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Investigation of ABO blood group expresser phenotype

A dissertation submitted to the University of Dublin for the Degree of Doctor of
Medicine

Diarmaid Ó Donghaile

Trinity College Dublin, May 2011

Haemostasis Research Group

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Declaration

I hereby declare that this thesis has not been submitted as an exercise for a Degree at this or any other University and that it is entirely my own work.

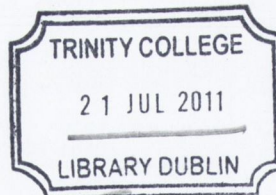
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Summary

The aims of this project were to study the variability of the ABO blood group expression on human platelets and to explore the underlying molecular mechanisms.

541 Irish blood donors were studied. The recently described high and low expresser phenotypes of platelet ABO blood group were identified in 5.4% and 4.4% respectively in group A₁ donors.

The molecular mechanisms accounting for this variability is unknown. Both ABO(H) determinants and sialic acid are terminal carbohydrates expressed on platelet glycoproteins. Therefore, I studied the expression of sialic acid on platelets to determine whether the phenotypes were a product of substrate competition. However, while variability in sialic acid expression was identified, this did not correlate with ABH expression.

I then compared donor ABO genotype with platelet ABO expression. Gene dosage was identified as the major predictor of High Expresser Phenotype (HXP) status, as 70% (n= 7 of 10) were homozygous A¹A¹. Nevertheless, the majority of A¹A¹ donors were not high expressers, suggesting that additional molecular modifiers are involved. Two HXP donors (genotype A¹A²) were type II HXP and expressed A antigen on 100% of platelets despite having only one functioning A¹ allele. Therefore, I sequenced all exons of the ABO gene and studied the 5' enhancer sequence of ABO enhancer region. Of the entire A₁ donor population only two had a novel A¹ allele, and were HXP. One homozygous A¹A¹ (Type I HXP) and one heterozygous A¹A² (Type II HXP) had four rather than the expected one repeat of a minisatellite sequence in the ABO enhancer. This is associated with a 100-fold greater transcriptional activity of the ABO glycosyltransferase gene. Therefore high expression of A antigen on platelets is likely to be multifactorial, but includes ABO gene dosage and enhancer repeat copy number at the enhancer sequence.

As the ABO blood group system is not limited to haemopoetic tissue, it is important to determine whether the extreme levels of blood group expression are also seen on Von Willebrand Factor (VWF) and whether it affects VWF antigen levels (VWF:Ag).

VWF:Ag levels were determined in all donors and compared firstly to donor genotype. Homozygous A¹A¹ donors had 12% higher levels than A¹O. Donors lacking an A¹ allele had significantly lower VWF:Ag levels which is consistent with similarly sized studies. However, while ABO blood group is a critical determinant of VWF:Ag levels, the level of platelet ABO blood group expression did not correlate with plasma VWF levels.

A antigen expression was then determined on VWF on 10 HXP and 43 A₁ donors. A on VWF was highest on HXP donors VWF (P=0.019). Therefore, while not having an effect of VWF:Ag levels, the Platelet HXP may influence the loading of A determinants on VWF.

In conclusion, I have identified the platelet expresser phenotypes in the Irish donor population. I have identified that ABO gene dosage and ABO enhancer repeats are the major predictors of HXP status. I have also shown that the expresser phenotypes may not be limited to haemopoetic tissue alone but may also influence the level of blood group expression on VWF.

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I dedicate this thesis to my parents, Charles and Veronica.

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List of abbreviations

L/l	Litre
°C	Degree Celsius
A transferase	α -1,3- <i>N</i> -acetylgalactosaminyltransferase
ATP	Adenosine triphosphate
B transferase	α -1,3-galactosaminyltransferase
bp	Base pair
BSA	Bovine serum albumin
CAT	Chloramphenicol acetyltransferase
CBF	Core binding factor
cDNA	Complementary deoxyribonucleic acid
CMP	Cytosine monophosphate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
EDTA	Ethylenediaminetetraacetic acid (disodium) salt
ELISA	Enzyme linked immunosorbent assay
FITC	Fluorescein isothiocyanate
Fuc	Fucose
FUT1	Fucosyltransferase gene
FVIII	Factor VIII
g	Gram
Gal	D-Galactose
GalNAc	N-acetylgalactosamine
GP	Glycoprotein
H transferase	α -1,2-fucosyltransferase

HLA	Human Lymphocyte Antigen
HPA	Helix pomatia
HXP	High expresser phenotype
IU	International units
KATO III	human gastric signet ring carcinoma cell line
kb	Kilo base-pair
LXP	Low expresser phenotype
<i>M</i>	Molar
MFI	mean fluorescence intensity
mg	Milligram
μg	Microgram
min	Minute
mL	Millilitre
μL	Microlitre
mM	Millimolar
μM	Micromolar
n	Number
NA	Not available
ND	Not done
NF-Y	Nuclear factor Y
ng	Nanogram
<i>N</i> -linked	Asparagine linked
No.	Number
np	Nucleotide position
NS	Not significant
<i>N</i> -terminal	Amino terminal
O-linked	Serine / threonine linked

PBS	Phosphate buffered saline
PBST	PBS + Tween 20
PCR	Polymerase chain reaction
PE	phycoerythrin
PPP	Platelet poor plasma
PRP	Platelet rich plasma
RFLP	Restriction fragment length Polymorphism analysis
SD	Standard deviation
SNA	Sambucus nigra agglutinin
SV 40	Simian virus 40
TBS	Tris buffered saline
UEA	Ulex europaeus
VWD	von Willebrand disease
VWF	von Willebrand factor
WP	Weibel-Palade bodies

Chapter 1

Introduction

1.1 Introduction

Human blood groups are antigens which have been identified through the immunological consequences of allogeneic blood transfusion. Red cells express numerous antigens that are found on membrane glycoproteins and glycolipids. These antigens are either added during red cell development, or else adsorbed from plasma onto the red cell membrane. Some blood group antigens are only found on red cells, while others are also expressed in other tissues and are thus more correctly termed histo-blood group antigens. Transfusion recipients lacking a particular red cell antigen which is present on the donor cells may generate an immune response against the antigen. The resulting alloantibody can cause a haemolytic transfusion reaction in subsequent transfusions. Similarly, the exposure of the maternal circulation to foetal blood during pregnancy may lead to antibody formation which may cause haemolysis in subsequent pregnancies (Klein, Anstee *et al.* 2005).

ABO is the most important system in transfusion medicine and is critical in the matching of donor recipient pairs in blood transfusion, bone marrow transplantation, and in solid organ transplantation. It was discovered by an Austrian pathologist, Karl Landsteiner, and described in 1901 (Landsteiner 1901). He mixed erythrocytes and serum from different members of his department, and observed that some mixtures formed clumps whereas others did not. Cumulatively, these findings suggested three groups; A, B and O with naturally occurring antibodies in the serum against the antigens the individual lacked. Prior to Landsteiner's discovery of the ABO system, transfusion was a dangerous practice associated with a significant risk of mortality (Giangrande 2000).

Despite our knowledge of their existence for over 100 years, a physiological function for ABO antigens has yet to be defined (Greenwell 1997). However, numerous association studies on ABO groups and human traits or diseases have been described.

Many have suggested statistical significance with spurious findings. The most trivial of these has suggested that Group A individuals have worse hangovers (Wiener 1970; Garratty 2000). Similarly, significant associations between ABO blood group and Intelligence Quotient (I.Q.) and socio-economic class have also been described, but clearly lack a rational scientific basis (Beardmore and Karimi-Booshehri 1983)

Nevertheless more recent work has identified plausible associations with infectious agents such as malaria, cancer and a risk of vascular thrombotic disease where a clear scientific mechanism is demonstrated.

Non-group O individuals have an increased incidence of arterial and venous thrombosis. This is due to increased Von Willebrand Factor levels, which are 25% higher in these groups.

Cellular adherence is key part of the pathogenesis of Malaria infection. Infection with *Plasmodium falciparum* may be less severe in group O patients. Erythrocytes infected with the parasites are more likely to bind to other erythrocytes bearing A or B antigens (Jenkins and O'Donnell 2006; Cserti and Dzik 2007).

1.2 Structure

The A and B blood groups are represented by the A and B antigens respectively whereas the O group consists solely of the H antigen which is a precursor common to A and B. The blood group antigens are sugar molecules formed by the sequential addition of sugars to a saccharide chain. The H antigen is an L-fucose, while the A and B groups are created by addition of a terminal N-acetyl-D-galactosamine or D-galactose respectively (Fig. 1.1) (King 2000). There are a potential six precursor carbohydrate chains beneath the H antigen, however the most important are type 1

chains, found in body fluids, and epithelia, and type 2 chains which are integral parts of the membranes and form the basis of ABO(H) on erythrocytes.

Red cells can also absorb some type 1 chain-bound blood group substance from the plasma. Once a precursor chain is fucosylated generating the H antigen, it is termed type 1H or type 2H. Glycan branching can also occur, leading to antennae with ABH bearing structures (Henrik and Sen-itiroh 1989).

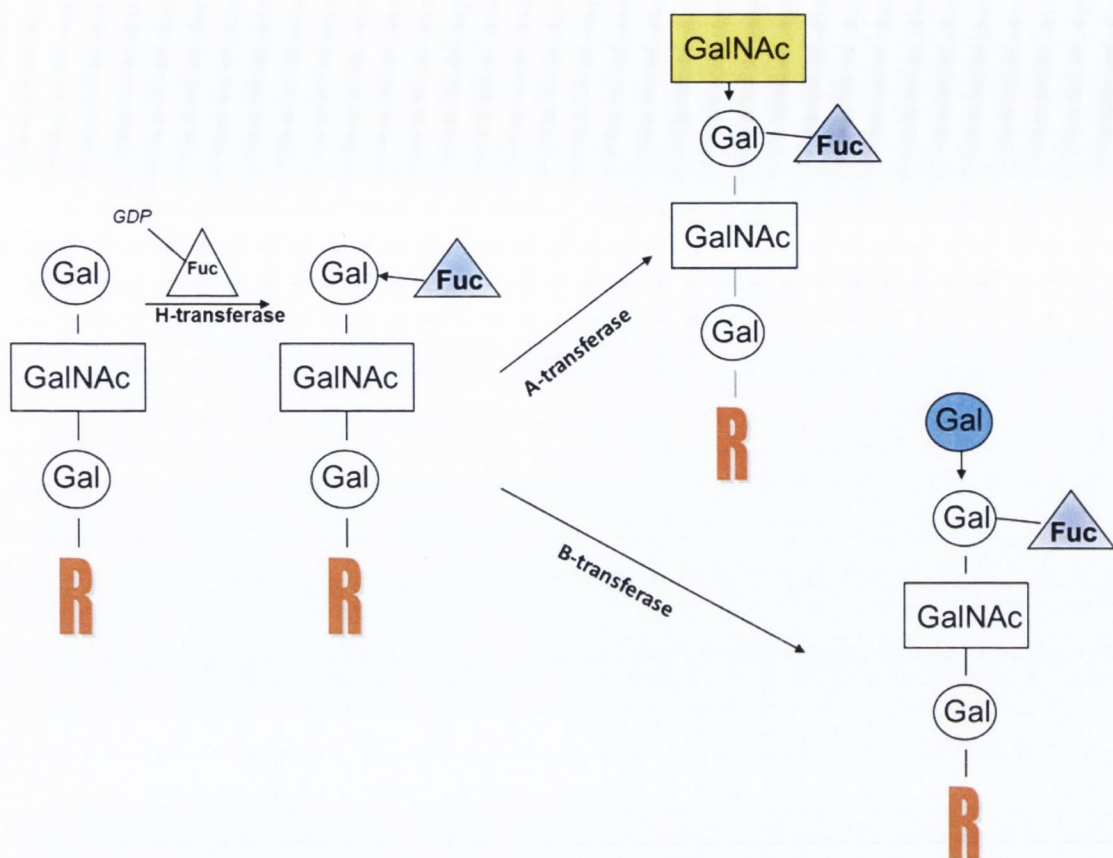


Figure 1.1

Synthesis of the ABH blood group determinants by the sequential addition of carbohydrate molecules. R is the precursor oligosaccharide chain, which may be linear or branched. First the H antigen is made by addition of a fucose (Fuc) by the H-transferase and then A or B by addition of N-acetylgalactosamine (GalNAc) to generate the A antigen and D-galactose (Gal) to form the B antigen.

1.3 ABO nomenclature

The ABO nomenclature is under consideration by the International Society for Blood transfusion (ISBT). In this thesis A₁ refers to the antigen. A₁ refers to the phenotype, as A₁ cells or A₁ individuals, while superscript A¹ refers to the allele. This numbering system is consistent with recent publications (Storry and Olsson 2009).

1.4 ABO glycosyltransferase

The ABO gene (*ABO*) does not generate the blood group antigens directly, but encodes a glycosyltransferase enzyme. The different ABO blood groups are due to specific variations in the coding sequence of the gene. These variations result in amino acid substitutions in the ABO glycosyltransferase which alter the substrate specificity of the enzyme or abolish enzymatic activity. The A and B antigens are generated by different affinities of the resulting enzyme for N-acetylgalactosamine and D-galactose. The H antigen is generated by the action of a different enzyme, a fucosyltransferase encoded by the *FUT1* gene on chromosome 19.

The *ABO* gene was cloned and sequenced by Yamamoto in 1990, and is located in chromosome 9q34.1-q34.2 (Yamamoto, Clausen *et al.* 1990). The gene is comprised of seven exons and consists of a coding region of 1065 base pairs. This encodes a 354 amino acid protein. Exon 6 and 7 are the largest exons and account for 77% of the full coding region. The transmembrane and N-terminal regions are coded by exons 2 and 4 respectively (Paulson and Colley 1989).

1.5 ABO glycosyltransferase gene sequence variation

1.5.1 A and B Alleles

Group A¹, B and O are the major alleles of this blood group system. Nevertheless there is extensive heterogeneity in sequence variation in the coding region. The B allele is differentiated from the A allele by seven single nucleotide polymorphisms; four of which translate into amino acid substitutions at residues 176, 235, 266 and 268 (Fig. 1.3). It is the residues at the third and fourth positions (266 and 268) that are critical in determining which sugar is utilised by the enzyme to generate A or B structures (Yamamoto, Marken *et al.* 1990; Yamamoto and McNeill 1996).

Additional sequence variation of the A and B alleles results in subgroups. The variations do not alter the sugar specificity of the enzyme but affect the enzymatic efficiency, resulting in decreased amount of blood group expression. The A² allele is the most common, in Europe, Near East and Africa (Tills, Teesdale *et al.* 1977; Daniels 1995). A single base deletion in exon 6 (1060delC) leads to a frame shift and an additional 21 amino acids being transcribed at the C-terminal. This leads to a 30-50 fold less enzymatic activity than the A₁ glycosyltransferase. Group A₃ is an A¹ allele but with an extra 871G>A substitution which is non-synonymous change from Asp to Asn at position 291 (Fig. 1.2). A_x and A_{el} are other rare subgroups. Similarly subgroups of B exist including B₃, B_x and B_{el} and each are associated with weak blood group B expression.

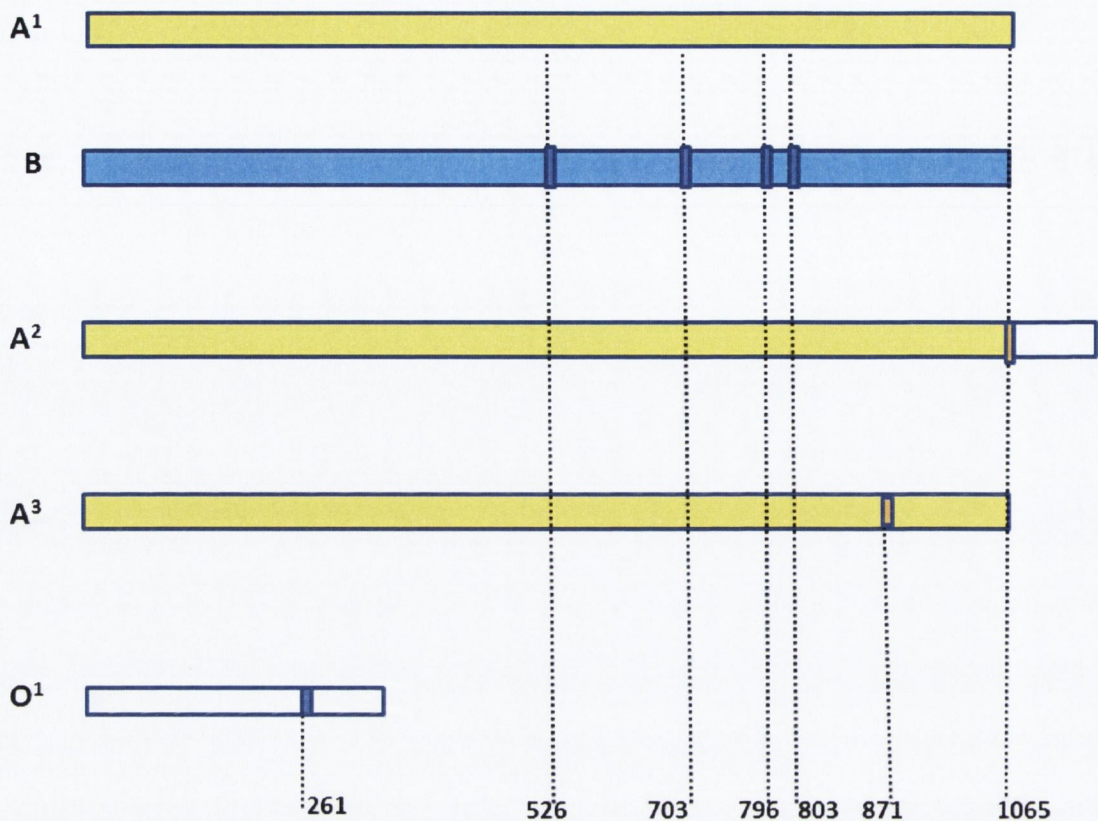


Figure 1.2

Representation of cDNA for each of the common *ABO* alleles. The yellow rectangle represents the A¹ consensus sequence of 1065 base pairs. The broken line and associated nucleotide positions indicate the position where mutations lead to amino acid substitutions. The clear rectangles represent the nonsense, non-A¹ consensus transcribed of A² (1060delC) and O (261delG) respectively. Group A₃ has the A¹ sequence with an additional single nucleotide polymorphism at position 871.

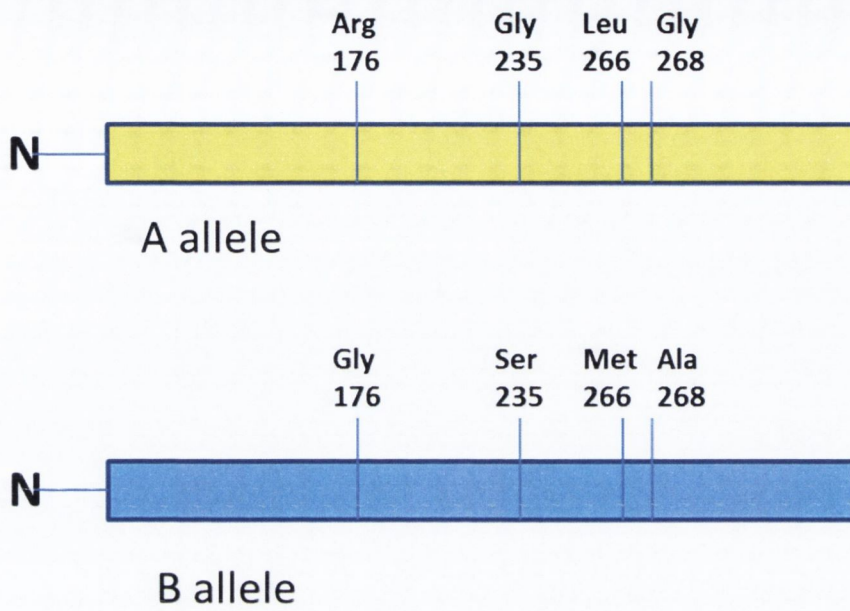


Figure 1.3

The protein products of the A and B glycosyltransferase genes are shown above. While each protein consists of 353 amino acids, there are four amino acid variations as indicated above

The O blood group, bearing the H antigenic determinant only, results from the absence of ABO glycosyltransferase activity. The predominant allele is derived from the A¹ allele but with a single G deletion at nucleotide 261 (261delG) in exon 6. This results in a reading frame shift and a premature stop codon (nucleotides 352-354) with a reading frame of 354 base pairs instead of the expected 1065. If translated the resulting protein would be truncated (116 amino acids) and non-functional. Therefore the base sugar structure (H antigen) is unmodified.

1.5.2 Regulation of ABO gene expression

The ABO gene has two promoter and enhancer sequences and a poorly defined 3' untranslated region. A proximal promoter is located between -117 and +31, while a distal promoter is located -832 to -667 relative to the transcription start site in exon 1 of the gene (Kominato, Hata *et al.* 2004). In addition, an N box sequence negative regulatory region has been identified upstream (-275 to -118) of the proximal promoter (Kominato, Hata *et al.* 2004). This N box binds to a nuclear factor RACP derived from Kato III cells.

Transcription of the ABO gene is also dependent upon binding of a transcription factor CBF/NF-Y to a minisatellite sequence (Kominato, Tsuchiya *et al.* 1997). This consists of a 43 base pair repeat unit located -3843 to -3672 bases upstream of the transcription start site. Interestingly, the number of repeats varies among ABO alleles. While the A², B and O¹ alleles contain 4 repeats, the group A¹ and O² alleles possess only one repeat (Fig. 1.4).

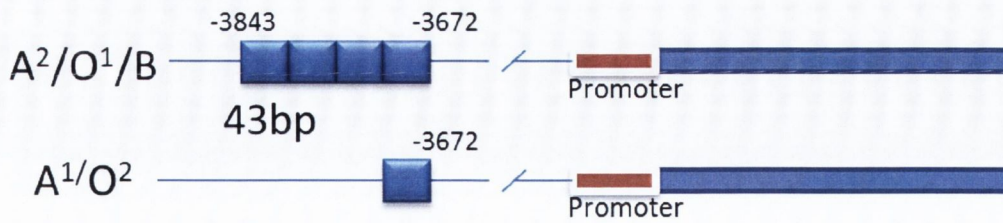


Figure 1.4

5' region of the *ABO* glycosyltransferase gene. The coding region is adjacent to the promoter on the right. Each unit of the ABO enhancer repeat sequence is represented by a single square.

In vitro expression studies suggest that the repeat copy number has a significant effect on A or B glycosyltransferase expression levels. Yu (Yu, Chang *et al.* 2000) studied the functional properties of minisatellite repeat numbers using a reporter gene assay, by introducing the enhancer of A¹ and B alleles upstream of the SV40 promoter of a pCAT3-promoter vector. These plasmids were introduced in to KATO III cells (a gastric cancer cell line). After 48 hours, expression levels of the reporter gene (CAT) were measured. Concentrations were 120-150 fold higher from the group B enhancer which contains 4 repeat elements. Moreover, this effect was further enhanced when the ABO gene promoter was added (~300 fold), demonstrating that the number of the repeat sequences clearly plays an important role in modifying ABO transcriptional regulation.

1.6 H determinant expression

The H determinant is the common precursor to A and B. The FUT1 gene on chromosome 19 encodes an alpha (1,2)-fucosyltransferase (H-transferase) and therefore ABO expression is dependent upon the functioning of this enzyme. Missense or nonsense mutations may lead to an absent or very weak H expression designated Bombay or Para-Bombay phenotype (Oriol, Candelier *et al.* 2000). The underlying sugar precursors remain un-fucosylated consisting of repeating and branched N-acetyllactosamine units which represent the Ii blood group system on red cells, which are precursors to the ABH antigens (Koscielak, Zdebska *et al.* 1979).

The I antigen is a branched form of the i antigen which is initiated by the I- transferase (Beta-1,6 N-acetyl glucosaminyltransferase). This becomes active after birth and accounts for the low ABH expression on neonatal red cells before 18 months of age (Hakomori 1981).

1.7 ABH on red cells

The ABO blood groups are borne on red cell membrane proteins with around two million antigen sites on each red cell. The main membrane glycoproteins involved are band 3 (anion transport protein) and band 4.5 (glucose transport protein), which express ABH antigens on their N-linked glycans (Anstee 1990). Glycophorins A, B and C are also membrane bound. While they predominantly express sialic acid residues a minor component also express ABH on O-linked glycans (Podbielska, Fredriksson *et al.* 2004).

Transcription of the H (*FUT1*) and *ABO* genes starts in immature peripheral blood progenitor cells and gradually decreases thereafter while H and A/B antigen

expression increases with cell differentiation and is maintained (Hosoi, Hirose *et al.* 2003). The majority of group A immature erythroblasts are A antigen positive however during further erythroid differentiation the binding diminishes on group A₂ cells but is maintained on group A₁ (Tulliez, Villeval *et al.* 1987).

Quantitative group A expression on mature red cells is known to vary significantly. First, it is well recognised that group A₂ red cells express 5 times less A antigen compared to group A₁ erythrocytes, due to the inefficiency of the A₂ transferase enzyme. Second, there is also variation of A expression within individuals of the same group A subgroup. For example, among group A₁ individuals, previous studies have also observed a 2-7 fold variation between the strongest and weakest A antigen expression levels (Smalley and Tucker 1983). Nevertheless, the biological mechanism(s) responsible for this inter-individual variation in expression levels has not been defined. However, Sharon *et al* demonstrated fluctuation in ABH expression during red cell aging in vivo. As cells age and get smaller A and B and to a lesser extent H expression decreases. It is also noteworthy that H expression on the red cells was homogenous while there were bimodal histograms of fluorescence of A on red cells labelled as 'low' and 'high' fluorescence (Fibach and Sharon 1994).

1.8 Platelet ABH expression

Platelets are small anucleate cells present in the circulation at a concentration of 150-400 cells x 10⁹/L. They form by budding from the membrane of megakaryocytes which are the large multinucleate precursor cells found in the bone marrow (Hoffbrand, Catovsky *et al.* 2005). Platelets are vital to primary haemostasis. Numerous platelet surface glycoproteins are involved in cell-to-cell interactions, via interactions with collagen on endothelial tissue, von Willebrand factor (VWF) interaction and interaction with fibrinogen and fibrin. The platelet glycoproteins include IIb/IIIa, Ib/IX, Ia/IIa, Ic, IV,

V, PECAM and CD109. These platelet surface glycoproteins also express ABH structures which are borne almost completely on type 2 precursor chains, and found particularly on GP IIb/IIIa (Santoso, Kiefel *et al.* 1991; Stockelberg, Hou *et al.* 1996) (Cooling, Kelly *et al.* 2005). In contrast to red cells, the contribution of adsorbed type 1 chain bearing ABO onto platelet surfaces is minimal, and therefore platelet ABH is predominantly due to endogenous production.

Nevertheless, there is also considerable heterogeneity in ABH antigen expression on platelet surfaces. Analysis of platelet A, B and H antigen expression by flow cytometry showed a broad distribution curve and high coefficient of variation (Dunstan and Simpson 1985; Dunstan, Simpson *et al.* 1985). Furthermore, considerable heterogeneity in ABH blood group antigen expression has also been reported on the surface of developing megakaryocytes. In vitro studies have shown populations of mature megakaryocytes exhibiting both weak and strong expression of A antigen and/or H antigen (Tulliez, Villeval *et al.* 1987).

Interestingly the expression of ABH on platelets is not a static phenomenon. For example expression of blood group increases during the storage of apheresis platelet products. Julmy *et al* demonstrated an increase in A expression among group A₁ platelets, noting that donors whose platelets expressed higher levels of A also had a greater potential for over expression during storage (Julmy, Achermann *et al.* 2003).

1.9 Platelet Expresser Phenotypes

While platelet ABH antigen expression is known to be heterogeneous, more recent studies have demonstrated that some individuals express unexpectedly high or low levels of blood group determinants. These variations were initially identified during investigations of platelet transfusion refractoriness. Typically in thrombocytopenic

patients receiving a platelet transfusion a post transfusion rise in platelet count (increment) is expected. Platelet refractoriness defines a clinical state whereby a patient's platelet count does not increase or does so minimally. This is most often mediated by immune or consumptive mechanisms, such as HLA alloimmunisation (e.g. from prior transfusion or pregnancy) where the transfused cells are rapidly cleared by recipient HLA antibodies (BCSH, (BCSH 2003)

In a Japanese study ten ABO incompatible transfusions were administered to a group O patient with high titre anti-A and anti- B antibodies. Two of these platelet units (both group B) did not result in any significant post transfusion increase in platelet count. Using quantitative flow cytometry these donors were found to have at least 20 times the amount of group B antigen expressed on their platelets compared to normal group B platelets. To determine whether such high expression was a common event, A and B antigen quantitation on randomly selected donors was performed. Within the population, both high and low expression were identified, designated high expresser phenotype (HXP) or low expresser phenotype (LXP) respectively. HXP (defined by ELISA absorbance, two standard deviations plus mean) was present at a frequency of 7% in the population (Ogasawara, Ueki *et al.* 1993).

Two subsequent studies in Caucasian populations have shown similar frequencies of HXP and LXP. Of 100 group A₁ and 100 group B blood donors, 7% and 4% respectively were designated HXP (defined in this study based upon the ratio of mean linear fluorescence intensity) (Curtis, Edwards *et al.* 2000). A second Caucasian study investigated 105 group A₁ donors, and instead assessed the percentage of platelets expressing A antigen. 4.8% were HXP defined by expression more than 75% A. In addition a further group of A₁ donors of low expression (LXP) with A<15% were also identified (Cooling, Kelly *et al.* 2005). Cumulatively, these emerging data demonstrate that despite considerable geographical variation in blood group frequencies, both the HXP and LXP are consistent phenomena. However, the biochemical basis underlying these two phenotypes remains unclear.

The classification of HXP has been further refined by examining platelet flow cytometry histogram patterns (Figure 1.5). Normal group A₁ donors demonstrate a characteristic bimodal peak of A expression. One platelet peak stains definitely positive for A antigen expression, whilst the second overlaps with the pattern of O and A₂. In contrast, platelets from group A HXP individuals demonstrate two distinct patterns of A antigen expression. Type I HXP is characterised by a skewed bimodal population of A expression on platelets, most of which express A antigen. In contrast type II high expressers are defined by the presence of a single sharply positive peak where all donor platelets express A antigen (Curtis, Edwards *et al.* 2000).

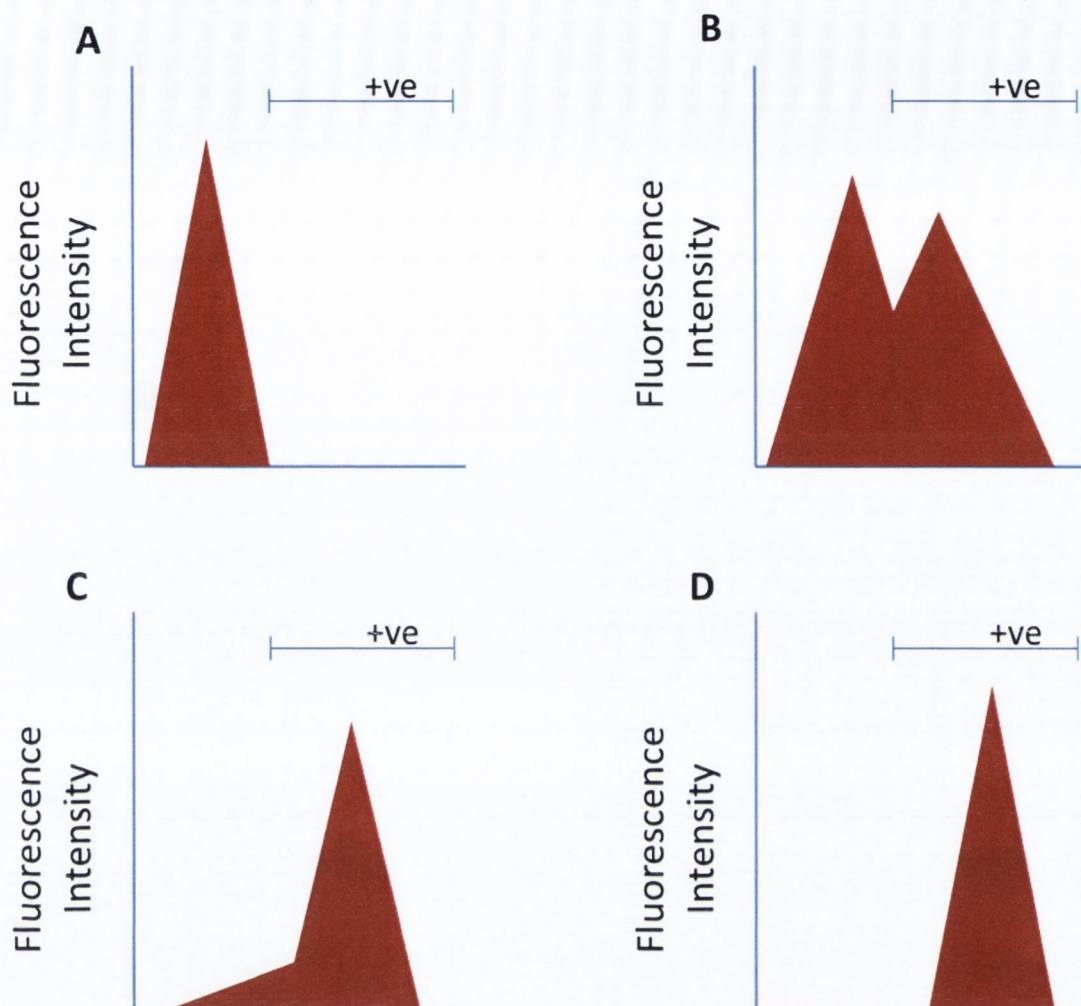


Figure 1.5

- (A) Representation of blood group expression on platelets. Group O and group A₂ express no A antigen.
- (B) Typical group A₁ bimodal distribution of A positive and negative within an individual.
- (C) Type I HXP is skewed positive with a weakly negative tail.
- (D) In type II HXP all platelets strongly express A antigen (D).

In contrast to the these novel data regarding the patterns of A and B antigen expression on platelets in HXP and LXP, the pattern of H expression on group A platelets is the subject of controversy. Studies by Cooling *et al* of group A platelets failed to detect H antigen on the proportion of A negative platelets within A₁ donors and not at all on group A₂, suggesting that the A₂ platelets are of a “Bombay-like” phenotype. Cooling *et al* suggest that this is due to megakaryocyte clones not expressing the FUT1 gene. However studies by Curtis *et al* have clearly demonstrated the presence of H antigen on all group A platelets, and failed to replicate the findings of Cooling (Curtis, Aster *et al.* 2006).

1.10 ABH on plasma proteins

ABH blood group determinants are also present on certain plasma proteins within the circulation and to date have been identified on factor VIII, von Willebrand factor (VWF) and α -2 macroglobulin. Human factor VIII and VWF have distinct functions in haemostasis but circulate as a complex, with the half-life of factor VIII dependent upon binding to VWF. VWF is heavily glycosylated and contains 22 glycosylation sites. Twelve are N-linked (on asparagine residues) and ten O-linked (on serine or threonine). Together this carbohydrate accounts for 19% of the total mass of VWF (Lenting, Westein *et al.* 2004). ABH determinants have been identified on the N-linked glycans of circulating VWF according to the blood group of the individual (Matsui, Fujimura *et al.* 1993). Interestingly, patients with the Bombay phenotype also lack ABH antigens on VWF (O'Donnell, McKinnon *et al.* 2005). Additionally ABH structures are only present on VWF derived from endothelial cells. Surprisingly, platelet derived VWF does not express ABH structures (Brown, Collins *et al.* 2002)

1.11 Von Willebrand Factor levels and ABO

VWF levels vary consistently according to ABO blood group. Numerous studies have shown that group AB have the highest and group O individuals have lowest levels. For example, in a study of 1117 volunteer blood donors, mean VWF levels in group AB individuals was 123.3U/dl, compared to only 74.8 U/dl in group O donors. (Gill, Endres-Brooks *et al.* 1987). Although this effect of ABO on plasma VWF-FVIII levels has been recognised for many years, the underlying molecular basis remains unclear. However, linkage analysis studies have shown that there is a direct effect of the ABO locus rather than an independent VWF regulatory region in linkage disequilibrium with the locus (Souto, Almasry *et al.* 2000).

1.12 Study aims

1.12.1 To determine the presence of the Expresser Phenotypes in the Irish blood donor population and explore the nature of ABH expression on platelets

- A. Blood group A expression on platelets will be examined in a cohort of normal group A Irish blood donors to determine the prevalence of both HXP and LXP and determine whether any regional variation occurs. In addition, expression of H antigen will also be examined to determine whether H is present on all populations of platelets and to characterise quantitative variation in H antigen expression in normal group A and group O individuals.
- B. ABH antigens and sialic acid are closely related. They are both terminal glycans and are expressed in close proximity on platelet glycoproteins. Therefore we will define whether platelet ABH expression can influence the expression of sialic acid.

1.12.2 To investigate the molecular mechanisms underlying high expression of blood group antigens on platelets

A cohort of HXP and LXP will be identified. Genetic mechanisms underlying the phenotypes will be investigated. As there has been limited investigation of HXP individuals the expresser phenotype cohort, the ABO glycosyltransferase gene will be sequenced in its entirety. The ABO enhancer region will also be studied.

1.12.3 Analysis of Plasma Proteins for ABH expression

It is not known whether the HXP is limited to platelets or whether it also can affect ABH expression on other plasma glycoproteins. We will study VWF to see if this is a tissue-specific phenomenon limited to platelets. ABH expression on plasma VWF will also be studied to determine whether HXP also influences the loading of ABH on VWF.

Chapter 2

Platelet ABO blood group expression

2.1 INTRODUCTION

ABO (H) determinants are present on the surface of the platelet membrane. They are the terminal carbohydrate antigens on saccharide chains attached to cell surface glycoproteins such as glycoprotein IIb/IIIa. These membrane glycoproteins are essential for the haemostatic function of platelets through cellular adhesion. However, the pattern of expression of blood group on platelets in individuals is not uniform, with inter-donor and intra-donor variation. Not all platelets within the circulation express similar levels of A or B antigen. As discussed in Chapter 1, recent studies have reported that some donors have particularly high or low expression of A and B antigen on their platelets, and have been designated High Expresser Phenotype (HXP) or Low Expresser Phenotype (LXP) respectively (Cooling, Kelly *et al.* 2005). Furthermore, the nature of H expression on platelets remains controversial (Curtis, Aster *et al.* 2006).

2.2 AIMS

In this study a healthy population of volunteer donors has been studied to determine the presence and prevalence of the expresser phenotypes in Irish Blood Donors.

Subsequently the controversy of the nature of H-expression on group A₁ and A₂ is explored. In addition the pattern of H expression on group O has not previously been described. This is critical as this is the substrate for the A/B antigens and thus directly related to the expresser phenotypes

2.3 METHODS

DONOR SPECIMENS

Platelets were obtained from donors that attended the National Blood Centre at the Irish Blood Transfusion Service, National Blood Centre at St. James's Hospital, Dublin. Donors who donate blood at the IBTS are specifically informed that unused portions of the collected blood may be used for quality control, audit or research and development purposes within the Irish Health System, or in other public laboratories, or in third level institutions, in a non-commercial fashion. They are then asked to sign a consent form to donation that specifies that they have read and understood the information provided. This consent has been formally reviewed by the St James's Hospital Ethics Committee, and has been accepted as adequate consent for ethics purposes, including for anonymous gene banking.

Group A and Group O platelet donors were selected by reviewing scheduled donor appointments. All available donors were included. As many donors return on a regular basis careful review was necessary to avoid unnecessary sample collections. Blood samples were collected over a one year period. Group A were selected due to the low prevalence of group B in the population (11%) (Dawson 1964).

A large bore 16 gauge intravenous cannula was inserted in to the antecubital vein in the donor's arm for the apheresis procedure 12 ml of whole blood was obtained in sodium citrate and 3 ml in EDTA [ethylenediaminetetraacetic acid] anticoagulated tubes. Samples in EDTA were frozen and stored for DNA extraction. Samples in citrate were processed to obtain platelet rich plasma (PRP) and platelet poor plasma (PPP). In total, samples were collected from 231 group A and 310 group O donors.

Samples were processed within 120 minutes of collection. Citrated whole blood was centrifuged at 170g for 10 minutes. PRP was carefully removed by Pasteur pipette to avoid platelet activation. The platelet count was measured using a Cell-Dyn 3200 analyser (Abbott laboratories, IS)

PPP was obtained from sodium citrate anticoagulated blood by centrifugation at 2000g for 10 minutes and the supernatant again centrifuged for 10 minutes. The PPP was then aliquoted and stored at minus 80 C.

GROUP A SUBGROUPING

Group A donor sub grouping was determined by standard red cell serology. Donor red cells were sub grouped by failure to agglutinate to anti-A₁, and were considered A₂. A 3% suspension of washed red cells in phosphate buffered saline (0.7%) was prepared. The samples were centrifuged at 1000g for 1 minute and supernatant removed. 1ml of blood grouping reagent, anti-A₁ lectin *Dolichos biflorus* (Alba Bioscience, Edinburgh), was added. The sample was incubated for 10 minutes and centrifuged to obtain a pellet. The tube was agitated. If the pellet resuspended this was interpreted as a failure to agglutinate to anti-A₁ and thus the sample was designated group A₂.

FLUOROCHROME LABELLED ANTIBODIES FOR FLOW CYTOMETRY

Phycoerythrin (PE)-labelled anti-human CD41a and Fluorescein (FITC)-labelled Mouse IgG1κ isotype control were obtained from BD Biosciences Pharmingen (San Jose, CA). FITC-labelled *Helix pomatia* (HPA, Anti-A) and FITC-labelled *Ulex europaeus* (anti-H) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO)

FLOW CYTOMETRY

10^6 platelets were added to 90 μ L of Phosphate buffered saline (PBS). 5 μ L of PE labelled anti-CD41a and 5 μ L of FITC labelled lectin were added to 10^6 platelets for a final volume of 100 μ L. Blood group A was detected by FITC-Helix pomatia (1 in 20 working dilution, determined as the saturation point of the lectin). H antigen, was detected by FITC-Ulex europaeus again at 1 in 20. Samples were incubated in the dark for 30 minutes. PBS was added to a final volume of 1 ml. Samples were immediately analysed on a BD FACSanto II (BD Biosciences, San Jose, CA) counting 10,000 per sample. Outcome was measured by mean per cent platelets positive for both CD41 and HPA or UEA. The mean fluorescence intensity of platelets positive for CD41 and UEA was also recorded. Each sample was analysed with isotype control. All group A were stained in parallel with UEA and HPA and were analysed in duplicate. Data was analysed by FACSDiva software (BD Biosciences, San Jose, CA)

DATA ANALYSIS

Expresser phenotypes were determined by quantifying the percentage of a donor's platelets expressing blood group antigen. In keeping with previous studies, HXP was defined as greater than 75% platelet positivity, and LXP defined as less than 15% platelet positivity, (Cooling *et al*).

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism Version 5.0 (Graphpad Software, San Diego, CA). Statistical tests are indicated in the appropriate section. A P value of <0.05 is considered significant. Mann-Whitney U test was used to test differences in median values

2.4 RESULTS

EXPRESSION OF A ANTIGEN ON PLATELETS

Blood group A expression was determined by quantitative flow cytometry on 231 group A (180 A₁ and 51 A₂) Irish Caucasian donors (Table 2.1). Expression was reported as percentage of platelets positive for A antigen within a given donor. Group A₁ donors exhibited a wide variation in expression (A, median 53.7%, interquartile range, 46.8-63.9%). In contrast A₂ donors express minimal A (median 0.3%, interquartile range, 0.2-0.6%) (Fig. 2.1). A positive correlation between signal fluorescent intensity and the percentage of platelets positive for A antigen was detected. ($P < 0.0001$, $r = 0.928$, Spearman) (Fig. 2.2). Repeat investigations were performed on 14 individuals to assess stability of the platelet A antigen expression. These repeat studies demonstrated that A expression levels were remarkably consistent (mean change, 9.5%). The intra and inter assay coefficient of variation were 1.16% and 12.45% respectively.

In total, 10 A₁ donors (5.5%) exhibited HXP (as defined by Cooling *et al*) with greater than 75% of their platelets expressing A antigen (Table 2.2). Analysis of the platelet A antigen expression pattern by flow cytometry in these individuals identified 8 HXP donors who exhibited type I HXP (bimodal distribution) whereas two individuals exhibited type II HXP (unimodal distribution) (Figure 2.3). Repeat testing of the type II HXP donors again demonstrated that this was a stable phenomenon. Both of these donors returned to donate platelets and were tested again, twice and three times respectively. In addition, eight A₁ donors had very low A antigen expression levels on their platelets (4.5-13.3%) consistent with a low expresser phenotype (Fig. 2.3).

Donor	All group A	A ₁	A ₂
Characteristics			
Total, no. (%)	231 (100)	180 (78)	51 (22)
Sex, no. (%)			
Male	182 (79)	143 (78)	39 (22)
Female	49 (21)	37 (76)	12 (24)
Age, X±SD			
Male	41±10.6	41±10.6	41±10.8
Female	42±10.0	42±10.1	41±10.0
Female	39±12.6	39±12.0	39±13.1

Table 2.1

Demographics of the study population of group A donors. The majority of platelet apheresis donors are male. Female donors who have been pregnant are not accepted as platelet apheresis donors by the IBTS. Pregnancy can lead to Human Leukocyte Antigen (HLA) alloimmunisation. These antibodies have been implicated in Transfusion Related Acute Lung Injury (TRALI), in recipients of these products. Therefore, male donors are favoured as apheresis donors, which is reflected in the numbers described above.

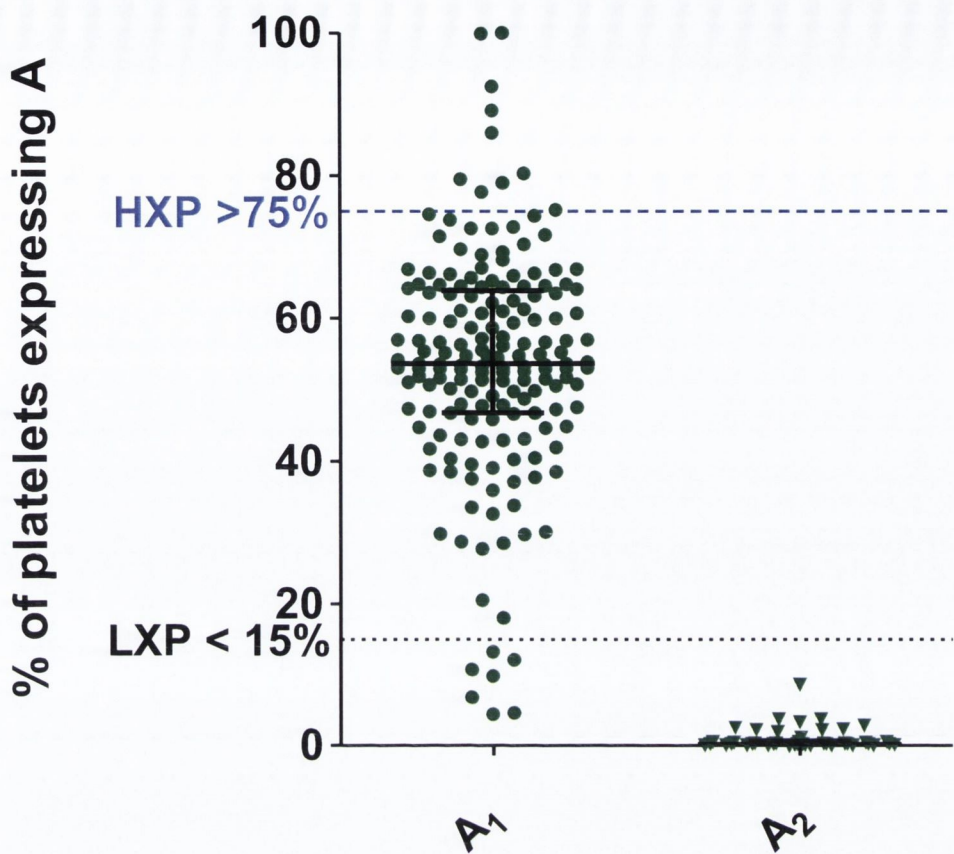


Figure 2.1

The expression of A antigen on group A donors is shown. Each point represents the percentage of platelets expressing A antigen in a given individual donor. The HXP and LXP populations are defined as greater than 75% and less than 15% A, respectively as illustrated. The median and interquartile range is indicated above.

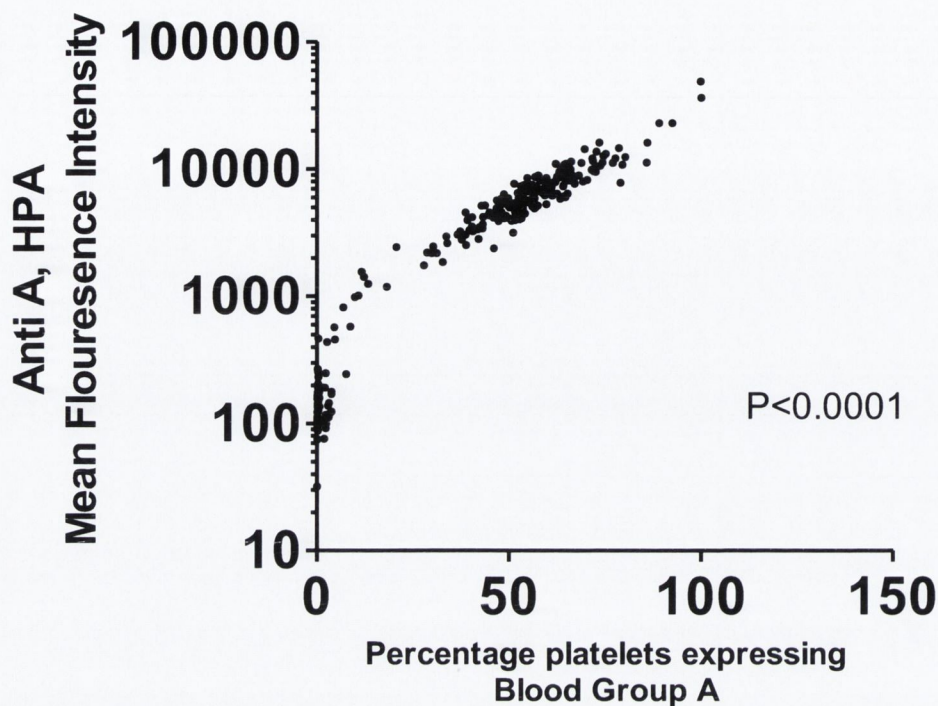


Figure 2.2

The mean fluorescence intensity of group A platelets measured against the percentage platelets positive for A antigen. Platelets were labelled with FITC conjugated anti-A as described in Methods. The fluorescence intensity demonstrates a positive correlation with the percentage of platelets expressing A ($P<0.0001$, $r=0.928$, Spearman).

Donor	Type I High	Type II High	Low Expresser
Characteristics	Expresser	Expresser	
Total, no. (%)	8 (4.4)	2 (1.1)	8 (4.4)

Table 2.2

Platelet expresser phenotype distribution in Irish Blood Donors. The overall prevalence of HXP (5.5%) is comparable to previous studies (5-7%).

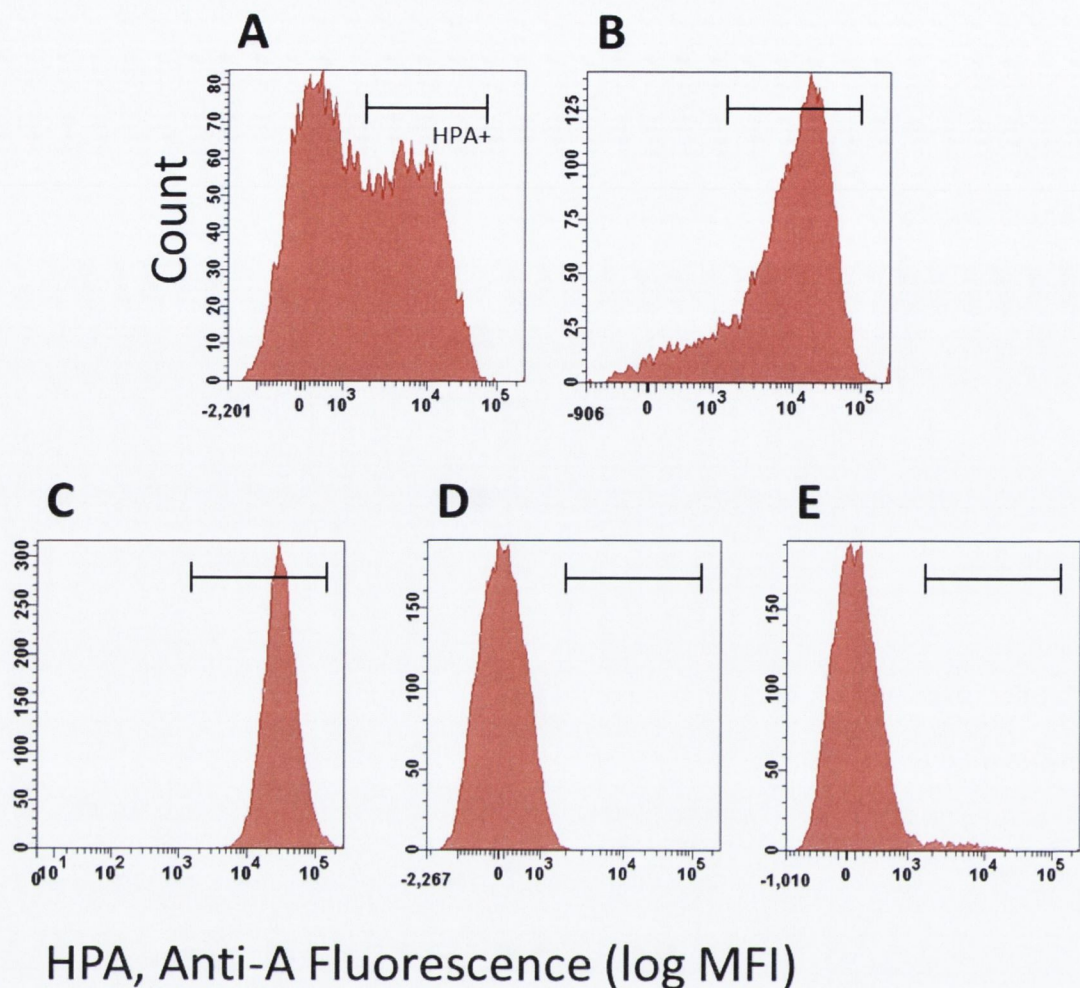


Figure 2.3

Expression of A antigen on group A donors. Group A₁ donors demonstrate a bimodal population of negative and positive populations (HPA+) within a donor's platelet population (Panel A). A type I HXP is also bimodal but predominantly positive (Panel B), while a type II HXP consists of a single uniform positive peak (Panel C). In contrast a group A₂ donor expresses no A antigen (Panel D), while a group A₁ donor of low expresser phenotype expresses minimal A, indicated by the weakly positive tail on the fluorescence histogram (Panel E)

EXPRESSION OF O(H) ANTIGEN ON PLATELETS

The expression of H antigen on group O donors was studied by quantitative flow cytometry using the lectin *Ulex europaeus* (UEA). This was to determine whether all platelets within an O donor expresses H antigen or whether a bimodal population and HXP similar to that of A antigen in A₁ donors exists.

We found that in 310 group O donors studied that all cells strongly expressed H antigen and no H negative population existed in any donor. Nevertheless, there was wide variation in the intensity of expression among donors (Fig. 2.4). The mean MFI was 54640 ± 16329 SD.

We then studied H antigen expression on 231 group A donors. Again all A₁ donors expressed H antigen with similar intensity as group O (Mean MFI, 51983 ± 18400 SD) and was present on **all** platelets expressed as a single positive unimodal peak (Fig. 2.4, Panel A).

One of the two type II HXP exhibited a marked reduction in expression of H antigen (MFI, 3993), (Fig. 2.5). Group A₂ donors also expressed H antigen and had a similar expression pattern to group A₁ donors. No 'Bombay-like' H negative population was seen, described by Cooling *et al.* The MFI of A and H correlated on A₁ donors ($P=0.04$), however no relationship existed between percentage of platelets expressing A ($P=0.5$).

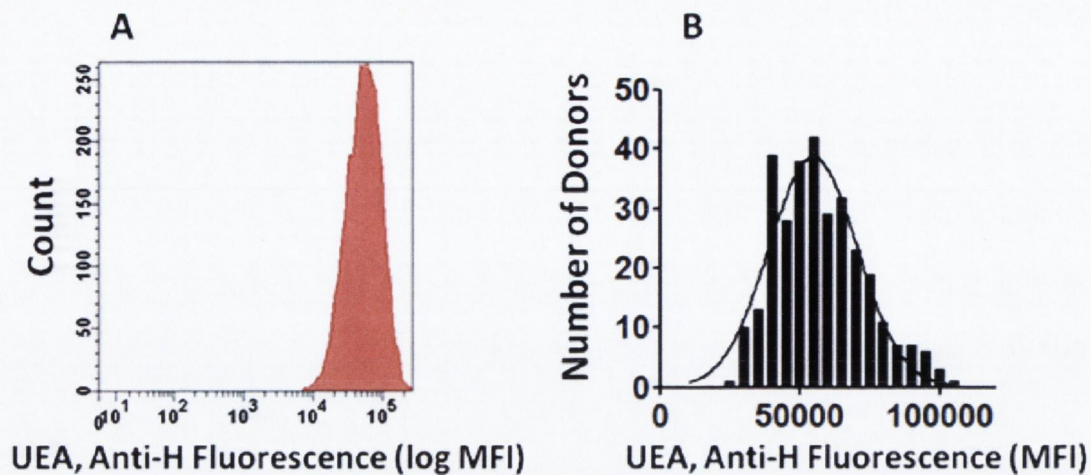


Figure 2.4.

The pattern of expression of H antigen on group O donors. All group O platelets express H antigen as indicated by a unimodal positive peak on the fluorescence histogram (A). The mean fluorescence intensity of H antigen varies between donors, by 2.3-fold (B)

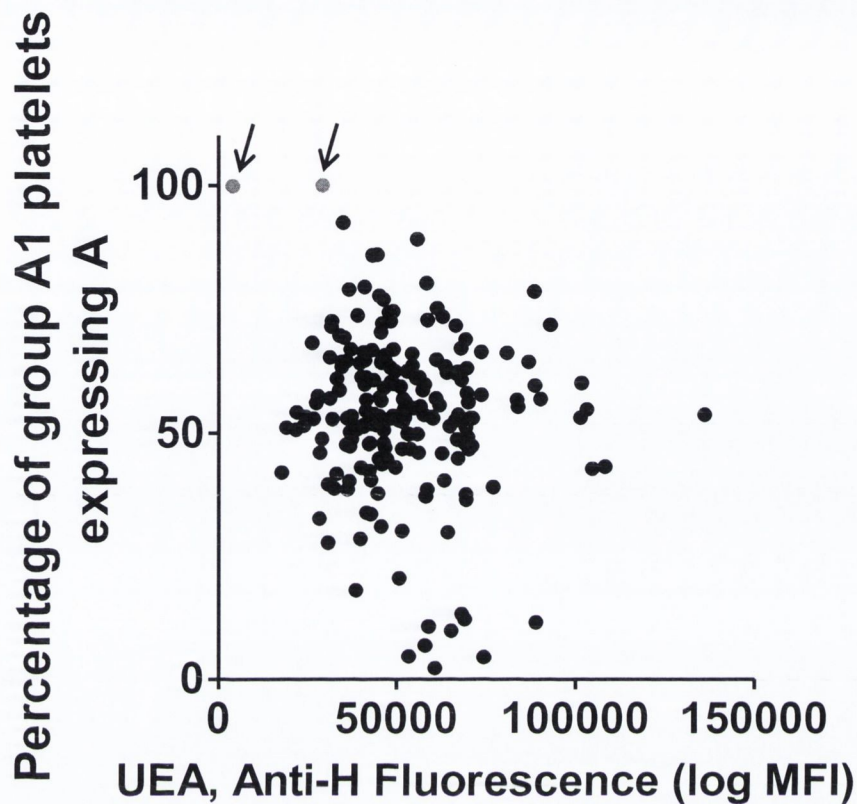


Figure 2.5

H expression on the surface of platelets in group A₁ individuals. H expression was measured by direct binding of the FITC labelled anti-H lectin, UEA. There is a wide distribution of H antigen expression on group A₁ donors. No correlation exists between H MFI and percentage of platelets expressing A ($P=0.51$, $r=-0.05$, Spearman). The two type II HXP are indicated in red, and with arrows. One has a marked reduction in H expression.

2.5 DISCUSSION

Blood transfusion services in times of shortage of platelet products may cross groups and transfuse ABO incompatible products. This is not ideal as it may lead to poor post transfusion platelet increments. For example, in a prospective study in thrombocytopenic children with malignancy or aplastic anaemia outcomes of mismatched A₁ and A₂ platelet transfusions were studied. Group A₁ transfusions were inferior as the A positive platelets were rapidly cleared from the recipients circulation while the A₂, lacking A, remained. In our study of 231 group A positive individuals, 22% of platelet donors subgroup as group A₂. As demonstrated in Fig. 2.1, group A₂ platelets express no or minimal A antigen and are thus group O compatible.

ABO mis-matched platelet transfusion also allowed the discovery of expresser phenotypes by Ogaswara, which has subsequently been confirmed by two separate studies. In this study the HXP and LXP has been identified in the Irish donor population and is the largest study population to date. Cooling *et al* describe a wide variation in A expression on group A platelets ranging from 8-87.5%. Similarly, in this study a similar variability is seen. The frequency of the HXP in Ireland is 5.5% which is in keeping with prior reports (range, 5-7%).

To exclude the possibility of platelet activation accounting for increased A expression we minimised platelet handling. Moreover, HXP donors were re tested on several occasions which clearly demonstrated that the HXP was a consistent phenomenon.

On red cells there is an inverse relationship between the expression of H and A antigen. Erythrocytes from A₂ individuals express more H and less A antigens compared to red cells from group A₁ donors. These findings are consistent with the reduced catalytic efficacy of the A transferase in group A₂ individuals, which ultimately means that less H antigen is converted into A (Daniels 1995). A similar phenomenon of

inverse correlation between A and H antigen expression levels would be expected on platelets. However previous reports have reached differing conclusions in this regard. Cooling *et al* reported that A and H were proportionately expressed in A₁ platelets. In particular, the A negative platelets identified by flow cytometry apparently also failed to express H antigen. Furthermore, group A₂ donors lacked H and A entirely and were consequently designated 'Bombay-like'. Finally, 45% of group O platelets were also found to lack H (range 45%-100%, H positive). These data raised the novel hypothesis that clonal variation in the expression of the FUT1 gene in some megakaryocytes might be responsible for determining quantitative ABH antigen expression on circulating platelets. Dunstan *et al* identified megakaryocyte populations with reduced or absent H expression and therefore reflects the pattern of ABH expression in platelets. In contrast however, we observed H antigen expression in all platelets derived from group A₁, A₂ or O individuals.

Noting that the expression of H and AB antigens are governed by independent molecular mechanisms, group O donors were studied to determine whether a HXP of H antigen exists which would provide high levels of substrate if a functional A/B glycosyltransferase were expressed. 310 group O donors were studied. All strongly expressed H antigens and no "Bombay-like" population was identified. There was a wide variation in levels of H expression measured by the mean fluorescence intensity of UEA binding. This may be an independent variable affecting to intensity of A expression on group A₁ platelets.

Nevertheless, the population of platelets in A₁ donors that are A negative remains unexplained. The N and O linked glycans on platelets are complex structures and are heavily sialylated. Therefore the H structures may be sterically unfavourable or alternatively the ABO glycosyltransferase may be in competition with a sialyltransferase for the penultimate oligosaccharide at the end of a type 2 chain such as on Glycoprotein Ia which is predominantly sialylated (Tsuji, Tsunehisa *et al*. 1983).

Our findings agree with Curtis *et al* who repeated the analysis of platelet A and H expression using their own methodology and that of Cooling *et al*. They also found that both A₂ and O platelets expressed similar amounts of H and that H is reduced where A antigen is over expressed. This is in the setting of a Type II HXP. This is not surprising considering the elevated A glycosyltransferase levels in Type II HXP which would be available to convert more A to H.

In reply, Cooling *et al* suggested that the findings may be related to their use of centrifuged washed platelets which may have activated them leading to increased ABH expression. Therefore in our study we avoided centrifugation, washing and used the same one-step labelling procedure as Cooling *et al*.

Similarly the contribution of genotype remains only partially explored. Jumly *et al*, 2003, did not find a relationship between A¹ genotype and the level of A antigen expression nor expresser phenotype. This is surprising as genotype has been shown to be associated with glycosyltransferase level and the quantity of A antigen expressed on Von Willebrand Factor (O'Donnell, Boulton *et al*. 2002).

Therefore the molecular mechanisms of the HXP and platelet blood group expression warrant further investigation. In conclusion, this study identifies the prevalence of HXP in Irish blood donors. The absence of a 'Bombay-like' phenotype in group A₂ platelets is also confirmed. Finally, group O platelets all express the H determinant to a variable degree but a HXP of H on group O is not seen.

Chapter 3

Sialic acid expression on platelets

3.1 INTRODUCTION

Sialic acids are monosaccharides found on terminal positions of cell surface glycoconjugates. They are generated by sialyltransferases which transfer the sialic acid from the donor substrate *CMP- sialic acid* to acceptor oligosaccharides which are otherwise capped with fucose or ABH determinants. The oligosaccharide chains may be both N-linked and O linked (Fig. 3.1). Importantly, sialic acid residues have a negative charge and thus play an important role in mediating the functional properties of many glycoproteins. They are recognized by sialic acid specific lectins, such as Sambucus Nigra Agglutinin (SNA). While acting as ligands, they also have a protective function by shielding underlying carbohydrates from lectin binding. Loss of sialic acid residues from erythrocytes exposes an underlying β galactose residue, which leads to red cell removal by recognition of the exposed residues by the lectin asialoglycoprotein receptor (Bratosin, Mazurier et al. 1995).

Sialic acid is also widely expressed as a terminal capping carbohydrate residue on the N- and O-linked glycans of many platelet surface glycoproteins (Tsuji and Osawa 1987). Furthermore, recent studies have shown that variation in platelet sialic acid expression is of direct physiological importance. In particular, sialic acid expression plays a critical role in modulating platelet clearance. Sorensen *et al* recently demonstrated that ST3Gal-IV^{-/-} mice have thrombocytopenia due to enhanced clearance through recognition of exposed terminal galactose residues on the platelet surface. However if the N-terminal domain of GP Ib is removed, the thrombocytopenia was significantly improved, suggesting that GPIb α played a major role in mediating the enhanced platelet clearance (Sorensen, Rumjantseva *et al.* 2009).

Interestingly, ABO(H) blood group determinants are also expressed as capping sugar residues. Moreover, ABH determinants and sialic acid are both expressed as terminal capping residues on a number of platelet membrane glycoproteins (GP) including GpIB, GpIIb and GpIIIa (Ogasawara, Ueki *et al.* 1993). Nevertheless, it remains unclear whether quantitative ABO(H) antigen expression influences sialic acid expression levels.

Similarly, erythrocytes express cell surface ABO(H) determinants and are also heavily sialylated. Importantly, Cohen *et al.* recently demonstrated that ABH antigen expression modulates α 2-6 linked sialic acid presentation on erythrocyte surfaces by stabilizing sialylated glycan clusters (Cohen, Hurtado-Ziola *et al.* 2009). For example on group A red cells, A antigens are present on the periphery of the cluster while sialic acids form a continuous cluster leading to a local increase in sialic acid concentration. This novel effect of ABO is not only of scientific interest, but also of direct translational significance since erythrocyte sialic acid mediates cell–cell interactions and interactions with pathogens including *Plasmodium falciparum* (Martin, Rayner *et al.* 2005)

To address the question whether ABO blood group may also regulate qualitative sialic acid expression on platelet surfaces, we have investigated the effect of HXP and LXP phenotypes on platelet sialic acid expression.

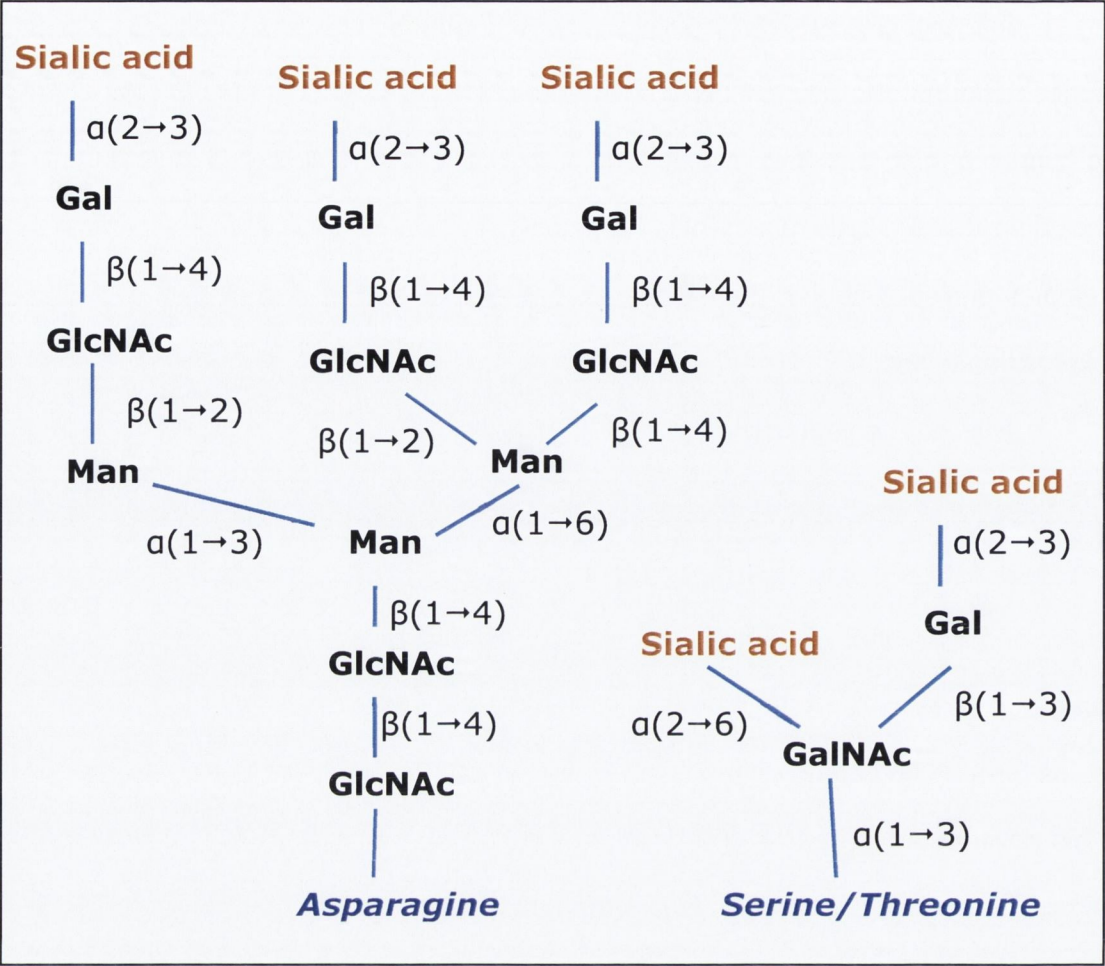


Figure 3.1.

Structure of sialylated oligosaccharides. N-linked are branched, and triantennary in this case, with a terminal $\alpha(2,3)$ linked sialic acid (N-acetylneuraminic acid). Sugars are Mannose (Man), N-acetyl-galactosamine (GlcNAc), and Galactose (Gal). Asparagine forms part of an amino acid chain (left).

O-linked oligosaccharide chains are linked to underlying serine or threonine residues. Sialic acid is attached to N-acetyl galactosamine (GalNAc) directly in an $\alpha(2,6)$ linkage or caps a galactose residue in an $\alpha(2,3)$ linkage.

3.2 METHODS

Platelet-rich plasma was prepared from blood samples collected from 117 healthy blood donors (72 group O and 45 group A). The group A donors were further subgrouped using anti-A₁ (32 group A₁ and 13 group A₂). Platelet expression of A antigen and H antigen were analysed by flow cytometry using FITC-labelled *Helix pomatia* (HPA, Anti-A) (Sigma-Aldrich; St. Louis, MO) and FITC-labelled *Ulex europaeus* (UEA, anti-H) (Sigma-Aldrich; St. Louis, MO), and Phycoerythrin (PE)-labelled anti-human CD41a (BD Biosciences Pharmingen; San Diego, CA) as described in Chapter 2. In addition, platelet α 2-6 linked sialic acid expression was analysed using FITC-labelled *Sambucus nigra* agglutinin (SNA) (Vector Laboratories Inc: Burlingame, CA).

3.3 RESULTS

Independent of ABO blood group, platelet sialic acid expression (as determined by SNA binding) demonstrated a single positive unimodal peak (Fig. 3.1C). This contrasts with the characteristic bimodal distribution of A antigen expression on group A platelets previously described (Chapter 2). Interestingly, platelet expression of A, H and α 2-6 linked sialic acid all varied widely in normal donors, even within specific blood groups (Table 3.1), (Fig. 3.2). Despite the ability of ABH determinants to regulate sialic acid presentation on erythrocytes, we found that ABO blood group did not affect sialic acid recognition by SNA on platelet surfaces (Fig. 3.3). Moreover, quantitative A antigen expression did not correlate with platelet SNA binding (Fig. 3.4).

Group	No.	Mean MFI, SNA
O	72	18727±6291
A ₁	32	19049±7271
A ₂	13	19364±4705

Mean values ± SD

Table 3.1.

The mean fluorescence intensity (MFI) of SNA is listed according to blood group.

No statistically significant difference exists between groups.

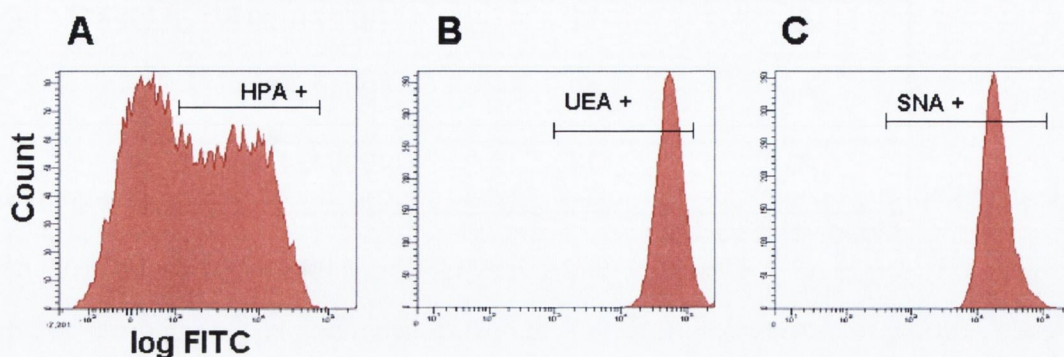


Figure 3.2

Fluorescence histograms showing characteristic examples of quantitative glycan expression on group A platelets. Group A₁ have a bimodal population of A positive and A negative platelets (A). In contrast both H antigen and sialic acid are expressed uniformly on all platelets (B,C).

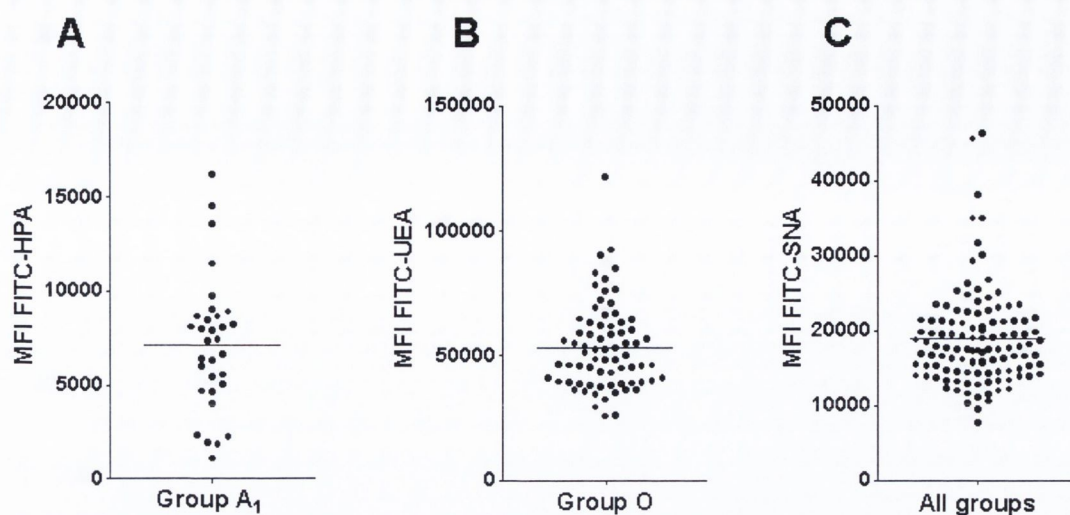


Figure 3.3

Mean fluorescence intensity (MFI) on group A and H antigen on group O varies widely between individual normal donors (A,B). This wide variation in expression in ABH glycans is mirrored by the expression of sialic acid (C).

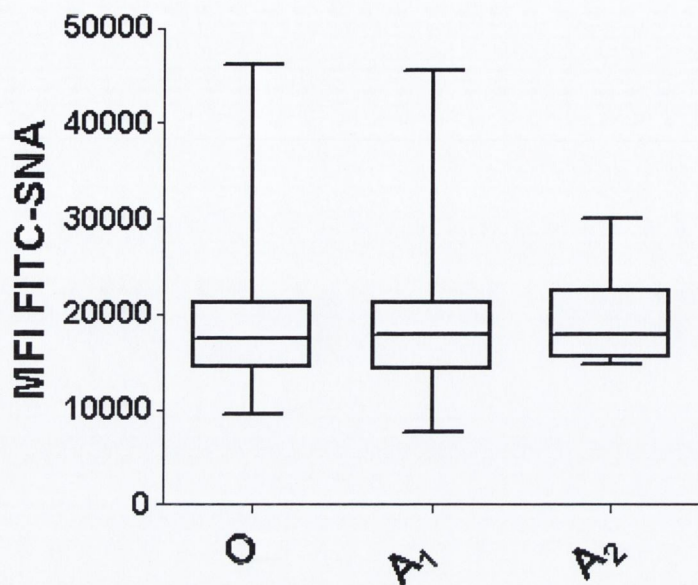


Figure 3.4

Platelet sialic acid expression does not vary with ABO genotype.

The MFI of sialic acid is similar on each blood group. Unlike red cells, the ABO blood group does not appear to influence sialic acid binding on platelets.

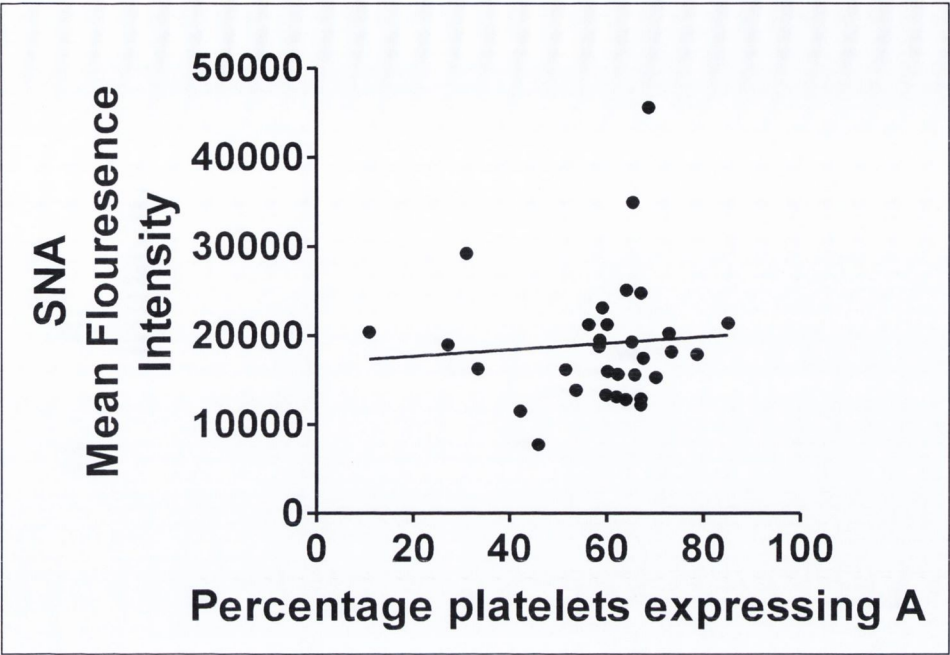


Figure 3.5

Platelet sialic acid expression does not correlate with platelet A antigen expression.

Comparison of percentage of platelets expressing A determinants and sialic acid expression (SNA, MFI). No correlation exists ($P=0.59$, $r=0.09$, Spearman).

3.4 DISCUSSION

In conclusion, Cohen *et al* have recently identified an entirely novel physiological function for ABH blood group determinants in regulating sialic acid presentation on erythrocytes. Our novel additional findings demonstrate that this novel effect is not replicated on platelet surfaces. This discrepancy may reflect the different glycoproteins involved, and/or the difference in quantitative ABH expression levels between platelets and erythrocytes respectively. In addition, our data also clearly demonstrates that significant quantitative variation in platelet ABH antigen, and perhaps more importantly α 2-6 linked sialic acid expression, does occur amongst normal individuals. Further studies will be required to define the molecular mechanism(s) responsible for these unexplained variations in terminal glycans expression, and whether they impact significantly upon platelet survival. Interestingly, no significant reduction in platelet sialic acid expression was observed in normal donors with ABO HXP phenotype. Similarly, LXP individuals demonstrated no increase in sialic acid expression

In this context, it is important to note that platelet glycoproteins are not uniformly glycosylated. Glycoprotein Iba is 40-60% carbohydrate consisting of galactose, galactosamine and sialic acid. The majority are O linked with a complex hexasaccharide structure, and may have sialic acid in both α 2-3 and α 2-6 linkages (Judson, Anstee *et al.* 1982; Tsuji, Tsunehisa *et al.* 1983). A minority of chains are N-linked and are sialylated bi, tri or tetraantennary structures (Tsuji and Osawa 1987; Korrel, Clemetson *et al.* 1988). Similarly GP IV is moderately glycosylated with O-linked bi and trisaccharide structures (Tsuji and Osawa 1986). Glycoprotein IIb/IIIa is a key protein mediating platelet-fibrinogen interaction. 18% of the glycoprotein is accounted by N-linked typical complex bi and triantennary structures. However they are again

capped with sialic acid (Tsuji and Osawa 1986). Consequently, defining the molecular basis responsible for quantitative variation in platelet surface sialic acid expression will pose significant technical problems.

Chapter 4

Molecular mechanisms of platelet expresser phenotypes

4.1 INTRODUCTION

The presence of both high expression and low expression of A antigen on the platelet surface has been shown by flow cytometry in healthy donors from an Irish population (Chapter 2). However the molecular mechanism responsible for the different expression patterns remains undefined. HXP individuals have been previously shown to have elevated levels of ABO glycosyltransferase levels, the product of the *ABO* gene, suggesting the *ABO* locus may play a direct role in determining expresser phenotype. Additionally studies of two HXP kindred have shown segregation of the HXP trait within the families in an autosomal dominant pattern (Curtis, Ogasawara). Limited molecular studies by Curtis *et al* of exons 5, 6 and 7 of *ABO* glycosyltransferase gene in one platelet HXP individual and exon 5 in another three HXP individuals have not shown any sequence variation. However extensive analysis of *ABO* genotype, regulatory elements and sequence has not previously been performed.

Possible mechanisms for high expression include gene dosage effects. Since the *ABO* gene is co-dominant, consequently A¹A¹ homozygous individuals express significantly more group A glycosyltransferase activity compared to group A heterozygotes. Sequence variation leading to amino acid substitution may alter enzyme efficiency or substrate affinity. Finally, variation in enhancer sequence may lead to increased transcription and elevated glycosyltransferase levels.

A comprehensive molecular analysis of the *ABO* locus was performed in all donors identified as HXP or LXP. Initially the effect of dosage at the *ABO* locus was determined. The entire coding region of the *ABO* gene was sequenced in all HXP and LXP donors. Finally the *ABO* enhancer sequence studied to look for variation in the expected minisatellite repeat number in all Group A donors and expresser phenotypes.

4.2 MATERIALS AND METHODS

DNA EXTRACTION

Genomic DNA was extracted by Gentra-Qiagen Autopure (Alameda, CA) from the EDTA anticoagulated samples. The DNA concentration was then quantified by NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

POLYMERASE CHAIN REACTION (PCR)

Oligonucleotide primers were synthesized by EurofinsMWG (Germany). All primer sequences used are shown in Appendix 1 together with the annealing temperatures. All PCR reactions were performed in a similar manner. Typically 100ng of genomic DNA was amplified in a 50 μ L reaction volume. Each reaction contained the relevant primers at a final concentration of 1 μ M each, 200 μ M of each deoxynucleotide triphosphate (Amersham Pharmacia Biotech, Pittsburg, PA), in 1x PCR reaction buffer (Promega, Madison, WI), and 1.25 units BioTaq polymerase (Labplan, Ireland).

Typical PCR amplification conditions consisted of an initial denaturing step (95°C for 5 minutes), followed by 35 cycles of 1 min at 95°C, 1 min annealing at the relevant temperature, and 1 mins at 72°C. After cycle 35 the solution was incubated for an additional 5 minutes at 72°C. An aliquot of each amplified product was analysed by electrophoresis on a 1% agarose.

ABO GLYCOSYLTRANSFERASE GENOTYPING

A¹, A² and O genotyping was performed by PCR RFLP analysis. ABO exons 6 and 7 of the ABO gene were amplified and the products digested by the relevant restriction enzyme as described below. Four different primers were used to amplify two fragments, each spanning a different polymorphic site of the ABO gene (Denise and Alexander 1996; Ogasawara, Bannai *et al.* 1996).

Primers ABO-1 and ABO-2 in conjunction with the restriction enzyme *KpnI* were used to differentiate O¹ alleles from A¹ and A² alleles. The post amplification PCR product using the primer pair was 187 base pairs (bp). On digestion with *KpnI* O¹ alleles yielded 141 and 46 bp products (Fig. 4.1). Primers ABO-3 and ABO-4 in conjunction with the restriction enzyme *PvuII* were used to differentiate A² alleles from A¹ and O¹ alleles. The undigested PCR product was 228bp, and upon digestion A² yielding 111, 76 and 41 base pair products (Fig. 4.2).

Restriction enzyme digestion was performed on a 25µL aliquot of each PCR product, using 20 units of the appropriate enzyme. The digested products were then resolved by electrophoresis on a 2% agarose gel. Individual donor genotype is therefore determined by exclusion. As O¹ alleles and A² alleles may be determined, the unidentified X allele is A¹ which is confirmed by serological confirmation of blood group at the Irish Blood Transfusion Service.

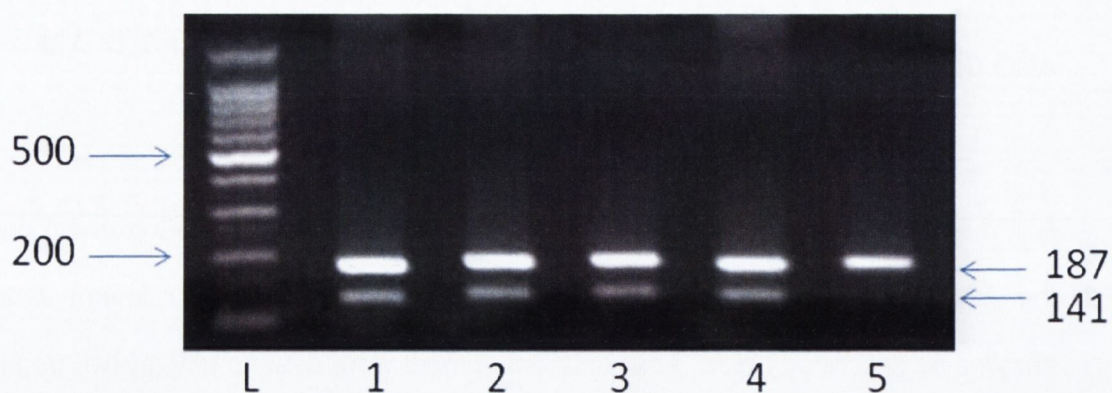


Figure 4.1

Agarose gel with digestion products of amplified sequence of exon 6 of ABO gene to differentiate O¹ and non O¹ alleles (X). A 100 base pair (bp) ladder was used (Lane L). Lane 1-4 are heterozygous X/O¹ as indicated by an undigested 187 bp product. The lower band is 141 bp represents an O¹ allele (the 46bp band is not visualised due to small size). Lane 5 is homozygous XX, and does not contain an O¹ allele.

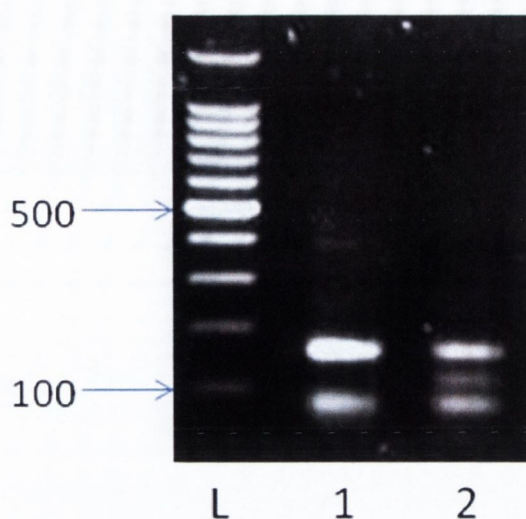


Figure 4.2

Agarose gel with digestion products of exon 7 of the ABO gene to differentiate A^2 and non- A^2 alleles (X). Lane 1 is XX, homozygous non- A^2 containing 2 bands 152 and 76 bp. Lane 2 is heterozygous X/A^2 with four digestion products representing the two alleles 152, 111, 76 and 41. The 41 bp product is not well visualised due to small size.

ABO ENHANCER REPEAT COPY DETERMINATION.

Analysis of a putative enhancer sequence of *ABO* was performed by PCR amplification. The enhancer is -3.7 kb upstream from the transcription initiation site and contains a minisatellite sequence composed of four tandem repeats of a 43 bp consensus unit. PCR primers F3 and R2 were used to amplify this region. A PCR product of 880 base pairs contains four repeats while a smaller 751 base pairs contains one repeat (Kominato, Tsuchiya *et al.* 1997).

ABO SEQUENCING

The *ABO* gene consists of seven exons. Primers were designed using Primer 3 software (Rozen and Skaletsky 2000). One set of primer pairs were required for the first six exons while the seventh and largest required two primer pairs. Each forward and reverse primer was synthesized with N-13 primer tags at the 5' end simplifying downstream sequence reaction.

PCR amplification was performed as described above with the addition of dimethyl sulfoxide (DMSO, Sigma-Aldrich, MO) to a final concentration of 5%. To check the presence and specificity of the PCR products a 2% Agarose gel was run (Fig. 4.3).

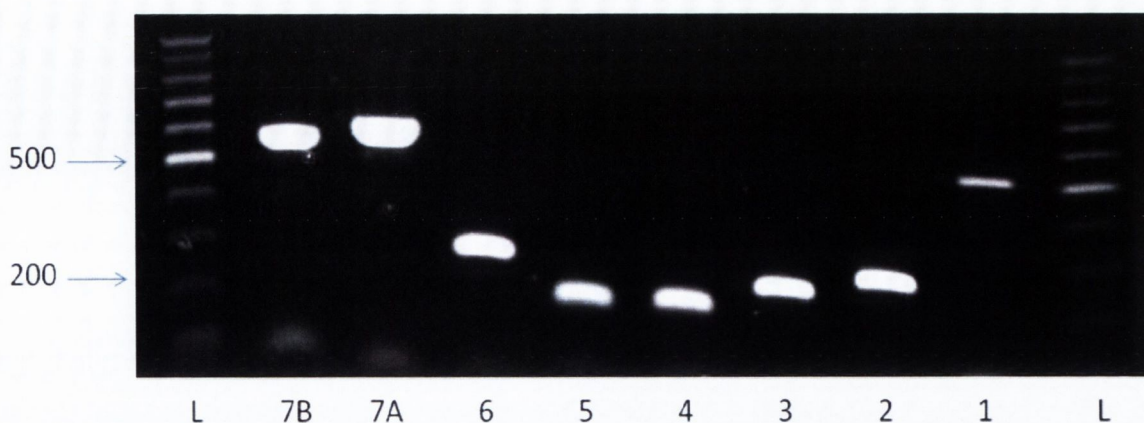


Figure 4.3

Agarose gel of the PCR products amplifying regions about each exon. Each band corresponds to an individual exon with the expected product size listed in Table 4. Note exon seven is amplified in two parts. In earlier experiments a single set of primer pairs were used to amplify the exon unsuccessfully and the primers were redesigned.

PCR products were purified prior to sequencing reactions by the use of MicroCLEAN (Microzone Ltd., W. Sussex, UK) as described by the manufacturer.

Briefly, after pulse vortex an equal volume of MicroClean reagent was added into each well containing PCR product. The plate was vortexed and incubated at room temperature for five minutes. It was then centrifuged at 2000g for 40 minutes. The supernatant was removed and each well containing purified PCR product was resuspended in 20µl of sterile distilled nuclease free water. Samples were run 2% Agarose gel to check the integrity of the DNA, and to ensure that the primer-dimer bands products have been removed.

SEQUENCING REACTION

Sequencing of the PCR products was performed by standard dideoxysequencing. Briefly 0.5ul of Big Dye terminator (v.3.1, Applied Biosystems) containing fluorescently labelled dideoxy-nucleotide mix was added to a final reaction volume of 20ul of 1x reaction buffer containing 3.2pmol of either N13f or N13r primer. Each PCR product was sequenced both in forward and reverse directions.

Cycle sequencing reactions were performed in a thermal cycler. The initial denaturing step (96°C for 1 minutes), was followed by 25 cycles of 10 seconds at 96°C, 10 seconds at 51°C , and 4 min at 60°C. After cycle 25 the solution was incubated for an additional 10 seconds at 60°C.

Prior to analysis the unincorporated nucleotides were removed, by ethanol precipitation of the products. Each sample pellet was then resuspended in 20µl of Hi-Di Formamide to each well containing sample. Sequence analysis was performed using the ABI3130 Genetic Analyser. The derived sequence data was compared with the human *ABO* reference sequence *NG_006669.1* by use of sequence alignment software (SeqScape, Applied Biosystems). All variations were verified by comparison

of both forward and reverse sequences with the reference sequence. Verified sequence changes were then compared to the ABO Blood Group Antigen Gene Mutation Database (Blumenfeld and Patnaik 2004).

4.3 RESULTS

ABO DETERMINES PLATELET ABO(H) ANTIGEN EXPRESSION

ABO genotype was determined for all 231 group A donors by PCR-RFLP analysis as described (Table 4.1). Expression of platelet surface A antigen expression was then analysed according to ABO genotype. A significant difference was shown between A expression according to genotype. Median A expression for genotype A^1O was significantly lower than that in A^1A^1 individuals (51.75% versus 71.9% respectively; $P < 0.001$, Mann Whitney). Overall, quantitative platelet A antigen expression varied in the order $A^2O^1 < A^1O^1 < A^1A^2 < A^1A^1$ ($P < 0.0001$, one-way ANOVA) (Fig. 4.4).

As described in Chapter 2, HXP and LXP were defined in accordance with Cooling *et al.* The ABO genotype of all HXP and LXP individuals was then assessed. Interestingly, 70% ($n=7$ of 10) HXP donors genotyped as homozygous A^1A^1 . Furthermore, A^1A^1 genotype accounted for all seven individuals with a type I HXP phenotype. However, the majority of A^1A^1 individuals (72%, 18 of 25) did not have a HXP phenotype.

In contrast, the two type II HXP donors identified were found to possess the A^2 allele (genotype A^1A^2). This observation was surprising given that the A^2 allele encodes an A transferase with significantly reduced A transferase enzymatic activity. All donors with a LXP platelet phenotype were found to have genotype A^1O^1 .

Genotype	Number of Donors
A^1A^1	25
A^1A^2	12
A^1O	143
A^2O	51
A^2A^2	1

Table 4.1
ABO genotype of group A donors.

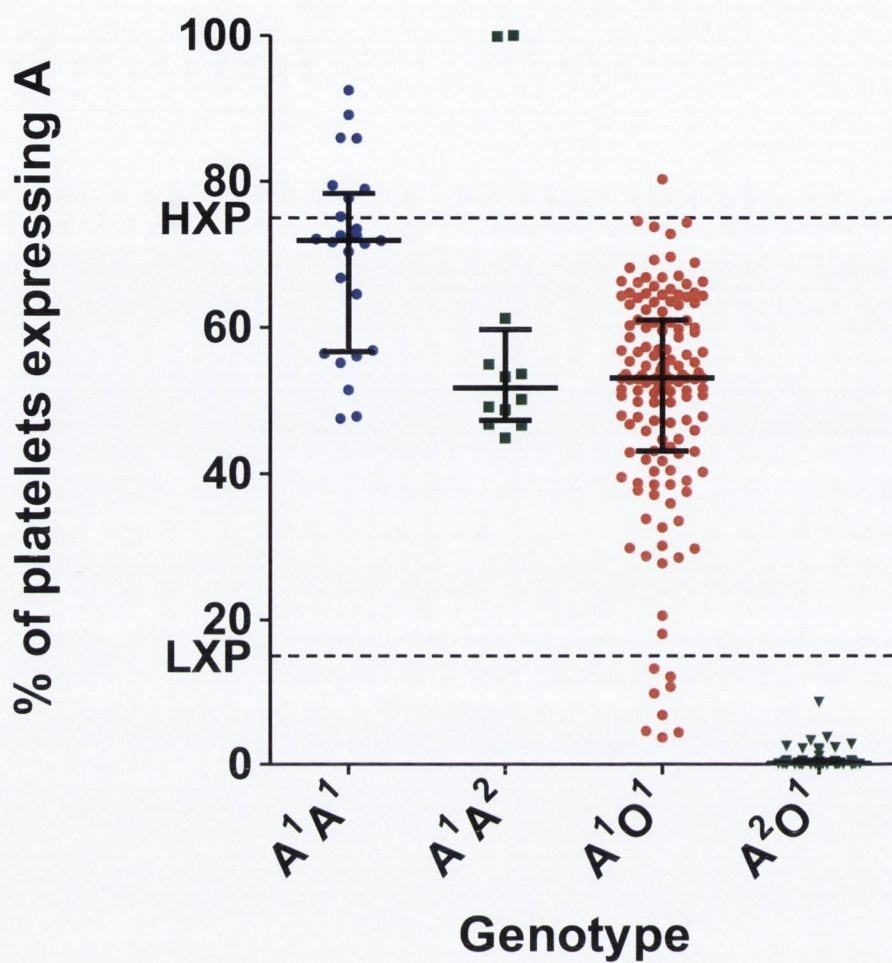


Figure 4.4

ABO genotype and platelet group A expression.

Donors are grouped by genotype, with median and interquartile range. The median of A¹A¹ (71.9) is significantly greater than A¹A² (51.75), $P=0.01$, and A¹O¹ (53.1), $P<0.001$, Mann-Whitney.

ABO GENE SEQUENCING

In order to determine whether sequence variations within the coding regions were associated with high and low expresser phenotypes, all exons and splice sites were sequenced in HXP and LXP individuals. Numerous variants were identified and are listed in Table 4.2. However all variants have been previously identified are not known to be associated with HXP or LXP. No novel mutations were identified with reference to the Blood Group Antigen Gene Mutation Database (Blumenfeld and Patnaik 2004). The ABO allele nomenclature is under consideration and variations exist. The A^1O^1 allele was identified in all A_1 in all expresser phenotypes and is the reference sequence. Similarly the A^2 allele was the common A^{20^1} allele. Three O alleles were identified O01 and O02 (also known as O1 variant) are common deletional O alleles characterised by the inactivating 261delG mutation. Similarly O26 is a deletional allele found in Europe with additional silent sequence variations (Yip 2002).

High Expresser			Low Expresser	
Genotype	Allele		Genotype	Allele
A ¹ A ^{2*}	A101/A201		A ¹ O	A101/O02
A ¹ A ^{2*}	A101/A201		A ¹ O	A101/O01
A ¹ A ¹	A101/A101		A ¹ O	A101/O26
A ¹ A ¹	A101/A101		A ¹ O	A101/O26
A ¹ O	A101/O01		A ¹ O	N/A
A ¹ A ¹	FAIL		A ¹ O	A101/O01
A ¹ O	A101/O01		A ¹ O	A101/O01
A ¹ A ¹	FAIL		A ¹ O	A101/O26
A ¹ A ¹	A101/A101			
A ¹ A ¹	A101/A101			
A ¹ A ¹	A101/A101			

Table 4.2

The sequence of the HXP and LXP donors is listed. For each individual the genotype identified by RFLP is listed with the allele. The type II HXP are indicated by an asterisk (*). One LXP sample was not available for analysis. Two type I HXP failed the sequencing reaction despite repeated attempt.

ABO ENHANCER TANDEM REPEATS INCREASED IN HXP

In addition to the coding region, the upstream enhancer sequence was analysed to determine whether there was any variation according to HXP or LXP. The A¹ and O² alleles typically have only one 43 bp repeat in the enhancer whereas A², B and O¹ alleles have 4 repeats (Fig. 4.5). In vitro studies have shown that a single repeat is associated with low transcriptional activity, at less than 1% the activity of alleles bearing four repeats (Kominato, Tsuchiya *et al.* 1997). All group A₁ donors were studied to determine repeat copy number (Table 4.3).

An unexpected four repeats were found in two donors with A¹ alleles. Both donors were high expressers. One type I (A¹A¹) was heterozygous and one type II HXP (A¹A²) homozygous for the four repeat allele.

ABO genotype	No.	1 repeat (homozygous)	1 and 4 repeats (heterozygous)	4 repeats (homozygous)
A^1A^1	25	24	1^a	
A^1A^2	12			1^b
A^1O	143		143	
A^2O	51		$2\#$	49

Table 4.3

ABO enhancer minisatellite repeat copy number is listed by genotype. HXP with A^1 alleles bearing four repeats are listed. Type I HXP(a) and Type II HXP (b). Two A^2O individuals have 1 and 4 repeats. They possess the uncommon O^2 allele which has one repeat rather than the expected four.

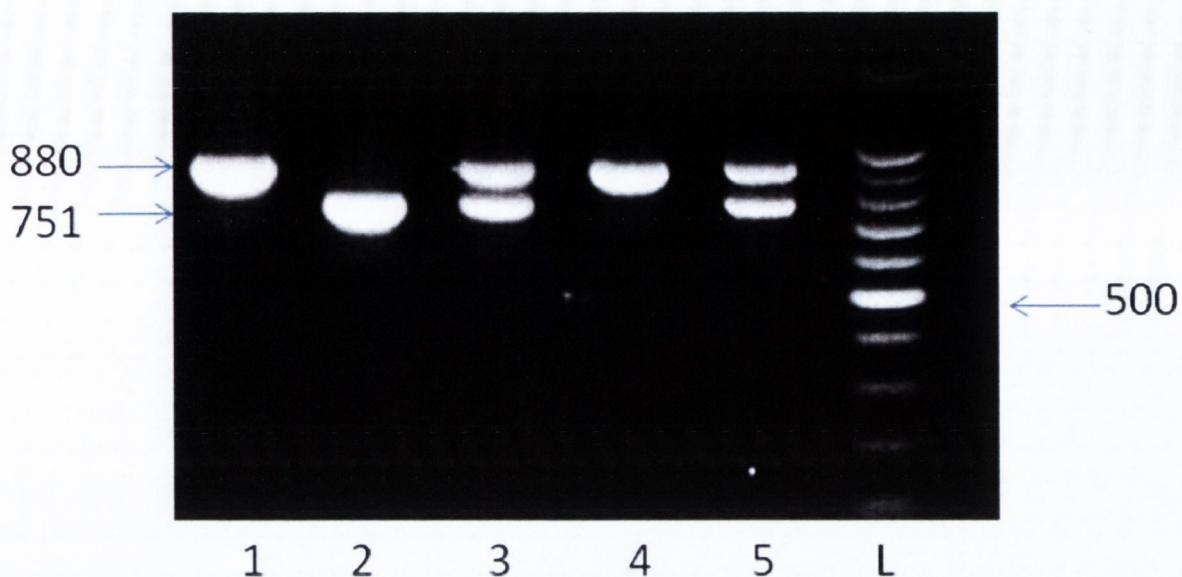


Figure 4.5

Agarose gel with PCR products amplified by primers F3 and R2, of the ABO gene enhancer region. Lane 1 and 4 are homozygous for 4 repeats of the minisatellite sequence. Lane 2 is Homozygous for a single minisatellite allele. Lane 3 and 5 are heterozygous

4.4 DISCUSSION

To determine a mechanism responsible for the expresser phenotypes, genetic factors influencing ABO glycosyltransferase levels were investigated. The ABO gene has a dosage effect upon the expression of ABO glycosyltransferase levels and the expression of A antigen per unit VWF (O'Donnell, Boulton *et al.* 2002). ABO genotype was determined for platelet donors and has shown a definite ABO gene dosage effect on the percentage of platelets expressing blood group A. It is the most important predictor of HXP status as 70% of HXP were A¹A¹. Conversely a significant minority approximately 28% of A¹A¹ individuals were HXP. Nevertheless, not all A¹A¹ individuals were HXP, suggesting other modifiers. Significantly the highest A expressers, both type II HXP, where A distribution is unimodal, had only one A¹ allele. In both these individuals 100% of platelets exhibited A antigen. A¹O¹ genotype is the major predictor of LXP status, appearing to form a subgroup of A¹O¹ donors.

Variation in the coding sequence of ABO can lead to amino-acid substitutions that cause a gain of function as described for B transferase in persons with B(A) red cell phenotype (Yamamoto) or conversely low expression. The coding regions of the ABO gene were examined in patients with HXP and LXP. Although variation was detected in the coding regions no association between sequence variation and expression status was determined. Therefore the enhancer region of the ABO gene was studied. *In-vitro* studies have shown that variation in copy number can increase by 300 fold the transcriptional activity of the ABO gene, in a gastric cell line. (Yu, Chang *et al.* 2000). However interestingly, only one study has investigated ABO transcript levels in haemopoetic tissue, comparing bone marrow and peripheral blood. A¹ and A² transcripts were present only in the bone marrow while B and O transcripts were present in both (Thuresson, Chester *et al.* 2008). It is suggested that this is evidence against a significant role for the enhancer, however the A² allele and A¹ have

4, and 1 repeat respectively and a reduction in transcripts in one only would be expected.

Expresser phenotype studies have not yet addressed *ABO* genotype, enhancer copy and platelet blood group expression. In particular the A¹ alleles in the HXP were studied to determine whether more than the expected one repeat would be identified that may enhance transcription that would contribute to the HXP. All group A individuals were studied and in two A¹ alleles four rather than one repeat was found. Significantly both were in HXP donors. One of the two A¹A² individuals with highest expression of A antigen on platelets was homozygous for the 4bp repeat. This suggests that transcription of A¹ allele was likely to be significantly increased.

The *ABO* gene is regulated in part by the binding of transcription factor CBF/NF-Y to this minisatellite sequence. In transient transfection assays where this sequence is mutated there is a dramatic loss in activity (Kominato, Tsuchiya *et al.* 1997). Transcription enhancement by minisatellite repeats is not limited to *ABO* and but also seen in the insulin gene. Binding sites for a transcription factor Pur-1 have been identified in a 5' minisatellite sequence. Transcriptional activity is dependent on length and copy number with longer being most active (Kennedy, German *et al.* 1995). Similarly the transcriptional activity of the *ABO* enhancer is increased with each repeat of a 43-bp unit when inserted individually upstream of a SV40 promoter of a pCAT30 promoter vector (Yu, Chang *et al.* 2000). Therefore an extra 3 repeats in an A¹ allele could substantially increase the rate of transcription at this allele and translate into the higher glycosyltransferase seen in type II HXP. Furthermore it is likely to compensate for the lack of a second A¹ allele in the A¹A² individual.

The second Type II HXP A¹ allele did not have this novel allele. However the platelet HXP is likely to have multiple molecular mechanisms and both *ABO* gene dosage and *ABO* Enhancer appear to contribute.

Further studies are necessary to determine these mechanisms, noting the complexity of the *ABO* gene and regulatory regions. To identify other regulator regions

that may influence transcription the up and downstream regions of the *ABO* gene have been sequenced. In the 5' region little allelic heterogeneity is seen while the 3' region is highly repetitive and more heterogeneous. There are numerous short repeats ($CA_{n=2-5}$) and longer repetitive sequences (Thuresson, Chester *et al.* 2008). The *ABO* gene promoter methylation status has been shown to influence the *ABO* gene expression. In vitro cell line studies have shown that demethylation of the *ABO* gene promoter can lead to transcription, and synthesis of A-antigens (Kominato, Hata *et al.* 1999). Therefore in further studies the expresser phenotypes may be studied in relation to the *ABO* 3'UTR for common motifs and the methylation status of the *ABO* gene promoter, and in particular in relation to low expression of blood group.

In summary, we have identified that the most significant factor in HXP is allele dosage. The positive and negative predictive values of A^1 allele homozygosity are 72% and 28% respectively. Furthermore we demonstrate that variation in enhancer repeat number in A^1 alleles is associated with HXP. Increased copy number is seen in two individuals in the A_1 study population and both were HXP. In addition to further genetic study, measurement of glycosyltransferase activity is necessary to determine the effect of the novel A^1 alleles.

Chapter 5

Relationship between platelet expresser phenotypes and VWF

5.1 INTRODUCTION

Although high and low expresser phenotypes were first described in relation to platelet surface ABO(H) antigen expression, it remains unclear whether these phenomena may also influence quantitative expression of ABH determinants in other tissues. ABO(H) blood groups have been demonstrated on three plasma proteins including VWF, Factor VIII and alpha-2 macroglobulin (Sodetz, Paulson *et al.* 1979; O'Donnell, Boulton *et al.* 2002). The relationship between ABO and VWF is particularly well-established. VWF is a multimeric plasma protein, stored in Weibel-Pallade bodies in endothelial cells from where it is released into the circulation. A separate stored pool exists within the alpha granules of platelets. VWF is critical to haemostasis. It plays a major role in primary haemostasis by binding to exposed collagen at the site of vessel injury, unravelling and binding platelets through surface membrane glycoproteins such as glycoprotein Ib (Ruggeri 2003) in response to increase in shear stress. VWF also has a significant role in coagulation, forming a complex with factor VIII which significantly increases FVIII half-life.

Plasma VWF levels in normal individuals vary over a wide range (typically 0.5-2.0 IU/ml). Nevertheless, deficiency of plasma VWF is responsible for the commonest inherited bleeding disorder known as Von Willebrand Disease (VWD) (Laffan, Brown *et al.* 2004). Conversely, excessively high plasma VWF levels constitute a risk factor for arterial thrombosis presenting as ischaemic heart disease (Rumley, Lowe *et al.* 1999).

Sixty-six per cent of the variation in VWF level is genetically determined, with 30% of this being explained by the ABO blood group. In particular group O individuals have VWF levels up to 30% lower than in non-group O individuals (Gill, Endres-Brooks *et al.* 1987; Shima, Fujimura *et al.* 1995; Souto, Almasry *et al.* 2000). Circulating plasma VWF and endothelial-derived VWF bears ABH antigens. In contrast, VWF derived from

platelets do not express ABO (Brown, Collins *et al.* 2002). In addition, bone marrow transplant recipients of ABO mis-matched marrow maintain the recipient blood group expression on circulating VWF (Matsui, Shimoyama *et al.* 1999) and platelet derived VWF similarly not express blood group.

Glycans account for 19% of the molecular weight of VWF and are both N and O-linked (Fig. 5.1). The N-linked glycans bear ABH structures depending upon the ABO blood group of the individual. These determinants are added in the post-golgi of endothelial cells before storage and release into the circulation. The oligosaccharide chain structure is complex, including mono (0.4%), bi (78.2%), tri (12.3%) and tetraantennary chains (2.3%) (Matsui, Titani *et al.* 1992).

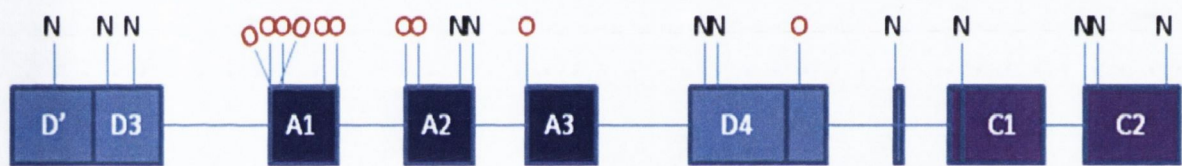


Figure 5.1
The positions of N and O linked glycans on a VWF monomer are shown. There are 12 N-Linked and 10 O-linked glycans (red).

In addition to blood group phenotype, VWF levels within a population have been described as varying according to ABO genotype, with VWF levels highest in A1A1 individuals (O'Donnell, Boulton *et al.* 2002; Morelli, de Visser *et al.* 2007). Previous studies have also examined quantitative A antigen loading on VWF, and have shown a dose dependent effect dependent on genotype (Morelli). However it is unknown whether the platelet high expresser phenotype of blood group extends to ABO (H) expressing plasma proteins. In this study, plasma VWF levels in normal Irish blood donors have been studied in conjunction with the ABO blood group genotype, and expresser phenotype. VWF antigen levels were determined for all blood group A and O

individuals and compared with platelet blood group expression. In particular HXP and VWF levels were studied with quantitation of A per unit VWF to determine whether HXP influences loading of A on VWF and whether it results in an elevated VWF level.

5.2 METHODS

VON WILLEBRAND FACTOR ELISA

VWF antigen (VWF:Ag) in citrated plasma samples, was measured by sandwich enzyme-linked immunosorbent assay (ELISA). 96-well ELISA plates (DAKO) were coated with rabbit polyclonal anti-human vWF antibodies (A082; Dako, Denmark) diluted 1:500 in 0.05M carbonate buffer (pH 9.6), overnight at room temperature. After washing with PBS containing 0.05% Tween, the plates were blocked for non-specific binding with a commercial blocking reagent (Tris buffer, sodium chloride and a protein mixture obtained by proteolytic degradation of purified gelatin; Boehringer Mannheim GmbH) for 15 mins. After further washings, dilutions of the test samples and reference plasma (Immuno) were added to the wells. Blanks were standard PBS. The samples were then incubated for 1 hour at room temperature. The plates were washed three times with PBS/Tween and then incubated with rabbit polyclonal anti-human vWF peroxidase conjugate, (P266; Dako, Denmark) diluted 1:500 in PBS/Tween, for 1 hour. After a further three washes, the plates were incubated with a substrate solution (20 μ l of 0.05M citric acid and 0.1M Na₂HPO₄ pH 5.0, 40mg ortho-phenylenediamine (Sigma) and 20 μ l 30% hydrogen peroxide). The reaction was stopped after 3 minutes with 150 μ l/well of 1M H₂SO₄, and the optical density measured at wavelength 492nm using an ELISA reader (Dynatech MRX). Dilutions of 100% Reference plasma (vWF:Ag 1.05 IU/ml; Immuno) were used to construct standard curves for calibration. All ELISA samples were tested in duplicate, using three replicate wells for each set of test

conditions. The intra-assay and inter-assay coefficients of variation were both less than 5% and the lower limit of VWF detection was 0.3mU/mL.

A ANTIGENIC DETERMINANTS ON VWF

Group A (GalNAc α 1 $\rightarrow$ 3 [Fuc α 1 $\rightarrow$ 2] Gal β 1 $\rightarrow$ 4 GlcNAc β 1 $\rightarrow$)) antigenic determinants on plasma was measured using a modified sandwich ELISA. ELISA plates were coated with rabbit anti-human vWF as described above. Each plasma sample was tested in duplicate at two dilutions. The plates were washed with TBS/Tween and then incubated with murine anti-A monoclonal antibody (Ortho Diagnostics), diluted 1:10 in TBS, for 1 hour. After a further five washes, the plates were incubated with goat anti-mouse IgM peroxidase conjugate (Sigma), diluted 1:1000 in TBS, for 1 hour. After another TBS/Tween wash, peroxidase substrate solution was added and incubated in the dark for 20 minutes. The reaction was stopped with 1M H₂SO₄ after 3 minutes, and the optical density measured at wavelength 492nm using an ELISA reader. Pooled group A plasma was assayed to produce a standard curve for each ELISA. The pooled normal plasma was assigned an arbitrary value of 1U/ml for the amount of A antigen expressed per unit VWF.

In group A individuals, the amount of A antigen detected varied significantly with the plasma concentration of VWF. Consequently, all results were corrected to amount of A antigenic determinant expressed per unit VWF (AVWF).

5.3 RESULTS

VON WILLEBRAND FACTOR ANTIGEN (VWF:Ag)

Plasma VWF:Ag levels were measured on all group O and group A individuals. In keeping with previous reports, VWF:Ag levels in group A₁ individuals were significantly higher than those in group A₂ and group O donors (Fig. 5.2) (Gill, Endres-Brooks *et al.* 1987). Median VWF:Ag levels for group O and A₁ were 0.78 IU/ml (interquartile range, 0.62-0.96) and 0.99 IU/ml (interquartile range, 0.84-1.17) respectively ($P < 0.0001$, Mann Whitney). The inter assay and intra assay coefficient of variation was 8.5% and 7% respectively.

VWF:Ag levels were then analysed according to ABO genotype. Median VWF:Ag levels were 4% higher in A¹A¹ compared with A¹O. However, in contrast to previous reports, this difference did not achieve statistical significance ($P = 0.3$, Mann Whitney) (Table 5.1). However donors lacking an A¹ allele, (i.e. group A²O and O), had significantly lower levels (Fig. 5.3). The median VWF:Ag for A¹O was 0.97 IU/ml (interquartile range, 0.84-1.17) compared with group O 0.78 IU/ml (interquartile range 0.62-0.96) ($P < 0.0001$, Mann Whitney). In comparison, two previous studies have reported significantly higher plasma VWF:Ag in homozygous A¹A¹ individuals compared to heterozygous A¹O¹ individuals. However in this context it is noteworthy that Souto *et al* only observed a mean VWF difference between A¹A¹ and A¹O of 4% whereas the mean difference in this study was 12%.

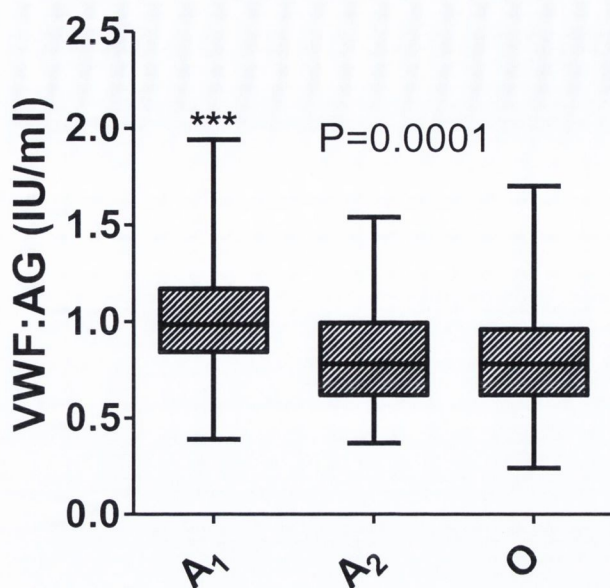


Figure 5.2

Effect of ABO blood group phenotype on plasma VWF:Ag levels. The box and whiskers plot describes the median, interquartile range and range.

Group A₁ (n=172), have significantly higher levels of VWF:Ag than A₂ (n=49) and group O (n=296).

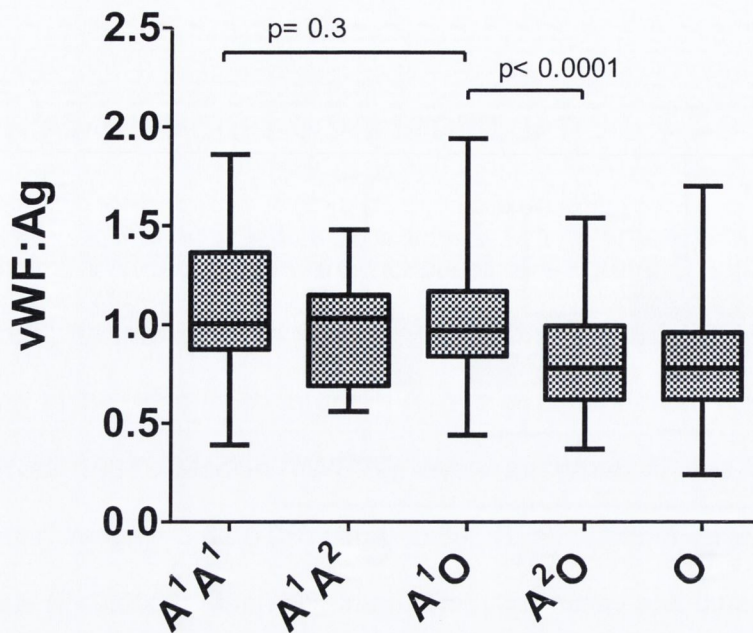


Figure 5.3

Effect of ABO genotype on plasma VWF:Ag levels.

The box and whiskers plot describes the median, interquartile range and range.

A¹A¹ (n=24) are not significantly greater than A¹A² (n=12) (P=0.3) or A¹O (n=142) (P=0.3).

Genotype	No.	Median IU/ml	Mean vWF:Ag IU/ml, $\pm$ SD
A ¹ A ¹	24	1.01, 0.88-1.37	1.08 $\pm$ 0.33
A ¹ A ²	12	1.03, 0.69-1.15	0.96 $\pm$ 0.28
A ¹ O	142	0.97, 0.84-1.17	1.01 $\pm$ 0.30
A ² O	49	0.78, 0.62-1.00	0.83 $\pm$ 0.29
O	296	0.78, 0.62-0.96	0.80 $\pm$ 0.26

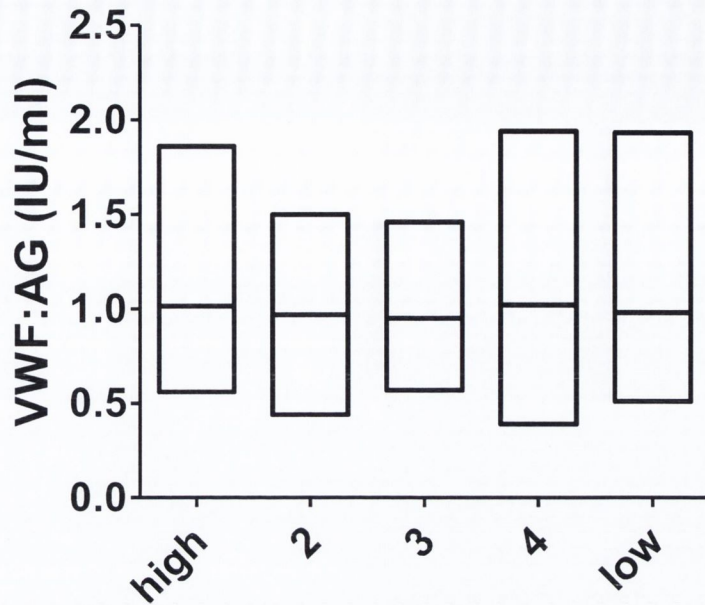
Table 5.1

Median and mean VWF:Ag levels according to ABO genotype. This includes the interquartile range.

While no statistically significant difference exists between A¹A¹ and A¹A² or A¹O, there is a 4% and 6% lower median VWF:Ag level respectively.

PLATELET A DETERMINANT EXPRESSION AND VWF:AG

Since ABO blood group is a critical determinant of plasma VWF:Ag levels, we further investigated whether HXP or LXP influenced plasma VWF levels. Although plasma VWF levels varied widely between normal donors, this variation was not influenced by either HXP or LXP respectively. The median VWF:Ag (IU/ml) for HXP was 1.00 (interquartile range, 0.79-1.24) (Mean, 1.00 ± 0.26 SD) and LXP 0.98 (interquartile range, 0.75-1.15) (Mean 0.95 ± 0.20 SD), $P=0.56$. Furthermore, no correlation was observed between the amount of A antigen expressed on platelet surfaces and plasma VWF:Ag levels. (Figs. 5.4 & 5.5). In conclusion, while Group O and A subgroup difference in VWF antigen levels is demonstrated as previously described, neither the Expresser Phenotypes nor the level of A blood group on platelets is a predictor of VWF:Ag levels.



Equal quintiles of % platelets expressing group A

high	2	3	4	low
1.015	0.9700	0.9500	1.020	0.9800

Figure 5.4

Platelet group A expression does not correlate with plasma VWF:Ag levels

Group A₁ donors are divided into five quintiles based upon the percentage of their platelets expressing blood group A. The box plot describes the median and range. The lower table is of the median VWF level of each quintile. High platelet A expression is not a predictor of VWF:Ag levels.

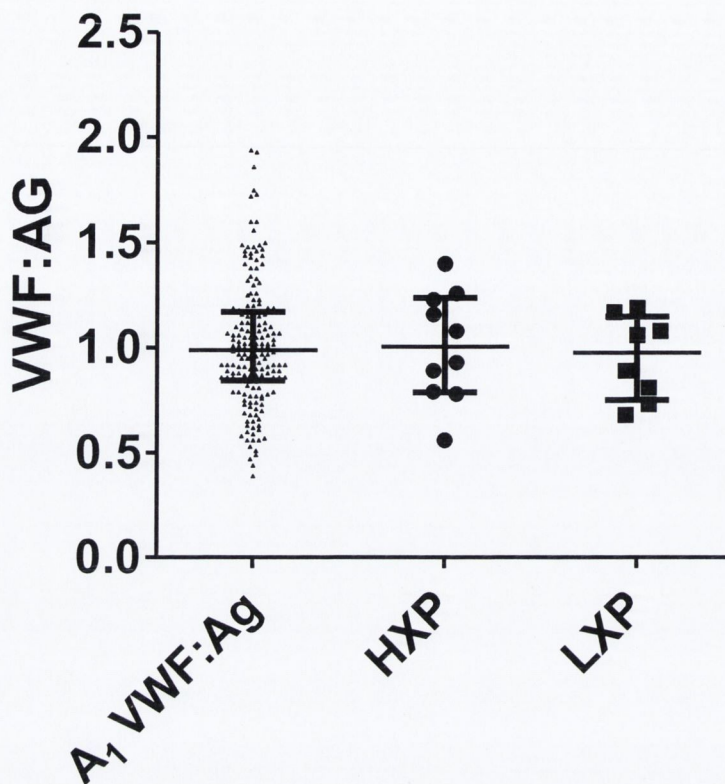


Figure 5.5

Relationship between expresser phenotypes and plasma VWF:Ag levels.

The median and interquartile ranges are indicated above.

No difference between median group A₁ and HXP VWF:Ag identified. (P=0.5, Mann Whitney). No difference exists between HXP and LXP VWF:Ag levels.

A ANTIGEN EXPRESSION IS INCREASED ON HXP VWF

To further investigate the molecular basis underlying platelet expresser phenotype, we examined whether HXP or LXP influenced quantitative loading of A antigen onto plasma VWF. To determine whether A antigen expression on plasma VWF was higher on HXP individuals, a modified sandwich ELISA was used. A antigen expression was determined on 10 HXP and 43 group A₁ donors. The amount of A VWF was significantly higher on HXP donors compared with group A₁ (median: 3.6 versus 2.5, Ratio 1.4) ($P=0.019$, Mann Whitney; Fig. 6). Again a single type II HXP outlier had very low A per unit VWF expression. This individual while having 100% A expression on platelets was the same donor found to have low VWF: Ag levels. There was no correlation however between percentage of platelets expressing A within donors and A-VWF ($P=0.23$, $r=0.186$, Spearman). The inter assay coefficient of variation was 17.24%.

Quantification of A per unit VWF was then analysed by genotype (Table 5.2). A¹A¹ individuals had significantly higher levels than A¹O ($P=0.016$, Mann Whitney). They were both significantly higher than A²O which has limited A₂ transferase activity ($P=0.0006$, $P<0.0001$ respectively, Mann Whitney). This is consistent with the dosage effect on the loading of A on VWF seen by O'Donnell et al, 2002 (Fig. 5.7).

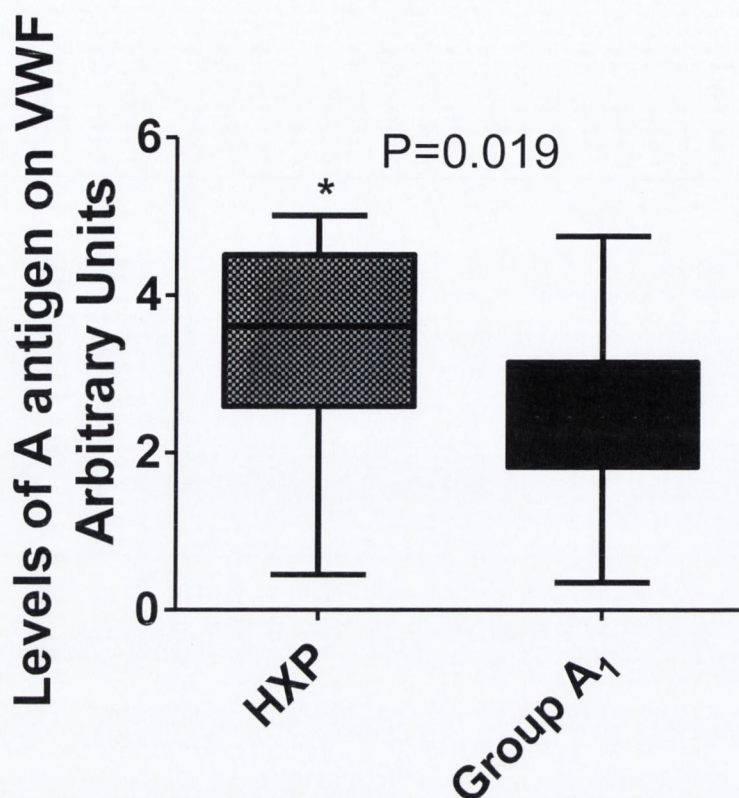


Figure 5.6

Effect of HXP on the loading of A antigen onto plasma VWF.

The box and whiskers plot describes the median, interquartile range and range.

The A-VWF of 10 platelet HXP individuals and 43 normal expresser group A₁ donors were measured as described in the methods. A antigen per unit VWF is significantly higher in the HXP group. This suggests that while the HXP may not influence VWF levels, it may influence the loading of A antigenic determinants on VWF.

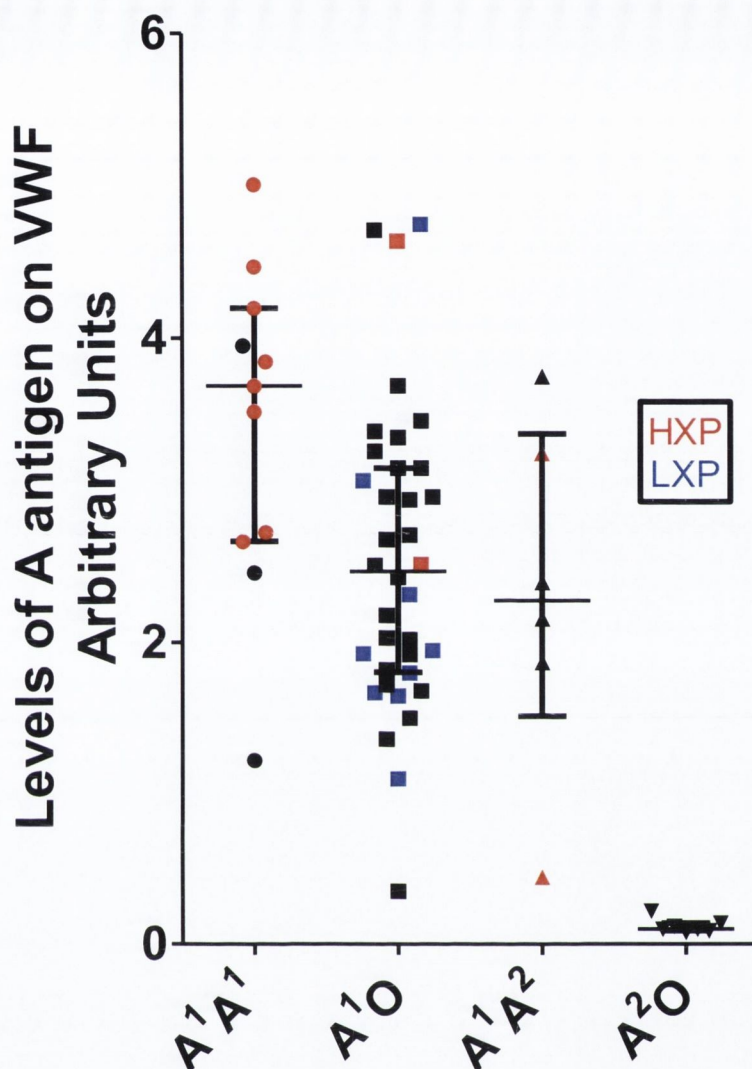


Figure 5.7

Effect of ABO genotype on the amount of A antigen expressed on VWF.

The box and whiskers plot describes the median, interquartile range and range.

HXP and LXP are highlighted in red and blue respectively. It is noteworthy that the majority of HXP are A¹A¹ and LXP are A¹O. Similarly a single type II HXP, having low VWF:Ag level (0.5 IU/ml) also has low A-VWF levels.

Genotype	No.	Mean A-VWF	Median A-VWF
A ¹ A ¹	11	3.44±1.08	3.69, 2.67-4.2
A ¹ A ²	6	2.31±1.15	2.28, 1.52-3.38
A ¹ O	36	2.53±0.99	2.47, 1.81-3.15
A ² O	7	0.12±0.05	0.10, 0.09-0.14

Table 5.2

Comparison of values of A-VWF with ABO genotypes

The mean ±SD, median with interquartile range are described.

5.4 DISCUSSION

In this chapter the potential relationship between the platelet HXP and VWF is explored. The ABO locus influence on plasma VWF:Ag levels is well described.

We confirm previous findings in that the blood group A cohort had significantly higher mean VWF levels than blood group O. The blood group A₁ cohort had approximately 26% higher median values of VWF:Ag. Although we demonstrated an effect of genotype on VWF levels this did not reach statistical significance. O'Donnell *et al* have previously shown a significantly raised VWF level in a cohort of A¹A¹ blood donors (n=14) compared to A¹O¹ donors with a difference of approximately 14%. Morelli *et al* also showed an approximately 8% higher level in a cohort of A¹A¹ genotype compared to A¹O¹ (significance not given). The underlying reason for the different findings is not clear. The Irish cohort is the second largest analysed to-date, and represents a relatively homogenous population. This is supported by our limited data on A antigen levels on VWF in the platelet HXP group. A significantly increased amount of A antigen was present on A-VWF in the HXP group as compared to the normal expressing group (Fig. 5.6) However no significant difference in median VWF levels was detected between HXP and normal expressers. However O'Donnell *et al*, found no correlation among A¹A¹, A¹O individuals and plasma VWF:Ag and A-VWF levels.

In this study the HXP is associated with significantly higher levels of blood group A antigen per unit VWF:Ag compared to group A₁, however this does not translate into higher levels of VWF. There are two potential hypotheses to explain this phenomenon. Firstly an association between platelet HXP and VWF:Ag exists. However the numbers in the HXP group are too small to detect a difference

considering the variability in VWF levels that exists in the normal range. Secondly the HXP is restricted to platelets. This is confirmed by the absence of an effect of HXP on VWF levels. However the increased A-VWF remains unexplained unless ABO dosage is studied. Seven of ten HXP are homozygous A¹. A per unit VWF has previously been shown to have highest VWF:Ag levels. Therefore the elevated A per unit VWF shown in this study may represent a gene dosage effect which is associated with elevated glycosyltransferase activity rather than the influence of the platelet HXP.

It is also noteworthy that a Type II HXP, clearly showing increased A expression on platelets did not have elevated A per unit VWF or raised VWF:Ag levels, but is consistent with the findings of Morelli *et al* as this donor also possessed an A¹ allele with four repeats (Morelli, de Visser *et al.* 2007).

Chapter 6

Discussion

6.1 DISCUSSION

Platelets are small anucleate cells which are a critical component of primary haemostasis. Their surface glycoproteins are terminally glycosylated by sialic acids and ABH antigens. We have investigated the nature of the ABH antigenic determinants in a healthy cohort in the Irish blood donor population and have identified the clinically important platelet expresser phenotypes. Furthermore the molecular mechanisms have been explored identifying ABO gene dosage and the ABO enhancer sequence as major predictors of the High Expresser phenotype. As von Willebrand factor also bears ABH antigenic determinants, we found that the platelet HXP is associated with increased loading of A antigen on the plasma protein but does not increase its plasma concentration. There remains a wealth of investigational opportunities to explore the nature of glycan expression on platelets and von Willebrand factor which is of direct clinical significance.

The ABO blood group system is of critical importance for Blood Banking. Inventory management is a particular challenge with increasing hospital demand due to new therapies requiring platelet support. This poses a problem when ABO matched platelets are not available. Unlike red cell components, ABO mis-matched platelet transfusion is permitted in times of shortage. There are negative outcomes reported by this practice however. Cardiac surgery cases may have poorer outcomes due to inflammation caused by immune complexes between Anti-A or B and soluble A/B antigens (Blumberg, Heal *et al.* 2001). HLA alloimmunisation poses a particular problem in frequently transfused patients, necessitating directed HLA matched donations. ABO mis matched transfusions are associated with early onset and higher titre of these antibodies (Carr, Hutton *et al.* 1990). However the most practical implication of mis match is poor platelet recovery after transfusion. For example Lee *et al* reported in a randomised trial a lower mean platelet count after the second

unmatched transfusion compared with matched (Lee and Schiffer 1989). In a recent study of paediatric transfusions ABO mismatched had a significantly lower one-hour percentage platelet recovery than matched at median 21% vs. 32% respectively. We now know that A₁ platelets have a bimodal population and A₂ platelets lack A antigen and Julmy *et al* have shown the rapid clearance of the A positive population from the recipients circulation, while A₂ platelets are as effective as ABO matched transfusions (Julmy, Ammann *et al*. 2009)

In this study we have found 22% of group A platelet donors are A₂ and therefore has potential to increase the inventory of O compatible platelets. They could also be the product of choice for A donor to O recipient bone marrow transplants as the product would be ABO compatible with the patients isohemagglutinins and plasma compatible with the donor graft (Cooling, Kelly *et al*. 2005). Subgrouping is not preformed anywhere except Norway, but A subgrouping is within capability of current automated blood grouping technology.

This study has identified the clinically important High Expresser phenotype of ABO blood group antigen, which is responsible for transfusion failure when ABO mismatched. HXP platelet products are of concern due to lack of platelet increment in patients who are bleeding due to severe thrombocytopenia. The prevalence in the Irish donor population is consistent to reports in Japan and the USA at 5.5% (range, 4%-8%) (Ogasawara, Ueki *et al*. 1993; Curtis, Edwards *et al*. 2000; Cooling, Kelly *et al*. 2005)

The HXP is not accounted by a single molecular mechanism. Studies first defining the HXP found elevated ABO glycosyltransferases levels in HXP donors and through limited family studies suggested an autosomal dominant pattern of inheritance. Therefore we considered that dosage of the *ABO* gene was likely to contribute. With extensive analysis of the *ABO* gene we have shown that genotype is the strongest predictor of HXP as 70% of HXP were homozygous A¹A¹. Genotyping of A donors therefore may be worthwhile with avoidance of transfusion of A¹A¹ in ABO mismatched

transfusions; as such donors have the highest level of platelets expressing A antigen. Therefore A¹A¹ would result in poorer platelet increments due to fast clearance as shown by *Julmy et al*, 2009.

Further work will be necessary to define the complete biological mechanism(s) involved. In addition to genotype, we have shown that the novel A¹ allele with an associated 4 minisatellite repeat within the ABO enhancer contributes to the HXP. The presence of such an allele in an individual is likely to lead to high transcriptional activity which would translate into elevated ABO glycosyltransferase levels. In further work it will be essential to measure levels in our HXP population but in particular in the A¹A² type II HXP. Furthermore, having identified 10 HXP individuals, family studies to confirm the pattern of inheritance of the phenotype and also to determine if the novel enhancer allele segregates similarly. In addition it would be helpful to explore the LXP which so far is associated with the A¹O genotype alone and whether it too is an inherited trait.

It remains undefined why type II HXP platelets express A on all platelets and why normal expressers have an A negative population, and yet express H antigen. *Cooling et al* had suggested that such platelets are “Bombay-like”. However both this study and *Curtis et al* do not confirm these findings suggesting another mechanism. Therefore we investigated the expression of sialic acid on platelets and found all platelets expressing sialic acid although to a variable extent. It is possible that the ABO glycosyltransferase is in direct substrate competition with sialyltransferases, as both cap the terminal sugars of the oligosaccharides expressed on platelet glycoproteins. In contrast to red cells the ABH on platelets is expressed on complex structures which could render some sites inaccessible to the ABO glycosyltransferase due to sialylation, leaving the H determinant unmodified.

Curtis et al postulated that the platelet expresser phenotype may extend to other ABH bearing proteins. HXP is not associated with elevated VWF:Ag levels but is associated with significantly higher levels of A antigen per unit VWF. However

the majority of HXP are A¹A¹ (70%) and dosage of the ABO gene is an independent predictor of A-VWF levels. Therefore our A-VWF findings may represent genotype rather than an independent effect of HXP. This is not surprising however as VWF itself is subject to tissue specific expression. ABH is expressed on VWF of endothelial origin but not on platelet VWF. However the increased loading of A on VWF in HXP donors may represent the alternative hypothesis that HXP is also a feature of other ABH bearing plasma proteins.

The ABH determinants are borne on complex carbohydrate structures and are distal to the *Ii* antigenic determinants. They are linear or branched repeats of N-acetyllactosamine. The *i* blood group is a linear structure found on neonatal red cells and accounts for the low expression of ABH in neonates. In early infancy a β -1,6-N-acetylglucosaminyltransferase (I-transferase) adds branches of these repeats onto the linear structure. This forms the *I* antigen. In doing so it increases the number of substrates for formation of ABH determinant formation and accounts for the increased ABH expression on adult cells (Jerzy, Ewa *et al.* 1979; Yu, Twu *et al.* 2001; Inaba, Hiruma *et al.* 2003). Similarly platelets express the *Ii* antigens, however little is known about the intensity of expression, or how the degree of branching may contribute to high expression of ABH and deserves further study (Dunstan, Simpson *et al.* 1984). Recent work has shown a lack of ABO(H) VWF:Ag differences in the first year of life and increasing levels in non-O groups after. This is suggested to be related to the *Ii* blood group development which may also influence the expression of ABH on VWF (Dieter, Christine *et al.*). Therefore in further investigations of the expresser phenotypes the activity of the I-transferase warrants investigation to determine its contribution to ABH expression on platelets and the expresser phenotypes.

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APPENDIX

APPENDIX

A.1 ABO GLYCOSYLTRANSFERASE GENOTYPING

PRIMER	SEQUENCE	PRODUCT SIZE
ABO-1	CATGTGACCGCACGCCT	187
ABO-2	TCGGCCACCTCACTGACTTA	
ABO-3	CCGTCCGCCTGCCTTGCAG	228
ABO-4	AAGTCACTGATCATCTCCAT	

Table 1
Primer sequences for amplification of parts of the coding region of the ABO glycosyltransferase gene

PCR CONDITIONS FOR ABO GLYCOSYLTRANSFERASE GENOTYPING

Genomic DNA was amplified in 50 μ L of a solution containing, a pair of oligonucleotide primers (50pM each), 200 μ M of each deoxynucleotide triphosphate (Amersham Pharmacia Biotech, Pittsburg, PA), 10X PCR buffer (Promega, Madison, WI), and 5U BioTaq polymerase (5u/ μ l) (Labplan).

The PCR protocol consisted of an initial denaturing step (95°C for 5 minutes), followed by 35 cycles of 1 min at 95°C, 1 min at 65°C , and 1 mins at 72°C. After cycle 35 the solution was incubated for an additional 5 minutes at 72°C. An aliquot of each amplified product was analysed by electrophoresis on a 1% agarose gel.

Restriction enzyme digestion was performed on a 25 μ L aliquot of each PCR product, using 20 units of the appropriate enzyme. The digested products were then resolved by electrophoresis on a 2% agarose gel.

A.2 ABO ENHANCER REPEAT COPY DETERMINATION

PRIMER	SEQUENCE
F3	CCCTTGCAACAGTTACAGGTGTACTCATCC
R2	CCGATGTGTTACTCACAAGACCAGGAGGTG

Table 2

Primer sequence for amplification of segment upstream of ABO gene in the enhancer.

Genomic DNA was amplified in 50μL of a solution containing, a pair of oligonucleotide primers (100pM each), 250μM of each deoxynucleotide triphosphate (Amersham Pharmacia Biotech), 10X PCR buffer (Promega), and 0.5 I/UTaq DNA polymerase (GibcoBRL).

The PCR protocol consisted of an initial denaturing step of 94°C for 5 minutes, followed by 35 cycles of 1 min at 94°C, 1 min at 62°C, and 1 mins at 72°C. After cycle 35 the solution was incubated for an additional 7 minutes at 72°C. The PCR products were then resolved by electrophoresis on a 2% agarose gel.

A.3 ABO GLYCOSYLTRANSFERASE GENE SEQUENCING

Reagents	Per reaction 25µl
H ₂ O	Up to total 24
10x _{NH4} Buffer	2.5
10mM dNTPs	2.0
50mM MgCl ₂	0.75
DMSO	1.25
DNA	1.0
12.25µM	0.5
Taq Polymerase	0.2

Table 3

Reagents used for ABO gene sequencing

PRIMERS	N-13 PRIMERS	PRIMER SEQUENCES	PRODUCT SIZE
Exon 1 N13F	<i>gtagcgcgacggccag</i>	ctctgtcccctcccgtgt	474
Exon 1 N13R	<i>cagggcgcagcgatgac</i>	cgaggagaggctggagac	
Exon 2 N13F	<i>gtagcgcgacggccag</i>	gtaaagtggggcaggtgaga	233
Exon 2 N13R	<i>cagggcgcagcgatgac</i>	tgatacttggggagctcagg	
Exon 3 N13F	<i>gtagcgcgacggccag</i>	accaacaggcagtcctcggt	199
Exon 3 N13R	<i>cagggcgcagcgatgac</i>	gagggggagttccagagt	
Exon 4 N13F	<i>gtagcgcgacggccag</i>	tgccctaaatcctgctcta	159
Exon 4 N13R	<i>cagggcgcagcgatgac</i>	gctgcaggacaattctgtga	
Exon 5 N13F	<i>gtagcgcgacggccag</i>	cgctttccatgcaaactcac	153
Exon 5N13R	<i>cagggcgcagcgatgac</i>	ggaaaccgccctctaatacc	
Exon 6 N13F	<i>gtagcgcgacggccag</i>	ctgccagctccatgtgac	233
Exon 6 N13R	<i>cagggcgcagcgatgac</i>	gtccacagtcactcgccact	
Exon 7-5' N13F	<i>gtagcgcgacggccag</i>	tgaatcgagcccaggactcg	541
Exon 7-5' N13R	<i>cagggcgcagcgatgac</i>	ggggccggcgctctgaggt	
Exon 7-3' N13F	<i>gtagcgcgacggccag</i>	caccctgcaccccggttcta	503
Exon 7-3' N13R	<i>cagggcgcagcgatgac</i>	ccgggaggggggtatctgt	

Table 4

Primer sequences used in sequencing reaction. One primer pair amplifies each exon with the exception of exon 7, which due to size requires two pairs.

Reagent	Per reaction (μl)
Big dye terminator v3.1 (PE Applied Biosystems)	0.5
5x Sequencing reaction buffer	5.5
N –13 Primer (3.2pmol/μl)	1
H ₂ O	12

Table 5
ABO gene sequencing Reaction Mix

Oral Presentations and Publications

Abstract leading to oral presentation

Defining the Molecular Mechanisms Responsible for the ABO High Expresser Phenotype

Ó Donghaile D, Jenkins PV, Mc Grath R, Preston L, Preston R, Murphy W, O'Donnell JS

Oral presentation at the Haematology Association of Ireland Annual Meeting, 2009

Abstract leading to poster publication

Defining the Molecular Mechanisms Responsible for the ABO High Expresser Phenotype.

Diarmaid O Donghaile, Vince P Jenkins, Rachel McGrath, Lisa Preston, Roger JS Preston, William G M

Blood (ASH Annual Meeting Abstracts), Nov 2009; 114: 2119.