Chemical basis of ABO subgroups

Insights into blood group A subtypes revealed by glycolipid analysis

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"Thus, it is essential to realise that every variant glycosyltransferase will result in a new range of unique glycoconjugate profiles in the tissues, yet may still serologically be phenotyped on red cells as simply group A".

Henry & Samuelsson 2000

ABSTRACT

Despite the ABO histo-blood group system being the most biologically significant in humans the chemical structures that define its various phenotypes still remain largely unresolved. Like all blood group systems there is a significant range in the amount of antigen present on the red cells of an individual and there exists a range of so-called "weak" phenotypes represented by decreasing expression of A or B antigens. There are a variety of known and speculative mechanisms that may result in these weak-subgroups/phenotypes. Mechanisms resulting in weak-subgroups can include glycosyltransferase catalytic domain mutations and mutations outside the catalytic domain. Mechanisms resulting in weak-phenotypes can include insufficient glycosyltransferase or precursor, secondary antigen acquisition, disruption in biosynthesis, glycosyltransferase redundancy or degeneracy, antibody sensitivity and specificity, chimera/transplantation/transfusion, infection, physiological changes and finally artificial manipulation.

Weak-subgroups/phenotypes are potential windows into the biochemistry of the ABO blood group system, due to the absence of dominating structures, and/or enhancement of trace antigens caused by a loss in normal competition.

The aim of this thesis was to gain insights into chemical basis of the ABO system by investigation of the mechanisms behind selected A weak-subgroups and/or A weak-phenotypes. A selected number of these were then biologically dissected and immunochemically and structurally investigated in details. Structural analysis of complex carbohydrate compounds is a delicate process where information from one technique is compiled with information from other techniques to finally elucidate a reliable identification of structure. It is the combination of analytical tools that allows for robust interpretation of results that give insights to the biosynthetic and genetic basis for the phenotypes.

In this thesis it was shown that the probable explanation between the A_1 and the A_2, apart from the quantitative aspects, is that the A-type 4 structure seems to be missing in the A_2 phenotype. TLC investigations into a range of weak-subgroups revealed a range of interesting anomalies, many of which have yet to be investigated.

Investigations on an individual A_3 phenotype revealed an absence of branched structures as a potential mechanism for the "mixed field" reaction. Also several new structures including extended p-Fs (para-Forssman) structures were found. Finally the A_{pae} phenotype revealed an unexpectedly discovery that this phenotype is caused by expression of the Forssman (Fs) antigen and not A antigens. This leads to a proposal to establish the 31st blood group system, tentatively named FORS.

Although the contribution of glycoproteins and polyglycosylceramide to the expression of weak ABO subgroups still remain uninvestigated the analysis of the glycolipids alone has revealed a variety of significant insights into blood group A subtypes/phenotypes.

Keywords: ABO, subgroups, glycolipid, para-Forssman, Forssman,

Fs synthetase

LIST OF PUBLICATIONS

I   **Svensson L**, Rydberg L, de Mattos L. C, Henry S. M (2009) Blood group A_1 and A_2 revisited: an immunochemical analysis. *Vox Sang 96:56-61*

II   **Svensson L**, Rydberg L, Hellberg Å, Gilliver L. G, Olsson M. L, Henry S.M (2005) Novel glycolipid variations revealed by monoclonal antibody immunochemical analysis of weak ABO subgroups of A. *Vox Sang 89:27-38*

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ABBREVIATIONS

Cer	ceramide
ESI-QTOF MS	electrospray ionization-quadrupole time-of-flight mass spectrometry
Fs	Forssman; $GalNAc\alpha GbO_4$
Fuc	Fucose
p-FS	para-Forssman; $GalNAc\beta GbO_4$
Gal	galactose
GalNAc	N-acetyl-galactosamine
GBGT1 gene	Forssman gene
Glc	glucose
GlcNAc	glucosamine
GbO_4	globoside
Gb_3	globotriaosylceramide
GTA	N-acetyl-galactosaminyl transferase ; glycosyl transferase A
hFsS	human Forssman synthetase
HPLC	high performance liquid chromatography
Lc-4	lactotetraosylceramide
MAb	monoclonal antibody
MS	mass spectrometry
nLc-4	neolactotetraosylceramide
NMR	nuclear magnetic resonance
^{1}H-NMR	proton NMR
2D NMR	two dimensional NMR
PAb	polyclonal antibody
RBC	red blood cell
TLC-EIA	thin layer chromatography-enzyme immuno assay

CONTENTS

1. OVERVIEW

Most simply the presence or absence of A and/or B antigens on the red cell membrane defines the ABO blood group system. However, the presence and detection of ABO antigens is a consequence of a complex interplay of known and unknown genetics, biosynthetics, environmental factors and finally the sensitivity and specificity of the diagnostic assay. All these factors together make the ABO blood group system diverse and complex.

In this thesis several individuals with phenotypes showing low expression of A antigens were examined to define, if possible, a chemical basis to their phenotype.

A brief history of the ABO system

40,000,000 to 1,000,000 years B.C. - An ancient ABO-like gene is believed to have existed as early as 40 million years ago with the evolution of the ABO gene starting at least 13 million years ago [1]. Several phylogenic studies suggest that A, B and O lineages developed between 1 to 4 million years ago [2].

1900 to 1920 – In 1901 Karl Landsteiner reported testing red blood cells and sera from six healthy men and discovery of the ABO blood group system, for which he earned the Nobel prize in 1930. In 1911 von Dungern and Hirschfeld reported the distribution of blood group A (47 %), B (11%), AB (6%) and O (36%) in Europeans, and separation of blood group A into A_1 and A_2. In 1926 and 1930 Yamakai, Lehres and Putkonen found soluble ABO blood group substances in secretions and could divide them into two groups, secretor and non-secretor [3]. In 1924 Bernstein proposed the theory of inheritance [4] that still holds true today.

1930 to 1980 – Several researchers isolated ABO blood group determinants from glycoproteins (reviewed by Morgan & Watkins 2000)[3] and the study of ABO glycolipids became popular, with most of the basic ABO structures being resolved during this period [5, 6].

1990 to 2010 – on the back of the new genetics revolution Yamamoto identified cDNA of the α1→3N-acetylgalactosaminyl transferase (A-transferase) [7] in 1990, and by doing so opened the door for genomic studies of ABO blood group system. For the next 20 years a wave of ABO gene discovery continued and by 2009 a total of 215 ABO alleles had been reported [8], and new alleles still continue to be identified and reported [9].

Biological significance

There is little doubt that the ABO blood group system and its associated antibodies have a relationship with micro-organisms and infection. However there is no definitive advantage of any one phenotype over another (unlike other blood groups antigens such as Duffy which can prevent life-threatening diseases such as malaria). The biological reason for the ABO polymorphism, yet alone any of its variants, remains unknown and it might be just a balancing evolutionary phenomenon, for example to maintain the heterozygote advantage for survival [2]. Despite its unknown biological origins or function, from a transfusion or transplantation perspective, it remains the most clinical significant blood group in humans.

2. ABO BLOOD GROUP SYSTEM

The ABO blood group system is defined by the presence or absence of two antigens (A and B) and is recognized as four major blood group phenotypes A, B, AB and O. The antigens are inherited according to Mendel's law, where one haplotype from each parent is inherited. The frequency in the European population is reported as blood group A 41.7%, B 8.5%, AB 3.0% and O 46.7% [10], but the frequency varies significantly in different ethnic groups.

ABO antigens exist on glycoproteins and glycolipids in red cell membranes and also on most cells and tissues in humans, and in animal tissues. The antigens are also present in the secretory fluids in the majority of humans. Thus the term "histo-blood group system" is a more accurate description than "blood group system". The antigens are unequally expressed among and within the different cells and tissues and in different species [11, 12]. Except for humans, only anthropoid apes, the orangutan and the gorilla have ABO antigens on their red cells, which suggest that the red cells are the last cells during evolution to obtain the ABO antigens [11].

A, B and H antigens are carbohydrate molecules built stepwise from saccharides such as galactosamine (GalNAc), glucosamine (GlcNAc), fucose (Fuc), galactose (Gal), and glucose (Glc). The synthesis is catalyzed by glycosyltransferases encoded by the ABO genes, and thus A and B antigens are secondary gene products.

The major alleles at the ABO locus are *A, B* and *O* and to-date a number of ABO blood groups variants have been reported, with approximately 250 different alleles registered in the Blood Group antigen gene Mutation database (BGMUT) [13]. This large variety and the continuing appearance of new mutations not only in the encoding ABO gene but also in the promoter and enhancer regions of the gene make genotyping difficult. Thus, genotype cannot always accurate predict the phenotype, and several allele variants may express the same phenotype [8].

Defining ABO "weak" phenotypes

Like all blood group systems there is a significant range in the amount of antigen present on the red cells of an individual. In most instances ABO antigen level is defined by the individuals ABO genotype, but in others the amount of antigen present can be determined by non-ABO genetics or environmental factors. Those phenotypes defined by ABO genes are known as subgroups of ABO and are represented by decreasing expression of A or B antigens. When the amount of antigen is very low compared with dominant phenotypes (e.g. A_1 or A_2) then the phenotype is usually referred to as "weak". To distinguish the ABO genetically

defined weak phenotypes from those defined by non-ABO genes or environmental factors, we define the former as ABO weak-subgroups and the latter as ABO weak-phenotypes. Many examples of individuals classified as A_3, A_m, A_x, A_{el}, etc, clearly have ABO gene mutations, but others do not, and thus should simply be defined as a weak-phenotype, and not as ABO with an alphanumeric subscript (e.g. A_{weak} vs $A_{x)}$. The A_{weak} phenotypes can be further defined as those with a genetic basis that is non-ABO and those caused by environmental factors. It should be appreciated that despite specific features being described for some serological phenotypes it may not be possible to distinguish ABO weak-phenotypes from ABO weak-subgroups. Until a genetic basis is resolved then weak phenotypes should be referred to simply as a weak-phenotype, e.g. A_{weak}. If a known ABO genetic basis for the weak-phenotype is identified then the sample can be classified on that basis. However, if the genetic basis is novel (and within the ABO gene) then we propose the use of only two terms – A_x or B_x when the phenotype can be serologically demonstrated, albeit very poor reactivity with direct agglutinating serological reagents; or A_{el} or B_{el} if the antigens can only be shown by very sensitive techniques (including absorption/elution) and not by direct agglutination serology. Like the blood group O phenotype there will be expected to be multiple genetic mechanisms resulting in the A_x/B_x or A_{el}/B_{el} phenotypes.

ABO genetics

The ABO gene is located at the long arm of chromosome 9q34 [14] and consists of seven exons and introns, covering approximately 20 kilobase pairs from the initiation to the stop codons. The nucleotide sequence of the *A* allele cDNA consists of 1062 base pairs, and encodes the enzyme protein [15-17]. The cDNA (the A^1 allele, [A101]) encoding the *N*-acetylgalactosamine transferase was cloned and sequenced by Yamamoto et al [7] and is considered as the consensus (index) gene against which all other variant of ABO genes are compared.

The ABO genes are very polymorphic both between and within the blood groups, however several main mutations in the genes are characteristic for some ABO blood groups. The A^2 allele [A201] has a substitution at nucleotide 467 (C>T) and a deletion (C) involving nucleotides 1059 to 1061, which extend the transferase with 21 amino acids, causing a less effective enzyme [18]. There are seven mutations, four of which change the amino acid in the enzyme, and separate the *B* allele [B01] from the A^1 allele. The amino acid changes 266 (Leu >Met) and 268 (Gly > Ala) are the most critical for B enzyme activity [19, 20].

The most common O^1 allele [O01] has a deletion at nucleotide 261, which leads to a stop codon and a truncated, inactive enzyme [21]. There are many other variants of *O* alleles not having this deletion but still causing inactive enzymes.

The majority of *ABO* alleles registered in the BGMUT database [13] are associated with the weak-subgroups. The genetically recognized weak-subgroups are A_{Int} (1

allele), A_3 (8 alleles), A_x (19 alleles), A_m (2 alleles), A_{finn}, A_{bantu}, and A_{el} (8 alleles). There is a large amount of genetic polymorphism within the same subgroups, which is exemplified by the number of reported variant alleles within the brackets. Another large group of blood group *A* alleles are unassociated with a specific phenotype and are simply named A^w (A^{weak}), with 29 alleles registered in the BGMUT database. The weak blood group A subgroups usually have an A^1 or an A^2 allele but with extra mutation/s combined with an O^1 or a variant *O* allele. Blood group B also express weak-subgroups (62 variant alleles registered) but are less well investigated. Blood group O also has a large number of different alleles (72 variant alleles) reported including alleles with weak expression of A or B antigen [22]. It can be argued that blood group *O* alleles resulting in low-level expression of A and/or B antigens are inappropriately named as *O* alleles, and only alleles that result in no-detectable A or B antigens should be classified as *O*.

ABO glycosyltransferases

ABO antigens are secondary products of ABO gene defined specific enzymes, so-called glycosyltransferases. N-acetylgalatosaminyltransferase (GTA) and galactosyltransferase (GTB) are the enzymes encoded by the ABO genes. GTA and GTB catalyze the transfer of GalNAc (using UDP-GalNAc) or Gal (using UDP-Gal) to the OH-3 position of the terminal Gal of the H structure (Fucα2Gal) to create A and B antigens, respectively [23-25]. Manganese ions (Mn^{2+}) are required as a co-factors and the disaccharide Fucα1-2Gal residue is the minimal required acceptor. These glycosyltransferases, and others that construct the requisite precursors, reside mostly in the endoplasmatic reticulum (RE) and Golgi apparatus. The glycosyltransferases are type II transmembrane proteins, and exist both membrane-bound and as soluble proteins in plasma and other body fluids. The membrane-bound enzyme has a short cytoplasmic N-terminal tail, a hydrophobic transmembrane domain, a stem region, plus a large C-terminal which constitutes the catalytic domain. The soluble transferases lacks the N-terminal and the hydrophobic transmembrane domain [23, 26].

ABO biochemistry

The defining glycotopes of the A and B antigens are the tri-saccharides GalNAcα3(Fucα2)Gal-R (figure 1, I) and Galα3(Fucα2)Gal-R (figure 1, II), respectively. H-antigen, Fucα2Gal-R (figure 1-III), is the requisite precursor and galactosylamine (GalNAcα3) or galactosyl (Galα3) with α1-3 linkage onto this H antigen become the A and B antigens respectively [3, 27]. Although the minimal structures representing the A and B antigens are clearly defined, these ABO determinants can be carried by a variety of inner cores, each of which imparts antigenic features to the glycoconjugate (table 1). The most frequent ABO antigens

found on red cells are carried by a type 2 core [28], and known either as A type 2 or B type 2. Other types of A antigens described on red cells are carried by type 3 and type 4 core chains [29-32] (table 1). Evidence for blood group A bearing type 6 (A-4-6) has also been reported [33]. The type 1 ABO structures carried by red cell are absorbed from the plasma onto the red cell and may also exist in a variety of forms depending on whether they have been modified by Lewis fucosylation (e.g ALe[b]) (see below).

I

GalNAcα1 → 3Galβ1-R
2
↑
Fucα1

II

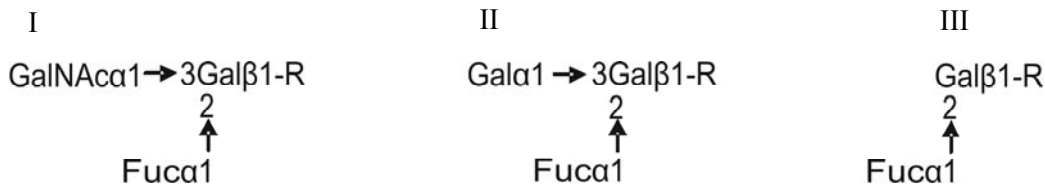Galα1 → 3Galβ1-R
2
↑
Fucα1

III

Galβ1-R
2
↑
Fucα1

Figure 1. The defining glycotopes of blood group A (I), B (II) and H (III)

Table 1. Inner core structures of the ABO blood groups

Precursor structures in human	Defining characteristic
Type 1	Galβ3GlcNAcβ - R
Type 2	Galβ4GlcNAcβ - R
Type 3	Galβ3GalNAcα3 - R
Type 4	Galβ3GalNAcβ3 - R

The majority of ABO antigens on red cells are linked to glycoproteins (approximately 70%), thus they very much influence the blood group activity on the red cells. However this thesis only describes glycolipids and very little will be mentioned about the unknown contribution of glycoproteins to the phenotype. Glycolipids are chosen because they are relatively structurally less complex than glycoproteins, easier to isolate to homogeneity, are usually representative of a single biosynthetic process, and can be relatively easily structurally resolved.

The ceramide part in the glycolipid is formed by a fatty acid in amide linkage to a sphingosine (figure 2), which is anchored in the cell membrane [34, 35]. The configuration of the carbohydrate chain of the four core structures (table 1) differs and can cause the ABO glycotopes to be presented differently on the surface membrane. Type 1 is nearly vertical to the cell membrane while type 2, 3 and 4 are bent more or less parallel in their minimum energizing state. Furthermore, rotations of the carbohydrate chains may cause the A determinant to point in different directions. This presentation of the antigen on the cell surface may affect the susceptibility for antibody and microbial binding [36].

Other than the A_1 and A_2 phenotypes the contribution of the various ABO antigen bearing structures to the serological phenotypes observed is largely unknown. But it will also be seen here (Paper I) that even the chemistry of the A_1 and A_2 phenotypes was ambiguous and required further resolution.
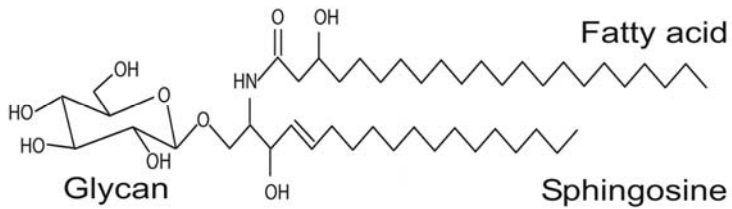
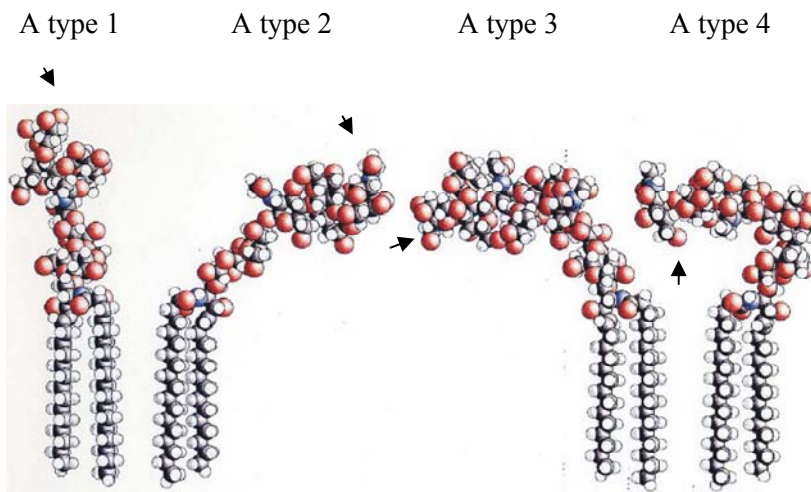Figure 2. The glycosylceramide shown consists of glucose, fatty acid and sphingosine.

Figure 3. Blood group A glycolipids with type 1, type 2, type 3 and type 4 carbohydrate chains illustrated in their minimum energy conformation. Published by Nyholm P-G (1989) J Molecular Recognition vol 2, No 3:103-113

Biosynthesis of ABO glycolipids

At each step in the biosynthesis of ABO antigens and carbohydrate chains, synthesis is facilitated by glycosyltransferases, which are competing for available precursors and substrates.

The biosynthesis of the ABO, I and P blood group system glycolipids (glycosphingolipids) originates with glucose (Glcβ1) linked to a ceramide (Figure 4). A galactose residue in β4 linkage (Galβ4) forms lactosylceramide (Galβ4Glcβ1-Cer). From this precursor two alternative pathways exist, one via lactotriaosylceramide towards type 1, 2, 3 precursors [28-30], and the other via globotriaosylceramide (Gb_3) towards type 4 precursor [32].

In more detail after lactotriaosylceramide (GlcNAcβ3Galβ4Glcβ1Cer) is formed biosynthesis splits into two alternative pathways: the addition of Gal with a β3 linkage leads to lactotetraosylceramide (Lc-4 or type 1 precursor, Galβ3GlcNAcβ3Galβ4Glcβ1Cer) [28], or a Gal in β4 linkage leads to neolactotetraosylceramide (nLc-4 or type 2 precursor, Galβ4GlcNAcβ3Galβ4Glcβ1Cer) [28]. Both of these precursors can be fucosylated by the H enzyme to form type 1 and type 2 H antigens. If type 2 H chain is terminated by the GalNAcα3 saccharide it forms the A-6-2 antigen [37], which can be extended with an Fucα2Galβ3 disaccharide creating H type 3 (Fucα2Galβ3GalNAcα3-R) which in term can be modified into A type 3 ("repetitive A" or A-9-3) (GalNAcα3[Fucα2]Galβ3-R) [11, 27, 30, 38] (table 1, and figure biosynthesis)

The biosynthesis of the type 4 pathway also originates from the lactosylceramide precursor but the addition of N-acetylgalactosamine (GalNAc) with β3 linkage leads to globotriosylceramide (Gb_3) and then globotetraosylceramide, Gb_4 (globoside also known as the P antigen) [39], (figure 4).

There are three alternative pathways that can extend Gb_4 into other structures. Elongation with the H-disaccharide forms H type 4 (globo-H) [40, 41]. The A_1 transferase can then create A type 4 (A-7-4, globo-A) by adding a α3GalNAc residue to the type 4 H precursor [40]. The A_2 transferase is not able to convert type 4 H precursor into A-7-4 [40]. The second alternative pathway is the addition of a GalNAc with β3 linkage creating the p-Fs (para-Forssman) glycolipid [42]. p-Fs is expressed in humans. In the third pathway the Fs synthetase catalyses the transfer of a GalNAc residue in α1-3 binding to Gb_4 [43-45]. Although Fs (Forssman) glycolipid is widely seen as an animal antigen a few publications indicate its expression in normal human tissue, but especially in tumors [46-48].
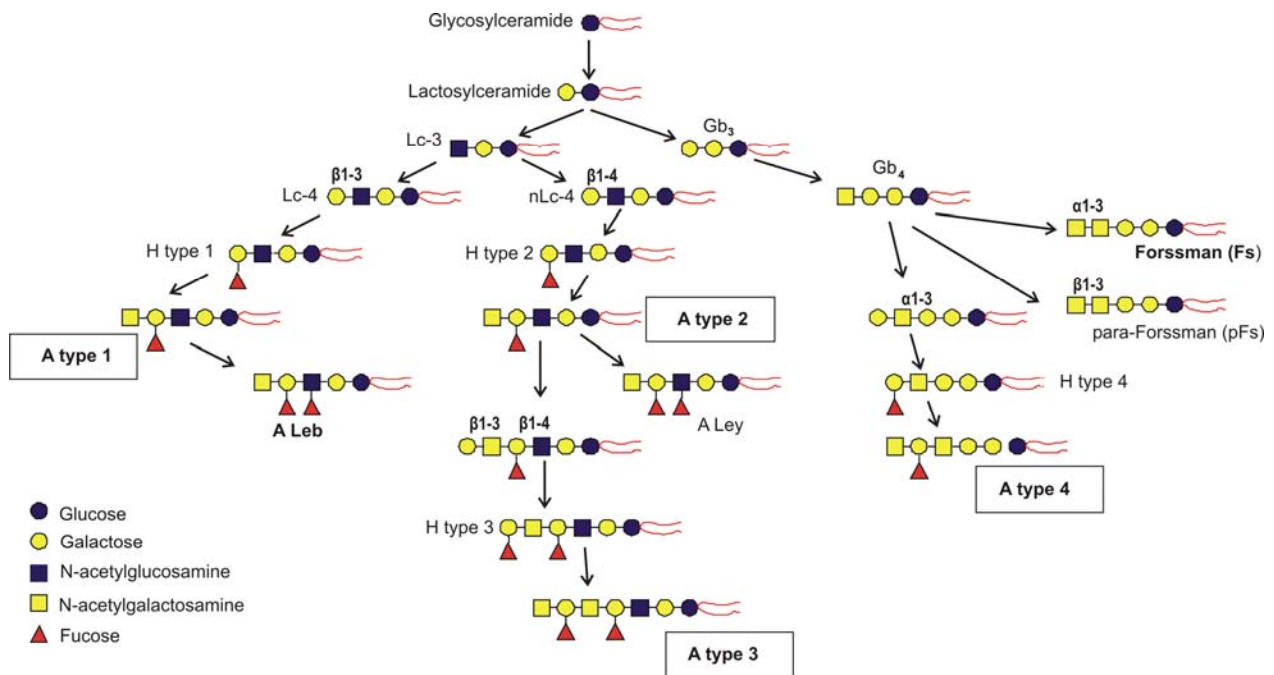
Figure 4. Schematic presentation of blood group A and competing pathways

ABO relationships with other blood group systems

The synthesis of ABO antigens and the actual structure of the ABO antigens are influenced by other glycosyltransferases (including those of other carbohydrate blood group systems). This section will only present a context relevant to this thesis. The reader is referred to the more comprehensive reviews [11, 49] if required.

The H blood group system or gene and its resultant transferase are responsible for synthesis of the requisite type 2 H precursor, without which the dominant type 2 A and B antigens on the red cell cannot be synthesized. It also is responsible for the synthesis of H types 3 and 4. In individuals of the Bombay phenotype no H type 2 antigen is synthesized and therefore these individuals cannot express their ABO genotype as A or B antigens despite having functional A- and B-glycosyltransferases. Individuals with partially inactivating mutations – e.g. para-Bombay may result in weak-ABO phenotypes (see below). Establishing a normal H antigen basis is easily done by use of the lectin *Ulex europeaus*, and anomalies can usually be resolved by genotyping for the H gene.

The secretor system is responsible for expression of H type 1 antigen (and H type 2 but only in the secretory compartment). The enzyme encoded by the H-gene cannot

utilize the type 1 precursor. H type 1 and associated A type 1 and B type 1 glycolipids can be found in the plasma (and secretions) of individuals with the secretor phenotype. These glycolipids will absorb into the red cell membrane and create low levels of type 1 ABO antigen expression - a mechanism which can result in a weak-ABO phenotype in an H deficient individual (see section 3). The secretor status of an individual can be easily determined by genotyping [50, 51]. The determination of ABO blood group substances in saliva is also often used to guide in the interpretation of subgroup, but due to the unreliability of this technique it should only be used for guidance and not for diagnosis.

The Lewis glycosyltransferase $\alpha 1,3/4$ fucosyltransferase (FUT3) is able to modify some A and B antigens into compound antigens such as ALe^b and BLe^b. These glycolipid antigens absorb from the plasma into the red cell membrane and can be readily detected on individuals of the Lewis-positive secretor-positive phenotypes. In general the Lewis modification of the ABO antigen reduces activity to most ABO reagents. In Lewis-negative secretor-positive individuals the type 1 ABO antigens of plasma remain unmodified and can also be detected on the red cell membrane. In determining the basis of a weak phenotype it is critical to know both the Lewis and Secretor phenotype/genotype of the individual so as to accommodate for the presence of absorbed glycolipids in the red cell membrane. Type 2 ALe^Y and BLe^Y structures if detected may also occur as a consequence of Lewis glycosyltransferase action.

The glycosyltransferases resulting in blood group P_1 and the glycolipid structure x_2 are also competing for the precursor neolactotetraosylceramide (nLc-4) and terminate the chain with $Gal\alpha 4$ and $GalNAc\beta 3$, respectively [52, 53].

The ABO antigens of the red cell have a further layer of structural complexity caused by the nature of the linear and or branched structures that carry them. These elongations are usually N-acetyllactosamine units ($Gal\beta 4GlcNAc\beta 3$). It is well recognized that the ABO antigens of the neonate are carried on linear (i antigens) while those of most adults are on branched (I antigens) structures [27, 54, 55]. The linear and branched structures may contain up $50 - 60$ carbohydrate molecules, so-called polyglycosylceramides [56]. The A, B and H are present on the terminus of these branched structures. Thus, the branching glycosyltransferases involved, although not directly affecting the ABH synthesis, may influence avidity to various antibodies and cause divergent blood typing [57]. It is believed that linearity in the carriers reduces the binding capacity of the antigen with antibody, a feature that has probably evolved as a consequence of protection of the neonate from ABO hemolytic disease [58].

ABO antibodies

The ABO blood group system is unusual in that antibodies are almost always present in individuals who lack the specific ABO antigens. The plasma of blood

group A, B, AB and O will contain anti-B, anti-A, neither anti-A nor anti-B, and anti-A,B antibodies, respectively. ABO antibodies can be detected by the age of three month and reach the maximum level at the age of 5 to 10 years [59]. The antibodies (also called natural antibodies) are mainly of IgM class, complement activating and hemolytic at 37˚C, but antibodies of IgG and IgA classes may also be present. After immunization IgG ABO antibodies will increase and IgA will also become present.

A characteristic of weak-subgroups and weak-phenotypes is often an unusual allo-antibody against ABO antigens (e.g. anti-A_1 is often seen in some weak A subgroups). Because of the lack of variability, and unreliability of antibody detection, these allo-antibodies should not be used to define a weak-subgroup or phenotype, but should instead only be used as an indicator of something unusual.

A_1 and A_2 Phenotypes

The two most common subgroups of blood group A are A_1 and A_2 expressing on average, 1 million and 250 000 A determinants, respectively [60]. The A_1 and A_2 glycosyltransferases require different pH optimum, K_m values and ions to transfer the N-acetylgalactosamine saccharide to the acceptor molecule [61]. The A^1(A101) allele is the reference allele [7], but has additionally eight variant alleles. The A_2 phenotype has an elongated protein making the enzyme less effective in creating A determinants [18]. Twenty variant alleles of A^2 have been published up to today (BGMUT database). In the European population approximately 20% have the A_2 phenotype, but frequencies vary in other ethnic populations. These two phenotypes are not weak-subgroups, however as the chemical basis of these phenotypes has been debated for many years, resolution of their chemistry (Paper I) was important to the study of the weak-subgroups/phenotypes.

3. ABO WEAK-PHENOTYPES AND WEAK-SUBGROUPS

The following section will review current knowledge of ABO weak-subgroups and weak-ABO phenotypes, and present known and speculative mechanisms that may result in these weak-phenotypes. As explained in detail above weak-subgroups have an ABO gene basis while weak-phenotypes are caused by non-ABO genes or environmental factors.

Mechanisms for ABO weak-subgroups

Mechanism1: Glycosyltransferase catalytic domain mutations – Glycosyl transferase catalytic domain mutation resulting in normal levels of a glycosyltransferase protein but with an impaired catalytic domain and function [62, 63]. This is often the result of a single genetic nucleotide mutation resulting in a glycosyltransferase with an impaired ability to react with either donor sugar and/or acceptor. This is probably the most widely recognized mechanism for causation of weak-subgroups and various glycosyltransferase mutations have been described. Constructs encoding subgroups A_3, A_x and A_{el} showed a 36 to 86 percent reduction A antigen-expressing cells and antigen expression on the cell surface (MFI, the mean fluorescence intensity) measured by flow cytometry. The greatest decrease in A activity occurred with the *ABO*Ael03* construct [64].

One usual example is the cisAB genotype, which has one allele encoding both for A- and B-glycosyltransferase activity. It is suggested that it is caused by a crossing-over event between an *A* and *B* allele or by mutations changing the transferase to be able to create both A and B epitopes [65, 66].

Mechanism 2: Glycosyltransferase mutations outside the catalytic domain - Glycosyltransferase mutation resulting in normal levels of a glycosyltransferase protein with a normal catalytic domain but with impaired functionality. Again this is probably the result of a genetic nucleotide mutation resulting in an aberrant glycosyltransferase protein. For example if the mutation in the glycosyltransferase was in its membrane anchor region it may affect its opportunity to react or may even affect its biological stability. Although no examples of this mechanism have yet been recognized for ABO subgroups, this mechanism has been described for weak Lewis antigen expression. This study showed that nine of ten individuals with homozygote mutation in the transmembrane domain expressed Lewis antigen in saliva but not on RBCs [67]. However this mechanism has also been investigated in vivo in HeLa cells where co- expression studies were performed. The wild type construct of A and B were co-expressed with mutations outside the catalytic

domain. The reduction of transferase activity was significant. This results suggest that amino acid changes outside the catalytic domain can cause weak A and B subgroups [64, 68]. An unstable enzyme with normal catalytic activity is described as the basis for the Se^w mutation [69].

Mechanisms for ABO weak-phenotypes

The following mechanisms potentially could result in ABO weak-phenotypes. It should be appreciated that in some circumstances a weak-phenotype may be observed as a consequence of inadequate blood grouping methodology rather than as a true reflection of antigen expression on the cells.

Mechanism 3: Insufficient glycosyltransferase - A normal glycosyltransferase is produced but its copy number is too low to cause normal activity. In this scenario the exon gene transcript would be expected to be normal, but mutations in regulatory genes would be expected. A study of Seltsam *et al* suggested that mutations give amino acid sequence variations in the CBF/NF-Y regulatory region may cause weak B subgroups [68]. Mutations in the promoter sequence, the regulator regions (up and downstream) and in splicing sites can all affect enzyme activity [17, 70].

Mechanism 4: Insufficient precursor – if the amount of H antigen available is too low then a subgroup will result despite normal AB glycosyltransferase activity. Such is seen with some para-Bombay phenotypes [71]. Partial H deficient phenotypes, such as para-Bombay, have partial inactivating point mutations in the FUT1 gene that can allow weak expression of H and subsequently A and B antigens on the red cells [8, 11, 72].

Mechanism 5: Precursor structure – the shape of the precursors will determine how the ABO antigen is presented. Cord blood samples are recognized as having weaker expression of A and B antigens as a consequence of the linearity of the I antigen. As a consequence precursor mutations may cause weak ABO antigen expression [58, 73] or influence avidity and affinity of highly specific monoclonal reagents.

Mechanism 6: Acquired antigens – it is well established that blood group glycolipids can be acquired from the plasma. Although these are genetically defined by the ABO genotype we have classified them as weak-phenotypes and not as subgroups due to this secondary acquisition. Some H deficient individuals may acquire a weak-ABO phenotype from the plasma, if they are ABH secretors (sometimes also referred to as para-Bombays). Additionally the transfusion of plasma can result in an acquired ABO phenotype if the plasma was from an ABO secretor [71].

Mechanism 7: Disruption of biosynthesis – if the Golgi apparatus is disrupted then less glycosylation is to be expected. Leucocyte adhesion deficiency (LAD) type II

syndrome is an example having a mutation in Golgi GDP-fucose transporter (GFTP) gene. This point mutation causes a defect in the fucose metabolism resulting in lack of H structures and sialyl-Lewis X on the cells. The lack of H antigen gives them a Bombay (O_h) phenotype on the RBCs. LAD type II leads to mental retardation and short status as adult [74].

Mechanism 8: Non-ABO glycosyltransferases making ABO antigens – most glycosyltransferases show redundancy and degeneracy [11]. This means that more than one glycosyltransferase may have the possibility to catalyze the transfer of the same carbohydrate molecule to the same acceptor (redundancy) and one enzyme could catalyze the transfer to two different receptors and create to different types of epitopes (degeneracy – mechanism 9).

Mechanism 9: ABO glycosyltransferases making wrong ABO antigens (degeneracy) – it is suggested that the B(A) phenotype is caused by a very effective B-transferase (α1-3 galactosyltransferase) capable of catalyzing the transfer of GalNAc(N-acetylgalactosamine) to the acceptor and therefore creating enough A determinants to cause agglutination with some anti-A reagents [59]. Although the ABO gene defines the glycosyltransferase responsible, we have classified this phenotype as an ABO weak-phenotype and not an ABO weak-subgroup. There are several reasons for this including a lack of structural work to conclude the inappropriate antigen is present and potential issues with defining the specificity of monoclonal reagents (e.g. see monoclonal antibody maps in Paper IV).

Mechanism 10: Non-ABO antigens reacting with ABO reagents - ABO antigens may be sufficiently related to non-ABO structures that they may react under some circumstances with ABO reagents. For example mapping of monoclonal ABO reagents has shown that these reagents can also detect several non-ABO antigens. If these antigens were present in sufficient quantities on red cells then they could result in an aberrant result. For example anti-A(B) ES15 MAb has been proven to be a suitable reagent to detect weak A subgroups on RBCs using flow cytometry [71], but using thin layer chromatography can be shown to react with non-ABO structures such as p-Fs (para-Forssman), Fs (Forssman), P_1 and probably x_2 (paper IV) Another example pertinent to this thesis is MAb anti-A 2-22 also reacting with the p-Fs structure (paper III).

Mechanism 11: Insensitive detection systems – simply put if the reagent or method being used is insensitive then apparent weak antigen expression will be seen. This is not a true weak-ABO but in the eyes of the observer it is. This problem can be caused by numerous factors including reagents too dilute, insensitive methodology, contamination and reagents with unusual specificities.

Mechanism 12: Chimera/Transplantation/Transfusion – unusual weak-phenotypes can occur as a result of a mixing of different ABO cell populations with the result often recognized as a mixed-field reaction. A variety of mechanism can cause this including chimeras, bone marrow transplantation, and transfusion.

Mechanism 13: Infection – although strictly speaking the deacetylated A antigen (the acquired B antigen) caused by a bacterial deacetylase is not a true ABO antigen, it does result in apparent weak expression of B antigen (with some reagents) [75].

Mechanism 14: Physiology - pregnancy may cause decreased ABO blood group expression on the cells, but it will return to normal levels post delivery. During certain medical conditions (e.g. cancer) the expression of antigens may also either decrease or sometimes result in the expression of an incompatible antigen.

Mechanism 15: Artificial in vitro manipulation – Man-made ABO weak-phenotypes can be created via a range of different mechanisms, including in vitro modification with glycosyltransferases, glycosidases, antigen masking agents (e.g. PEGylation), genetic manipulation (transfection of precursor cells) and KODE technology [76, 77].

4. AIMS

The quantitative and serological differences of the ABO weak-subgroups and weak-phenotypes are well established, but their qualitative and structural basis are still largely unexplored. Weak-subgroups are potential windows into the biochemistry of the ABO blood group system, due to the absence of dominating structures, and/or enhancement of trace antigens caused by a loss in normal competition. All the same they are challenging phenotypes not only because they are rare but also by definition their ABO antigens are expressed in low levels.

The aim of this thesis was to gain insights into chemical basis and the mechanisms behind selected A weak-subgroups and/or A weak-phenotypes. The specific aims were:

- to establish a baseline by resolving the structural difference between blood group A_1 and A_2.
- to investigate the immunochemical and structural differences, if any, of the weak subgroups of A.
- to speculate on the potential the mechanisms resulting in weak ABO subgroups and phenotypes.

5. METHODOLOGY AND CONSIDERATIONS

The primary constraint of these studies was the availability of material. The weak ABO phenotypes are rare and relatively large volumes of blood are needed to isolate trace glycolipids for structural analyses. The collection of adequate volumes of blood may take up to several years because a donor is only allowed to donate 3 or 4 units of blood per annum.

All methods have their own strengths and weakness and no single technique gives an unambiguous answer. In order mitigate these risks a range of confirmatory, complementary and contrasting methods were used to assign identities to glycolipids. The methods used in this thesis such as hemagglutination, flow cytometry, TLC-EIA, MS, NMR and DNA genotyping separately may not always give the correct interpretation but in combination these assays makes the data and interpretations reliable and more secure. A serological phenotype is primarily defined by antibody agglutination of native RBCs. However testing the isolated RBCs antigen (as glycolipids) on a TLC plate and immunostained with the same antibody, may show a different profile to agglutination serology. Thus, structures identified chemically do not necessarily contribute to the serological phenotype when present on the RBC membrane. Quantitatively there may be insufficient antigens presented on the RBCs for agglutination yet on the TLC plate the concentration (also further enhanced by purification and fractionation) and accessibility of the antigen is adequate, or even enhanced. As the presentation of the antigen on the cell surface and its presentation on TLC plates are different this may affect the affinity and avidity of the antibodies that react. It is also possible that steric hindrance caused by larger structures (or charged structures) on the intact cell membrane may affect the ability of antibodies to detect short carbohydrate antigens, conversely other glycolipids on TLC plates may similarly cause interference (e.g. globoside).

Likewise ABO phenotype and genotypes are not automatically concordant. Some ABO weak-phenotypes only have the common A^1 or A^2 alleles (see above). As a consequence genotyping was used as a guide to interpreting phenotypes and more importantly to determine and assign Lewis and secretor types.

Blood samples – Single and multiple donations of RBC units were collected from single blood donors with defined ABO subgroup by serological testing and genotyping [62, 78], Lewis [51], and Secretor [50] status. If the routine ABO genotyping did not reveal any mutations previously associated with ABO subgroups, sequencing of the gene including exons, introns, and splicing sites was performed. Samples were always washed free of plasma to preclude the contribution to the glycolipid profiles of plasma antigens.

Reagents and antibodies - Historically polyclonal antibodies (PAbs) were used routinely to define the weak subgroups. PAbs are prepared from immunized serum of humans or animals, recognizing multiple epitopes and are less standardized and concentrated than most monoclonal reagents. PAbs are also more tolerant to variations in saline concentration and pH than MAbs [79]. Historically weak phenotypes were more frequently detected by PAbs than with todays modern MAbs, primarily because the latter are more standardized and concentrated. Many ABO MAbs of today are formulated as blends and the clones used are selected to be broadly reactive with all appropriate ABO antigens. Probably many of the stronger historical weak-subgroups would now go undetected today, or simply type as normal ABO phenotypes.

High specificity MAbs being those that are selective against the ABO antigen they recognise, are not usually selected for routine serological reagents (or are used in blends) make for powerful research tools, and are as used here. Additionally the cross-reactivity profiles of these specific reagents can be used to extend the detection capabilities of a reagent, but this is only of value if these cross-reactivity profiles are known and recognized, otherwise they are a risk to interpretation. For example a monoclonal reagent showing only anti-A specificity with the flow cytometry technique has been shown to be a good candidate to detect weak A subgroups is the reagent anti-A(B) ES15 MAb [71]. But by the TLC and immunostaining (TLC-EIA) technique strong reactivity can also be seen with p-Fs (para-Forssman), Fs (Forssman) and probably also with x_2 structures. These non-ABO structures have a terminal GalNAc residue and different core structures. Structures with a terminal Gal residue such as B and P_1 also showed reactivity with anti-A(B) ES15 using TLC-EIA. Interpretation of reactivity of this, and all other MAbs, therefore need to be carefully considered in light of known and potentially unknown reactivity.

All the same it should be appreciated that we can never know the full activity profile of a particular Mab and in some circumstances reactivity may be misinterpreted due to unknown reactivity. For these reasons we always used a panel of well-characterized monoclonal anti-A, -AB and -H antibodies (MAbs) to make interpretation of the results as secure as possible [38, 80-84]. Consensus was required to give certainty in defining antigens on the basis of activity with Mab reagents.

In paper IV we also used anti-Fs MAbs to investigate the unexplained rare weak ABO subgroup A_{pae}. The monoclonal rat anti-Fs antibody (clone M1/22.25.8HL) used in the initial analyses was not commercially available for the final experiments. However the cell line was still available so a subculture of the hydridoma clone was prepared as recommended by the manufacturer [85, 86]. These newly produced anti-Fs MAbs showed the identical binding to Fs glycolipids as the "old" commercial produced MAb.

Isolation and purification of glycolipids - The method used to extract glycolipids from the RBCs membrane is based on the method developed by K A Karlsson [87]. Modifications were made to decrease the loss of glycolipids, which to some extent may occur in every step of the procedure (for more information see paper II).

The first step of extraction (at 72˚C) using isopropanol, methanol and a chloroform/methanol mix gave a total lipid fraction. The initial extraction was followed by mild alkali methanolysis of the ester linkages of phospholipids and triglycerides [87]. Dialysis followed to remove salts and water-soluble residues of phospholipid degradation. Open silica column chromatography was use to fractionate the lipids and remove cholesterol and fatty acid methyl esters. Ion exchange chromatography was used to separate the neutral (non-acidic) and acidic glycolipids. The removal of sphingomyelin was performed changing the chromatographic properties of the neutral glycolipids by acetylation to a nonpolar chromatographic interval followed by further fractionation on silica chromatography, deacetylation and dialysis.

Most of the total neutral glycolipids were further purified by high performance liquid chromatography (HPLC) or medium pressure liquid chromatography (MPLC) and open silica column chromatography to get as pure glycolipid fractions as possible. Fractionation was deliberately limited to reduce the risk of losing valuable and rare glycolipids [88], and as a consequence always resulted in heterogenous partially fractionated samples. For this reason the structural analyses had to be done on mixtures of glycolipids and not on ideal pure glycolipid fractions.

Acidic glycolipids and polyglycosylceramides were not included in these studies because of their loss and or exclusion by the methodology used. Any contributions to the phenotype these structures may have had, are therefore unknown.

Thin layer chromatography – enzyme immuno assay (TLC-EIA) – On silica gel TLC plates coated with silica gel and using solvent systems for neutral glycolipids (i.e. chloroform:methanol:water 60:35:8;v/v/v) glycolipids are migrating according to polarity and will be separated primarily by number of carbohydrate residues although migration is also affected to some degree by the hydrocarbon chain lengths and hydroxylation of the ceramide. However some divergent migrations can sometimes be seen i.e. p-Fs being a pentasaccharide migrates in the region of 6 sugars.

Chemical and immunostaining combined give good information about the size of the structure (migration on the plate) and the glycotope they may carry. Anisaldehyde is used as a semi-quantitative chemical staining method to detect glycolipids, revealing a characteristic green colour. Monoclonal or polyclonal antibodies of mouse or human origin usually of IgM or IgG type and lectins are used in the immunostaining and give information on the glycotope. TLC-EIA is based on the method of Schnaar [89].

Mass spectrometry (MS) – a large number of techniques are available today. With glycolipids this technique reveals mass (molecular weight) information that can be use to determine: type of saccharide, number and sequence of saccharides (hexose, hexosamine and fucose), branching points and ceramide type. Only rarely does this technique assist in determining type glycosidic linkages (α or β) and therefore cannot resolve isomeric structures of the basis of mass (e.g p-Fs and Fs). Particular and valuable advantages of this technique are its requirement for small quantities of sample and ability to resolve impure fractions.

In paper III nano-ESI-QTOF MS (nano-electrospray ionization-quadrupole time-of-flight mass spectrometry) was used to screen the fractions of glycolipid mixtures and for structural elucidation CID (collision-induced dissociation) tandem MS experiments were performed [90, 91]. In paper IV nano-LC/MS was used to structurally verify the Fs structure. First the ceramides were removed by hydrolysis with *Rhodococcus* endoglycoceramidase II [92]. The characterization of carbohydrate sequence, particularly isomeric forms, was simplified by the separation on porous graphitized carbon column, involving hydrophobic and polar interactions, before the MS experiments [93].

Nuclear magnetic resonance (NMR) spectrometry – Proton NMR (1D and 2D) gives information about the monosaccharides, sequence, and the glycosidic linkage of a carbohydrate structure [94]. The degree of hydroxylation and occurrence of double bonds of the ceramide can also be defined. However a substantial limitation of this technique is that relatively large amounts of reasonable pure glycolipids are needed for good resolution of diagnostic signals.

Nomenclature of glycolipids – The nomenclature used in this thesis is as recommended by IUPAC-IUB (http://.chem.qmul.ac.uk./iupac/misc/glylp.html) and reported by Chester 1998 [95]. The abbreviation, for example A-6-2 refer to blood group A, number of carbohydrate molecules (6 monosaccharides) and the type of core structure (type 2) [33].

Additional analyses - In collaboration (paper IV) with PhD student Annika Hult, Department of Laboratory Medicine, Lund University performed gel column analysis of RBCs and flow cytometry for the serology testing; modelling of the human Fs synthetase; haemagglutination of Fs positive cells (A_{pae}) with *E.coli*; and pedigree study of the two families having the weak subgroup A_{pae} phenotype.

Table 2. The blood group A glycolipid structures discussed in this thesis.

A-4-6	GalNAcα3(Fucα2) Galβ4Glcβ1Cer
A-6-1	GalNAcα3(Fucα2)Galβ3GlcNAcβ3Galβ4Glcβ1Cer
A-6-2	GalNAcα3(Fucα2)Galβ4GlcNAcβ3Galβ4Glcβ1Cer
A-7-1 (ALe^b)	GalNAcα3(Fucα2)Galβ3(Fuc4)GlcNAcβ3Galβ4Glcβ1Cer
A-7-2 (Ale^y)	GalNAcα3(Fucα2)Galβ4(Fuc3)GlcNAcβ3Galβ4Glcβ1Cer
A-7-4	GalNAcα3(Fucα2)Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer
A-8-2	GalNAcα3(Fucα2)Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer
A-9-3	GalNAcα3(Fucα2)Galβ3GalNAcα3(Fucα2)Galβ4GlcNAcβ3Galβ4Glcβ1Cer
A-10-2	GalNAcα3(Fucα2)Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer
A-11-3	GalNAcα3(Fucα2)Galβ3GalNAcα3(Fucα2)Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer
A-12-2	GalNAcα3(Fucα2)Galβ4GlcNAcβ3[GalNAcα3(Fucα2)Galβ4GlcNAcβ6]Galβ4GlcNAcβ3Galβ4Glcβ1Cer
A-14-2	GalNAcα3(Fucα2)Galβ4GlcNAcβ3Galβ4GlcNAcβ3[GalNAcα3(Fucα2)Galβ4GlcNAcβ6]Galβ4GlcNAcβ3Galβ4Glcβ1Cer
H type 1	Fucα2Galβ3GlcNAcβ3Galβ4Glcβ1Cer
H type 2	Fucα2Galβ4GlcNAcβ3Galβ4Glcβ1Cer
Lc-3	GlcNAcβ3Galβ4Glcβ1Cer
nLc-4	Galβ4GlcNAcβ3Galβ4Glcβ1Cer
i	Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer
I	Galβ4GlcNAcβ3[Galβ4GlcNAcβ6]Galβ4GlcNAcβ3Galβ4Glcβ1Cer
Para-Forssman	GalNAcβ3GalNAcβ3Galα4Galβ4Glcβ1Cer
Forssman	GalNAcα3GalNAcβ3Galα4Galβ4Glcβ1Cer
Le^a	Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer
Le^b	Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer
P	GalNAcβ3Galα4Galβ4Glcβ1Cer
P$_1$	Galα4Galβ4GlcNAcβ3Galβ4Glcβ1Cer
X$_2$	GalNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer

6. PRESENT WORK

The following papers describe the study of blood group A glycolipids (< 20 sugar residues) isolated from RBC membranes of individuals with interesting A weak phenotypes. Due to methodological limitations glycoproteins, >20 sugar residues (polyglycosylceramides) and acidic glycolipids were not experimentally considered, although they would most likely contribute to the serology of the weak phenotypes observed. However, it remains unknown if, or to what extent, glycosylation of the glycoproteins would differ from that of the glycolipids.

Historically subgroups were defined using polyclonal antibodies (PAbs) and were therefore more frequently detected than by today's use of highly potent and specifically formulated commercial blends of monoclonal antibodies (MAbs). In this work panels of characterized unblended MAbs with different sensitivity and crossreactivity profiles for glycotopes were used (see Methodology). Even though the MAbs used were considered well characterized the identity of the glycolipids to which they bound on TLC plates may be affected by avidity and affinity of the antibody, glycolipid concentration, steric configuration of the glycolipid, and unknown specificity. To overcome this weakness a variety of reagents were always used, glcyolipids were loaded over concentrations ranges and identities by TLC-EIA alone were always only tentative. To assign identity a range of techniques including genomic typing and sequencing, agglutination tests, flow cytometry, TLC-EIA, NMR and MS, were used.

Structural glycolipid differences between A_1 and A_2 subgroups (paper I)

The qualitative differences between blood group A subgroups A_1 and A_2, if any, have probably been debated since these subgroups were described in the early 20th century. The major difference between these subgroups is the lower level of A antigen expressed in A_2. Most text books on blood group serology also report that the blood group A structures A type 3 and A type 4 are expressed predominantly in A_1. However, some published papers suggest that these structures also are expressed in the A_2 subgroup. In order to understand the basis of weak-subgroups it was essential to first understand the basis of the two major subgroups of A. As the literature was ambiguous the glycolipids of A_1 and A_2 individuals were reanalyzed.

In *paper I* glycolipids from four A_1 and four A_2 individuals were isolated. Subgroup A_1 was as expected found to have about four to five times more A antigen in the RBC membrane, and to compensate for this two and a half more A_2 total glycolipids were loaded for the TLC-EIA analysis, e.g. 20 μg and 50 μg

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respectively. Higher loadings were not practical as the dominant glycolipid globoside could cause interference. The Aweak-I sample with phenotype A_3 (*paper II*) was also included as a control, loaded at 50 µg. To be able to compare our results with earlier reports, the same MAbs as in the original publications such as HH4 (anti-A type 2), TH1 (anti-A type 3), HH5 (anti- A type 3+4), KB 26.5 (anti-A type 3+4), BE2 (anti-H type 2), HH14 (anti-H type 3), AH21 (anti- A type 1) and HH3 (anti-ALe^b) (for references see Methodology) were used.

The TLC-EIA results found that A type 2 and A type 3 glycolipids showed almost identical pattern in the A_1 and A_2 phenotypes albeit in smaller amount (figures 1& 2, paper I). As expected from predicted biosynthesis A_2 showed larger amounts of H type 2 and 3 precursors. A type 4 is a smaller component of the RBC membranes, but A-7-4 was clearly present in A_1 but not in A_2. Interestingly, the Aweak sample expressed A-7-4, which might indicate that the absence of A-7-4 in A_2 is due to steric hindrance between the glycosyltransferase and the type 4 precursor, presumable as a result of the elongation of the A_2 transferase. The Lewis and secretor phenotypes did not influence to glycolipid profiles of the A_1 and A_2 subgroups.

It was therefore concluded that A type 3 glycolipids are similarly expressed in A_1 and A_2, but in lesser amount in the latter. The only visible glycolipid difference between the two subgroups is the presence of A-7-4 in A_1 and it is undetectable or expressed in very small amount in A_2.

Glycolipid variations in weak A subgroups (paper II)

In *paper II* a panel of nine A subgroups were studied, including the phenotypes A_3 (n=2), A_x (n=1), A_{el} (n=5) and $A_{finn}B$ (n=1). Glycolipids were isolated from a single blood unit of RBCs from each individual. All individuals were serologically and genetically defined for ABO, Lewis, and Secretor (paper II, table 2). If the ABO genotyping was inconclusive, exons 1 to 7 were sequenced. The genotype does not always reflect the result of the product expressed on the RBC membrane [8]. The phenotype A_3 illustrates this; having the common A^1 or A^2 alleles combined with a normal O allele, but expressing less A antigen and showing the mixed field phenomena. The two A_3 individuals (Aweak-I and Aweak-II) in this paper revealed mixed field reactions with anti-A and anti-AB MAbs and had allo-anti-B in their serum. Aweak-I, had an A^1 allele without mutations and a normal glycolipid profile, except for an apparent lack of an extended structure (≥ 14 sugar residues) (paper I and II) (Figures 3 and 4, plate 7 and 9). Additionally an A_3 individual, not included in this study, also showed this lack of extended structures when tested against MAb anti-A type 2 (2-24). No clear characteristics explaining the A_3 phenotype, was found for the Aweak-I individual. However, the expression of subgroups may be affected by genes outside the ABO genome or by other events during the biosynthesis.

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In contrast to Aweak-I, the Aweak-II expressed an A^2 allele with a substitution 539G>A in exon 7 (encoding the catalytic domain). This mutation appeared to be a new mutation not previously reported. Anti-A_1 was present in the serum. Aweak-II also lacked a less extended structure (about 12 sugar residues) (Figures 3 and 4, plate 7 and 9). This A_3 individual also showed peculiar antibody binding in the migration zone for hexaglycosylceramide on the TLC plate. A total of sixteen different MAbs were tested against the total glycolipid fraction and the reactivity's was from negative to moderate positive. Interestingly, the MAb anti-A 2-24, favoring the A type 2 determinant, did not bind in this region despite glycolipids with type 2 chain being the most common ABO blood groups antigen on the RBC membrane [27, 37]. The known blood group A bearing glycolipids in the region are A-6-2 and A-6-1, but A-6-1 can be excluded in this case because of the non-secretor status of the individual. An A type 2 glycolipid may also be excluded because of the negative result with MAb 2-24. We could not see any clear-cut correlation between these odd reactions and the mixed field phenomena. The atypical binding pattern was not resolved and we suggested that a novel glycolipid was causing the positive reactions in the hexaglycosylceramide region.

Additional speculations were the absence of extended structures in both A_3 individuals might indicate that the branching of the glycolipid structures is absent or partial. Alternatively the A transferase, with the 539G>A mutation, might be inefficient glycosylating some H structures.

The phenotype A_x Le(a-b-) secretor (Aweak-III) showed moderate reactivity against the anti-A and anti-AB MAbs and the binding pattern was identical to the A_2 control even when the glycolipids were loaded at the same concentration. Stronger anti-A reactions were seen with the TLC-EIA technique compared to the RBC serology, which gave a 1+ reaction. The genotyping and sequencing revealed an A^2 allele with a G>C substitution at nucleotide 203 in exon 4. This mutation was the first mutation outside the exons 6 and 7 proven to affect the A transferase activity and has been seen in the subgroups [62]. However, the results suggest that this variant of A_x phenotype might be an A_2 with low level of A antigens probably caused by a mutation located before exon 6.

The four A_{el} samples (Ael-I to IV) included in this study, showed the expected serological characteristics for a weak-subgroup, i.e. negative results against anti-A and anti-A,B antiserum; positive absorption/elution of anti-A antibodies; and only anti-B antibodies in serum. All four samples had the A^{el} combined with O alleles. Ael-I revealed an O^1 allele with a 467C>T mutation in exon 7, but the remaining Ael's had an O^{1v} allele.

At high concentrations of total glycolipids, reactions against some MAbs appeared, indicating very low level of blood group A glycolipids. Interestingly, an A or A-like structure (*rm* 2.8) was detectable in all A_{el} samples, except for Ael-IV. This structure was migrating in the same region as glycolipids with 9 to 10 sugar residues when analyzed with MAb anti-AB 2-39. MAb 2-39 is of immunoglobulin

class IgG3 and therefore might be able to better detect the structure than IgM. However, this glycolipid structure was also seen in approximately the same amount in A_1, A_2, Aweak-I, -II and –III phenotypes independent of Lewis or secretor status. It appears this structure becomes relatively dominant structure in the A_{el} samples due to the very low amount of other blood group A bearing glycolipids. Due to a lack of sufficient glycolipids for structural analysis we could not make any conclusions, but this particular glycolipid might be a glycotope contributing to the A_{el} phenotype.

The single $A_{finn}B$ Le(a+b-) non-secretor sample with a A^{finn}/B genotype did not react convincingly with any anti-A MAb except for very weak reactivity with MAb 1401 at high concentration loading. Some similarity to Aweak-II, in not reacting in the hexaglycosylceramide region with MAb anti-AB 2-41, was seen. The $A_{finn}B$ phenotype expressed only trace of A antigen but was not further investigated.

In conclusion, despite no structural analysis, this paper showed unmistakable qualitative variations influencing the phenotypes when using a range well-characterized MAbs.

A variant of the blood group A_3 phenotype (paper III)

Paper III - is a continuation of the investigation of the Aweak-II individual in *paper II*, expressing a blood group A_3 Le(a+b-) phenotype. This heterozygote genotype has an A^2 allele with a G539A mutation combined with an O^1 allele, registered in BGMUT (Blood group antigen gene mutation base) [13] as A304. In this paper this subgroup is named A304 A_3.

In addition to a low levels of A antigen the common A_3 phenotype has a characteristic "mixed field" agglutination. In mixed field agglutination both small aggregates and free cells exists, thus there are a few small agglutinates in a background of free RBCs [96]. It has been proposed that only the anti-A agglutinating RBCs have weak enzyme activity in the membrane, but not the unagglutinated RBCs. However, the N-acetylgalactosamine transferase in serum was active and with no detectable aberrations in the chemical properties to the fully functional A_1-enzyme [97]. Evidence for two populations of RBCs has been reported by Heier 1988 and recently Hult et. al. has shown, using flow cytometry, that the typical A_3 phenotype actually has two population [71, 98]. Unfortunately, because we used total glycolipids isolated from the red cell membranes it was impossible to separate two populations in our study. However, serology of this individual does not support dual populations as the agglutinated and unagglutinated cells when reanalysed each show mixed field agglutination (Figure 1, paper III). It had been shown in an early study of the A_3 phenotype that a repeated agglutination of the unagglutinated RBCs with fresh anti-A resulted in a further agglutination [99]. We were able to confirm this result using serologic gel card technique. Thus,

the repeated agglutination with fresh MAb anti-A brought out a new mixed field reaction (Figure 1, paper III).

Total neutral glycolipid of A304 A_3 showed a peculiar antibody binding pattern i.e. in the migration zone of hexaglycosylceramide on the TLC plate reactions varied from none to moderate. The usually dominating A-6-2 glycolipid seemed to be absent. In the migration zones of more extended glycosylceramides ($\geq$8 sugars) the reactions were as expected. Overall identical reactions to the result in *paper II* were seen.

In an extended TLC-EIA, a purified p-Fs (GalNAcβGbO$_4$) glycolipid fraction was included, because it has similar migration as hexaglycosylceramide [42, 100]. Surprisingly, some of the anti-A MAbs reacted clearly with GalNAcβGbO$_4$ (p-Fs). Interestingly, the p-Fs fraction showed the same reaction pattern as the total A304 A_3 glycolipid fraction. Thus, the strong glycolipid reactions seen against some anti-A MAbs, in particular anti-A 2-22 MAb, may be due to crossreactivity against p-Fs glycolipids (if the TLC migration is appropriate).

The total neutral glycolipids were fractionated by MPLC (medium pressure liquid chromatography) into seven fractions. These fractions hold mixtures of glycolipids of increasing size. TLC-EIA performed on these partly purified fractions gave a more distinct result, and very small amounts of A-6-2 glycolipids (A hexaglycosylceramide with a type 2 chain) were visible (Figure 3 plate II, paper III). Positive reactions with MAb anti-A type 2 (2-24), not binding the p-Fs (GalNAcβGbO$_4$) glycotope (Figure 3 plate IV, paper III), confirmed this reaction. MAb anti-A 2-22 was strongly reactive in all fractions indicating blood group A type 2 glycolipids with 8 to 12 sugar molecules and possible also extended p-Fs glycolipids (Figure 3 plate III, paper III). A small amount of A glycolipids with type 3 chain was also defined (Figure 3 plate III, paper III). Type 1 A structures could be excluded, because A304 A_3 was secretor negative. Thus, no A substances were absorbed from the plasma. The blood group A precursors H type 2 and 3 showed no abnormalities in their expression.

Structural analysis of all fractions were achieved by MS (Nano-ESI-QTOF and tandem MS) and proton NMR. The very small level of the A type 2 structure was confirmed by MS and ^{1}H-NMR. A type 3, p-Fs, and extended p-Fs glycolipid structures were also verified. All relevant structures found are presented in table IV, paper III.

In conclusion this A304 A_3 variant of blood group A_3 had very low levels of A-6-2 glycolipids; presence of A-8-2, A-10-2, A-9-3 and A-11-3; and presence of p-Fs (GalNAcβ3GbO$_4$) and its extended forms. The p-Fs glycolipids were not considered as a factor involved in the mixed field phenomena, because the structure has been seen in other blood groups [42, 100]. The other striking feature, was the absence of an extended glycolipid structure ($\geq$12/14 sugars), was not further investigated. However, branching structures migrating in this region on the TLC plate but there was no evidence for branching glycolipids in the MS spectra.

Thus, the absence of branched structures may contribute to the mixed field reaction together with low abundance of A antigen. An obvious weak point in this study was that the most likely branched structures, glycoproteins and polyglycosylceramides were not analyzed, so their impact on the red cell membrane of the A_3 phenotype is not known.

Blood group A subgroup-A_{pae} (paper IV)

Investigations into the A_{pae} phenotype unexpectedly revealed the presence of the Forssman antigen (*paper IV*). Because the Forssman glycolipid is not an ABO antigen it was not discussed above in the introduction. Therefore a short summary of the current knowledge on the Forssman antigen is presented here.

Johan Forssman, professor in Bacteriology and Pathology in Lund 1900, detected the Forssman (Fs) antigen by injecting an extract from guinea pig kidney into rabbits. The immunological reaction that followed created an antiserum that haemolysed sheep erythrocytes. The same reaction was found to occur in horse kidney, goat and cat erythrocytes but not in bovine, pig, rabbit and human tissues. This observation was published 1911 and the antigen was named Forssman antigen and published 1911 [101].

Several researchers, including Landsteiner, tried to structurally determine the Fs antigen and during the period from 1930's to 1960's it was established that the Fs antigen was a glycolipid and was initially incorrectly described as an anomeric isomer of globoside. After some competition between the groups of Yamaka and Hakomori during the 60's, in 1971 Hakomori's group finally published the correct structure, as globoside with an additional GalNAc, thus being GalNAcα3GalNAcβ3Galα4Galβ4Glcβ1Cer [43, 45]. Globoside is the precursor for the Fs antigen, thus Fs synthetase catalyses the transfer of GalNAc to GalNAcβ3Galα4Galβ4Glc1Cer (globoside) in a α1 to 3 linkage. Some years later Karlsson et al. verified the structure by MS without any degradation of the glycolipid and thus conclusively established the number and type of sugars [102].

Even though it was established that Fs is a glycolipid there are studies indicating that the Fs antigen also is present on glycoprotein. Slomiany et al found glycoprotein in dog gastric mucus contained *O*-glycosidically linked carbohydrate chains bearing the Fs epitope [103, 104]. They also proposed that the Fs glycolipids were connected to the mucosa membrane while the glycoprotein antigen was associated with the secretion. The Fs glycotope on glycoprotein has also been found in human cytoplasma of colon globlet cells using rabbit-IgG anti-Fs [105].

The Fs antigen is widely seen as an animal antigen found in sheep, horse, cat, dog, canine, and equine, but is not expressed in human. The distribution of Fs antigens is

expressed unequally between species but also within the same cells and tissue of the same species. But there is evidence reported for normal Fs expression in humans, for example; in human gastric and colonic mucosa [46, 106]; in human lung (0.03 µg/g dry tissue weight) [107]; in human colon globlet cells [105]; in human kidney (6.4 µg/g dry tissue weight) [48]. In the latter study glycolipids were extracted from a transplanted $A_1Le(a+b-)$non-secretor kidney. The neutral glycolipids were reactive against anti-Fs MAb and the Fs structure was verified by MS. This result might be of clinical importance in the terms of transplantation because Fs antigen is an alloantigen and preformed anti-Fs antibodies exist in the majority of humans. The preformed antibodies probably are a response to the exposure of the Fs antigen expressed in animals, bacteria and viruses, similar to ABO antibodies. The human antibodies are mainly of IgM type (5/6) and are independent of the blood groups A, B or O [106]. Strokan et al showed that human anti-Forssman antibodies reacting with sheep erythrocytes and organs are both IgG and IgM antibodies [108].

Several studies have shown the presence of Fs antigen in human cancer such as cancer in lung, colon and stomach. Hakomori tested normal gastrointestinal mucosa and tumor derived from the mucosa, obtained from 21 individuals, for Fs activity. Interestingly, five individuals with Fs positive normal mucosa did not express Fs antigen in the tumor and vice versa, thus Fs negative individuals expressed Fs antigen in the tumor, which was confirmed by Young 1979. He showed that a patient with 22 µg Fs antigen per 100 mg dry residue from normal mucosa expressed <0.01 µg in the tumor. In contrast the patients (5 individuals) with Fs negative normal mucosa (<0.01 µg) expressed 4.5 to 78.5 µg in the tumors. The blood groups included in his study were B (5 individuals) and O (1 individual) [46] [109]. These result indicated Fs antigen to be a cancer-associated antigen at least in terms of gastrointestinal mucosa tumours.

The Fs synthetase gene in humans, GBGT1, is located on the chromosome 9q34, the same as blood group A and B transferase genes and they have approximately 42% homology between amino acids sequences [44]. The human GBGT1 gene has about 83% identical amino acids to the animal GBGT1 gene, i.e canine. Xu et al found mRNA in several human tissues and cells, but the encoded protein was inactive [110]. By expression of different designed construct, i.e. combining human:dog and dog:human in COS-1 cells the conclusion made was that a mutation in exon 7, encoding the catalytic domain, probably inactivating the enzyme in humans.

The Fs glycolipid is also known as a microbe receptor, for example the P-fimbriated *Eschericia coli* prs-adhesin, which are known to bind to GalNAcα3GalNAc [111]. Another study showed that an active Fs synthetase is protective against Shiga toxin, because it will modify the Shiga toxin receptor into Fs [112] and as result the susceptibility for the toxin will decrease. The authors made the conclusion that "these results demonstrate that variability in glycolipid expression among species may have a profound effect on susceptibility to an

infectious agent and provide insight into the molecular evolution of glycosyltransferase gene expression".

It can be concluded that Fs structures are expressed in animals, and probably to some extent in humans. Naturally occurring anti-Forssman is predominantly IgM but IgG can be seen in the serum of a majority of human individuals. The Fs antigen may also be clinical important as a receptor for bacteria, virus, toxin and parasites and might be important to consider at allo/ xeno transplantations and blood transfusions.

Paper IV - The blood group A subgroup A_{pae} was first reported by Robert Stamps at NHS Blood and Transplant, Sheffield, UK 1987 [113]. Family studies confirmed the inheritance of this weak-subgroup. The main defining feature of blood group A_{pae} is strong reactivity with the lectin *Helix pomatia*, which has specificity for α-N-acetylgalactosamine (αGalNAc). Three out of 18 human anti-A and eight of 18 human anti-A,B PAbs agglutinated A_{pae} RBCs, but no agglutination was caused by a range of anti-B or AB-serums tested. Thus, this study indicated some characteristics associated with blood group A weak phenotypes but others unrelated to group A, such as:

i). Serum contained anti-A and anti-B with normal titer for a blood group O and saliva expressed only H not A or B substances.

ii). No activity of A transferase was detected in the A_{pae} serum.

iii) the A_{pae} individuals were genotyped as homozygote blood group O (*i.e.* O^1/O^1 and O^1/O^{1v}).

To define what antigenic structure(s) were causing the abnormal serological pattern and this A-like blood group, Robert Stamps (UK) sent blood units donated by two A_{pae} individuals from unrelated English families for glycolipid analysis.

In *paper IV* extended structural, serological, and genomic studies and glycosyltransferase modelling were performed on the A_{pae} individuals.

Initially total neutral glycolipids were isolated from red cell membranes. The glycolipids were tested against *Helix pomatia* and a variety of anti-A and –A,B (n=14) MAbs using TLC-EIA technique. Only three of the MAbs showed reactivity *i.e.* anti-A A003 and "Lorne", showed weak reactivity in the migrating regions of 6, 8/9 and 10/12 sugars. Although these reactions are still not clarified, there was no conclusive evidence for any blood group A antigens. One of the MAbs was an anti-AB (clone ES-15) which appeared to bind both Fs ($GalNAc\alpha GbO_4$) and p-Fs ($GalNAc\beta GbO_4$) structures. (Figure paper IV)

Helix pomatia stained an area in the lower edge of the GbO_4 (globoside) band. This stained band was separate from globoside, which gave evidence for a crucial tetra- or pentaglycosylceramide structure. Even though Fs glycolipid is generally seen as an animal structure not normally expressed in human, its TLC migration and

aberrant reactivity suggested it as a candidate structure. Subsequent anti-Fs MAb analysis showed a strong reactivity with this aberrant pentaglycosylceramide. Total neutral glycolipids were then separated into fractions by open column chromatography to obtain sufficient material of adequate purity for structural analyses (Figure paper IV). HPLC was not used because of, as in paper III, the possibility in losing relevant glycolipids especially those expressed in trace amounts. Most glycolipid mixes are difficult to resolve and all HPLC fractions contained mixes of glycolipids of different lengths. One fraction, which eluted with the solvent mix CM 65:35 was selected for MS and NMR analyses, because it contained the anti-Fs/*Helix pomatia* reactive glycolipid in a fair amounts and had the least amount of globoside and other unwanted glycolipids. Both mass spectrometry and 2D NMR verified that the *Helix pomatia* positive pentaglycosylceramide was Fs glycolipid. Other glycolipids were present but differentiated from Fs and included p-Fs and x_2.

Anti-A,B ES-15 MAb and anti-A 2-22 are reactive with p-Fs, and indicated that the positive reactivity in 6 sugar region was due to p-Fs glycolipids. Likewise the positivity against longer structures may be due to extended p-Fs structures (*paper III*), but that is speculative and needs to be further investigated.

At this stage collaboration was established with Professor M. Olsson and PhD student A. Hult at the Division of Hematology and Transfusion Medicine, Dept. of Laboratory Medicine, Lund University, because of their skills and knowledge in the area of genomics in connection with blood groups. Based on the glycolipids findings they tested A_{pae} RBCs against anti-Fs MAb and random donor plasma with flow cytometry and serological techniques. The A_{pae} RBCs were reactive with anti-Fs MAb, which confirmed the expression of the Fs structure on red cells membrane. The frequency of positive reacting plasma was about 10%. However, the majority of the human population has preformed antibodies against the Fs structure. The low percentage positive individuals among the donors may be due to low titer and/or the affinity and avidity of the antibody for native RBC's. Strong positive reacting plasma was absorbed with A_{pae} RBCs and the eluted antibodies were tested against A_{pae} and various blood group RBCs. Reactivity with A_{pae} cells was seen, but not with other blood groups. Gel card technique confirmed this result. The DNA profile was retested including sequencing of ABO exon 1 to 7 and the upstream CBF/NF-Y-binding enhancer region. The homozygote *O* genotype was confirmed on both individuals and no aberrations were detected.

To explain the active synthetase and expression of Fs in A_{pae} individuals the exons 1-7 of the Fs synthetase gene, GBGT1 gene, were sequenced. The nucleotide in position 887 in the human GBGT1 gene is a guanine (G) and in the animal gene adenine (A) except for primates, having a guanine as in humans. The A_{pae} individuals revealed a heterozygote substitution at nucleotide 887 changing G to A and the amino acid 296 for Arg to Gln. Thus, something has forced the GBGT1 gene of the A_{pae} individuals to mutate and created an "animal-like" Fs synthetase gene and an enzymatic functional protein. Two hundred and fifty six individuals

were tested for the 887G>A SNP. All were negative, indicating a low frequency of the Fs positive genotype among humans, at least in this Scandinavian population. Saliva DNA obtained from family members of both families confirmed the inheritance of the pheno-/ and genotype as described by Stamps et al [113].

Additionally another mutation was identified, 363C>A, creating a stop codon. This mutation was not located on the same allele as mutation 887G>A and appeared in family members bearing the unmutated 887G human GBGT1 gene as well. The affect of this mutation has not yet been clarified.

The mRNA of GBGT1 is detectable in a lot of human tissues, such as heart, placenta, kidney, spleen, pancreas, small intestine and leucocytes [110], but expression in human erythroid cells if any was unknown. The GBGT1-mRNA was measured in the two A_{pae} blood samples, in 5 random donors and in CD34 positive bone marrow cells. The blood samples showed mRNA in about the same quantitative, but the level of transcript in the bone marrow cells was 4 times higher implying expression of mRNA in hematopoietic cells.

A three dimensional model of the assumed human Forssman synthetase (hFsS), showing an overall similarity with the human GTA, was created. This model showed that the position of amino acid Arg296 in the hFsS is located in the same position as amino acid His301, which is catalytically important for GTA. However, as the arginine is not suitable to form a hydrogen bond to the glycosyl donor, but with a glycine in this position it can easily allow for the GalNAc molecule. Consequently, a hydrogen bond is more likely to be formed which may explain why the enzyme becomes active.

The Fs glycolipid is known as a microbe receptor and of that reason the A_{pae} RBCs were tested by agglutination against P-fimbriated *Eschericia coli*, the K 12 *E. Coli* laboratory strain HB101, unmodified or expressing either prs or pap adhesin. Clear agglutination was seen with prs-adhesin, which is known to bind to GalNAcα3GalNAc [111].

The conclusion of *paper IV* is that the A_{pae} phenotype is not a weak subgroup of A nor is it a weak-phenotype. Instead it is the result of aberrant phenotyping, where certain A antigen reactive reagents also detect the Fs antigen – an antigen not previously known to present on human red cells. Why some humans, have a substitute mutation at nucleotide 887 of the GBGT1 gene which gives the opportunity to create a functional GBGT1 synthetase is not known. These A_{pae} phenotype individuals have the same 887G>A SNP as the animals, instead of the human/primate 887G. The susceptibility of the $GalNAcGbO_4$ antigen to bind microbes such as *E. Coli* prs-adhesin has been proven, which may be of clinical importance in the area of microbiology. This study was done on red blood cells only, and we do not yet know to what extent Fs structures are expressed on other organs and tissues in humans or if they are taken up by the red cells from plasma. A transplanted human kidney is reported to express Fs glycolipids [48]. However, Fs is an apparent low frequency antigen in humans and most humans have anti-Fs

antibody in their serum, which may cause problems at organ transplantation and blood transfusions.

We will propose the Fs antigen to the International Society of Blood Transfusion terminology committee to be designated as new blood group system – the 31st since Landsteiner discover ABO in 1901. The criteria for a blood group system are:

1.   independency of other blood group antigen

2.   be expressed on red blood cells

3.   be inheritable

4.   presence of naturally-occurring antibodies

All these criteria have been fulfilled and if accepted we have proposed the name FORS, after its discoverer Professor John Forssman.

7. CONCLUDING COMMENTS

Structural analysis of complex carbohydrate compounds is a delicate process where information from one technique is compiled with information from other techniques to finally elucidate a reliable identification of structure. To perform these studies and to identify blood group active glycolipids in very small amounts, and in extremely complex mixtures, and then to get definitive results requires tedious, meticulous preparative work, advanced analytical tools like MS and NMR, and access to a network of experience and knowledge.

In this thesis carbohydrate glycolipid antigen expression in weak A phenotypes/subgroups was studied by thin layer chromatography, mass spectrometry and NMR. Initially the age-old issue of the difference between the A_1 and the A_2 subgroup was investigated to establish a base-line. It was shown that the probable explanation, apart from a quantitative aspect, is that the A-type 4 structure seems to be missing in the A_2 phenotype. TLC investigations into a range of weak-subgroups revealed interesting anomalies, many of which have yet to be investigated. Focussed investigations were carried out on an individual with the A_3 phenotype and it revealed that one possible mechanism is probably an absence of branched structures. Further studies are still necessary to solve the A_3 phenotype. This study also found several new structures including extended p-Fs (para-Forssman) structures and an association of p-Fs with blood group B, which also remains to be resolved. Finally the question regarding the A_{pae} phenotype was addressed by unexpectedly discovering this phenotype is caused by expression of the Forssman (Fs) antigen and not A antigens. These studies lead to a proposal to establish the 31st blood group system, tentatively named FORS.

To conclude, we have shown that analysis of glycolipids extracted from few units of blood can be sufficient to determine the structural composition of key antigenic components, and yield information relevant to the basis of the phenotype. It is the combination of analytical tools that allows for robust interpretation of results that give insights to the biosynthetic and genetic basis for the phenotypes. The new knowledge on weak ABO subgroups/phenotypes, coming forth from this work highlights how very little we know about the mechanisms and purpose behind the weak subgroups. Thus, many more of questions need to be answered in the future.

Last but not least, the contribution of glycoproteins and polyglycosylceramide are still important to consider in the connection with the expression of weak ABO subgroups. Further study will be essential to get the complete picture and better understand the weak subgroups. All the same analysis of the glycolipids alone has revealed a variety of significant insights into blood group A subtypes/phenotypes. Extension of these studies promises to reveal further biochemical treasures.

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