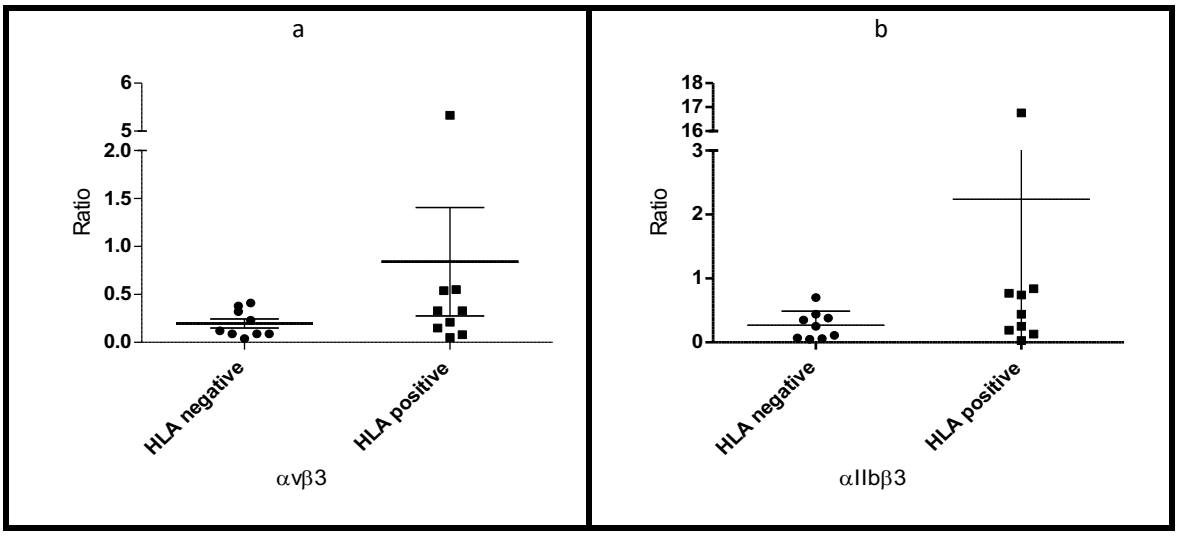

Figure 4.1. Sample ratios obtained after dividing the mean fluorescent intensity (MFI) of each sample by the mean+4SD of six negative control sera (AB sera from untransfused donors). All samples were tested with transfected HPA-1a versions of αvβ3 cells (a) and αIIbβ3 cells (b). All ratios below 1 are classed as negative. There was no significant difference between the ratios of HLA antibody positive samples to HLA antibody negative samples (αvβ3 p=0.29, αIIbβ3 p=0.18), indicating that HLA antibodies present in sera do not bind to the expressed integrin or to HEK293 cells. (Please note that the Y axis is different, and this is a representation).

4.3 First cohort of ICH and non-ICH samples tested with HPA-1a/HPA-1b versions of αvβ3 and αIIbβ3 cells

The experiment in Section 4.2 was repeated using samples from 11 mothers with confirmed NAIT and presence of HPA-1a antibodies, six of whom had an infant with ICH. These samples were tested against cells expressing αvβ3 and αIIbβ3 with either the HPA-1a protein of the β3 protein or the HPA-1b protein (Leucine to proline at amino acid 33). Therefore, the assay should be capable of detecting both HPA-1a and HPA-1b antibodies.

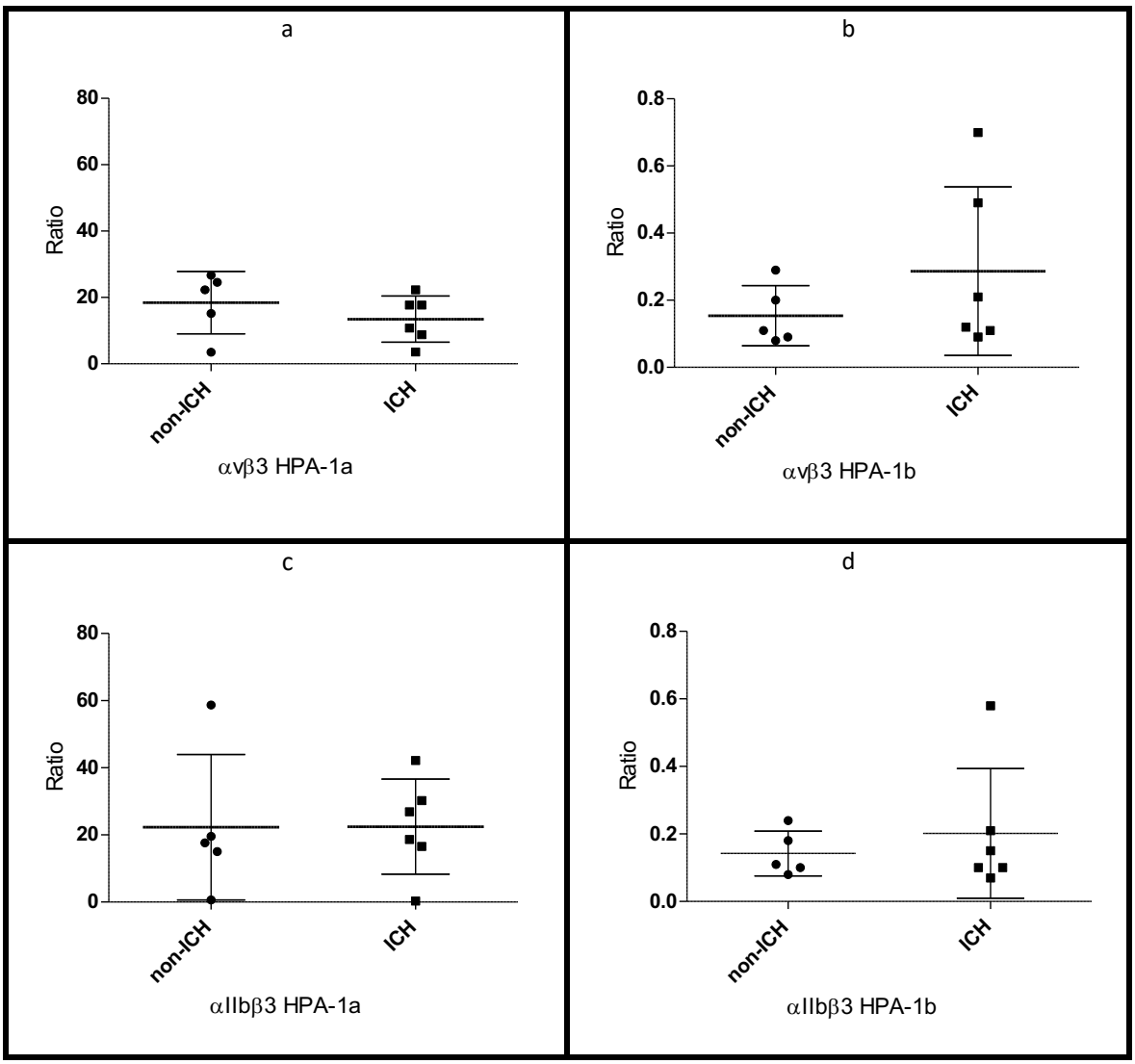

Figure 4.2. The ratios of two patient cohorts tested against HPA-1a and HPA-1b antibodies, one cohort whose fetus/baby which had ICH (n=6) and one which did not have ICH (n=5). All samples with HPA-1a antibodies were positive against the αvβ3 protein; one patient from each cohort was negative when tested against αIIbβ3 proteins. None of the samples containing HPA-1a antibodies reacted with the HPA-1b version. Ratios were calculated by dividing the mean fluorescent intensity (MFI) of each sample by the mean+4SD of six negative control sera (AB sera from untransfused donors). All ratios below 1 are classed as negative. Graphs a (αvβ3) and b (αIIbβ3) display the results for the HPA-1a versions of the proteins, whilst c and d show the HPA-1b versions.

All the samples were positive for the αvβ3 integrin which had the β3 HPA-1a protein and negative for the β3 HPA-1b protein when assessed in comparison to the negative controls (Figure 4.2). Two samples did not meet the positive threshold for the αIIbβ3 integrin (Figure 4.2). Control serum containing HPA-1b antibodies were tested against the β3 HPA-1b variant of αvβ3 and αIIbβ3 and had a positive reaction, ensuring that HPA-1b antibodies could also be detected if present (data not shown). Comparing ICH and non-ICH cohorts (Mann-Whitney U test) showed no significant difference in the ratios for binding to the αvβ3 (p=0.36) or αIIbβ3 (p=0.79) integrins (Figure 4.2).

4.4 Implications of Section 4.3 and future experiments

Section 4.3 highlighted that if an HPA-1a antibody is present in the serum then positive reactions are demonstrated against cells expressing the HPA-1a version of both the αvβ3 and αIIbβ3 integrins. Consequently, this assay cannot be used to demonstrate the presence of antibodies specific to an epitope on the αvβ3 integrin complex only. Therefore, HPA-1a antibodies should be absorbed out using cells expressing αIIbβ3 before testing for the possible presence of specific αvβ3 antibodies. Platelets cannot be used for absorption as they express both αvβ3 and αIIbβ3 integrins (Durrant *et al.,* 2017). Santoso *et al.,* (2016) utilised αIIbβ3 coupled to amino-link beads for absorptions, it was decided to modify this approach by attaching a biotinylated recombinant HPA-1a version of the recombinant β3 polypeptide (66.3kD) to streptavidin beads (Stafford *et al.,* 2009).

The first part of the experiment was to develop the absorption methodology to remove αIIbβ3 antibodies from the patient sera. After each absorption, the resin was pelleted, and supernatant removed and stored. After washing, the bound antibody was acid-eluted from the β3 coupled resin. The resin was regenerated between each sample so that it could be used multiple times for a number of samples (Section 2.13).

To determine if antibody had been absorbed onto the resin, five sequential eluates from each of three sequential absorptions were assessed for protein concentration as measured by spectrometry at A_{280}nm, and by Western Blotting. Protein concentration is displayed in Figure 4.3 showing that the amount of protein in each eluate decreases with each absorption. Figure 4.4 shows the Western Blot performed on the separate eluates (x5) for

each round of absorption (x3) and a sample of the combined eluates. The molecular markers largest product is 172kDa (blue) and the next is 81kDa (blue), IgG is around 120kDa which can be seen between the two described marker bands (Figure 4.4).

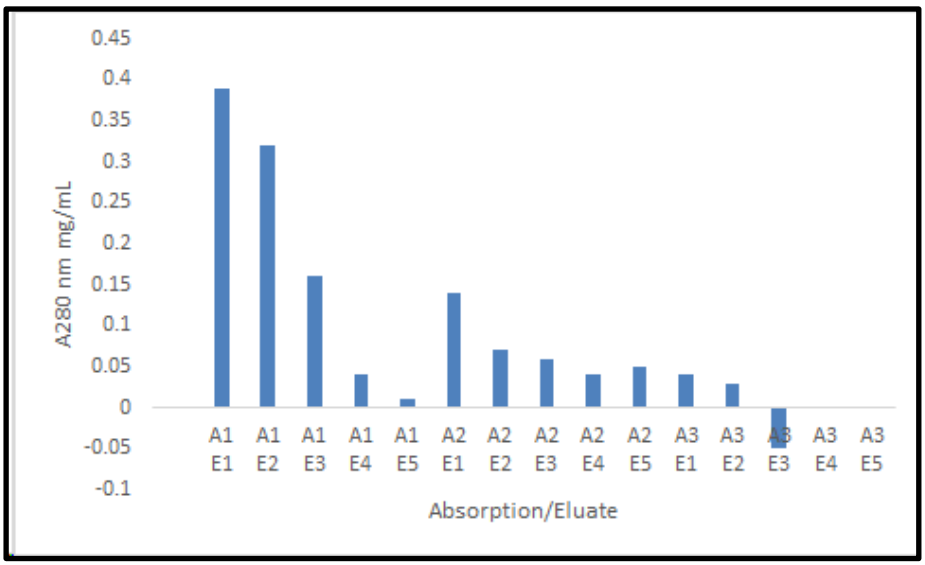

Figure 4.3. Protein concentration after each 'round' of serum absorption (A) and the resin regeneration for one sample. The resin was regenerated after absorption by stripping off non-covalently bound protein with 0.1M glycine-HCl pH 3.0, repeated five times before using it for another round of absorption. Three rounds of absorption were performed on the same sample. Eluted protein (E) was measured at A_{260}nm. As expected, absorption one, eluate one contained the most eluted protein as shown on the graph. After each round of absorption there is a drop in the amount of protein and by absorption number three eluate three there is no protein being stripped from the resin.

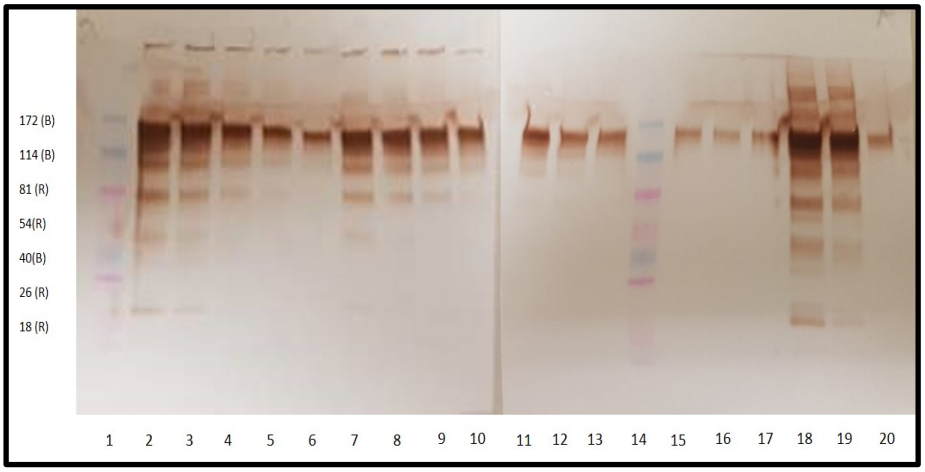

Figure 4.4. Western Blot analysis performed on the separate eluates (x5) for each round of absorption. This was to assess if there was a visible decrease in the amount of IgG after each absorption. Lane 1 and 14 is the molecular weight marker (kDa, B-Blue bands, R-Red bands in ladder), lanes 2-6 are absorption round 1 eluate 1-5. Lanes 7-11 are absorption round 2 and eluates 1-5. Lanes 12-16 are absorption round 3 eluates 1-5. Lane 17 is a molecular weight marker, lanes 18-20 is the concentrated form of the combined eluates from each round of absorption.

The eluates from each absorption (x3) were combined, concentrated and buffer exchanged into PBS using a centrifugal concentrator (30kD molecular weight cut-off) and further

analysed using the platelet immunofluorescence test (PIFT) (Section 1.7.5 and 2.15) to assess whether αIIbβ3 HPA-1a antibodies had been removed from the original control test serum sample. The PIFT assay was used instead of the transiently transfected recombinant cell lines (Chapter 3) to enable the 3 pooled eluates to be investigated quickly as this is a routine test. Table 4.1 show negative and positive control sera which had been tested with HPA-1a1a and HPA-1b1b platelets. MFIs obtained from the negative controls were used to calculate the assay positive cut-off (mean + 4 SD). The supernatants from absorptions 2 and 3 and the pooled eluate from each round of absorption were tested by the PIFT assay. The MFI was above the positive cut-off value for both absorption samples and the 3 pooled eluates (supernatant from absorption 1 was not tested due to experimental error).

Serum/Absorption/Eluate	**HPA type of frozen platelet: 1a1a,2b2b,3a3a,5b5b MFI**	**HPA type of frozen platelet: 1b1b,2a2a,3b3b,5a5a MFI**
AB serum 1	1,009	1,186
AB serum 2	992	1,152
AB serum 3	731	896
AB serum 4	756	911
AB serum 5	1041	1,256
Mean + 4SDs above is positive	1,503	1,743
HPA-1a control	5,539	610
Absorption 2	13,512	11,521
Absorption 3	9,267	6,247
Eluate from Absorption 1	23,738	2,562
Eluate from Absorption 2	2,423	1,809
Eluate from Absorption 3	677	581

Table 4.1. Analysis using PIFT to detect if HPA-1a antibodies are present after absorption methodology. The table displays the median fluorescent intensity obtained from the PIFT assay using frozen-thawed platelets of known HPA type. The mean+4SD of the negative AB serum was used as a positive cut-off. The HPA-1a antibody positive control showed a positive reaction when incubated with the HPA-1a platelets. Absorptions 2 and 3 and the pooled eluates 1 and 2 gave positive results against both HPA-1a and HPA-1b platelets, suggesting the presence of additional HLA antibodies in the control test serum sample.

The PIFT assay (Table 4.1) and Western Blot (Figure 4.4) suggested that three absorptions were sufficient to remove HPA-1a antibodies present in the control test serum sample. However, the PIFT results suggest that HLA antibodies were contributing to the positive results as reactions were obtained against HPA-1b1b (HPA-1a negative) platelets. This sample was tested using an HLA antibody screening kit, LSM12 analysed on a Luminex™ 200 Fluoroanalyzer and found to be pan-reactive for HLA Class I antibodies confirming that the PIFT assay is unsuitable for validating the removal of αIIbβ3 antibodies.

4.5 Absorption studies using transiently transfected cells for antibody detection

Section 4.2 showed that HLA antibodies do not affect results when using HEK293 cells. Therefore, transiently transfected HEK293 cells expressing αvβ3 and αIIbβ3 integrins were used (Chapter 3).

Samples from the study cohort were selected based on the volume of serum available for each patient. In total three ICH sera and three non-ICH sera were selected, and each was absorbed three times with peptide conjugated resin (Figure 4.4). When these sera were tested (neat and diluted 1/4) against the transfected HPA-1a versions of αvβ3 and αIIbβ3 HEK293 cells, each serum was positive prior to absorption and negative following absorption with both integrins (Figure 4.5). There was no difference between the ICH and non-ICH cohort in binding to either integrin suggesting the removal of HPA-1a antibodies from both cohorts and the lack of antibodies directed against the αvβ3 integrin in either cohort (Figure 4.5). Even though the non-ICH had higher ratios to both integrins than the ICH sample at neat and 1/4 dilution.

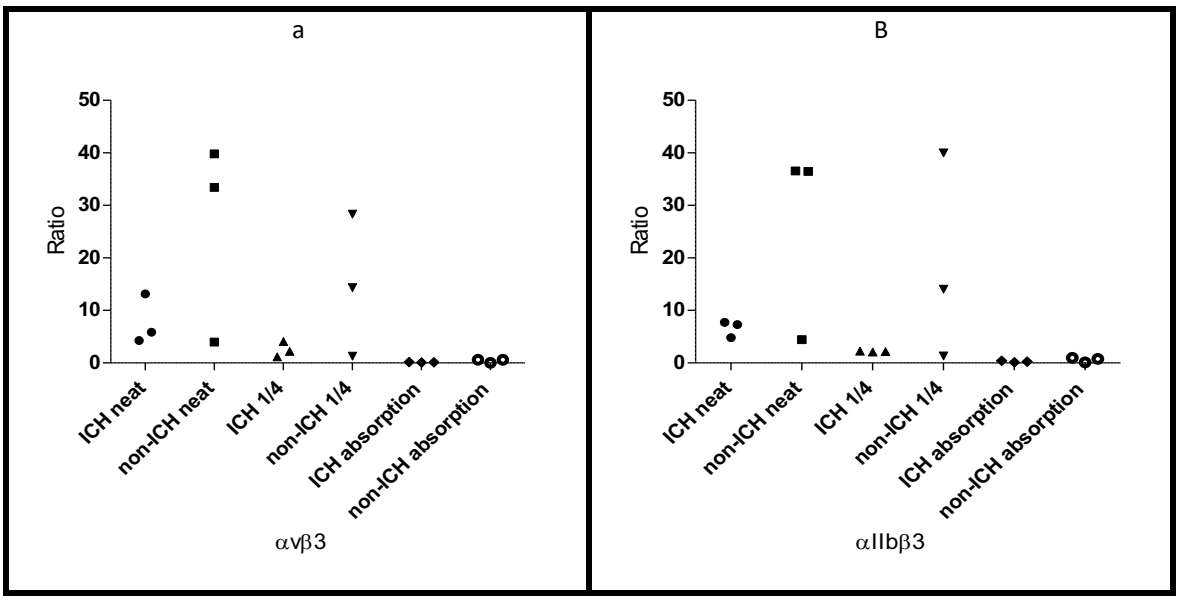

Figure 4.5. Results of testing neat and 1/ 4 dilution and after three absorptions. Three samples from each cohort were tested pre-absorption (neat and diluted 1/ 4) and post 3x absorptions. Each sample was incubated with the HPA-1 versions of either αvβ3 (a) or αIIbβ3 (b) cells and stained using a PE-conjugated anti-human IgG before analysis on a flow cytometer. Absorbed sera all had a ratio <1 and were negative against αvβ3 and αIIbβ3. Ratios were calculated by dividing the mean fluorescent intensity (MFI) of each sample by the mean+4SD of six negative control sera (AB sera from untransfused donors). All ratios below 1 are classed as negative.

4.6 Antibody titre experiments using HEK293 cells transfected with HPA-1a positive αvβ3 and αIIbβ3 integrins

These experiments were performed to assess if there was a difference in antibody titre between the two patient cohorts (ICH versus non-ICH) and were tested against the two different αvβ3 and αIIbβ3 HPA-1a positive integrins.

Five sera from both cohorts were tested neat and diluted 1/4 (Figure 4.6). The dilution was to detect a possible 'prozone' effect. Prozone is a phenomenon whereby a high titre antibody blocks the binding of other antibody molecules which can result in a false negative or a 'weak' reaction (Weinstock & Schnaidt, 2012). The sera were tested in triplicate to account for test variation (Table 4.2).

Sample Numbers	αvβ3 Integrin testing in triplicate			αIIbβ3 Integrin testing in triplicate		
	αVβ3 T1	αVβ3 T2	αVβ3 T3	αIIbβ3 T1	αIIbβ3 T2	αIIbβ3 T3
6245 ICH	4.22	4.27	8.62	4.44	7.73	8.84
6245 1/4	2.76	2.22	3.11	2.54	2.27	2.95
5481 ICH	3.49	5.34	7.71	9.18	9.06	11.11
5481 1/4	4.56	5.87	9.52	5.72	7.85	9.45
4311 ICH	1.99	5.02	3.7	1.55	8.23	3.43
4311 1/4	2.41	2.41	0.936	3.02	2.22	3.04
6886 ICH	3.91	4.34	10.2	4.57	7.46	15.04
6886 1/4	3.59	3.74	3.74	3.51	4.08	4.88
5665 ICH	5.01	3.52	9.06	7.04	5.91	10.41
5665 1/4	3.81	4.48	5.5	4.42	4.63	5.57
1546 non-ICH	4.4	6.33	10.9	4.99	6.46	16.7
1546 1/4	2.11	2.19	3.07	2.91	3.51	3.39
0178 non-ICH	3.85	6.26	10.2	6	7.02	10.04
0178 1/4	3.2	3.43	4.69	3.62	2.54	3.89
2864 non-ICH	2.98	4.98	9.6	5.83	6.24	11.23
2864 1/4	2.76	2.01	3.13	3.07	2.93	3.79
2728 non-ICH	2.43	6.94	11.9	9.76	8.23	18.21
2728 1/4	3.45	5.94	6.86	4.64	5.8	9.7
3698 non-ICH	2.49	3.99	6.86	4.83	4.43	6.41
3698 1/4	1.69	1.33	1.48	1.54	1.36	1.45

Table 4.2. Results of testing ICH and non-ICH serum at neat and 1/4 dilution to assess the 'prozone' effect. Displayed are the ratios of the MFI divided by the cut-off of 4SD above the mean of the negative controls, a ratio above 1 is positive. These samples were tested in triplicate (T1-T3). Only one sample (5481) had a repeatedly higher positive ratio when diluted at 1/4 against αvβ3 expressing cells but not with the αIIbβ3 expressing cells. Highlighted in yellow are the ratios for samples which are higher on dilution than the neat sera. As can be seen these are not reproducible across the triplicate testing suggesting experimental variation. Sample 4311 gave a negative reaction in one assay, highlighted in grey.

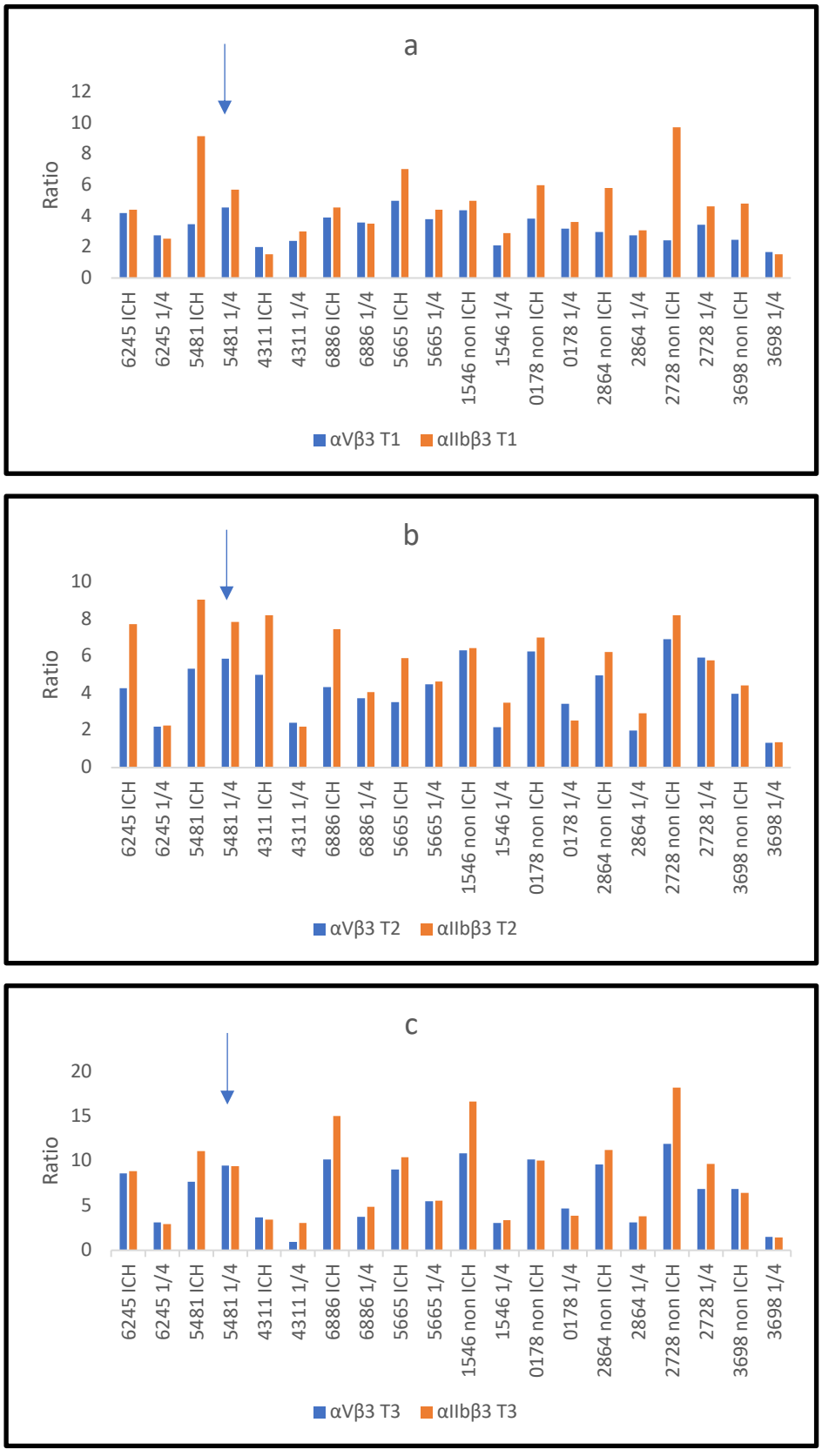

Figure 4.6. Testing ICH and non-ICH serum at neat and 1/4 dilutions to detect a possible 'prozone' effect. Each sample was incubated with either αvβ3 or αIIβ3and stained using a PE-conjugated anti-human IgG before analysis on a flow cytometer These graphs show the ratios of the samples which are calculated using six negative control sera (AB sera from un-transfused donors) mean+4SD a ratio above 1 is positive. Samples were tested in triplicate (T1-T3). Each triplicate is displayed as a separate graph; a is test 1, b is test 2 and c is the results from the third set of tests. One sample (5481 -blue arrow indicating) repeatedly yielded a higher positive ratio at 1/4 dilution with the αvβ3 expressing cells but not with the αIIbβ3 expressing cells.

A prozone effect, indicating a strong antibody, might only be seen in ICH. However, only one sample from the ICH cohort (sample number 5481) reproducibly gave a higher positive ratio in the dilution against the αvβ3 integrin and not against the αIIbβ3 integrin (Table 4.2 and Figure 4.6). This suggested that this sample had a high titre antibody that could differentiate between the αvβ3 and αIIbβ3 integrins. Other than sample 4311, all samples were positive at neat (undiluted) and 1/4 for both αIIbβ3 and αvβ3 expressing cells (Table 4.2). There was no statistical difference observed when comparing the ICH versus non-ICH cohort to αvβ3 and αIIbβ3, did not show any statistical difference of binding to the different integrins at neat or 1/4 dilution (Mann-Whitney U test). Table 4.2 and Figure 4.6 shows that a prozone effect was present in one sample (5481).

Kruskal-Wallis analysis of neat sera comparing all groups and integrins gave p=0.0002 with higher ratios for both cohorts to αIIbβ3 expressing cell, comparing αvβ3 integrin between the two cohorts gave a p=0.0015; the αIIbβ3 integrin for the two cohorts yielded p=0.05 with higher reactivity for both cells in the non-ICH cohort. However, no differences were detected in these three testing modes at 1/4 dilution, and this together with the poor reproducibility of testing, cast doubts on the reliability of the results which were positive on single occasions. Further statistical work was performed on the neat dilutions to try and elucidate where the differences between the groups were. This was performed using Mann-Whitney U test, comparing each testing triplicate test result for ICH v non-ICH for both integrins. Table 4.3 shows that this did not reveal clear differences were, suggesting variation in results in different experiments (day of testing) might have accounted for apparent differences between the cohorts.

Parameter	Neat p value	1/4 p value
T1 αvβ3 ICH v T1 αvβ3 non-ICH	0.55	0.17
T2 αvβ3 ICH v T1 αvβ3 non-ICH	0.22	0.31
T3 αvβ3 ICH v T1 αvβ3 non-ICH	0.17	0.84
T1 αIIbβ3 ICH v T1 αIIbβ3 non-ICH	0.42	0.69
T2 αIIbβ3 ICH v T1 αIIbβ3 non-ICH	0.25	0.69
T3 αIIbβ3 ICH v T1 αIIbβ3 non-ICH	0.42	0.84

Table 4.3. Results of Mann-Whitney U statistical analysis for each of the neat (undiluted) groups of ICH and non-ICH. This was performed using the data from the triplicate testing ratios obtained from neat and 1/4 dilutions for ICH and non-ICH groups. The above displayed was only for neat sera testing with both αvβ3 and αIIbβ3 cells to try and interrogate which may cause a significant difference when analysed together by Kruskal -Wallis analysis.

To determine a suitable dilution, range for further testing, titrations studies were performed on one serum sample from each cohort (5481-ICH and 2728-non-ICH). These samples were tested at neat, 1/2, 1/4, 1/16, 1/64, 1/256 and doubling dilutions to 1/2056 (Figure 4.7). Antibody titre, defined as the most dilute value that had a positive reaction with the αvβ3 and αIIbβ3 expressing cells, was 1/16 for both cohorts.

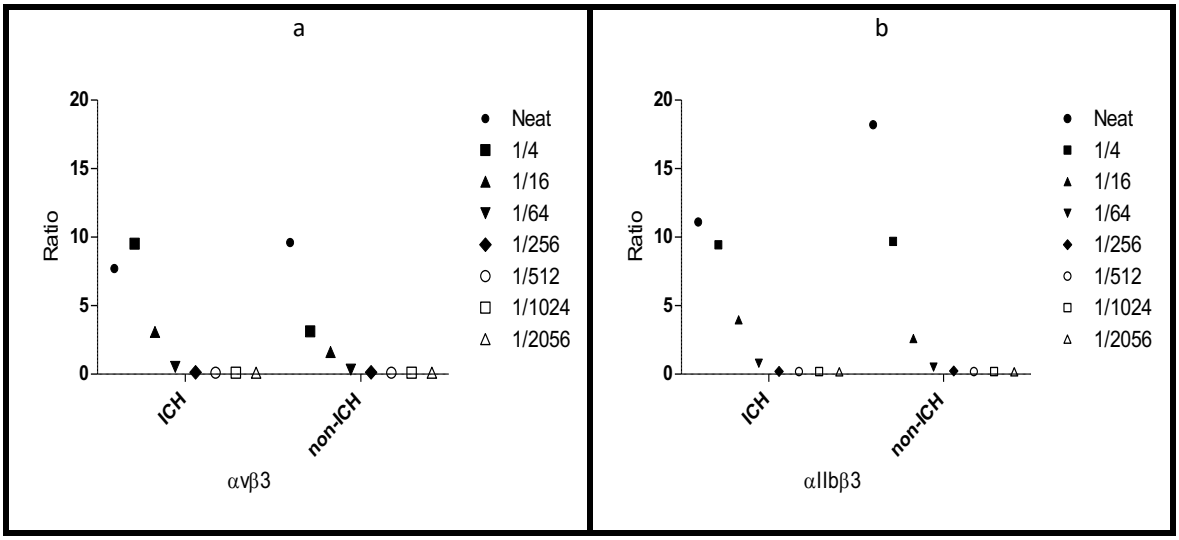

Figure 4.7. Results of titration studies from neat to 1/2056. A sample was chosen from each cohort to test at a number of dilutions against each of the integrins. Displayed are the ratios of the mean fluorescent intensity (MFI) divided by the mean of the negative controls plus 4SD. All ratios above one are classed as positive. For both the ICH and non-ICH sample the antibody titre was 1/16 to both αvβ3 (a) and αIIbβ3 (b) expressed cells.

To achieve higher precision, another series of assays was performed using doubling dilutions from neat to 1/64, in duplicate and on different days. Six samples from each

cohort were included, these were selected based on the amount of serum left in the aliquot.

No difference in titre was seen between the two cohorts. There were no major differences in titre between the αvβ3 and αIIbβ3 expressed cells (Figure 4.8). One sample from the non-ICH cohort (3139) was negative with both integrins. Five samples had the same antibody titre with both αvβ3 and αIIbβ3 cells, whilst another five samples resulted in a higher antibody titre with αvβ3 than αIIbβ3 cells. Only one sample gave a higher titre with αIIbβ3 cells and one sample from each cohort had an antibody titre of 1/64. Table 4.4 shows the p-values for the multiple Kruskal-Wallis analysis performed for each cohort, which included the dilutions and duplicate testing, there was no significant differences found between the cohorts. Interestingly sample 6828 had a noticeably higher reaction when comparing ratios to the αIIbβ3 cells than the αvβ3 cells which was reproducible (Figure 4.8).

Dilutions	**p-value of testing both cohorts against αvβ3/ αIIbβ3 cells**
Neat	0.74
1/2	0.88
1/4	0.98
1/8	1.00
1/16	1.00
1/32	1.00
1/64	1.00

Table 4.4. Results of Kruskal-Wallis statistical analysis between ICH and non-ICH cohorts. This was performed using the ratios obtained from duplicate testing of serum serial dilutions from neat to 1/64.

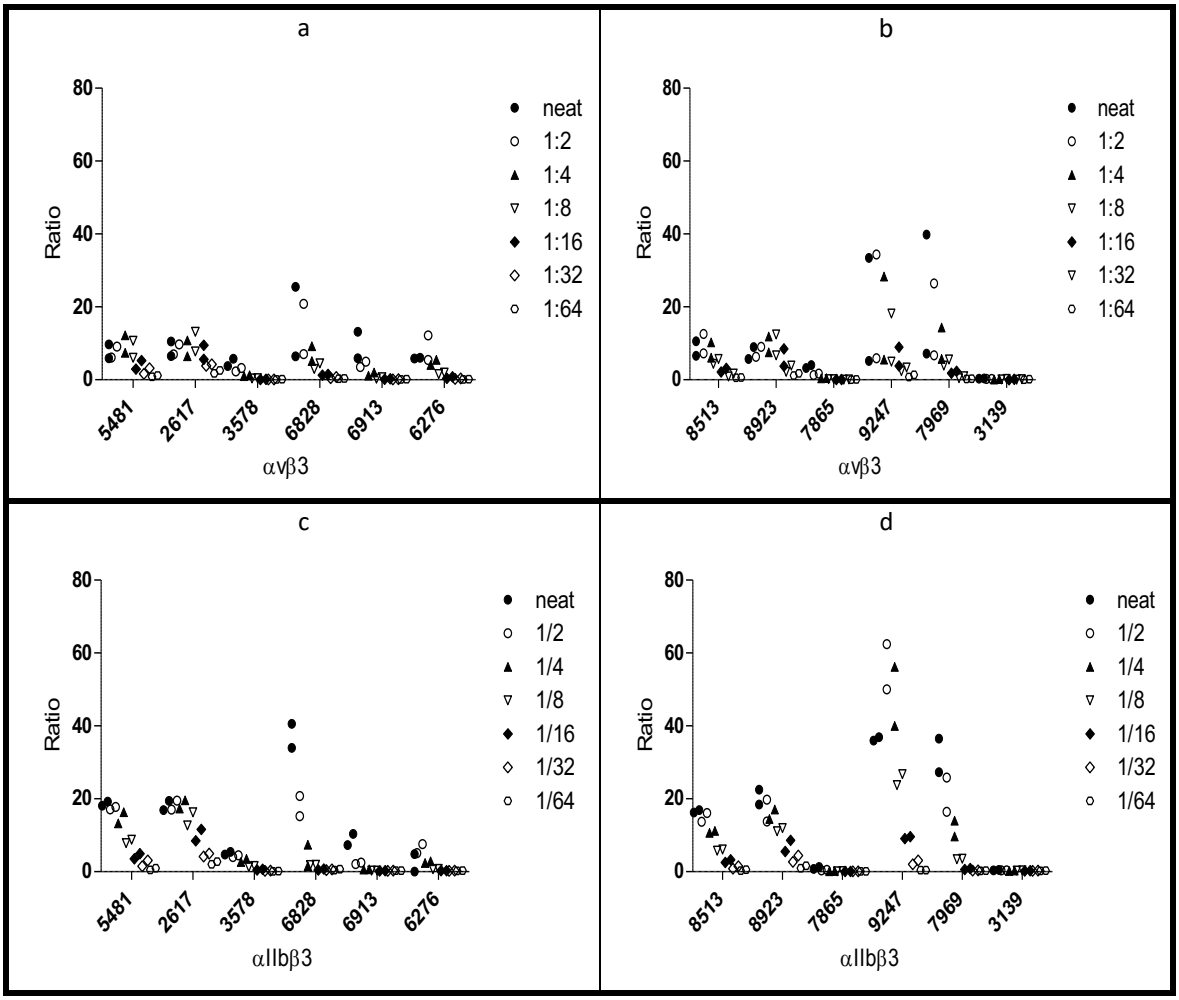

Figure 4.8. Titre testing of samples against the different expressing cells. Each sample was incubated in duplicate (different days) with either αvβ3 or αIIbβ3 expressing cells and stained using an RPE-conjugated anti-human IgG before analysis on a flow cytometer. These graphs show the ratios of the samples which are calculated using six negative control sera (AB sera from un-transfused donors) mean+4SD. A ratio above one is classed as positive. Graphs a and c show the ICH tested with the αvβ3 and αIIbβ3 expressed cells, whilst b and d are non-ICH samples for the cells. There were no significant differences in dilutions between the two cohorts or in binding of αvβ3 and αIIbβ3 integrins.

4.7 Summary and discussion

The primary aim of this chapter was to investigate if the presence of specific αvβ3 antibodies could be used to predict ICH in the fetus or new-born in NAIT cases by testing sera from two patient cohorts (ICH and non-ICH). An additional aim was to test whether there was a difference in HPA-1a antibody titre to αvβ3 or αIIbβ3 between the two cohorts which may also aid in predicting ICH in NAIT cases. This was assessed using HEK293 cells transiently expressing either HPA-1a versions of αvβ3 or αIIbβ3 integrin, as validated in Chapter 3. Table 4.5 indicates the clinical conditions of all the samples tested in this Chapter. Some information is missing such as sex of the fetus, platelet count and transfusion history. This is a problem with using retrospective data.

Cohort	Sample number	Platelet count at birth	Platelet Transfused	Sex of newborn
ICH	6625	3	Y	M
	6828	9	Y	M
	0213	9	Y	UNK
	0341	120	N	M
	5665	12	NS	M
	7563	48	Y	UNK
	3578	15	Y	UNK
	6886	9	NT	F
	6276	UNK	UNK	UNK
	2617	17	Y	F
	5481	12	N	M
	6245	UNK	UNK	F
	6913	UNK	UNK	UNK
Non-ICH	1546	63	UNK	M
	2651	10	Y	M
	8513	62	N	M
	0178	9	Y	M
	2651	12	Y	F
	2864	UNK	Y	F
	3139	15	Y	M
	3698	9	Y	F
	7865	11	Y	UNK
	7969	64	N	UNK
	9247	69	Y	M
	8923	82	N	M

Table 4.5. Medical history of samples tested in this chapter. UNK means this information is unavailable. This includes all samples tested within this chapter. Further information is not available.

The first objective was to investigate if HLA antibodies present in serum would react with HEK293 cells regardless of integrin expression. Section 4.2 reports that samples containing HLA antibodies but not HPA-1a antibodies do not generate positive reactions against cells expressing αvβ3 or αIIbβ3. Thus, transfected HEK293 cells can be used to screen for specific αvβ3 and αIIbβ3 antibodies without false positive reactions due to HLA antibodies. Unexpectedly, one of the control donors from the TRALI panel had HPA-1a antibodies, which was confirmed by standard laboratory techniques. This indicates that NAIT is under-reported when an HPA-1a antibody was detected in such a small pool of donors by chance, but there was no clinical follow-up to know if the baby had a low platelet count or any clinical symptoms.

Objective 2 was to investigate whether there was a difference between the two patient cohorts when testing with the individually expressed αvβ3 or αIIbβ3 HEK293 cells. HPA-1a and HPA-1b versions of the β3 integrin were co-expressed with both αv and αIIb integrins and used to determine whether specific antibodies reacting only with αvβ3 could be identified. Samples from the ICH and non-ICH cohorts showed that they bound to the HPA-1a version but not the HPA-1b version of the protein (Section 4.3). There was no notable difference between the ICH and non-ICH groups when binding to HEK293 cells expressing αvβ3 or αIIbβ3 (Section 4.3, Figure 4.2).

Santoso *et al.,* (2016) postulated that NAIT cases presenting with ICH, may be due to HPA-1a antibodies reacting with a compound antigen formed by the complex of the αv and β3 subunits in a similar way that is accepted that some HPA-1a antibodies only recognise compound antigens carried on the αIIbβ3 complex. Compound epitopes are observed in some blood group systems e.g., anti-f (ce) and anti-G (CD) in the Rh system (Pourazer, 2007). NAIT samples containing HPA-1a antibodies will bind to the HPA-1a β3 subunit on both the αvβ3 and αIIbβ3 complexes making it difficult to distinguish antibodies binding to the αvβ3 complex only. Therefore, this group established a methodology whereby antibodies directed to the β3 subunit and the compound αIIbβ3 were absorbed out leaving antibodies reacting only with the αvβ3 complex. The group were then able to establish that αvβ3 antibodies were present in their ICH cases and not in the non-ICH cases.

Objective 3 was to design a system which would remove all HPA-1a antibodies, except those reacting with the αvβ3 complex, from patient sera used in this study. This was to establish if there was a difference between the ICH and non-ICH cohorts and whether the presence of αvβ3 antibodies could predict ICH cases. A 66.3kD β3 biotinylated peptide subunit was attached to streptavidin resin which could be regenerated and reused to remove the HPA-1a antibody.

PIFT and Western Blot were used to determine the number of absorptions required to remove αIIbβ3 HPA-1a antibodies which indicated that three rounds of absorptions were required per sample (Section 4.4). Three patient serum samples from the ICH and non-ICH cohort were absorbed using β3-resin and tested with the HEK293 cells expressing the αvβ3 and αIIbβ3 integrins. The absorbed samples from both cohorts were negative when tested

with cells expressing either αvβ3 or αIIbβ3 (Section 4.5). Only a small number of samples were tested; however, these results were not able to support the findings of Santoso *et al*., (2016) that compound αvβ3 antibodies are present in NAIT cases presenting with ICH.

The final objective for this chapter was to assess if there was a difference of HPA-1a antibody strength in the two patient cohorts which could be linked to predicting severity in NAIT. Multiple serum samples from each cohort were tested at a range of dilutions against HEK293 cells expressing either αvβ3 or αIIbβ3 (Section 4.6). In this study, no difference in antibody titre or specific integrin binding was demonstrated between the two cohorts.

The literature is conflicted on whether antibody titre can be used to predict severe NAIT. This could be explained by various laboratories using different methodologies, such as ELISA or MAIPA or differing reagents to determine titre (Kjaer *et al.*, 2019). One study highlighted this by comparing ELISA and MAIPA and showed there were differences in results between the two methods (Bessos *et al*., 2008a). A recent study has stated that if a quantitative antibody test is performed, it should be by MAIPA (Kjaer *et al.*, 2019). This current study used an immunofluorescence test using membrane-bound transient transfected proteins, which is an alternative method of stably expressing recombinant heterodimeric integrins. To produce a consistent reagent for use in the PIFT, stable cell lines would need to be produced, the intact protein dimer on the cell surface would provide antigen presentation which would possibly mimic *in vivo* environment without the hindrance of HLA antibodies. Therefore, differences in results could be accounted by differences in methodology which is supported in the current literature (Kjaer *et al*., 2019). There are differences in the literature between prospective and retrospective studies. The current study supports no link between antibody titre and severity, consistent with other retrospective studies (Proulx *et al*., 1994; Kille *et al.*, 2007; Ghevaert *et al*., 2007). However, prospective studies have supported the link with titre and decreased platelet counts at birth (Williamson *et al.,* 1998, Bessos *et al*., 2008, Killie *et al.,* 2008). A problem with retrospective studies is there are inevitably variations in sampling methodology -: timing, storage, number of freeze/thaws, which could all affect antibody integrity, and may not be fully documented. Prospective studies have all the relevant information, and the sample integrity is known such as date bled and receipt into the laboratory. Other studies noted differences in weeks' gestation and link with titre and severity (Killie *et al*., 2008; Bertrand

et al., 2011; Jaegtvik *et al*., 2000). Although this information was not available for this study, it may aid in explaining the differences in retrospective and prospective studies.

In summary, this chapter has shown that HPA-1a versions of αvβ3 and αIIbβ3 expressing HEK293 cells react with known HPA-1a antibody positive sera from ICH and non-ICH NAIT cases. The HPA-1a antibody sera did not react with HPA-1b versions of the two integrins. Limited absorption studies on the different cohorts could not identify if there were αvβ3 antibodies present in addition to β3 and αIIbβ3 antibodies, perhaps due to absorption methodology. Investigations did not indicate any differences in antibody titre within the two cohorts to be able to predict ICH.

Chapter 5: Results Chapter 3

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5.1 Introduction

The main objective of the experiments presented in this chapter is to establish if there is a difference in HPA-1a antibodies, both total IgG and/or any of the IgG subclasses, between intracranial haemorrhage (ICH) and non-ICH cohorts. Additional cohorts of post-transfusion purpura (PTP) and platelet refractory patients were investigated to help establish differences in IgG subclass for HPA-1a antibodies and whether the corresponding data could potentially be used to be able to predict which NAIT cases are at risk of ICH.

There has been limited research on the different IgG subclasses involved in NAIT. IgG1 and IgG3 are reported to cause NAIT and HDFN as these are T cell-dependent antibodies (Bruhns *et al*., 2008). One study found an increased concentration of IgG3 in post-delivery sera in NAIT-affected pregnancies (Proulx *et al*., 1994). Functional assays have shown that complement is not activated in NAIT and therefore antibody-dependent cell-mediated cytoxicity (ADCC) causes platelet destruction (Proulx *et al*., 1994). One study reported that the IgG1 subclass is the predominant alloantibody in platelets, in addition IgG3 is present in combination with IgG1 (Kumpel & Manoussaka, 2011).

PTP patients are usually female who have previously made HPA-1a antibodies (possibly from undiagnosed NAIT). It is a condition with unknown pathogenesis that causes a catastrophic thrombocytopenia 7-9 days' post transfusion. It is thought that firstly there is an alloimmune response by HPA-1a antibodies to donated platelets followed by an autoimmune response which destroys the patient's own platelets (Li *et al*., 2012). In addition, there are patients referred to the laboratory who are refractory to platelet transfusions and are found to have HPA-1a antibodies. The definition of refractory is the failure to increment over $10x10^9$/L of platelets on two occasions with blood group-compatible apheresis platelets (single units) (Stanworth *et al*., 2015).

Antibodies generated in solid organ transplantation have been extensively studied to try and understand the mechanism of organ rejection. Lowe *et al.,* (2013) have studied the IgG subclass distribution in kidney organ transplant patients and compared these with sensitising events. They revealed that HLA sensitised by pregnancy or previous transplants resulted in a serum IgG antibody subclass concentration in the order IgG1>IgG3>IgG2>IgG4.

This supports the literature in NAIT, with IgG1 and IgG3 being found to be the predominant subclasses in serum, consistent with the hypothesis that pregnancy was the sensitising event.

An additional study in kidney rejection analysed IgG subclasses to establish if antibody mediated rejection and graft loss could be predicted, they showed that IgG4 was indicated (Khovanova *et al*., 2015). Higher levels of donor specific HLA antibodies of IgG4 subclass in patient serum was a risk factor for both antibody mediated rejection and graft loss and it was hypothesised that this could be a biomarker to predict these outcomes and be added to a risk stratification for decision making to transplant against these antibodies (Khovanova *et al*., 2015). The assumption was that this is a mature response that has undergone class switching and affinity maturation and the antibodies would have a higher affinity to their respective antigens than other subclasses (Khovanova *et al*., 2015). Due to the limited number of studies on antibody subclass in NAIT, this concept has not been investigated. A multiparous female could potentially have a more mature HPA-1a antibody response in NAIT, which could indicate IgG4, such as in the case of kidney rejection.

Luminex™ bead-based technology was used in the present study to assess if there is a difference in IgG subclass between different patient cohorts namely, NAIT babies with ICH, NAIT babies with no ICH, PTP and platelet refractory patients. All patients in the defined cohorts possessed HPA-1a specific antibody detected by MAIPA. Recombinant β3 fragment proteins incorporating the HPA-1a and HPA-1b antigens and a further GPVI protein were produced, validated and attached to Luminex™ beads by Chong *et al.,* (2011). The same recombinant proteins were available for this study and were attached to Luminex™ beads as described in the above paper. The novel aspect of this study was the use of different conjugated secondary antibodies specific to each subclass.

Luminex™ technology allows a rapid antibody detection method which can test multiple samples in under 3 hours (Section 2.17 for methodology). Briefly, samples were incubated with beads and following washes, the antibody conjugate was added for a further incubation and further washes and then analysed on the Luminex™. Analysis was performed using either the HPA-1a or HPA-1b bead against the inert bead (GPVI), together

with negative AB serum. For a test batch to be valid, a positive result had to be obtained with HPA-1a or HPA-1b beads using known test sera.

5.2 Establishing a positive cut-off for multiplex beads

Chong *et al.,* (2011) developed methodology and validated the production of HPA-1a, HPA-1b and inert GPVI beads for use in HPA antibody detection and determined the positive/negative cut-off threshold for the assay based on the One Lambda™ equation used for HLA screening kits. This method of calculation was employed in the current study and the first series of experiments aimed to establish the positive/negative cut-offs for total IgG and for each of the IgG subclasses to be used for all subsequent testing.

The transfusion acute lung injury (TRALI) risk reduction samples described in Section 4.2 were used to establish positive cut-offs for total IgG and each of the IgG subclasses. These samples were all from females who had recently returned to the apheresis panel following pregnancy. Median fluorescence intensity (MFI) output from the Luminex™ instrument was used in the calculation. The LABScreen One Lambda™ equation to obtain ratios used in this study was:

$$\text{Ratio} = \frac{\text{Sample MFI for HPA-1a or HPA-1b/ Sample MFI for GPVI}}{\text{AB serum MFI for HPA-1a or HPA-1b/ AB serum MFI for GPVI}}$$

For example:

Sample 4311 (see Table 5.2)

$$\text{Ratio } 1.12 = \frac{\text{Sample MFI of 13886 for HPA-1a bead/Sample MFI of 9424 for GPVI bead}}{\text{AB serum MFI of 4474 for HPA-1a bead/AB serum MFI of 3388 for GPVI bead}}$$

Ratios were calculated for the 22 TRALI risk reduction samples and the mean and standard deviations determined. The cut-off is the mean plus 3SDs. Table 5.1 shows the value for total IgG and each of the IgG subclasses used as the positive cut-off in all patient testing.

IgG Class	HPA-1a	HPA-1b
Total IgG	1.58	1.67
IgG1	3.63	2.19
IgG2	2.28	1.62
IgG3	3.13	2.58
IgG4	2.26	2.01

Table 5.1. Establishing positive cut off values for the Luminex™ Assay. Female TRALI risk reduction samples who had returned to the apheresis panel after pregnancy were used to establish the positive cut-off ratios for total IgG and each subclass. The beads were incubated with samples and a negative control AB serum was included to calculate the ratios. The mean and SD were established for each subclass and the cut-off is the mean plus 3 SDs. This positive cut-off range was used for all patient testing.

5.3 Patient antibody testing

Samples from the different cohorts were tested randomly over different days in duplicate and sometimes triplicate and the mean ratios taken. In each group there were samples that did not react with the HPA-1a beads when tested for total IgG, these samples were removed from all analyses including subclass analysis. On reviewing the raw data, it was noted that in some samples, there was a positive shift in the MFI value with the HPA-1a beads, but when the ratios were calculated these samples fell below the positive cut-off value. This is due to the samples binding non-specifically with the HPA-1b and GPVI beads (which is classed as high background). Table 5.2 is an example of the data obtained from these samples which were excluded. If one of the duplicates failed to reach the positive cut-off and the other was over the threshold, the average was taken. Table 5.3 details the final number of samples tested and included in the analysis from each group and the samples that were removed.

Sample Number	Median Fluorescent Intensity			Ratio	
	GPVI	HPA-1a	HPA-1b	HPA-1a	HPA-1b
Negative	3388	4474	3690	NA	NA
4311	9424	13886	9230	1.12	0.90
4234	14496	19467	14600	1.03	0.91
3578	771	1401.5	645	1.39	0.76

Table 5.2. An example of the raw data of samples which was not included in the analysis. Displayed are the raw data (MFI) and calculated ratios for samples which did not meet the positive threshold due to high background for total IgG, as indicated by the reaction to both GPVI and HPA-1b beads, when analysed on a Luminex™ 200 instrument.

Patient group	Number of Samples	Number Excluded	Number included for analysis
Intracranial haemorrhage	20	5	15
Non-intracranial haemorrhage	26	3	23
Post transfusion purpura	9	2	7
Platelet refractory	25	10	15

Table 5.3. The number of samples which were selected for this study and the numbers which did not react with the HPA-1a beads and were excluded from subsequent analysis.

5.4 Intracranial haemorrhage cohort

All samples were tested for total IgG and IgG subclasses in the same assay. Figure 5.1 displays the HPA-1a average ratios; data on HPA-1b are not included as the value of ratio was negative for this group. Some samples were removed from the analysis if they had false-positive ratios for HPA-1b beads in the relevant subclass analysis (IgG1, IgG2 and IgG3). Data is available in Table 5.4 relating to the results for HPA-1b ratios and analysis. Table 5.4 displays the mean, range and standard deviation (SD) for total IgG and all the subclasses from this cohort. In relation to HPA-1a antibodies, IgG1 had the highest mean ratio whereas the biggest range of ratios was for IgG3, though the majority of HPA-1a antibody ratios (except IgG2) had a high SD, indicating the variability in the samples tested. Thirteen out of the fifteen samples from the ICH group were positive for IgG1, but only 6, 5 and 2 for IgG2, IgG3 and IgG4 respectively. Interestingly, the raw MFI data for IgG2-IgG4 was very low, <500 in most cases for all cohorts which indicates that these antibody subclasses are not present. These results indicate that for patients with ICH, the predominant IgG subclass is IgG1.

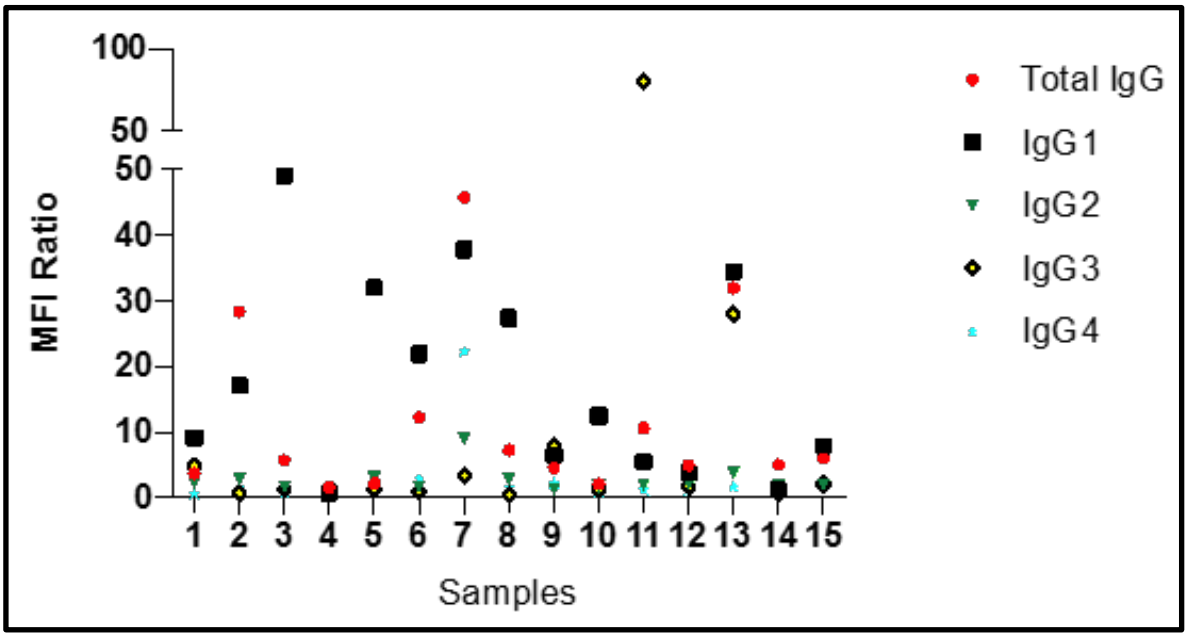

Figure 5.1. Ratios for HPA-1a reactivity in the ICH cohort. These data were obtained by incubating the sera with the Luminex™ beads and adding an IgG conjugate for total and each IgG subclass and analysed on a Luminex™ analyser. Ratios were calculated using the standard equation for Luminex™ HLA screening kits. The Y-axis scale reflects the variability in ratios for the different subclasses.

Calculations	Total IgG		IgG1		IgG2		IgG3		IgG4	
	HPA-1a	HPA-1b	HPA-1a	HPA-1b	HPA-1a	HPA-1b	HPA-1a	HPA-1b	HPA-1a	HPA-1b
Mean	11.51	0.88	17.83	1.10	2.64	1.00	9.05	0.98	2.89	0.78
Min	1.61	0.69	0.77	0.42	1.18	0.64	0.46	0.18	0.73	0.38
Max	45.82	1.01	49.14	1.39	8.86	1.29	80.05	1.40	22.38	1.33
SD	13.17	0.08	15.16	0.25	1.86	0.21	20.85	0.30	5.42	0.28

Table 5.4. An overview of the statistics for total IgG and each of the subclasses obtained of the ICH cohort. Ratios were calculated using the standard equation for Luminex™ HLA screening kits. Total IgG and IgG1 had the highest ratios. The data for IgG2 and IgG4 had low raw data MFI values which make the ratios seem in the table above higher than what they should be in a 'normal' distribution.

5.5 Non-intracranial haemorrhage cohort

Figure 5.2 shows the HPA-1a mean antibody ratios for the samples and not the results for the HPA-1b bead testing. Some samples were removed from the subclass analysis if they had positive HPA-1b bead result (IgG1 and IgG3). Table 5.5 displays the mean, range and standard deviation (SD) for total IgG and all the subclasses from this cohort, including the HPA-1b testing results. IgG3 had the highest mean ratio for HPA-1a antibodies, followed by IgG1 and total IgG. Again, the biggest range of ratios were for IgG3, but this subclass had the highest SD of 67.69, followed by IgG1, which also had a high SD of 42.05, showing the variability of samples. Twenty out of the twenty-three samples from this cohort were

positive for IgG1, whereas 12 were positive for IgG2, 10 were positive for IgG3 and 7 for IgG4. As displayed in Figure 5.2, there were a number of samples which had a higher HPA-1a bead ratio to IgG3 than to IgG1.

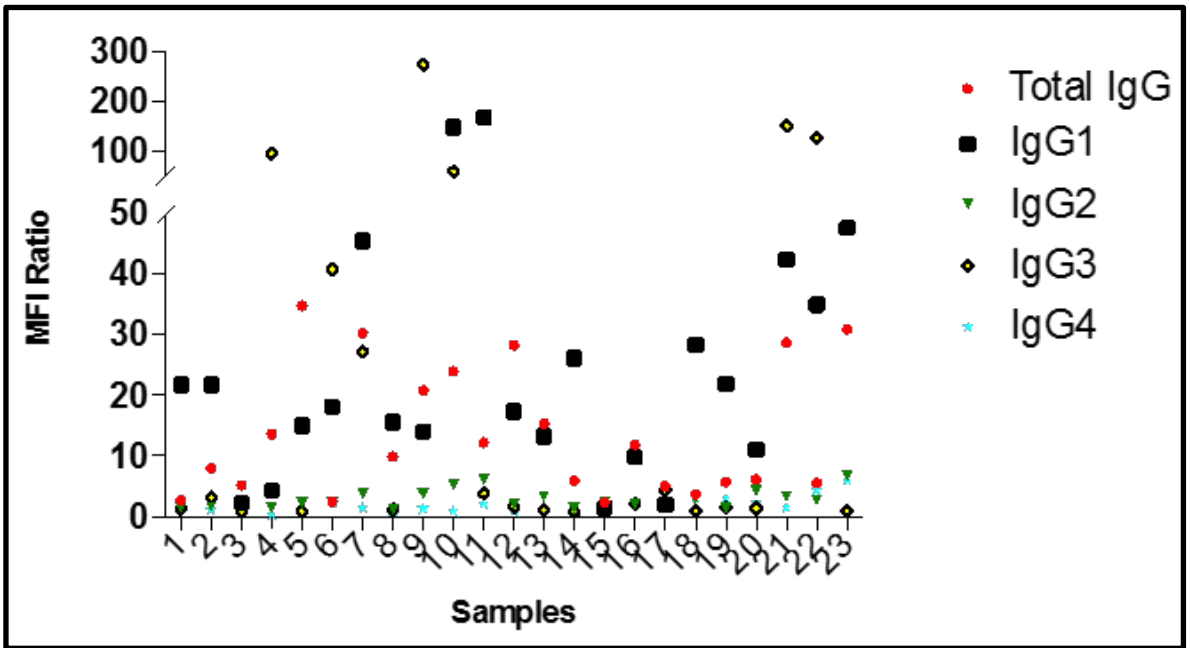

Figure 5.2. Ratios for HPA-1a reactivity in the non-ICH cohort. This followed incubating the sera with the Luminex™ beads and adding an IgG conjugate for total and each IgG subclass and analysed on a Luminex™ analyser. Ratios were calculated using the standard equation for Luminex™ HLA screening kits. The Y-axis scale reflects the variability in ratios for the different subclasses.

Calculations	Total IgG		IgG1		IgG2		IgG3		IgG4	
	HPA-1a	HPA-1b	HPA-1a	HPA-1b	HPA-1a	HPA-1b	HPA-1a	HPA-1b	HPA-1a	HPA-1b
Mean	13.63	0.90	31.79	1.08	2.79	0.98	34.76	1.27	1.88	0.88
Min	2.27	0.63	1.40	0.33	1.10	0.70	0.74	0.72	0.43	0.50
Max	34.78	1.10	167.70	1.73	6.67	1.28	274.06	2.17	6.00	1.42
SD	10.75	0.12	42.05	0.33	1.56	0.14	67.69	0.35	1.30	0.28

Table 5.5. An overview of the statistics for total IgG and each of the subclasses obtained for the non-ICH cohort. Ratios were calculated using the standard equation for Luminex™ HLA screening kits. IgG3 and IgG1 had the highest mean HPA-1a ratios.

5.6 Post-transfusion purpura

Only 9 samples were available from the PTP cohort due to the rarity of the condition after the introduction of leucodepletion of donated blood. Two samples were removed from the analysis as these samples did not react with the beads for total IgG. Figure 5.3 displays the HPA-1a average ratios for antibody binding, whilst Table 5.6 has a range of statistics from each of the samples. Four samples were positive for IgG1; one sample had a ratio of 234.32

for the IgG1 subclass, whereas all the other samples were had a ratio under 22.46. Similarly, four samples were positive for IgG2, but only 1 sample was positive for IgG3 and 3 were positive for IgG4.

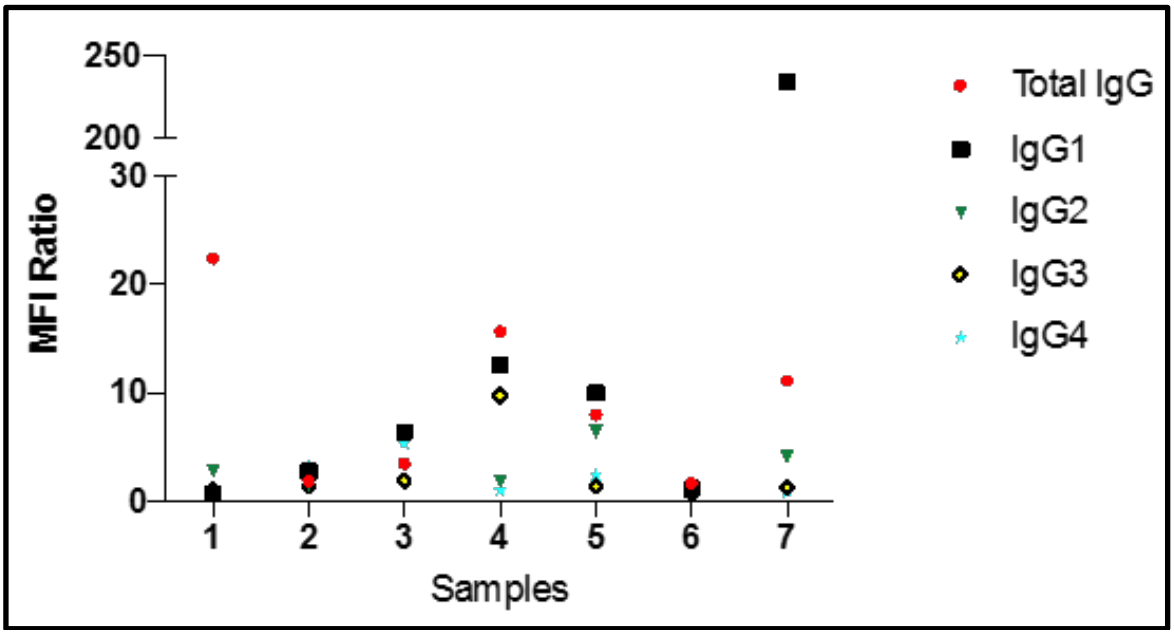

Figure 5.3. Ratios for HPA-1a reactivity in the PTP cohort. This followed incubating the sera with the Luminex™ beads and adding an IgG conjugate for total and each IgG subclass and analysed on a Luminex™ analyser. Ratios were calculated using the standard equation for Luminex™ HLA screening kits. The Y-axis scale reflects the variability in ratios for the different subclasses. IgG2, IgG3 and IgG4 results had very low raw data values and may not be valid.

Calculations	Total IgG		IgG1		IgG2		IgG3		IgG4	
	HPA-1a	HPA-1b	HPA-1a	HPA-1b	HPA-1a	HPA-1b	HPA-1a	HPA-1b	HPA-1a	HPA-1b
Mean	9.18	0.96	38.27	1.10	2.99	0.96	2.50	1.24	2.07	1.00
Min	1.68	0.57	0.71	0.72	0.92	0.55	0.75	0.67	0.50	0.50
Max	22.46	1.20	234.3	1.34	6.44	1.20	9.76	1.62	5.33	1.44
SD	7.81	0.21	86.56	0.23	1.90	0.23	3.22	0.31	1.75	0.37

Table 5.6. An overview of the statistics for total IgG and each of the subclasses obtained for the PTP cohort. Ratios were calculated using the standard equation for Luminex™ HLA screening kits. Total IgG and IgG1 had the highest ratios. The data for IgG2, IgG3 and IgG4 had low MFI raw data values, thus inflating the calculated values.

5.7 Platelet Refractory

There were 15 samples included in the analysis for the platelet refractory cohort. Platelet refractory patients quite commonly make antibodies to the less frequent HPA antigens such as HPA-1b or HPA-2b and not frequently to HPA-1a (Porcelijn, Huskes & de Haas, 2020). A high number of this cohort (10) did not react to the beads when using total IgG conjugate. In total there were five samples positive for IgG1, 4 for IgG2 and IgG3 and 7

samples were positive for IgG4 HPA-1a antibodies. IgG1 antibodies had the highest HPA-1a bead ratios and the largest variability between samples with a SD of 21.01. In this cohort as in the other cohorts, the raw MFI values for IgG2-IgG4 were low. Figure 5.4 displays the HPA-1a antibody mean ratios from each of the samples which shows four samples have a high ratio in the IgG1 subclass, and one sample has a high IgG2 ratio. Table 5.7 displays the HPA-1 and HPA-1b statistics from all the testing of this cohort.

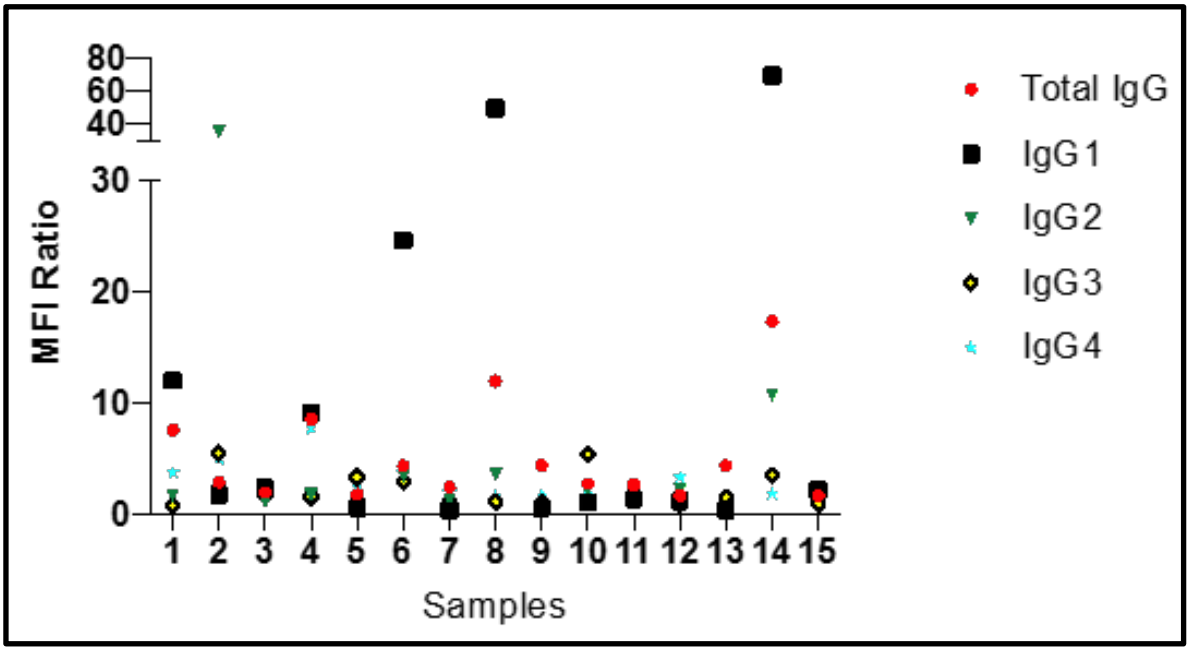

Figure 5.4. Ratios testing for HPA-1a reactivity in the HPA refractory cohort. This followed incubating the sera with the Luminex™ beads and adding an IgG conjugate for total and each IgG subclass and analysed on a Luminex™ analyser. Ratios were calculated using the standard equation for Luminex™ HLA screening kits. The Y-axis scale reflects the variability in ratios for the different subclasses. IgG2, IgG3 and IgG4 results had very low raw data values and may not be valid.

Calculations	Total IgG		IgG1		IgG2		IgG3		IgG4	
	HPA-1a	HPA-1b	HPA-1a	HPA-1b	HPA-1a	HPA-1b	HPA-1a	HPA-1b	HPA-1a	HPA-1b
Mean	5.10	0.91	11.77	0.86	4.60	0.93	2.21	1.30	2.76	1.12
Min	1.66	0.73	0.34	0.66	0.67	0.71	0.73	0.69	0.50	0.75
Max	17.36	1.09	69.31	1.29	35.63	1.17	5.52	2.17	7.75	1.50
SD	4.51	0.11	20.80	0.17	8.93	0.15	1.58	0.43	1.79	0.21

Table 5.7. An overview of the statistics for total IgG and each of the subclasses obtained for the platelet refractory cohort. Ratios were calculated using the standard equation for Luminex™ HLA screening kits. IgG1 had the highest HPA-1a mean ratios but the largest SD indicating the variability in testing. The data for IgG2, IgG3 and IgG4 had low MFI raw data values which make the values seem above higher than what they should be and may not be valid.

5.8 Comparing cohorts

The ICH cohort had the highest ratio for total IgG HPA-1a (45.82), followed the non-ICH (34.78), PTP (22.46) and platelet refractory (17.36) cohort. Observationally the platelet refractory cohort had lower means for all results compared to the other cohorts. Statistical analysis of all patient groups was performed by Kruskal-Wallis, which revealed significant differences in total IgG overall ($p=0.0267$), due to differences between the non-ICH and platelet refractory groups ($p=0.015$; (Dunn's post-hoc test). Similarly, there was a difference in IgG1 levels between groups ($p=0.0114$), again due differences in levels in the cohorts of non-ICH versus platelet refractory patients ($p=0.009$). There was also a difference in IgG2 overall (p-<0.0001), due to differences between the platelet refractory group and each of the other cohorts (ICH - $p<0.0001$; non-ICH - $p<0.0001$ and PTP $p=0.0016$). There were no differences in either IgG3 ($p=0.5530$) or IgG4 ($p=0.146$) between any of the cohorts.

The highest HPA-1a ratio was in the IgG3 subclass in the non-ICH group (274.06); such ratios resulted in a large SD (67.69). The non-ICH cohort had the highest number of positive samples for IgG3 compared to the other cohorts. All patient groups had low MFI raw data for subclasses IgG2, IgG3 and IgG4, in some cases under twenty. As a comparison, the data for total IgG was >1000 MFIs.

5.9 Summary

Chong *et al.,* (2011) developed recombinant proteins and optimised an assay for the detection of antibodies to HPA-1a and HPA-1b. This methodology was utilised in the current study and positive cut-offs were established as described in the paper. Some study samples did not meet the positive cut-off criterion due to only a small MFI shift between the inert bead (GPVI) and the HPA-1a bead, even though the shift was towards the HPA-1a bead (Section 5.3). This high background could be due to a number of factors: other serum proteins blocking the beads, the age of the samples or storage conditions; sample age ranged from 2010-2018; long term storage is at -40^{o}C. There is no evidence in the literature that these conditions affect Luminex™ assay results, but from personal observation within the laboratory a number of samples do have high backgrounds to HLA beads which require a repeat sample.

There was a difference in total IgG, IgG1 and IgG2 between the patient groups. Kruskal-Wallis analysis indicated that this was between non-ICH and the platelet refractory cohorts for total IgG and IgG1; for IgG2 there were statistical differences between the ICH, non-ICH and PTP cohort versus the platelet refractory cohorts. These results indicate that platelet refractory patients have a lower titre antibody compared to the NAIT cases. Platelet refractory patients make HPA antibodies due to platelet transfusions; if they are not being stimulated frequently with the antigens these antibodies could be more transient. During NAIT the immune system is being stimulated for months which may increase the antibody strength and avidity.

The raw data from analysis of IgG2, IgG3 and IgG4 were low in the majority of tests for all cohorts and therefore the ratios and whether samples are positive/negative should be considered with caution, including the results of the statistical analyses of the data for IgG2. Another possibility is that these antibody subclasses may not be present in these patient groups, or at least not in these limited number of samples. These were the first experiments of this kind to use Luminex™ technology, therefore controls were not available for each of the IgG subclasses for HPA-1a or for HPA-1b beads, but rather only for total IgG.

The main aim of the work presented in this chapter was to investigate if there is an isotype bias of IgG subclass in four patient groups, ICH, non-ICH, PTPs and platelet refractory using the highly sensitive Luminex™ technology commonly used in H&I laboratories for HLA antibody detection. The predominant HPA-1a antibody subclass is IgG1 in each of the cohorts tested, which is in agreement with published reviews (Kumpel & Manoussaka, 2011; Sachs, 2020). However, the results suggest that using this method to measure the relative abundance of different IgG subclasses in NAIT is unlikely to be useful in predicting ICH in the fetus/newborn.

In summary, the methodology described here provides a reliable screening assay for HPA-1a and HPA-1b for total IgG and IgG1, which could be used clinically, in conjunction with MAIPA, to diagnose NAIT. However, the results from the analyses presented in this chapter do not support utility in predicting severity of disease. The data suggest that detection of IgG2, IgG3 and IgG4 antibodies has no clinical use, as these antibodies were not found to be associated with NAIT severity, however, it could be the assay requires further optimisation for these subclasses.

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Chapter 6: Discussion

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6.1 Discussion

Recently, there have been a number of reviews about predicting severity and risk stratification in NAIT (Kjaer *et al.,* 2019; Kjeldsen-Kragh & Bengtsson, 2020; Sachs, 2020). These reviews discuss potential association of clinical severity risk with αvβ3 specific antibodies, HPA-1a IgG subclasses and antibody titres, but there is little recent relevant literature, and the majority of review publications cite the same original papers (Proulx *et al*., 1994, Mawas *et al.,* 1997, Williamson *et al.,* 1998, Kille *et al.,* 2007; Ghevaert *et al*., 2007, Bessos *et al*., 2008, Killie *et al.,* 2008). The most recent investigations relate to detecting αvβ3 antibodies, HPA-1a antibody fucosylation and avidity/affinity of antibodies, but these are already five years old (reviewed in Sachs, 2020). The risk stratification used currently, is obstetrics history; essentially a mother who has had a previous child with ICH, may be offered intravenous immunoglobulin (IVIg) during the pregnancy, though this depends on the country and region (Lieberman *et al*., 2018; Kjeldsen-Kragh & Bengtsson, 2020). However, an HPA-screening in pregnancy (HIP) study conducted in the Netherlands 2017-2020, which is yet to report, collected prospective data throughout pregnancy and clinical detail such as ICH presence, infant platelet counts and whether the baby had transfusions at birth (Winkelhorst *et al.,* 2020). Prospective observational studies such as these will help inform treatment throughout pregnancy and at birth.

The present retrospective study reported here, aimed to investigate whether αvβ3 specific antibodies, HPA-1a antibody titres and the relevant IgG subclasses could be used in the English population to predict the severity of clinical sequelae in NAIT. A number of hypotheses were tested to assess if NAIT severity might be predictable.

6.1.1 Hypothesis 1: The presence of αvβ3 antibodies in NAIT maternal samples is associated with development of ICH in the fetus or newborn.

The first objective to test this hypothesis was to clone the αv protein for co-transfection with β3 so the αvβ3 heterodimer could be transiently expressed into HEK293 cell and used to screen for antibodies.

The αIIb and the β3 constructs had previously been validated within the laboratory (Poles *et al.*, 2019) and the same approach was used to produce the αvβ3 integrin. The protein

expression was confirmed using a combination of monoclonal antibodies specific to αvβ3 or αIIbβ3, together with polyclonal HPA-1a patient sera (Chapter 3). Initial experiments demonstrated that the transfection efficiency was under 20% but with increasing the transfection incubations to 48 hours, this increased the efficiency to over 40%. An observation made with samples with HPA-1a antibodies, that when they bound to the αvβ3 expressing cells, the number of cells were reduced, which could be due to the cells being killed during the experiment, this was not the case with the αIIbβ3 cells (Section 3.8). This may be due to HPA-1a antibodies having a higher affinity to αvβ3 than αIIbβ3. This phenomenon is evidenced when the laboratory detects high titre human neutrophil antigen-3a antibodies using the recombinant HEK cells (personnel observation, Wozniak *et al.,* 2011).

The HPA-1b versions of both αvβ3 and αIIbβ3 were produced to allow detection of both HPA-1a and HPA-1b antibodies. Both HPA-1a and HPA-1b isoforms of αIIbβ3 would be useful as a screening technique clinically, which would replace the use of frozen platelets, assuming a stable cell line could be produced. Platelets contain HLA on their cell surface which can interfere between the differentiation of HLA and HPA antibodies. Current guidelines recommend the use of two different assays to detect platelet antibodies; the MAIPA and PIFT are used routinely (Petermann *et al.,* 2018, https://www.transfusionguidelines.org/red-book). A screening method that detects only HPA antibodies would be an advantage as a second technique to MAIPA. This could also be developed for other HPA antigens such as HPA-3, as antibodies to this antigen can sometimes only be detected by the PIFT in certain cases and not by the MAIPA (Porcelijn, Huiskes & Haas, 2020).

Objective two; to develop an absorption method to remove the αIIbβ3 antibodies from patient serum, then test against αvβ3 expressing cells using the ICH and non-ICH cohorts to see if αvβ3 antibodies were present in ICH cases.

Recombinant β3 protein was attached to resin and incubated with samples from ICH and the non-ICH cohorts. Pilot testing on a limited number of samples from each cohort showed all samples were negative against both αvβ3 and αIIbβ3 expressing cells. This is in contrast to the study by Santoso *et al.,* (2016) which reported all ICH cases possess αvβ3 antibodies.

The lack of antibody reactivity with the αvβ3 cells may be due to over-absorption and removal of all antibodies (Section 4.5), but as the αvβ3 is a compound antibody that reacts with the heterodimer, that is unlikely. Recently, work has been published demonstrating the World Health Organisation's HPA-1a standard only reacts with the β3 part of the protein and not with either αvβ3 or αIIbβ3 heterodimers. Therefore, this could support these results if all the samples in this study contained only β3-specific antibodies which had been absorbed out resulting in failure to detect any antibodies (Bayat *et al.*, 2019).

The third objective was to compare the presence of αvβ3 antibodies between the two cohorts.

As stated in objective two, all samples which had been absorbed using the β3 conjugated resin were negative for antibodies when screened with αvβ3 and αIIbβ3 cells in both cohorts (Section 4.5). Due to the lack of controls for absorbing antibodies from sera, this absorption approach would be hard to obtain laboratory accreditation for from bodies such as the United Kingdom Accreditation Service, to ISO15189:2012 standards due to lack of controls and external quality assurance schemes. The approach presented here was not able to test the hypothesis properly due to the lack of a robust methodology within this study and the limited testing. Interestingly, there has still only been one published study indicating that αvβ3 antibodies are linked to ICH in NAIT. In this study all ICH cases were linked to αvβ3 antibodies (Santoso *et al*., 2016). Reviews quote this paper, but no further research has been published either supporting or disputing this observation.

Future work for hypothesis 1:

- Modify the absorption methodology to ensure αvβ3 antibodies can be detected, this could be by attaching the αvβ3 to the resin and incubating with the sera and eluting the antibody from the resin and testing. This is an improvement to the method in this study, as this will detect the αvβ3 antibodies only if present and not potential antibodies to β3.

- Stable cell lines could be produced for both HPA-1a and HPA-1b αIIbβ3 integrin of to enable an alternative screening assay capable of HPA antibody detection without

hindrance of HLA, such an assay could replace the PIFT assay This would require the addition of other cell lines which expressed HPA-3 and HPA-5 to be a viable option.

6.1.2 Hypothesis 2: A high HPA-1a IgG titre causes severe NAIT.

The fourth objective was studying the effect of prozone on both ICH and non-ICH samples.

This arose when the absorption studies showed no difference between the ICH and non-ICH cohorts for sample binding to αvβ3 expressing cells. It was possible the findings of Santoso *et al.,* (2016) were due to antibody titres: a high antibody titre sample may not have had all the antibody absorbed and differences in affinity between αIIbβ3 and αvβ3 might allow residual binding. The same samples were chosen for the absorption studies and the initial 1/4 dilution studies to indicate if prozone was affecting the results by blocking the secondary conjugated antibody. Only one sample from the ICH cohort gave reproducibly higher results at a 1/4 dilution, and to αvβ3 cells only. Other apparent differences were not reproducible using triplicate testing taking into account testing variance and laboratory conditions (Section 4.6). This has indicated that prozone is not interfering in the methodology in these immunofluorescence tests and is not implicated in ICH NAIT cases and the hypothesis was not supported in this study.

The limitations of this investigation are that the samples may, in certain cases, have been stored for multiple years and sample integrity may decline over time due to freeze/ thaw cycles which may reduce reactivity which may affect the reliability of the results.

The fifth objective was to establish a range of serum dilutions which could be used to determine HPA-1a antibody titre.

This objective would optimise titre testing for use clinically and save time on serial dilutions. This was tested in the experiments presented in Section 4.6 by using cases of ICH and non-ICH and comparing antibody titres using two cell lines transiently expressing either αvβ3 or αIIbβ3 integrins. Testing was limited by sample volume and availability of HEK293 cells and integrin DNA for transfection. The first experiments used doubling dilutions up to 1/2056 dilution. Both samples tested had a 1/16 HPA-1a antibody titre, indicating that the doubling dilution did not need to be performed lower than 1/64. ICH and non-ICH samples had a

range of different titres but there were no statistically significant differences between the two groups when analysed using Kruskal-Wallis (Section 4.6). This retrospective study on limited sample numbers (6 ICH and 6 non-ICH cohort in duplicate, over multiple days) does not support the use of HPA-1a antibody titres to predict NAIT severity, as also concluded by Kjaer *et al.*, (2019).

A recent systematic review has shown that the most reliable method to quantify antibody titres is to use the MAIPA with the WHO HPA-1a standard which could allow inter-laboratory comparisons (Kjaer *et al.,* 2019). This review noted that immunofluorescence tests did not find a link between antibody titre and severity and concluded that this methodology may not be suitable. This study commented about the differences in prospective and retrospective studies. Retrospective studies have a bias as samples have been selected due to the fetus/newborn being affected by NAIT and therefore it would have a higher antibody titre, whereas prospective studies are blind to the clinical outcomes. The review did conclude that maternal antibody titres could be linked to fetal/newborns platelet count but could not be used in risk stratification for ICH due to the lack of studies (Kjaer *et al.,* 2019; Kjeldsen-Kragh & Bengtsson, 2020).

Future work for hypothesis 2:

- Perform antibody titration studies on all methods of platelet antibody detection to determine which assay is the most sensitive and reproducible.

- Repeat antibody titre work on the quantitated MAIPA including the WHO control as this has recently been published this is the most reliable method to determine antibody concentration and can be compared between different laboratories (Kjaer *et al.,* 2019).

6.1.3 Hypothesis 3: There is a difference in the IgG subclasses during gestation that can be used to predict NAIT severity.

The overall objective here was to establish a laboratory assay to detect and differentiate all IgG subclasses of HPA-1a antibodies.

This was achieved by using previously published methodology, Luminex™ beads, to detect HPA-1a and HPA-1b (Chong *et al.,* 2011) and adapting it to detect all the IgG subclasses. The different IgG secondary conjugates were recommended by one of the authors of a study in transplant immunology (private communication) (Lowe *et al.*, 2013). They validated each IgG monoclonal conjugate to the different IgG subclasses and selected the best available conjugates that did not cross-react with other IgG subclasses.

In this study, it was thought that there would be a more mature immune response in the PTP and ICH cohorts and therefore that the IgG response would be IgG4; akin to observations in patients with kidney rejection (Khovanova *et al*., 2015).

There was a significant difference in the ratios of total IgG and IgG1 subclass between the non-ICH and platelet refractory cohort ($p=0.0267$ and $p=0.0144$, respectively). There was a difference observed in IgG2 ($p<0.0001$) between the ICH, non-ICH, PTP cohorts versus platelet refractory cohort. There were no differences observed in the IgG3 and IgG4 subclasses across all cohorts (Section 5.8).

The results indicate that IgG1 is the predominant subclass but there was not any evidence to suggest that this can be linked to NAIT severity. Therefore, this study could not find differences in IgG subclasses that can be used to predict NAIT severity.

A limitation of this study was the lack of controls available to ensure all the IgG subclass conjugates were performing as expected. The lack of controls is due to the rarity of Platelet Immunology laboratories and the lack of investigation of HPA-1a antibody subclasses. For IgG2, IgG3 and IgG4 the raw data values were very low in the majority of samples tested, indicating these subclasses are not present. Some samples had higher raw data to ensure confidence, but these were very limited (Sections 5.4 & 5.5).

The literature is sparse on IgG subclass investigations and the majority were performed over 20 years ago (Sachs, 2020). One study indicated that IgG3 was linked to NAIT severity but this also had low numbers in the study (Mawas *et al.,* 1997). A recent review noted that IgG2 and IgG4 were not detected in previous studies, which supports the findings of the current work and therefore the low MFIs may be due to the absence of antibody in the patient serum (Sachs, 2020) rather than poor performance of the assay. Indeed, this study used a more sensitive technology than was unavailable in previous studies and within the laboratory there is evidence that Luminex™ can detect HPA-1a antibodies which have not been detected by MAIPA (Stanton *et al.*, 2018).

Future work for hypothesis 3:

- Optimise this Luminex™ system for all IgG subclasses to ensure the detection method is capturing all the subclasses if they are present. Studies should be performed using higher concentrations of the conjugated secondary antibodies.

- A minimum threshold of median fluorescent intensity should be developed to indicate a valid test. Other methods of calculating the positive cut-off for the Luminex™ assay should be explored. There were a number of samples which had a positive MFI shift but did not reach a positive threshold. These samples would be examined to assess whether age was a factor and be re-tested on commercial kits or MAIPA to ensure the HPA-1a antibody could still be detected.

- Once further work has established the cut-offs for the Luminex™ assay this should be introduced as a screening method as Luminex™ HLA antibody detection has been shown to be more sensitive than other methods such as ELISA for HLA antibody detection (Metzner *et al.,* 2017; Stanton *et al.,* 2018). The laboratory has previously shown that Luminex™ has detected HPA-1a antibodies which could not be detected by MAIPA (Stanton *et al.*, 2018).

- Develop Luminex™ bead technology to detect other HPA antibodies as this has increased sensitivity over the MAIPA and other laboratories are adopting this technology for platelet antibody detection (Metzner *et al.,* 2017).

6.2 Conclusion

This retrospective study failed to find any association between αvβ3 antibodies, titres or IgG subclass and clinical severity in NAIT. Current treatment during pregnancy will rely on previous obstetric history, but lately the free fetal DNA sampling may reduce any unnecessary treatment interventions if the father is heterozygous but is currently only available for HPA-1 and HPA-5 (Kjeldsen-Kragh & Bengtsson, 2020)). Newer technologies such as surface plasmon resonance may find previously undetected antibodies due to low avidity, but within the UK, clinical conditions without a detectable antibody will be treated cautiously, especially if the mother has an HPA-1b1b genotype.

One of the major limitations of a retrospective study, is the availability, and trustworthiness, of clinical data available. This study used information held in local databases which stated if fetal scans had identified whether ICH was present, but for the non-ICH cohort this information may not have been added to the database. Earlier samples from the cohort used to state if there were no brain abnormalities, but this information was not available for later cases. No information was available whether the samples in this study was pre-birth or post-birth or what pregnancy number which could have affected the outcome of this study.

Currently the risk of ICH or fetal death will still be based on obstetric history and predicting future risk depends on the paternal partners HPA genotype. If NAIT is implicated, the fetus will be treated accordingly, from obstetric history and the maternal HPA genotype (e.g., HPA-1b1b) even if an antibody has not been detected.

6.3 General recommendations

Establish a reliable database for storing medical records on NAIT mothers to include the serum sample date, number of weeks' gestation, whether diagnosis was *in utero* or post-birth, or pre-natal, number of pregnancies, whether a brain scan was performed and the results of laboratory tests. To hold information such as platelet count at birth, and whether the new-born received platelets and if the pregnancy was treated with IVIg. This would help in potentially informing how to treat future NAIT cases in both a prospective and for any retrospective studies.

England to participate in studies/collaborate in studies such as the Netherland HIP study (Winkelhorst *et al.,* 2020). This study was collecting prospective data throughout pregnancy of women identified with HPA-1a antibodies. It would be worthwhile for England to collect data like this to inform on treatments/interventions in the future.

6.3.1 Recommendations specific to hypothesis

Hypothesis 1

The Platelet Immunology community should investigate as a whole on whether αvβ3 can be linked to ICH in NAIT cases. If all laboratories investigated this, the case numbers would be more significant, and the power would be increased to indicate relevance; than the low numbers of individual laboratories would be reporting on and the results would be more compelling.

Hypothesis 2

The international Platelet Immunology community should work together and start quantifying HPA-1a antibodies in a prospective study using the quantitated MAIPA. Samples can be sent as part of the International Platelet Immunology Workshop and the results compared. This would help inform if antibody titre can be used to predict NAIT severity. A number of parameters should be agreed such as methodology, timing of samples and information such as gravida.

Hypothesis 3

Use more sensitive methods such as Luminex™ based technology to prospectively test for the IgG subclasses in HPA-1a NAIT cases and add this criterion to International Platelet Workshops to help identify controls for this methodology.

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Appendix 1

Higher Specialist Scientific Training Module C1 Innovation Business Case

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Risk assessing the severity of neonatal alloimmune thrombocytopenia (NAIT)

Lay Summary

NAIT affects 1 in 2000 births every year. This is caused by the mother making an antibody to a platelet antigen which the fetus has and that is missing in her. Antibodies from the mother cross the placenta and attack the fetal platelets which cause their destruction. Affected fetuses/newborns can present with blood spots or in the worse case, have intracranial haemorrhages (ICH) which can lead to death or learning disabilities. The most common antibody in the Caucasian population which accounts for 85% of cases is human platelet antigen-1a (HPA-1a). Currently there is no reliable test to predict the severity of NAIT.

The purpose of this innovation proposal is to develop a testing strategy to predict NAIT severity so that treatment can be tailored to the individual. Hopefully this will decrease the cases of ICH and it manifestations. Also decrease the cost of treatment and exposure to the current treatment strategy.

Executive Summary

The literature has conflicting evidence on whether platelet antibody concentration can be used to predict NAIT severity. There is no conclusive evidence showing if the different IgG subclasses have a differing effect in NAIT severity. Newer bead-based technologies are akin to those used in solid organ transplant antibody having been developed for detecting platelet antibodies. These are easier to use and the result can be used to compare over multiple measurements throughout the pregnancy. A feasibility study is required to investigate whether NAIT severity can be predicted in our local population by quantifying IgG antibody and subclass concentration. Another business case will be submitted if initial results indicate that this study should be implemented into routine testing.

The study aims to use bead-based technology to measure the total amount of IgG and to perform IgG subclass antibody concentration. It has the potential to be able to devise a risk stratification of NAIT severity which can be used throughout the pregnancy to aid clinicians with their treatment plan. Additional testing would be required throughout an affected pregnancy than current strategies. The extra cost of this testing would be off-set from money saved from prescribing intravenous immunoglobulin (IVIg) treatment if shown not to be needed. Currently diagnosed pregnant NAIT cases can be treated with IVIg from 20th week gestation. Being able to predict severity may mean that some pregnancies may not need IVIg or others may have to start treatment earlier. Predicting ICH can reduce the life-long medical requirements and social care if mental disabilities results. Reducing IVIg administration even by a week would save unnecessary fetus exposure and costs.

Background

NAIT affects 1 in 2000 births every year (Arnold, Smith & Kelton, 2008). This is caused by the mother making an antibody to a platelet antigen which the fetus has and that is missing in her. The maternal IgG platelet specific antibodies cross the placenta and attack the fetal platelets which cause their destruction (Sachs, 2013). NAIT can affect first pregnancies and the severity normally increases during affected subsequent pregnancies (Bussel & Primiani, 2008). This can result in the fetus or newborn having a low platelet count which can cause

rashes, bleeding and in more severe cases ICH (Wienzek-Lischka *et al*., 2017). If during pregnancy a mother is likely to have a NAIT child the treatment involves weekly infusions of IVIg to reduce the chances of ICH within the fetus or newborn (Bussel Primiani, 2008). This reduces the maternal HPA antibodies by unknown mechanisms and therefore reduces the amount of antibody crossing the placenta to affect the fetal platelets. The effects of this treatment to the newborn are unknown and IVIg is an expensive treatment costing $1,000-$2,000 per week (Mella &Eddleman, 2014). Newborns can be treated at birth with neonatal HPA-1a negative platelets to help increase their platelet count and decrease risk of ICH.

Currently there are no laboratory tests that can reliably predict NAIT severity (Espinoza *et al*., 2013). The testing strategy detects an antibody but does not quantify the amount present. Standard protocols do not monitor the antibody throughout gestation or the antibody concentration. There have been a limited number of studies which have not been able to conclusively link the HPA-1a antibody concentration with NAIT severity (Bessos *et al*., 2009). Some studies indicate antibody titre and affinity can predict NAIT severity, whilst others have not confirmed these findings (Killie *et al*., 2008; Proulx *et al*., 1994). This may be due to a variety of reasons such as low numbers included within the studies, methods used and the samples tested are at different time-points during gestation. There has been a limited study on whether differing IgG subclasses are associated with NAIT which have not provided conclusive evidence (Proulx *et al*., 1994, Mawas *et al*., 1997). These studies were performed prior to 2000 and there has not been any recently published data on IgG subclasses in NAIT.

The current immunoassay used within our Platelet Immunology laboratory is based on the original 1987 monoclonal antibody immobilisation of platelet glycoprotein assay (MAIPA) (Kiefel *et al*., 1987). The MAIPA is robust at detecting antibodies but is time consuming and requires a large number of reagents. A disadvantage with the MAIPA is that low avidity antibodies can be lost in the method due to numerous wash steps and potentially reported as false negative (Zdravic *et al*., 2016). Newer technologies are available such as bead-based assays analysed by Luminex™ machines. Recombinant HPA molecules are attached to a micro-bead which has a unique combination of fluorescent dyes so that the bead can be identified when passed through the Luminex™ analyser. This method can be modified to assess the different IgG subclasses. The Luminex™ technique has fewer steps than the MAIPA and therefore low avidity antibodies should not be 'lost' from the test (Porcelijn *et al*., 2014). Newer technologies such as surface plasmon resonance have been used to detect low avidity antibodies but are still very much a research tool (Bakchoul *et al*., 2013).

Luminex™ technology is used in the detection of human leucocyte antigen (HLA) antibody testing in solid organ transplantation and has revolutionised the testing. In organ transplantation the output from the machine the median fluorescent intensity (MFI) has been used to indicate antibody strength (Lachmann *et al*., 2013). HLA antibody profiles can be assessed if desensitisation protocols to remove antibodies are working by using this value. The MFI value has been used to give a risk stratification of deceased donor transplants outcomes.

There has only been limited testing using the HPA bead-based PakLx kits to show if results are comparable to MAIPA (Porcelijn *et al*., 2014). Ideally the bead-based HPA kits should be applied to monitoring the antibody concentration over the pregnancy to gain knowledge

of the potential effects throughout gestation. Additional testing of IgG subclass concentration will identify if there are changes throughout gestation. Further knowledge can be gained by studying the effects of the different IgG subclasses throughout gestation to identify if there are changes within these antibody profiles. IgG subclasses transfer across the placenta at different time-points and different rates which must be taken into consideration when analysing results. Testing these parameters will gain insight which may aid in developing a risk stratification model which can be used to predict NAIT severity.

Innovation Proposal

Establishing a testing procedure that will reliably predict NAIT severity by measuring HPA-1a IgG antibody concentrations and for each of the four IgG subclasses. This is not currently performed within the NHS.

Impact

Being able to predict NAIT severity within the fetus/newborn would mean that clinicians are being provided an evidenced-based approach on the treatment of NAIT mothers during pregnancy. Monitoring quantitatively HPA-1a antibody concentration and IgG subclass concentration throughout gestation can lead to knowledge of when to trigger an intervention. Affected patients would be treated with individual care packages instead of the generic treatment approach using IVIg as soon as an antibody is detected. This would reduce any unnecessary exposure to the newborn if the risk of NAIT severity was deemed as low.

Objectives include:

- Measuring IgG HPA-1a antibody concentration at multiple time-points throughout gestation.
- Measuring IgG subclasses in HPA-1a antibodies at multiple time-points throughout gestation.
- Using the data derived from the above objectives to identify at what time-points should HPA antibodies should be measured throughout gestation in an affected pregnancy.
- Compile a risk classification model using the results obtained.
- Establish a testing strategy for NAIT referrals throughout gestation based upon results.
- Engaging with stakeholders to discuss results of innovation.

Option Appraisal

Option 1 – Do nothing and keep current testing strategy. This option will not give any additional knowledge of how the maternal antibody profile for total IgG concentration and IgG subclasses changes throughout gestation. Current testing and treatment strategy will stay the same.

Option 2 – Perform this study using MAIPA. This would be time consuming and hard to test patient samples as a large quantity of serum would be required. Previous studies using the

MAIPA have shown conflicting results in predicting NAIT severity. Low avidity HPA-1a antibodies may be reported as negative due to the wash steps which would bias the study.

Option 3 – Use PakLx Luminex™ kits to screen for total IgG concentrations and the four IgG subclasses at multiple time-points. This is the more expensive option but low avidity HPA-1a antibodies can be detected with this method. Low amounts of serum are required and therefore multiple tests can be performed.

Option 4 – Use surface plasmon resonance to perform the testing. This method is still a research tool and not enough evidence available to introduce into a diagnostic laboratory. No costs are available for this test and a biosensor would be required for detection.

Preferred option: Option 3 – Bead-based assays are used in HLA antibody screening and which risk classifications have been formed. Measuring the HPA-1a IgG antibody concentration and the IgG subclass concentration are easier using this methodology and comparable over-time. Technique is faster and does not rely on internal reagent validation and the use of cell panels. This method has not been used by published studies in measuring IgG and subclass concentrations in predicting NAIT severity.

Financial Appraisal – Please see Appendix 1 for analysis of costs for the innovation. Being able to predict NAIT reliably has the advantages of decreasing ICH and the resulting mental disabilities. Mental disabilities can have life-long effects which require medical and social support, which was not accounted for in this brief. Delaying the prescription of IVIg by at least one week would cover the cost of additional tests based on one referral requiring five tests (total IgG concentration and four IgG subclasses).

Current State and Future State

Current State	Future State
People Mothers referred for NAIT investigations which have the potential to have an affected fetus/newborn. Presently reports states supports/does not support diagnosis of NAIT.	People Mothers referred for NAIT investigation which have an affected fetus/newborn have a report comment which offers a prediction of NAIT severity. This will allow clinicians to give appropriate treatment packages.
Process Referrals are currently tested by MAIPA to identify any platelet-specific antibodies. No antibody quantification is performed but may have multiple referrals throughout gestation to ensure antibody is still detected.	Process Referrals from mothers still pregnant are analysed for total IgG and to measure each of the subclasses. Multiple samples from the same pregnancy can aid in identifying if antibody stasis is changing. If one IgG subclass is shown to indicate severity only that type can be screened for.
Technology Currently using MAIPA which takes 6 hours based on the original methodology published in 1987. Technically demanding and requires a high quantity of serum and validated reagents.	Technology Using PakLx commercial kits with Luminex™ technology which will be modified to test all different IgG subclasses. Not technically demanding and takes 3 hours with little serum used.
Further Information Antibody quantification is not standard practice using immunoassays.	Further Information For antibody quantification and subclass analysis to become standard practice on all samples referred.

Appendix 2

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Royal College of Pathologist part 2 oral examination results

The Royal College of Pathologists
Pathology: the science behind the cure

College Reference Number: **1005823X**

Candidate Number: **S268**

Jayne Suzi Johnson

20 November 2020

Dear Miss Johnson

FRCPath Part 2 Oral Examination in Histocompatibility & Immunogenetics (old format) – Autumn 2020

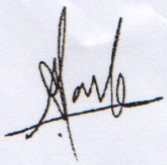
I am pleased to inform you that you have satisfied the Examiners in the Part 2 Examination.

However, as you are aware, you are not yet eligible to become a Fellow of The Royal College of Pathologists as your Part 2 Project has not yet been approved.

We look forward to receiving the project in due course. If you have any queries about your project, please contact exams@rcpath.org.

Congratulations on your success in this examination.

Yours sincerely



Dr Sanjiv Manek
Clinical Director of Examinations

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Appendix 3

A Units and C1 Credits for Appendix to DClinSci Thesis

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A Units and C1 Credits for Appendix to DClinSci Thesis

Alliance Manchester Business School (AMBS) A Units		
Unit Title	**Credits**	**Assignment Word Count**
A1: Professionalism and Professional Development in the Healthcare Environment	30	Practice Paper – 2000 words A1 – Assignment 1 – 1500 words A1 – Assignment 2 – 4000 words
A2: Theoretical Foundations of Leadership	20	A2 – Assignment 1 – 3000 words A2 – Assignment 2 – 3000 words
A3: Personal and Professional Development to Enhance Performance	30	A3 – Assignment 1 – 1500 words A3 – Assignment 2 – 4000 words
A4: Leadership and Quality Improvement in the Clinical and Scientific Environment	20	A4 – Assignment 1 – 3000 words A4 – Assignment 2 – 3000 words
A5: Research and Innovation in Health and Social care	20	A5 – Assignment 1 – 3000 words A5 – Assignment 2 – 3000 words

Life Sciences - Unit C1

C1: Innovation Project - Credits 70. Literature Review, Lay Presentation and Innovation Proposal