Studies of glycosphingolipids in infection, immunity and differentiation

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Cover illustration: MS^2 and MS^3 spectra of a branched Le^y/H type 1 undecasaccharide (Fucα2Galβ4(Fucα3)GlcNAcβ6(Fucα2Galβ3GlcNAcβ3)Galβ3GlcNAcβ3Galβ4Glc).

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Abstract

Cell surface glycoconjugates play a role in many biological processes such as responses to microbial infections, cell-cell interactions, differentiation, and inflammatory responses. The present work is focused on structural characterization of glycosphingolipids with potential roles in adhesion of *Helicobacter pylori* and *Vibrio cholerae*, differentiation of human pluripotent stem cells, and as blood group determinants.

In the first study, the structural binding requirements of *Helicobacter pylori* BabA adhesin revealed a different carbohydrate binding potential than previously defined. Adhesion of *H. pylori* generalist, specialist and BabA deletion mutant strains were examined using mixtures of glycosphingolipids. An unexpected binding by specialist and generalist *H. pylori* to the hexaosylceramide region of porcine intestinal non-acid glycosphingolipids was found. After isolation and characterization by mass spectrometry and proton NMR, the binding-active glycosphingolipid was determined as Globo H hexaosylceramide (H type 4). Further binding studies demonstrated that the generalist strain, but not the specialist strain, also recognized Globo A heptaosylceramide (A type 4). Non-secretors have an increased risk of peptic ulcer disease although they express little or no H type 1 sequences, and thus no Le^b. However, these individuals have a functional FUT1 enzyme that may produce the Globo H sequence, suggesting that Globo H hexaosylceramide might have a role in *H. pylori* adhesion to the gastric epithelium of non-secretor individuals.

In the second study the carbohydrate binding potential of *Vibrio cholerae* was investigated. Binding-active glycosphingolipids, detected by the thin-layer chromatogram binding assay, were isolated and characterized by antibody binding, mass spectrometry and proton NMR. Thereby, three different binding modes were identified; the first was complex glycosphingolipids with GlcNAcβ3Galβ3/4GlcNAc sequence, the second glycosphingolipids with terminal Galα3Galα3Gal sequence, and the third lactosylceramide and related glycosphingolipids.

V. cholerae with non-functional chitin binding protein GbpA bound to glycosphingolipids in the same manner as the wild type bacteria, demonstrating that the GbpA is not involved in glycosphingolipid recognition.

In the third study the non-acid glycosphingolipids of human embryonic stem cells were structurally characterized. Chromatogram binding assays, mass spectrometry and proton NMR demonstrated the presence of type 2 core chain glycosphingolipids (*neo*lactotetraosylceramide, H type 2 pentaosylceramide, Le^x pentaosylceramide, and Le^y hexaosylceramide), and blood group A type 1 hexaosylceramide, along with the previously characterized glycosphingolipids with type 1 and type 4 core chains. Thus, the glycosphingolipid diversity of human embryonic stem cells is more complex than previously appreciated.

The PX2 antigen is assumed to belong to the GLOB blood group system and has until further notice been assigned to that blood group. However the enzymatic machinery involved in PX2 synthesis has not been determined. In the fourth study, glycosphingolipids isolated from blood group AP_1^k erythrocytes, App erythrocytes and *B3GALNT1* transfected MEG-01 cells were

characterized by antibody binding and mass spectrometry. The *B3GALNT1* transfected MEG-01 cells had an increased expression of PX2. No P antigen or PX2 were found in the AP_1^k erythrocytes, while the App erythrocytes expressed PX2, but no P1 and P antigens. The conclusion from these experiments is that the P synthase also is responsible for synthesis of the PX2 antigen.

Keywords: Glycosphingolipids, mass spectrometry, *Helicobacter pylori* BabA, *Vibrio cholerae*, human embryonic stem cells, glycosyltransferase, PX2.

Sammanfattning på svenska

Glykokonjugat på ytan av celler deltar i många biologiska processer, såsom cell-cellinteraktioner, differentiering, inflammation samt infektioner. Målet med denna avhandling har varit att karaktärisera strukturen hos glykosfingolipider som har potentiell betydelse för bindning av *Helicobacter pylori* och *Vibrio cholerae* till sina målvävnader, differentiering av humana pluripotenta stamceller, samt som blodgruppsantigen.

I den första delstudien studerades bindningsspecificiteten hos BabA adhesinet från ”magsårsbakterien” *Helicobacter pylori*. Initialt studerades bindning av specialist, generalist, och BabA knock-out *H. pylori* stammar till glykosfingolipidblandningar. Därvid upptäcktes en bindning av specialist och generalist *H. pylori* till en neutral hexaosylceramid från gristarm. Den bindningsaktiva glykosfingolipiden isolerades och karaktäriserades med masspektrometri och proton NMR som Globo H hexaosylceramid (H typ 4). Ytterligare bindningsstudier visade att generalist *H. pylori*, men inte specialist *H. pylori*, också binder till Globo A heptaosylceramide (A type 4). Förmågan att binda till blodgruppsantigen på typ 4-kedjor kan ha betydelse för adhesion till magsäckspeitelet hos ”non-secretor” individer, vilka har en förhöjd risk att utveckla magsårssjukdom.

I nästa studie gjordes en kartläggning av *Vibrio cholerae*-bindande glykosfingolipider. Flera interaktioner med komplexa neutrala glykosfingolipider påvisades vid screening av glykosfingolipider från olika källor. Efter isolering och karaktärisering av bindningsaktiva glykosfingolipider med masspektrometri och proton NMR fastställdes tre olika bindnings-mönster. Den första var bindning till komplexa lakto/*neo*lakto strukturer, det andra glykosfingolipider med en terminal Galα3Galα3Gal sekvens, och den tredje laktosylceramid och relaterade glykosfingolipider. Det kitin-bindande proteinet GbpA från *V. cholerae* är ett potentiellt adhesin. Dock visade bindningsstudier med mutant *V.cholerae* med inaktiverat GbpA att detta protein inte är involverat i bindning till glykosfingolipider.

I den tredje studien karaktäriserades de neutrala glykosfingolipiderna från humana embryonala stamceller. Flera glykosfingolipider med typ 2 kedja, såsom *neo*laktotetraosyl-ceramid, H typ 2 pentaosylceramid, Le^x pentaosylceramid och Le^y hexaosylceramid, identifierades. En A typ 1 hexaosylceramid påvisades också, liksom tidigare beskrivna glyko-sfingolipider med typ 1 och typ 4 kedjor. Vidare karaktäriserades glykosfingolipider med kort kolhydratkedja som galaktosylceramid, glukosylceramid, laktosylceramid, galabiosylceramid, globotriasylceramid och laktotriasylceramid. Detta visade att uttrycket av glykosfingolipider i humana embryonala stamceller är mer komplext än man tidigare ansett.

I den fjärde undersöktes den genetiska bakgrunden till blodgruppsantigenet PX2. För att testa detta studerades uttrycket av glykosfingolipider i humana erytrocyter med blodgrupperna App och AP_1^k, samt i celler transfekterade med genen för glykosyltransferaset P synthase (*B3GALNT1*). Glykosfingolipiderna karaktäriserades med antikroppsbindning och masspektrometri. Transfektion med *B3GALNT1* gav ett förhöjt uttryck av PX2. I AP_1^k erytrocyterna saknades P och PX2 antigen, medan App erytrocyterna uttryckte PX2, men inte

P1 and P antigen. Sammanlagt visar detta att det *B3GALNT1*-kodade P-syntaset även producerar PX2 antigenet.

List of papers

I. **Benktander J**, Ångström J, Breimer ME, Teneberg S. Re-definition of the carbohydrate binding specificity of *Helicobacter pylori* BabA adhesin. 2012. J Biol Chem 287, 31712-24.

II. **Benktander J**, Ångström J, Karlsson H, Teymournejad O, Lindén S, Lebens M, Teneberg S. The repertoire of glycosphingolipids recognized by *Vibrio cholerae*. 2013. PLoS One, 8, e53999.

III. Barone A, **Benktander J**, Ångström J, Aspegren A, Björquist P, Teneberg S, Breimer ME. Structural complexity of non-acid glycosphingolipids in human embryonic stem cells grown under feeder-free conditions. 2013. J Biol Chem 288, 10035-50.

IV. Westman JS*, **Benktander J***, Storry JR*, Peyrard T, Hult AK, Hellberg A, Teneberg S, Olsson ML. Genetic basis of PX2, a recently acknowledged glycolipid blood group antigen. Submitted.

* Contributed equally to this study.

Contents

1. Abbreviations

BabA	Blood group antigen-binding adhesion
Cer	Ceramide
ESI	Electrospray ionization
Fuc	Fucose
Gal	Galactose
GalNAc	*N*-acetylgalactosamine
Glc	Glucose
GlcNAc	*N*-acetylglucosamine
hESC	Human embryonic stem cells
Hex	Hexose
HexNAc	*N*-acetylhexosamine
LacCer	Lactosylceramide
LC/MS	Liquid chromatography mass spectrometry
MS	Mass spectrometry
MS/MS and MS^2	Tandem mass spectrometry
m/z	Mass-to-charge
NeuAc	*N*-acetylneuraminic acid/Sialic acid
NMR	Nuclear magnetic resonance
RT	Retention time

2. Background

2.1. Glycobiology

Glycobiology encompasses the studies of glycans in all its forms. A glycan, or the more common name carbohydrate, is a molecule composed of carbon, hydrogen and oxygen. The simplest glycan unit is a monosaccharide. When monosaccharides are joined together to form saccharide chains they are referred to as oligosaccharides, while those having >10 saccharide units are called polysaccharides.

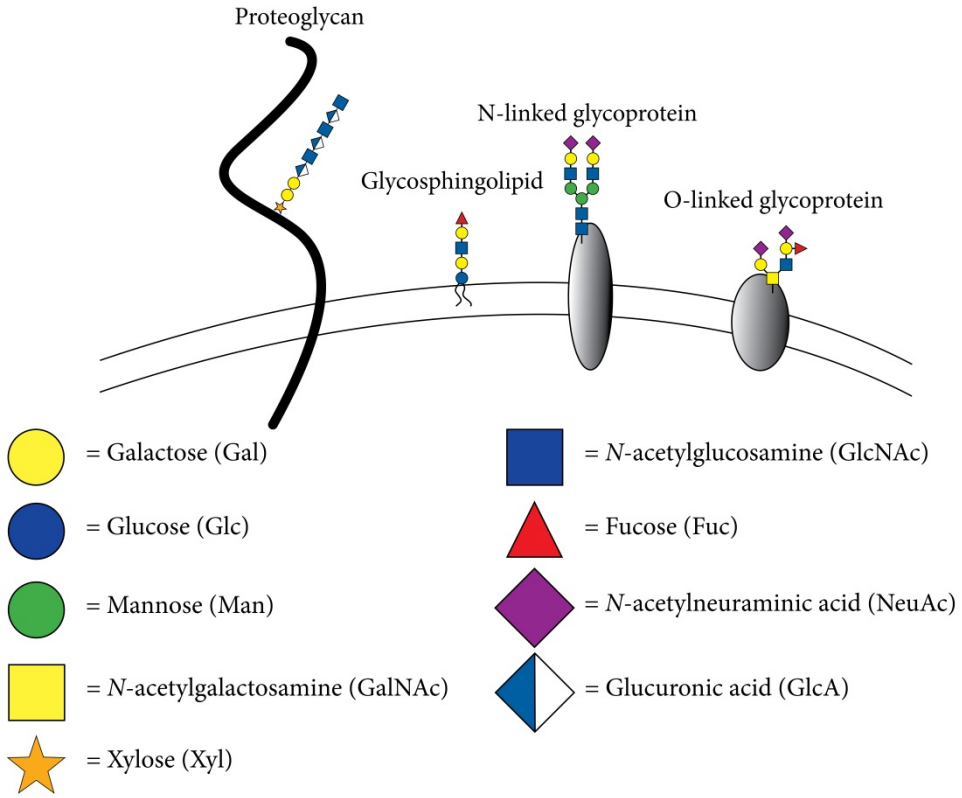

Figure 1: Mammalian cell membrane glycoconjugates (Modified from Fuster and Esko, 2005, [1]) with symbols and abbreviations of common monosaccharides [2].

Glycans can be found as free saccharides, as in mammalian milk, but often the glycan is linked to a non-carbohydrate moiety. Those structures are generally named glycoconjugates. There are many types of glycoconjugates, such as glycoproteins, glycolipids and proteoglycans (Figure 1).

The major types of glycoproteins comprise N- and O-glycans. The glycans are directly attached to the amino acids of the proteins. N-glycans are attached to asparagine side-chains. The N-glycans are derived from a dolichol-oligosaccharide precursor and are generally classified into three types; oligomannose, complex and hybrid type.

O-glycans are linked to the side-chains of serine or threonine. The majority of O-glycans consists of one of four core structures, all starting with a GalNAc attached to the protein. Mucins are heavily glycosylated O-glycan proteins.

Finally, the proteoglycans are heavily glycosylated proteins. Large glycosaminoglycan (GAG) chains are linked to serine via a tetrasaccharide linker. The proteoglycan proteins have a wide variety of numbers of glycosaminoglycans attached, some only have one while others have more than 100 GAGs. The glycosaminoglycans structure is composed of repeating disaccharide units of acetylated or sulfated hexosamines, linked to either a galactose or an uronic acid. [3].

There are also different glycolipid classes, though the focus in this thesis will be on glycosphingolipids.

2.1.1. Glycosphingolipids

Glycosphingolipids are amphipathic molecules consisting of a polar carbohydrate linked to a hydrophobic ceramide moiety (Figure 2). The ceramide part anchors the glycosphingolipid into the cell membrane with the carbohydrate chain sticking out from the membrane. The ceramide consists of a long-chain base and a fatty acid. The most common long-chain bases are sphingosine, sphinganine and phytosphingosine, all with 18 carbon chains [4, 5]. In some tissues long-chain bases with 20 carbon chains are present [6]. The fatty acid chains are more variable and consist of 14-30 carbons atoms, or even longer, although fatty acids with 16-24 carbons are most commonly found. The fatty acids can also be unsaturated and have α-hydroxy groups [3].

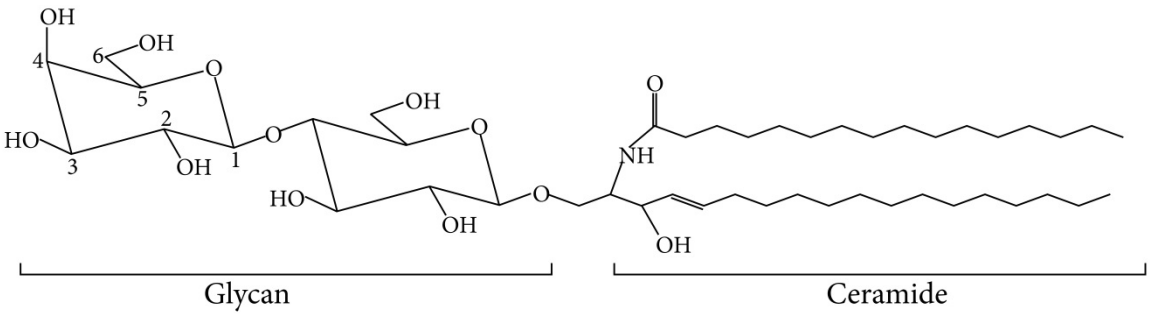

Figure 2: Structure of a lactosylceramide d18:1-16:0 glycosphingolipid. The carbon number assignment is shown on the monosaccharide to the left. Shorthand nomenclature for fatty acids and bases: d18:1 represents a sphingosine and t18:0 represents a phytosphingosine. The number before the colon refers to the carbon chain length and the number after gives the total number of double bonds. Fatty acids with a 2-hydroxy group are denoted by the prefix h.

Glycosphingolipids are heterogeneously distributed in the outer leaflet of the cell membrane due to stabilizing hydrogen bounds. They are therefore assembled into a formation called “lipid rafts” containing sphingolipids, cholesterol and specific membrane proteins [7]. There are also glycosphingolipids assembling without cholesterol in microdomains called “glycosynapses”, that are involved in carbohydrate dependent interactions [8].

Glycosphingolipids is generally characterized as either acid or non-acid. The main acid glycosphingolipids are of two types; the gangliosides, which have sialic acids and the sulfated glycosphingolipids, where the saccharide is modified with sulfate.

The non-acid glycosphingolipids includes all glycosphingolipids that have no charged moieties and the majority of the oligosaccharides are constructed from one of the major core-structures. Some of the vertebrate major glycosphingolipid core structures are shown in Table 1.

Table 1: Common core structures of vertebrate glycosphingolipids.

Core structure series	Abbreviation	Structure
Lacto	Lc	Galβ3GlcNAcβ3*Galβ4GlcβCer
Neolacto	nLc	Galβ4GlcNAcβ3Galβ4GlcβCer
Globo	Gb	GalNAcβ3Galα4Galβ4GlcβCer
Isoglobo	iGb	GalNAcβ3Galα3Galβ4GlcβCer
Ganglio	Gg	Galβ4GalNAcβ4Galβ4GlcβCer

* The carbohydrate sequence defining each core is underlined.

Another glycosphingolipid core is the athro sequence (GalNAcβ4GlcNAcβ3Manβ4GlcβCer), found in several classes of invertebrates [3]. Different types of glycosphingolipids are expressed differently depending on species [9], individual [10], tissue and cell type [11].

2.1.2. Glycosphingolipid biosynthesis

The synthesis of the glycosphingolipids starts at the cytoplasmic side of the Golgi apparatus [12, 13] where the GlcCer synthase adds a glucose in β-linkage to the hydroxyl group at position 1 of the ceramide. The ceramide is synthesized earlier in the endoplasmic reticulum and is then transported to the Golgi [14]. Further additions of glycans to the glucosylceramide occurs after switching to the luminal side of the Golgi [15] with the help of an uncharacterized flippase [16]. In some cases a galactose is added to the ceramides. This happens at the luminal side in the endoplasmic reticulum [17], though the downstream synthesis pathway is very limited for galactosylceramide, therefore this pathway will not be further described.

After synthesis of glucosylceramide, a galactose is added in the Golgi lumen by the LacCer synthase. The generated lactosylceramide has multiple possible synthesis pathways, one is where the Gb3 synthase adds a galactose in α4-linkage to lactosylceramide creating the globotriaosylceramide, which is the precursor of the globo-series glycosphingolipids. The iGb3 synthase is also a possible synthesis pathway, where the iGb3 synthase transfers a galactose in a α3-linkage, leading to the isoglobo-series. This is however not normally found in humans since the gene of the iGb3 synthase is inactivated [18]. The precursor of the ganglio-series, gangliotriasylceramide, is another possible synthesis, where GM2/GM3 synthase add a β4-linked *N*-acetylgalactosamine to lactosylceramide. The lactosylceramide can also be substituted with *N*-acetylglucosamine in a β3-linkage by the Lc3 synthase, generating the lactotriaosylceramide glycosphingolipid [19]. From the lactotriaosylceramide precursor the lacto-series and the neolacto-series is generated either by an addition of a Galβ3 (lacto) or a Galβ4 (*neo*lacto) (Figure 3A).

Aside from the synthases that divides the glycosphingolipids into the different core structures, a lot of glycosyltransferases modify the terminal ends of glycosphingolipid carbohydrate chains. A common addition is fucosylation by the blood group O and Lewis enzymes (Figure

3B). Addition of one or more sialic acids by the different sialyltransferases creates a ganglioside, while addition of a sulfate group creates a sulfated glycosphingolipid. The regulation of glycan structures expressed by a cell is not entirely understood, but is known to be influenced by kinetic parameters of the glycosyltransferases and organization in the Golgi. It is also possible that glycans are regulated by transcriptional regulation of the transferases. However, to express a specific glycan the right combination of glycosyltransferases must be expressed [20].

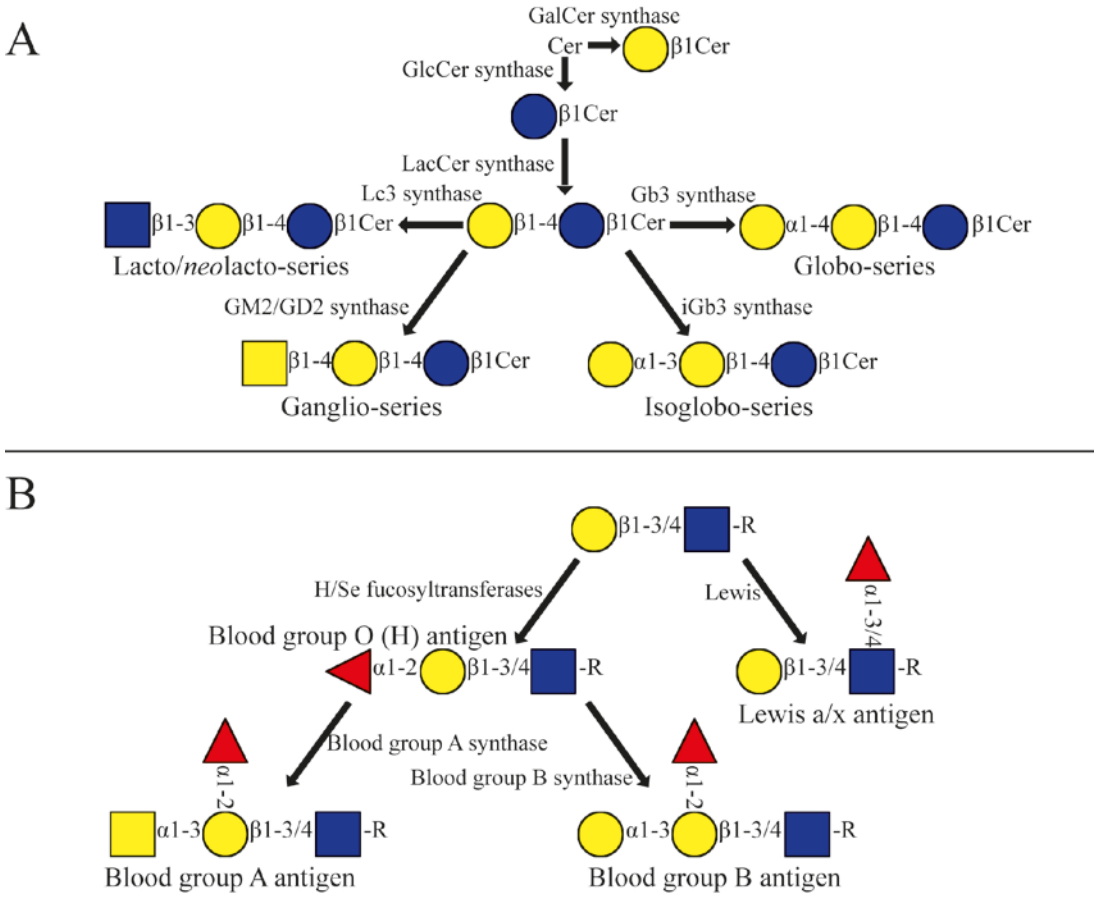

Figure 3: *A)* Simplified overview of synthesis pathways for the basic core structures of the glycosphingolipids. The glycosphingolipid synthesis is initiated by the addition of either a Gal or a Glc to the ceramide. The enzymes responsible for this are the GalCer synthase (UDP-galactose:ceramide galacosyltransferase) and GlcCer synthase (UDP-glucose:ceramide glycosyltransferase). The LacCer synthase (β-1,4-galactosyltransferase 5) continues by transferring a Gal to the glucosylceramide. From lactosylceramide, several synthesis pathways are possible which generates the precursors of the different core-structure series. The Lc3 synthase (β-1,3-N-acetylglucosaminyltransferases 5) transfers a GlcNac to the lactosylceramide, thus creating the precursor of the lacto/neolacto series. Another possibility is that the GM2/GM3 synthase (β-1,4-N-acetyl-galactosaminyltransferase) adds a GalNAc creating the gangliotriaosylceramide. Aside from these the Gb3 synthase (α-1,4-galactosyltransferase) and iGb3 synthase (α-1,3-galactosyltransferase) can transfer a Gal in a α4- or α3-linkage, respectively, initiating the globo-series and the isoglobo-series [reviewed in [19]. *B)* Basic overview of the synthesis of the ABO and Lewis blood group systems. The *FUT3* gene encodes the Lewis enzyme (fucosyltransferase 3) that is an α-1,3/4-fucosyltransferase that adds a fucose to a type 1 or type 2 chains. However, several fucosyltransferases, such as FUT4, FUT5, FUT6 and FUT7, can also act on the type 2 chain and produce the Lewis antigens [21]. There are two genes responsible for the creation of the H antigen, the secretor gene and the H gene. The secretor gene encodes the FUT2 enzyme that adds Fucα2 to both type 1 and type 2 chains, while the H gene that encodes the FUT1 enzyme, which preferably adds Fucα2 to type 2 chains. The A transferase (α-1,3-*N*-acetyl-galactosaminyltransferase) and B transferase (α-1,3-galactosyltransferase) adds GalNAcα3 and Galα3, respectively, to the galactose in the H antigen (both type 1 and 2) [22].

2.1.3. Functions of glycosphingolipids

The specific function of each glycosphingolipid is generally hard to define since they often have widespread functions, which overlap with other glycosphingolipids and components. One hypothesis is that the basic function of the glycosphingolipids is as a low-cost method of protecting the cell membrane with a carbohydrate layer (a.k.a. glycocalyx) [23]. According to this hypothesis the glycosphingolipids can be divided into two groups according to function; those who give the cell a protective carbohydrate coat with practically no other function, and those who also have a specified function.

Another hypothesis [24] attempts to explain the variety of glycosphingolipids as the result of an evolution in response to host-pathogen or host-symbiont interactions that drives the organisms to express new glycotransferases, capping the glycans with new saccharides. Other secondary functions are believed to have appeared later in evolution.

The gangliosides have more defined functions, mainly connected to the nervous system, cell-cell adhesion and recognition [25]. Several phenotypical defects are found in ganglioside-synthase knock-out mice [reviewed in [26], which generally affects brain development. Less defined functions has been found in the non-acid glycosphingolipids, but some are still hinted, such as involvement in cell-stage specific signaling and cell proliferation [23].

2.2. Microbial glycan adhesion

To attach to cellular surfaces or extracellular matrices bacteria often express adhesion proteins called adhesins. In many cases the adhesins bind the glycan part of glycoconjugates such as glycoproteins (*e.g.* mucins) or glycolipids, which enables infection and invasion by the bacteria. Binding to carbohydrates also has the advantage of mediating infection to specific hosts and tissue types [27, 28].

Adhesins can be of different types, like fimbriae, which are organelles extending from the bacterial surface. The adhesins of bacterial fimbriae are with either the tip protein or the major structural subunit [29]. There are also adhesins which are integral cell wall proteins of the bacteria [30]. Similar to adhesins, bacterial toxins also take advantage of carbohydrate recognition. Toxins such as the *Vibrio cholerae* cholera toxin (CT) [31], *Bacillus thuringiensis* crystal toxin (Bt toxin) [32], *Clostridium tetani* tetanus toxin [33], *Escherichia coli* heat-labile enterotoxin (LT) [34], *Clostridium difficile* toxin A [35] and *Shigella dysenteria* Shiga toxin [36] bind to carbohydrate structures.

2.2.1. Helicobacter pylori

In the 1980ies Warren and Marshall discovered that a previously unknown bacterium was connected with acute and chronic gastritis [37]. The bacterium was first given the name *Campylobacter pylori*, which later was changed to the present name *Helicobacter pylori*. *H. pylori* infection is nowadays also associated with peptic ulcers and gastric carcinogenesis [38]. Overall 47 % of all humans are positive for *H. pylori* infection. An incidence between 11% and 69% is found throughout different parts of the world, with low prevalence in developed countries and high in developing countries [39] .

H. pylori normally resides in the gastric mucus layer, though some bacteria adhere directly to the gastric epithelial cells [40]. Two *H. pylori* adhesins, the blood group antigen-binding adhesin BabA, and the sialic acid-binding adhesin SabA, were previously characterized [38], and recently a GalNAcβ1-4GlcNAc binding adhesin termed LabA (lacdiNAc-binding adhesin) has been found [41]. The BabA adhesin bind primarily to the Lewis b determinant (Fucα2Galβ3(Fucα4)GlcNAc-R), and also to the H type 1 determinant (Fucα2Galβ3GlcNAc-R) [42, 43]. Strains expressing not only BabA but also vacuolating cytotoxin VacA and cytotoxin-associated antigen CagA are generally associated with a more severe disease [44]. SabA binds sialylated glycan structures such as sialyl-Le^x (NeuAcα3Galβ4(Fucα3)GlcNAc-R). SabA mediated adhesion is suggested to be most prevalent during chronic inflammation [45].

The BabA adhesin of *H. pylori* adapts to the expression of fucosylated glycans among the local population. In populations from Europe and USA where blood group A, B and O are all widespread, strains called “generalists” express a BabA that is able to bind to determinants of these blood groups. In South American native population where only blood group O is prevalent, local *H. pylori* BabA does not have the ability to bind A and B type epitopes. The strains with this BabA with more narrow specificity are called “specialists” [46].

2.2.2. Vibrio cholerae

Vibrio cholerae was first described in 1854, and since then the strains and their characteristics have changed considerably. There have been seven or eight cholera pandemics since 1817, before that cholera was only found in the eastern parts of the world. It is believed that the classical biotype was the cause of the six first pandemics, whereas after 1961, when the seventh pandemic began, the El Tor strain took over [47]. In 1992 in Chennai in India, a new type of strain named *V. cholerae* O139 Bengal was isolated from a cholera epidemic [48]. That strain was first believed to be the start of an up-coming eight cholera pandemic, but since no further outbreaks have been found, many find it unlikely. More than 200 serogroups of *V. cholerae* have so far been discovered, with only two of them (O1 and O139) causing contagious cholera [49]. The O1 strains are further divided into two biotypes (classical and El Tor) and three serotypes (Ogawa, Inaba and Hikojima) [50].

V. cholerae infects the small intestine after ingestion of the bacteria. A cholera infection is characterized by severe watery diarrhea which can be potentially fatal, because of dehydration and loss of electrolytes. An estimate of 3 to 5 million people is affected yearly by cholera, leading to approximately 100.000-120.000 deaths (WHO, 2012). The diarrhea is mainly caused by the cholera toxin, an AB_5-toxin with one A-subunit and five B-subunits [51]. The B-subunits mediates attachment to the small intestine epithelium by the ligand ganglioside GM1 (Galβ3GalNAcβ4(NeuAcα3)Galβ4Glcβ1Cer)[52]. Cholera toxin binding to GM1 is a well-known fact, but the requirements for adhesion of *V. cholerae* bacterial cells to the human intestinal epithelium have until now not been investigated. A potential candidate adhesin is the chitin binding protein GbpA that has been found to bind chitin surfaces, human intestinal cells and mouse intestine [53, 54]. However, no carbohydrate structure required for GbpA binding to the human small intestine has been identified.

2.3. Human embryonic stem cells

Embryonic stem cells have the distinguishing ability of pluripotency, meaning that the cell has the ability to transform into all cell types in the adult body. Pluripotent stem cells are also able to proliferate indefinitely while retaining their diverse transformation ability. Human embryonic stem cells (hESC) are obtained by taking cells from the inner cell mass, called epiblast or embryoblast, from the blastocyst, that is formed five days after fertilization [55, 56].

A number of carbohydrate markers are used for the identification of undifferentiated stem cells, as the SSEA-3 (stage-specific embryonic antigen 3), SSEA-4 [57], TRA-1-60 [58] and TRA-1-81 [44]. The SSEA-3 and -4 markers are glycosphingolipids (globopentaosylceramide and sialyl-globopentaosylceramide, respectively). Both of these have the globo core with the Galα4Gal structure, which is basically only found in glycosphingolipids [59].

The glycosphingolipids of stem cells has mainly been studied using mouse embryonic cells, and there were only two previous studies of the glycosphingolipids in hESC [60, 61]. These studies identified the non-acid glycosphingolipids globotetraosylceramide, globopentaosyl-ceramide (SSEA-3), globo H hexaosylceramide and H type 1 pentaosylceramide, as well as the acid glycosphingolipids GM3, GM1, GD1a or GD1b, sialyl-globopentaosylceramide (SSEA-4) and disialyl-globopentaosylceramide.

2.4. The P1PK and GLOB blood group systems

Aside from the common blood groups ABO and Rh, there are a number of minor blood group systems such as the GLOB and P1PK blood group systems. In total, 35 blood group systems have been defined (2015) [62].

The P1PK blood group system, previously called the P system, includes the P1 (Galα4Galβ4GlcNAcβ3Galβ4Glcβ1Cer) and P^k antigen (Gb3; Galα4Galβ4Glcβ1Cer). Both the P^k and P1 antigen is synthesized by the *A4GALT*-encoded enzyme. However, due to single-nucleotide polymorphisms in the gene, P1 is not always expressed [63]. A later addition to the P1PK blood group is the NOR antigen (Galα4GalNAcβ3Galα4Galβ4Glc-β1Cer). It was added after the discovery of a mutation were glutamic acid is replacing glutamine at amino acid position 211 in the *A4GALT*-encoded enzyme, inducing the expression of NOR [64, 65]. The P antigen (Gb4; GalNAcβ3Galα4Galβ4Glcβ1Cer), the predominant non-acid glycosphingolipid in human erythrocytes [66], belongs to the GLOB blood group system. This is associated with the P1K1 blood group, since its precursor is the P^k antigen, but is synthesized by the *B3GALNT1*-encoded P synthase [67] (Figure 4).

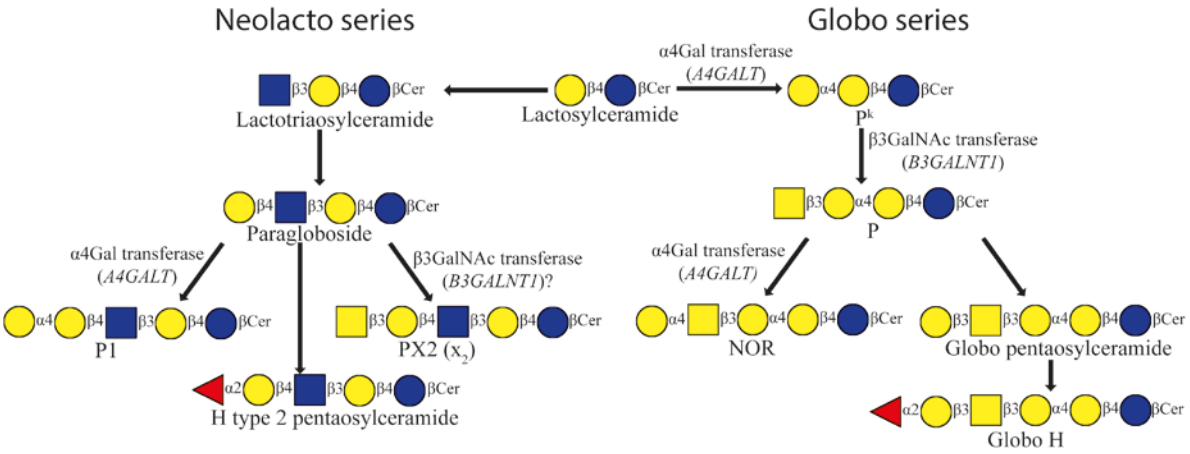

Figure 4: Basic overview of P1PK and GLOB synthesis pathway. The α4Gal transferase, encoded by *A4GALT*, transfers a Gal to the terminal Gal of lactosylceramide and paragloboside in a α4-linkage. *B3GALNT1* encodes a β3GalNAc transferase that adds a GalNAc to the terminal Gal of P^k in a β3-linkage.

Besides the P antigen, the x_2 pentaosylceramide (GalNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) has been included in the GLOB blood group system, as of 2011, under the name PX2 [68]. The genetic background of PX2 is not yet known, but it was still included in the GLOB blood group until further notice.

Table 2: General outline of P phenotypes, their expressed antigens and functional genes.

Phenotype	**Expressed antigens**	**Functional genes**
P_1	P, P^k, P1	*A4GALT, B3GALNT1*
P_2	P, P^k	*A4GALT*, B3GALNT1*
P_1^k	P^k, P1	*A4GALT*
P_2^k	P^k	*A4GALT**
p	None	*B3GALNT1*

* Indicates an *A4GALT* gene with single-nucleotide polymorphism resulting in inability to synthesize P1.

There are five different phenotypes of P1PK and GLOB blood groups; the common P_1 and P_2, the rare P_1^k and P_2^k phenotypes, and the p phenotype [69]. The antigens expressed by respective phenotype are shown in Table 2. The null phenotypes of P1PK or GLOB blood group systems have a slightly increased risk of intravascular hemolytic transfusion reactions and spontaneous abortions, caused by natural antibodies against the missing P antigens [70].

3. Aims

The overall aim of this thesis was to characterize glycosphingolipids with potential roles in microbial adhesion, differentiation of human pluripotent stem cells, and as blood group determinants.

Specific aims:

1. To characterize the glycosphingolipids involved in adhesion of *Vibrio cholera* and *Helicobacter pylori*.
2. To characterize the glycosphingolipids of human embryonic stem cells.
3. To characterize the glycosphingolipids of human blood group AP_1^k and App erythrocytes, in the course of an investigation of the role of P synthase in synthesis of the x_2 glycosphingolipid.

4. Methods

4.1. Extraction and purification of glycosphingolipids

The method used for extraction and isolation of glycosphingolipids was developed by K-A. Karlsson, and has been used for over 30 years [71]. It mainly uses polarity to separate the glycosphingolipids on silic acid columns, but also applies ion-exchange chromatography. With this method it is possible to purify glycosphingolipids with glycans ranging from 1 to 18 saccharide units.

The tissues or cells are prepared for extraction by lyophilization. After this the sample is extracted with mixtures of chloroform and methanol in a Soxhlet apparatus. The extracts are then pooled and dried. Thereafter, the lipid extract is subjected to a mild alkaline methanolysis, which will break the ester bonds of phospholipids and as a result release the glycerol linked fatty acids. This is followed by dialysis for 3-5 days to remove the salt. Fatty acids, ceramide and other non-polar lipids are separated from glycosphingolipids by silicic acid column chromatography, utilizing increasing methanol to chloroform ratios. Next DEAE (diethylaminoethyl) ion exchange chromatography is used to separate acid and non-acid glycosphingolipids. Non-acid glycosphingolipid are eluted with chloroform and methanol, while the acid glycosphingolipids are eluted with an excess of salt (LiCl). Then the non-acid fraction is acetylated, giving a decreased polarity since the hydroxyl groups on the saccharides are caped with non-polar acetate groups. The acetylated glycosphingolipids are separated on a silicic acid column to remove sphingomyelin, which only gets a small change in polarity compared to the glycosphingolipids. After this the acetylated glycosphingolipid fraction is deacetylated with a mild alkaline treatment, which restores them to the original state. After dialysis, the glycosphingolipids are subjected to another DEAE were alkali-stable phospholipids with amine groups (such as plasmalogens), which have turned acidic by acetylation, are separated along with remaining acid glycosphingolipids. Finally a silicic acid column is used to remove remaining fatty acids and other non-polar molecules (Figure 5).

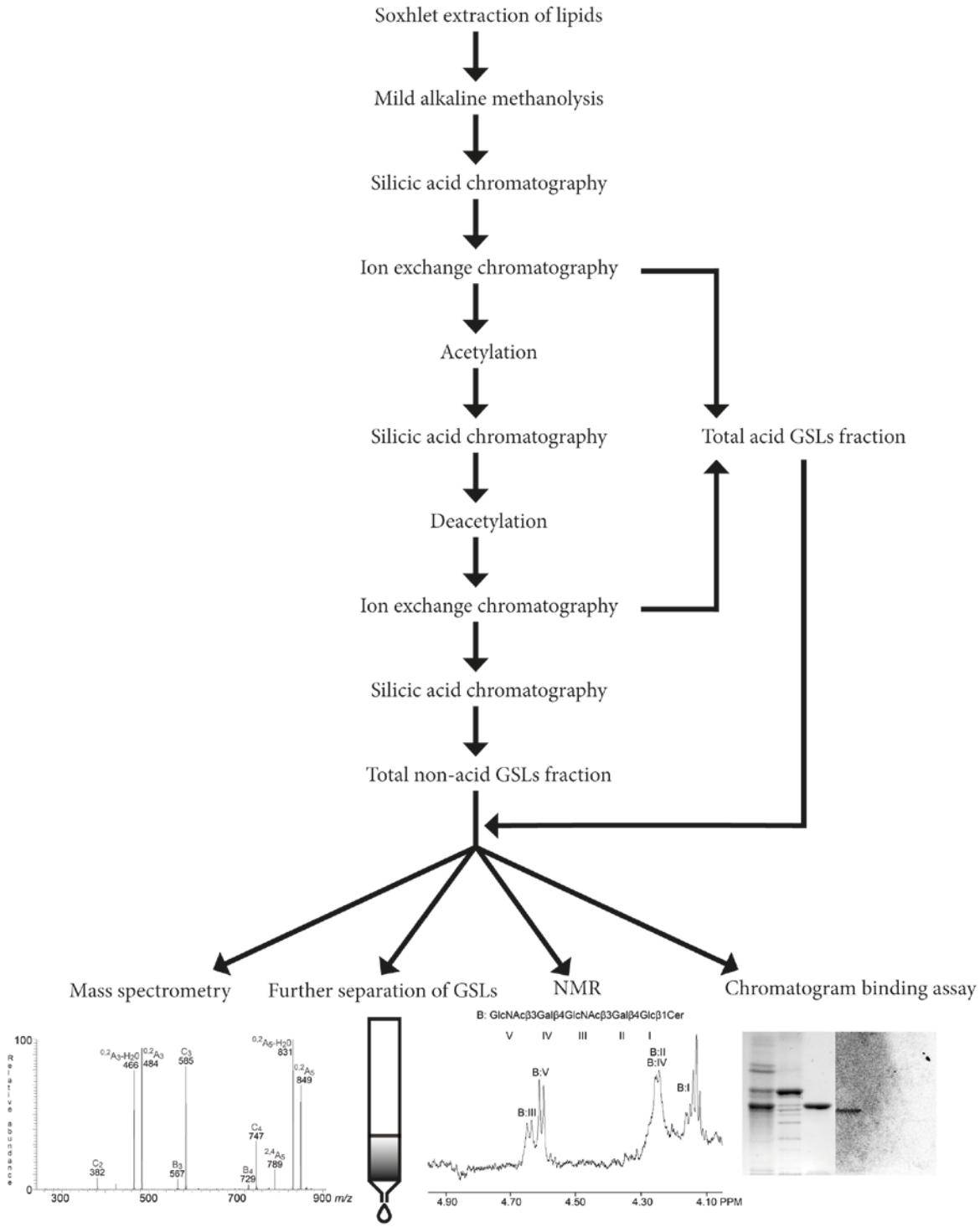

Figure 5: General scheme for glycosphingolipid (GSL) purification and characterization.

Iatrobeads (Iatrobeads 6RS-8060; Iatron Laboratories, Tokyo) column chromatography is used for further separation of the glycosphingolipid fractions, since Iatrobeads have a smaller particle size which allows a more fine-tuned separation. The fractions are eluted by increasing levels of methanol to chloroform or by mobility in chloroform/methanol/water (60:35:8, by volume). The fractions collected are pooled according to their mobility on high performance thin-layer chromatography (HPTLC) plates after visualization by anisaldehyde staining. The

fractions are also pooled according to the results from antibody, lectin and bacterial binding assays.

4.2. Structural characterization of glycosphingolipids

Structural characterization of glycosphingolipids involves definition of the types of carbohydrates, the carbohydrate sequence, the linkage positions, linkage anomerity and the ceramide composition. These data are mainly obtained by mass spectrometry, proton NMR spectroscopy, degradation studies and chromatogram binding assays. The data obtained is best interpreted in combination, due to the different information obtained from each method. Mass spectrometry is for example unable to predict the types of saccharides and anomerity of linkages, but is very good at determining carbohydrate sequences and some linkage positions. Chromatogram binding assays with specific antibodies and lectins may confirm the presence of certain carbohydrate epitopes in the samples, thus filling in the blind spots of mass spectrometry. NMR is a good method for determining the types of monosaccharides and linkage anomerity, but has more difficulty to find the correct carbohydrate sequence, therefore it works very well in combination with mass spectrometry. However, NMR is not always possible since it requires a larger quantity of pure sample.

4.2.1. Thin-layer chromatography, detection and binding assays

Thin-layer chromatography is a method to separate the glycosphingolipids depending on their polarity, and at the same time visualizing them. It can either be done on aluminium, plastic or glass-backed plates covered by silica gel [72]. Samples are eluted with mixtures of chloroform, methanol and water as mobile phase. Chemical detection of glycosphingolipids may be done with different reagents as orcinol [73], anisaldehyde [74] or resorcinol [75].

Chromatogram binding assay (CBA) is used for detecting specific epitopes/antigens of samples on thin-layer chromatograms and can therefore be used for characterization. This method may also be used for establishing the binding specificity of binding agents. In short the procedure starts by coating the dried thin-layer chromatograms in plastic. After that, it is possible to bind radiolabeled bacteria, viruses, lectins, antibodies and various proteins to the glycosphingolipids on the chromatogram [76-78]. The binding is usually visualized by a x-ray film reacting to radioactivity [79, 80], or enzyme-conjugated antibodies with visualizing substrate [81].

4.2.2. Mass spectrometry

In the late 1880ies physicists described mass spectrometry, but it was not until 1913 that Sir Joseph John Thomson, who invented the first mass spectrometry instrument, could demonstrate the use in analytical chemistry. Organic substances could be detected in the late 1940ies, and in 1952 a method for combining gas chromatography (GC) and MS was invented, which made MS useful in biochemistry. Although GC-MS is excellent when analyzing many compounds, the downside is that the molecules analyzed are required to be thermally stable, which limits the number of compounds that can be analyzed.

The soft ionization methods, electrospray/ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) appeared in the yearly 1990ies and enabled studies on larger biomolecules such as proteins [82, 83]. These methods also supported coupling to liquid chromatography (LC) and capillary electrophoresis (CE) [84].

Mass spectrometry is basically an analytical technique creating positively or negatively charged gas-phase ions which are separated according to their mass-to-charge ratio (*m/z*). Electrospray ionization (ESI) is the ionization method focused on in this thesis. Electrospray is enabled by adding a voltage to the needle that holds the sample liquid. This will cause the liquid to adopt an elliptical shape. At a certain voltage the elliptical shape changes to a pointed cone, called Taylor cone [85]. This cone will generate a spray of small charged droplets. The small droplets will shrink in size due to evaporation of neutrally charged solvent molecules and charged ions will be formed when the number of charges is accumulating in the droplets. There are currently two different theories explaining the ESI ionization mechanism more in detail: the ion evaporation model and the charge residue model [86].

A linear ion trap quadrupole (LTQ) mass analyzer was used in this thesis. LTQ utilizes an electric potential to guide the ions from the electrospray into an ion transfer capillary. The ion transfer capillary leads the ions into a series of two quadrupoles and one octapole before reaching the linear ion trap. During this time, ions are transferred down a pressure gradient while increasing their kinetic energy. The ions are then trapped inside the ion trap with an electric potential which is able to eject ions of specific *m/z* by voltages of matching radiofrequencies. The ions ejected are detected by a detector defining the intensity of each *m/z*. Inside the ion trap, collision induced dissociation (CID) is managed by increasing the ion motion leading to dissociation into fragments from the parent ions, after gaining enough energy from colliding with helium gas. The fragment ions are ejected in the same manner as described before [87].

The precursor ions collided in the collision cell of the mass analyzer gives, after detection, a spectrum of the fragments called MS^2 or MS/MS spectrum. A MS^2 fragment can be further investigated by repeated collisions in the collision cell, creating new fragments shown in a MS^3 spectrum. For glycans, MS^2 mainly yields the fragment ions C, B, Z, Y and the ring-cleavage fragments A (Figure 6). For underivatized oligosaccharides, the A fragments are found in many types and indicate different linkages, as *e.g.* ^{0,2}A-fragments which indicate 1-4 linkages and ^{0,3}A-fragments which indicate 1-6 linkages [88].

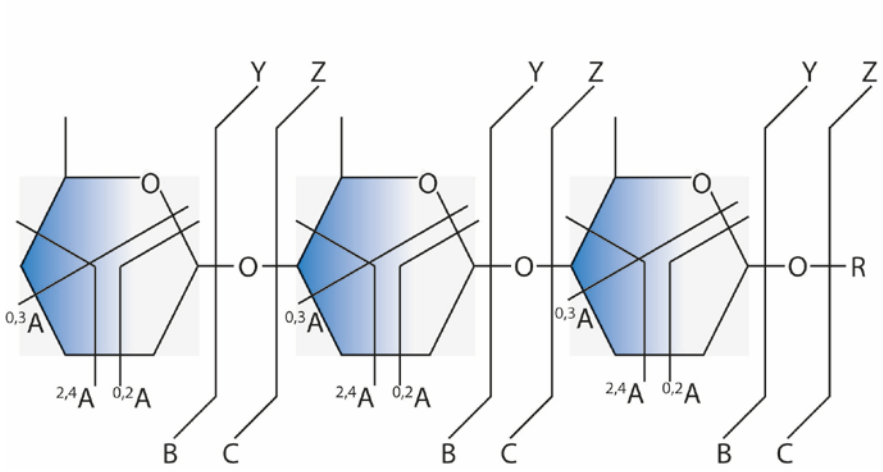

Figure 6: MS/MS fragments created in glycan analysis. R denotes the reducing end.

The ions obtained from ESI/MS in negative mode, yields the deprotonated ions $[M\text{-}H^+]^-$, were M is molecular mass. For positive mode, protonated ions $[M+H^+]^+$, lithium adducts $[M+Li^+]^+$ and sodium adducts $[M+Na^+]^+$ can be generated. Ions of high molecular weight often have more than one charge which decreases the *m/z* as a result, *e.g.* an ion which is doubly charged has half of the *m/z* value of a singly charged ion of the same type [89].

4.2.3. Mass spectrometry of glycosphingolipids

The first mass spectra of a glycosphingolipid were obtained in 1970 by using electron ionization (EI) on a trimethylsilylated glucosylceramide [5]. Permethylation, *i.e.* chemical permethylation of the hydroxyl groups, enabled glycosphingolipids to be analysed on MS by increasing the stability and volatility of the molecules [90]. Also combined permethylation and reduction of glycosphingolipids helped in improving MS analyses [91]. In 1983 underivatized glycosphingolipids could be analyzed with the relatively soft ionization method fast atom bombardment (FAB) in negative ion mode [92]. This was further improved in the early 1990ies when ESI-MS started to be applied to glycosphingolipid MS analysis [93]. Derivatized glycosphingolipids *e.g.* permethylated intact glycosphingolipids are still used, but not to the same degree since many of the instruments previously used are not manufactured today. A promising technique is thin-layer chromatogram coupled mass spectrometry (TLC-MS), that uses thin-layer chromatograms to separate the glycosphingolipids, with a possibility to detect with both chemical staining and overlay assay, in combination with mass spectrometry to structurally characterize the glycosphingolipids. It can be applied as an indirect method were the thin-layer plate is scraped combined with preparation, or as a direct method were the plate is directly scanned after addition of a liquid matrix, as TLC-IR-MALDI-MS [94, 95].

An option when analyzing glycosphingolipids is to remove the ceramide part. Enzymes such as ceramide glycanases and endoglycoceramidases enable cleavage of the glycosphingolipids into ceramides and glycans. By analyzing the oligosaccharides thereby obtained, liquid chromatography separation of the different saccharides is more efficient, and more information, such as linkage positions, can be obtained [88, 96]. However, the obvious

disadvantage of removing the ceramide is that no information about the ceramide profile is obtained. Nevertheless, depending on the experiment, determination of the ceramide type is not always necessary [97]. Endoglycoceramidase II from *Rhodococcus* spp. was used for hydrolyzing glycosphingolipids in this thesis. A weakness with this enzyme that must be noted is that endoglycoceramidase II has restricted hydrolytic capacity on glycosphingolipids in the globoseries and some specific gangliosides [98]. Ideally the ceramide glycanase from *Macrobdella decora* would be used, which has a broader activity [99]. However this enzyme is no longer commercially available.

The glycosphingolipid-derived oligosaccharides are analyzed by capillary-LC/MS, with separation on a column packed in-house with porous graphite particles [96]. The oligosaccharides are eluted with a gradient of water to acetonitrile. The eluting saccharides are then analyzed in negative ion mode on a LTQ linear quadrupole ion trap mass spectrometer.

For analysis of native glycosphingolipids, they are first dissolved in 3:1 methanol:acetonitrile (by volume) and then separated on a column packed in-house with polyamine II particles by elution with an acetonitrile to water gradient. The eluting saccharides were analyzed in negative ion mode on a LTQ linear quadrupole ion trap mass spectrometer.

4.2.4. Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a technique able to determine the atomic structures of large molecules in solution. NMR uses the fact that some atoms nuclei are naturally magnetic. This property is called spin. The atom utilized in the case of glycosphingolipids is mainly the hydrogen nucleus, ^{1}H. The spin of the ^{1}H nucleus can have the states, α and β, which have different energies, α having the lowest. The difference in the energy between the states increases with applied magnetic field.

By applying a radiofrequency of electromagnetic radiation, the nuclei will resonate between α and β state, yielding resonance spectra. Atoms in the surrounding area of the ^{1}H-atoms will give local magnetic fields contrasting the applied field that can be seen in the spectra. The different frequencies gained is called chemical shifts and given in the unit; parts per million (ppm) [100].

NMR of glycosphingolipids is nowadays a non-destructive method that uses DMSO and D_2O as solvents [101]. The temperature is important due to variations in chemical shifts, so references should ideally be run at the same temperature [102].

1D NMR is used to find the saccharide composition and anomeric linkages, but for determination of the carbohydrate sequences, the data from both 1D NMR and MS are combined. On the other hand, 2D NMR spectroscopy methods, as correlated spectroscopy (COSY) [103], are able to determine the connectivity between saccharaides, with the disadvantage of requiring more time.

4.3. Flow cytometry

Flow cytometry is a method for cell counting in heterogeneous cell suspensions. The cells are recorded by light scattering and fluorescent features while moving through a laser/light beam in a liquid channel. This enables the cells to be counted, sorted, detected by size and classified by fluorescent fluorochrome-linked molecules such as antibodies. The sorting enables collection of subpopulations of cells in the samples. Multiple fluorochromes can be used at the same time, which facilitates sorting and analysis against several parameters, which are analyzed by software, displaying it in the form of single to three parameter histograms [104]. In clinical work flow cytometry is used for diagnostics and monitoring of *e.g.* cancer, immune deficiencies and cell-based therapies [105].

5. Results and discussion

For structural characterization of glycosphingolipids a combination of different techniques is used to get reliable information. The tools include mass spectrometry, proton NMR, thin-layer chromatography, chromatogram binding assays, as well as prior knowledge of the pathways of glycan synthesis. The identification of glycosphingolipids is a central theme in this thesis, used for description of bacterial binding receptors in Paper I and II, and used for mapping glycosphingolipid expression in Paper III and IV.

The carbohydrates expressed on cell surfaces influences the cells susceptibility to pathogens, differentiation and immune response. Thus, the characterization of glycans is important to understand these areas.

5.1. Glycosphingolipids in infection

5.1.1. Paper I: Re-definition of the carbohydrate binding specificity of Helicobacter pylori BabA adhesin.

H. pylori specialist and generalist strains BabA adhesins have different tolerance for modifications of the Le^b and H type 1 epitope Fucα2Gal. Specialist strains do not tolerate any substitutions, while the generalist BabA can tolerate substitutions in 3-position with αGal as in blood group B antigens, and αGalNAc as in blood group A antigens [46]. The structural binding requirements of BabA were here further investigated.

When binding of *H. pylori* generalist, specialist and BabA deletion mutant strains were examined using mixtures of glycosphingolipids, an unexpected binding of specialist and generalist *H. pylori* to the hexaosylceramide region of porcine intestinal non-acid glycosphingolipids was found. The initial hypothesis was that the binding-active compound was a Le^b hexaosylceramide, although no Le^b epitopes have so far been reported in pigs. However, after isolation and characterization by mass spectrometry and NMR the binding-active glycosphingolipid was determined as Globo H hexaosylceramide (H type 4). Further binding studies demonstrated that the generalist strain, but not the specialist strain, also recognized Globo A heptaosylceramide (A type 4).

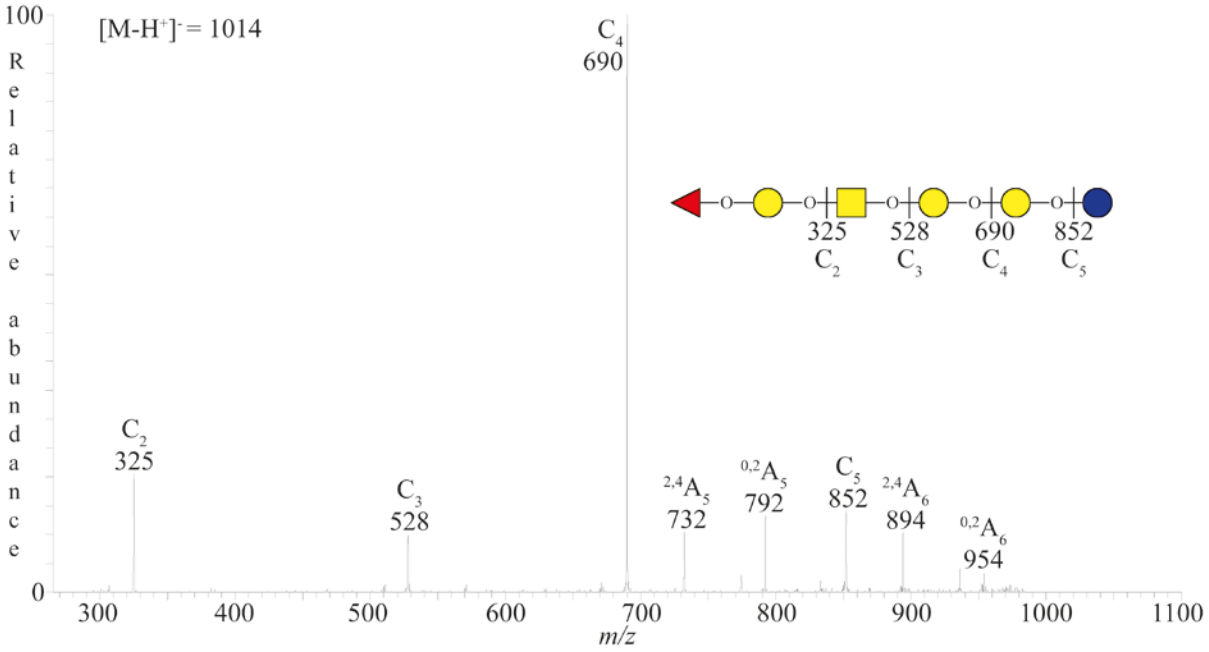

Figure 7:. MS^2 spectra of $[M-H^+]^-$ ion at *m/z* 1014 from blood group O porcine small intestinal epithelium. C-type ions predict the structure as Fuc-Hex-HexNAc-Hex-Hex-Hex, while the $^{0,2}A$ ions at *m/z* 792 and *m/z* 954 defines 1-4 linkages between saccharide 4→5 and 5→6. This suggests the Globo H pentasaccharide (Fucα2Galβ3GalNAcβ3Galα4Galβ4Glc).

The novel binding activities of BabA were most likely due to conformational similarities of the terminal disaccharides of H type 1 pentaglycosylceramide and H type 4 hexaglycosylceramide, and the terminal trisaccharides of A type 1 hexaosylceramide and Globo A heptaosylceramide, respectively, as demonstrated by molecular modeling (Figure 8).

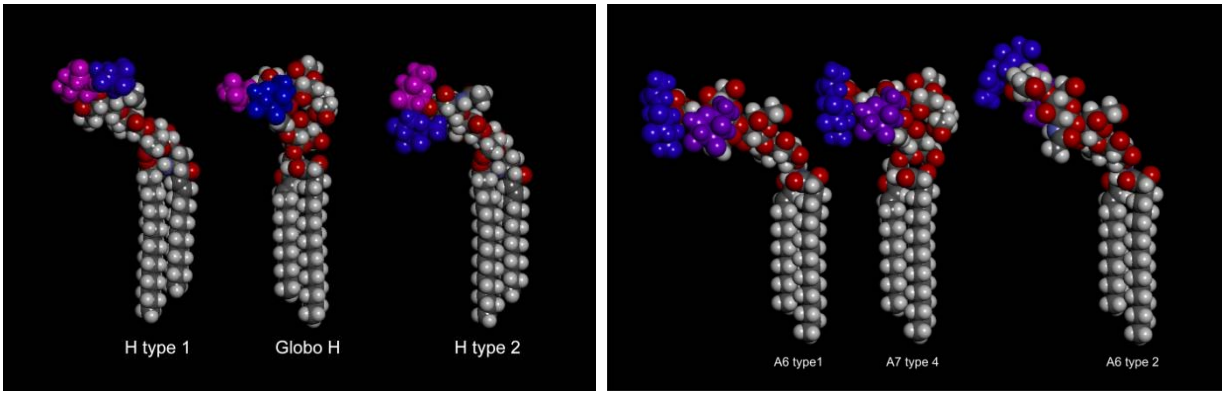

Figure 8. Minimal energy conformations predicted using CHARMm force field within the Discovery Studio molecular modeling package (Accelrys, Inc., San Diego CA), with initial points of torsion angles from literature [106, 107]. Glycosphingolipids with blood group H antigens (Left). Glycosphingolipids with blood group A antigens (Right).

In addition, a number of low migrating fractions of porcine intestinal epithelium were bound by *H. pylori*. These compounds were identified as branched deca- and undecaglycosylceramides with at least one H type 1 branch. These branched glycosphingolipids explains the *H. pylori* binding in the slow-migrating regions of the chromatogram binding assays. Notable is a novel undecasylceramide with one Le^y on the 6-branch and a H type 1 on the 3-branch (Fucα2Galβ4(Fucα3)GlcNAcβ6(Fucα2Galβ3GlcNAcβ3)Galβ3GlcNAcβ3Galβ4Glcβ1Cer).
The results from chromatogram binding assays are summarized in Table 3.

Table 3: Summary of the glycosphingolipid binding of *H. pylori* generalist and specialist strains. The BabA knock-out strain was tested to exclude non-BabA binding.

Abbreviation	***H. pylori* J99 (generalist)**	***H. pylor*i S831 (specialist)**	***H. pylori* J99/BabA-**
A tetraosylceramide	-	-	-
Le^a pentaosylceramide	-	-	-
Le^x pentaosylceramide	-	-	-
H type 1 pentaosylceramide	+	+	-
H type 2 pentaosylceramide	-	-	-
H type 4 (Globo H) hexaosylceramide	+	+	-
Le^b hexaosylceramide	+	+	-
Le^y hexaosylceramide	-	-	-
B type 1 hexaosylceramide	+	-	-
B type 2 hexaosylceramide	-	-	-
A type 1 hexaosylceramide	+	-	-
A type 2 hexaosylceramide	-	-	-
B type 1 heptaosylceramide	+	-	-
A type 1 heptaosylceramide	+	-	-
A type 2 heptaosylceramide	-	-	-
A type 4 (Globo A) heptaosylceramide	+	-	-
A type 1 octaosylceramide	+	-	-
A type 1 nonaosylceramide	+	-	-
A type 2 nonaosylceramide	-	-	-
A type 3 nonaosylceramide	-	-	-
Le^a octaosylceramide	-	-	-
Le^x octaosylceramide	-	-	-
Branched H type 1 decaosylceramide	+	+	-
Branched H type 1/H type 2 decaosylceramide	+	+	-
Branched Le^y/H type 1 undecaosylceramide	+	+	-
NeuGc-*neo*lactohexaosylceramide	+	+	+

It should be noted that Le^b was the best ligand for both the generalist and specialist *H. pylori* strains. However, non-secretor individuals have low amounts or no Le^b antigens on their epithelial surfaces, since the precursor of the Le^b sequence, *i.e.* the H type 1 sequence, is not formed due to lack of a functional FUT2 enzyme. Still non-secretor individuals have an increased risk of peptic ulcer disease [108]. The finding that BabA also binds to GloboH offers resolution of the seeming contradiction since the Globo H sequence can still be formed by FUT1 [109, 110], and might thus function as an adhesion factor for BabA-expressing *H. pylori*.

Infection with *Helicobacter pylori* is correlated with an increased risk for gastric cancer and lymphoma, and is regarded as a paradigm for carcinogenesis mediated by an infection-induced chronic inflammation. Although the prevalence of *H. pylori* is decreasing in developed regions, the prevalence is still high in developing countries [39]. Furthermore, we are today facing a situation with increasing resistance against antibiotics. Novel therapeutic strategies are thus urgently needed. The BabA adhesin is an important target for future actions against *H. pylori*, both as vaccine candidate and as target for anti-adhesion strategies.

5.1.2. Paper II: The repertoire of glycosphingolipids recognized by Vibrio cholerae.

V. cholerae O1 pathogenic strains of classical and El Tor biotypes were examined for binding to various glycosphingolipid mixtures, to identify potential glycan binding epitopes. Detected binding active glycosphingolipids were characterized by mass spectrometry and NMR.

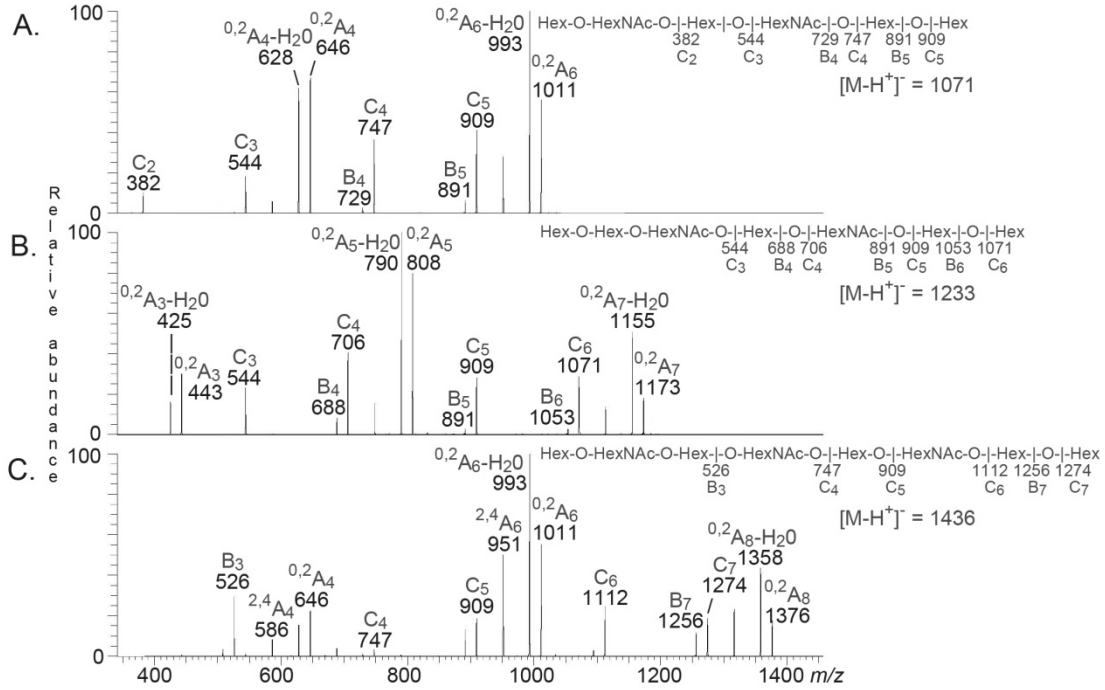

Figure 9: Mass spectrometry of oligosaccharides derived from *V. cholerae* El tor binding glycosphingolipids of rabbit thymus. *A)* MS^2 of $[M-H^+]^-$ ion at *m/z* 1071 with interpretation formula. *B)* MS^2 of $[M-H^+]^-$ ion at *m/z* 1233 with interpretation formula. *C)* MS^2 of $[M-H^+]^-$ ion at *m/z* 1436 with interpretation formula.

A number of *V. cholerae* binding glycosphingolipids were isolated and characterized (Figure 9), and binding of the bacteria to reference glycosphingolipids was also examined (Table 4). From these experiments we were able to discern three types of glycosphingolipid binding classes.

Glycosphingolipids with a GlcNAcβ3Galβ3/4GlcNAc sequence were binding in the binding pattern called lacto/*neo*lacto-binding (Figure 10). However certain substitutions obstruct the binding, such as terminal NeuAcα3 on *neo*lactohexaosylceramide, or Fucα3 on any of the GlcNAcs. Some differences were seen between the lacto and *neo*lacto sequences, the most notable were that a Fucα4 substitution is tolerated on the innermost GlcNAc for lacto core whereas not for *neo*lacto core. The conformation and/or torsion angle may be the cause, or it could be that two different laco/*neo*lacto binding adhesins is causing these binding patterns. This is somewhat supported by the fact that the classical strain is unable to bind lacto terminated glycosphingolipids, while binding complex *neo*lacto glycosphingolipids. Consequently, more studies to identify the adhesin(s) involved are needed.

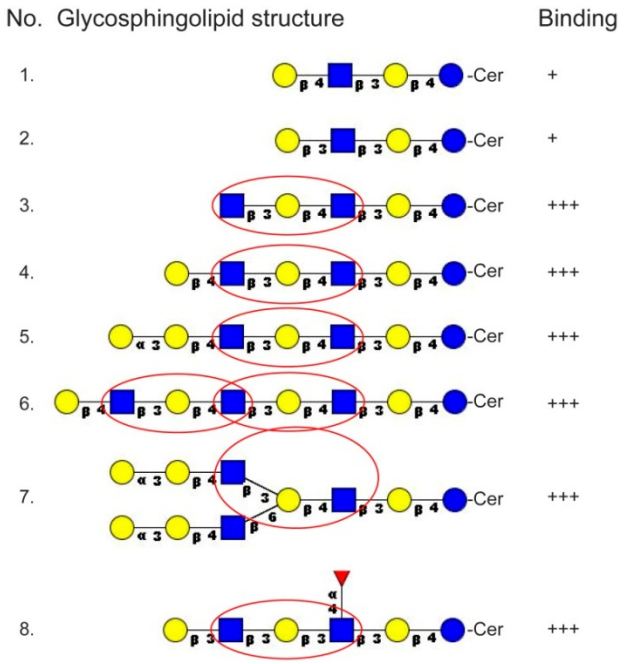

Figure 10: Summary of *V.cholerae* El Tor binding to lacto and *neo*lacto glycosphingolipid. The saccharide sequences in the red circles are required for high affinity binding. +++ denotes strong binding on thin-layer chromatograms while + denotes an occasional binding.

The second binding specificity is the Galα3Galα3Gal-binding. The glycosphingolipids Galα3-Galα3Galβ4GlcNAcβ4Galβ4Glcβ1Cer and Galα3Galα3Galβ4Glcβ1Cer were recognized by *V. cholerae*, while Galα3Galβ4GlcNAcβ4Galβ4Glcβ1Cer was not. Thus, the Galα3GalαGal sequence is the minimum binding epitope. The enzymes α1,3-galactosyltransferase or the iGb3 synthase could be producing these structures [111, 112], but these enzymes are non-functional in humans. That suggests that this binding specificity is not involved in adhesion of *V. cholerae* to the human small intestinal epithelium.

The third binding type is the lactosylceramide-binding. This type of binding represents binding to lactosylceramide, galactosylceramide, isoglobotriaosylceramide, gangliotriaosyl-ceramide and gangliotetraosylceramide. Binding to this battery of glycosphingolipids has previously been reported for both pathogens and indigenous flora [113]. The lactosyl-ceramide-binding does most likely not have a major role in human infection, because none of these glycosphingolipids have been found in the human intestinal epithelium [114].

Table 4: *V. cholerae* El Tor binding glycosphingolipids. Bold character type designates the structural binding motifs.

Trivial name	Structure
I. Neolacto binding	
Neolactotetra	Galβ4GlcNAcβ3Galβ4Glcβ1Cer
	GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer
Neolactohexa	Galβ4**GlcNAcβ3Galβ4GlcNAc**β3Galβ4Glcβ1Cer
B7	Galα3Galβ4**GlcNAcβ3Galβ4GlcNAc**β3Galβ4Glcβ1Cer
Neolactoocta	Galβ4**GlcNAcβ3Galβ4GlcNAc**β3Galβ4GlcNAcβ3Galβ4Glcβ1Cer
	Galα3Galβ4GlcNAcβ6(Galα3Galβ4**GlcNAcβ3**)**Galβ4GlcNAc**β3Galβ4Glcβ1Cer
II. Lacto binding	
Lactotetra	Galβ3GlcNAcβ3Galβ4Glcβ1Cer
Lacto-Lea	Galβ3**GlcNAcβ3Galβ3**(Fucα4)**GlcNAc**β3Galβ4Glcβ1Cer
III. Lactosylceramide binding	
GalCer	Galβ1Cer
LacCer t18:0-h16:0-h24:0	Galβ4Glcβ1Cer
Isoglobotri	Galα3Galβ4Glcβ1Cer
Isoglobotetra	GalNAcβ3Galα3Galβ4Glcβ1Cer
Gangliotri	GalNAcβ4Galβ4Glcβ1Cer
Gangliotetra	Galβ3GalNAcβ4Galβ4Glcβ1Cer
IV. Galα3Galα3Gal binding	
	Galα3Galα3Galβ4Glcβ1Cer
Galα3-B5	**Galα3Galα3Gal**β4GlcNAcβ3Galβ4Glcβ1Cer

V. cholerae with non-functional chitin binding protein GbpA bound to glycosphingolipids in the same manner as the wild type bacteria, demonstrating that the GbpA is not involved in glycosphingolipid recognition. A recent study came to a similar conclusion when examining the binding of GbpA on glycan arrays [115].

5.2. Glycosphingolipids in differentiation

5.2.1. Paper III: Structural complexity of non-acid glycosphingolipids in human embryonic stem cells grown under feeder-free conditions.

Glycosphingolipids from two human embryonic stem cell (hESC) lines called SA121 and SA181, were characterized after culture in feeder free conditions. These culture conditions eliminated the risk of glycosphingolipid contamination from the mouse embryonic fibroblast feeder cells. The resolution of our analysis was increased by using a large amount of starting material (~1×10^9 cells), allowing separation of non-acid from acid glycosphingolipids, and fractioning of non-acid fractions. The large amount of starting material also enabled chromatogram binding studies.

The glycosphingolipids in the non-acid fractions were first analyzed by a thin-layer chromatogram with chemical staining which showed glycosphingolipids migrating from the monosaccharide to hexasaccharide regions. The initial chromatogram binding assays indicated the presence *neo*lactotetraosylceramide, H type 1 pentaosylceramide, globopenta-osylceramide and Globo H pentaosylceramide.

The non-acid fraction from each cell line were separated by Iatrobeads column chromatography into four fractions The three fractions containing fast-migrating glycosphingolipids fractions were analyzed by mass spectrometry in native form by separating on a hydrophilic interaction liquid chromatography column (polyamine II) coupled to the mass spectrometer [116, 117]. Results are summarized in Table 5.

The fractions containing slow-migrating glycosphingolipids were further tested by monoclonal antibodies in the chromatogram binding assay. A binding of antibodies against the blood group A determinant in the hexaosylceramide region was here obtained. In the slow-migrating region, glycosphingolipids were recognized by anti-Le^x and a highly cross-reactive anti-Le^y antibody.

These fractions were digested by *Rhodococcus* endoglycoceramidase and the oligosaccharides thereby released were analyzed by LC-ESI/MS using graphitized carbon columns to separate isomeric saccharides. The base peak chromatogram obtained showed molecular ions ranging from trisaccharides to hexasaccharides. Additionally, some minor oligosaccharides were detected by examination of selected ions.

MS^2 of the molecular ions gave a tentative identification of globotri, lactotri, globotetra, lactotera, *neo*lactotetra, H type 1 penta, H type 2 penta, Le^x penta, globopenta, Le^y hexa, globo H hexa and A type 1 hexa saccharides. The interpretation was guided by comparison of MS^2 spectra and retention times of reference oligosaccharides. The identity of several of the glycosphingolipids was also confirmed by proton-NMR and chromatogram binding experiments (Table 5).

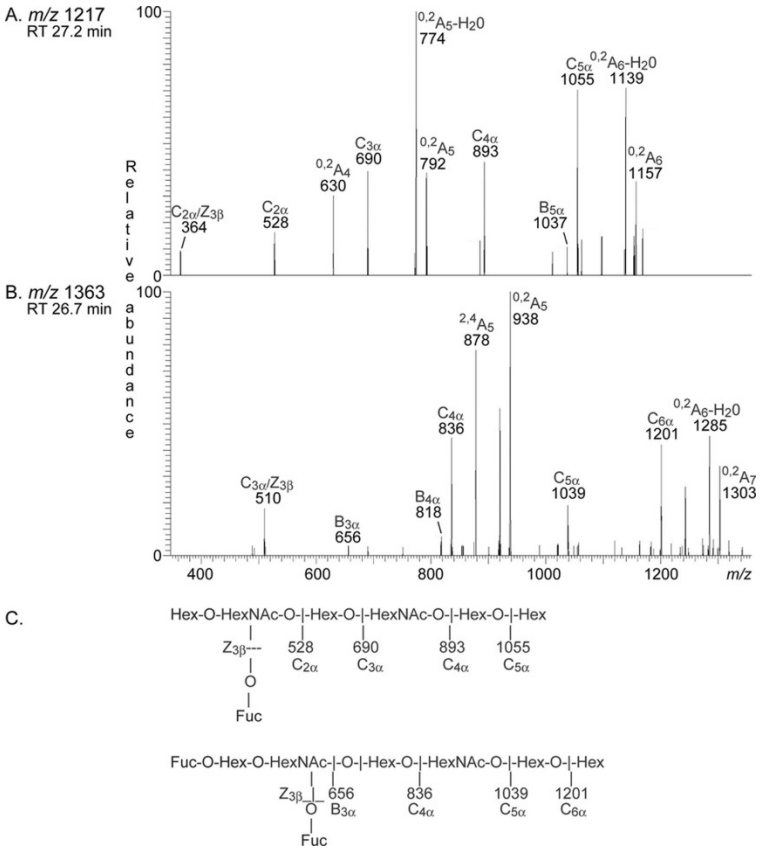

Figure 11: MS^2 of Endoglycoceramidase II yielded oligosaccharides from human embryonic stem cells. *A)* MS^2 of the $[M-H^+]^-$ ion at *m/z* 1217 *B)* MS^2 of the $[M-H^+]^-$ ion at *m/z* 1363 *C)* Structural interpretation formulas.

Finally, the fractions containing the slow-migrating glycosphingolipids were separated one more time with Iatrobeads chromatography, and the fractions obtained were digested by *Rhodococcus* endoglycoceramidase II, followed by analysis of the released oligosaccharides by LC-ESI/MS. The mass spectrometry revealed the same oligosaccharides as described above, and two additional ions at *m/z* 1217 and *m/z* 1363 were found in the two most slow-migrating fractions. By MS^2 of these ions a Le^x heptasaccharide (*m/z* 1217) and a Le^y octasaccharide (*m/z* 1363) were tentatively identified (Figure 11). These structures could not be confirmed with NMR due to low amounts of sample but is supported by chromatogram binding assays.

Table 5: Results from characterization by chromatogram binding assay (CBA), MS, and proton NMR, of non-acid glycosphingolipids in human embryonic stem cells.

Glycosphingolipid	**SA121 cell line**			**SA181 cell line**		
	CBA	**MS**	**NMR**	**CBA**	**MS**	**NMR**
Previously found hESC glycosphingolipids						
Globotetraosylceramide	ND	+	+	ND	+	+
Globopentaosylceramide/SSEA-3	+	+	+	+	+	+
Globo H hexaosylceramide	+	+	+	+	+	+
Lactotetraosylceramide	ND	+	+	ND	+	+
H type 1 pentaosylceramide	+	+	+	+	+	+
Novel glycosphingolipids found in hESC						
Glucosylceramide	ND[a]	+[b]	+	ND	+[b]	+
Galactosylceramide	ND	+[b]	+	ND	+[b]	+
Lactosylceramide	ND	+[c]	+	ND	+[c]	+
Galabiaosylceramide	ND	+[c]	+	ND	+[c]	+
Globotriaosylceramide	ND	+	+	ND	+	+
Lactotriaosylceramide	ND	+	−	ND	+	−
Neolactotetraosylceramide	+	+	−	+	+	−
H type 2 pentaosylceramide	−	+	−	−	+	−
A type 1 hexaosylceramide	+	+	−	+	+	−
Le^x/SSEA-1 pentaosylceramide	+	+	+	+	+	+
Le^y hexaosylceramide	+	+	−	+	+	−
Le^x/SSEA-1 heptaosylceramide	+	+	−	+	+	−
Le^y octaosylceramide	+	+	−	+	+	−

[a] ND, not determined. [b] Glucosylceramide and galactosylceramide give identical mass spectra. [c] Lactosylceramide and galabiaosylceramide give identical mass spectra.

Thus, several new glycosphingolipids previously not found in hESC were identified, as *e.g.* the blood group A type 1 hexaosylceramide. Since the ABO system is a strong immunological barrier, the blood groups should be taken into consideration when hESC are generated for future clinical applications.

5.3. Glycosphingolipids in immune recognition

5.3.1. Paper IV: Genetic basis of PX2, a recently acknowledged glycolipid blood group antigen.

This study was initiated to investigate the cause of an unexpected reaction of the plasma of a group A_1B person with the P_1^k phenotype with pp erythrocytes. The working hypothesis was that the p phenotype erythrocytes express a glycosphingolipid not found in P_1^k/P_2^k erythrocytes, and that such persons have natural antibodies against this glycosphingolipid antigen. Thorn *et al.* [118] have previously shown that erythrocytes of p phenotype have an increased amounts of the x_2 glycosphingolipid (GalNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer). This is a potential P antigen since the terminal disaccharide (GalNAcβ3Gal-R) is the same as the P glycosphingolipid. This is also supported by an earlier study that identified the x_2 glycosphingolipid following observations of additional reactivity by rabbit P sera on erythrocyte membranes [119]. In order to accurately determine the role of x_2 in the P1PK and GLOB system, a characterization of glycosphingolipids expressed on pp and P_1^k erythrocytes was done.

Thus, non-acid and acid glycosphingolipid fractions from human App and AP_1^k erythrocytes were isolated, and after separation fractions with glycosphingolipids migrating in the diglycosylceramide region and below were obtained. These fractions were characterized by chromatogram binding studies and mass spectrometry. In a similar manner, the acid glycosphingolipid fraction from App erythrocytes was separated into the two fractions with glycosphingolipids migrating in the sialyl-neolactotetraosylceramide region and below.

The non-acid fraction from App erythrocytes was hydrolyzed by *Rhodococcus* endoglycoceramidase II and the oligosaccharides obtained were analyzed by LC-ESI/MS. The base peak chromatogram (Figure 12A) had a molecular ion at m/z 909, and MS^2 of this ion (Fig. 12B) allowed identification of the x_2 pentasaccharide (GalNAcβ3Galβ4GlcNAcβ3Galβ4Glc).

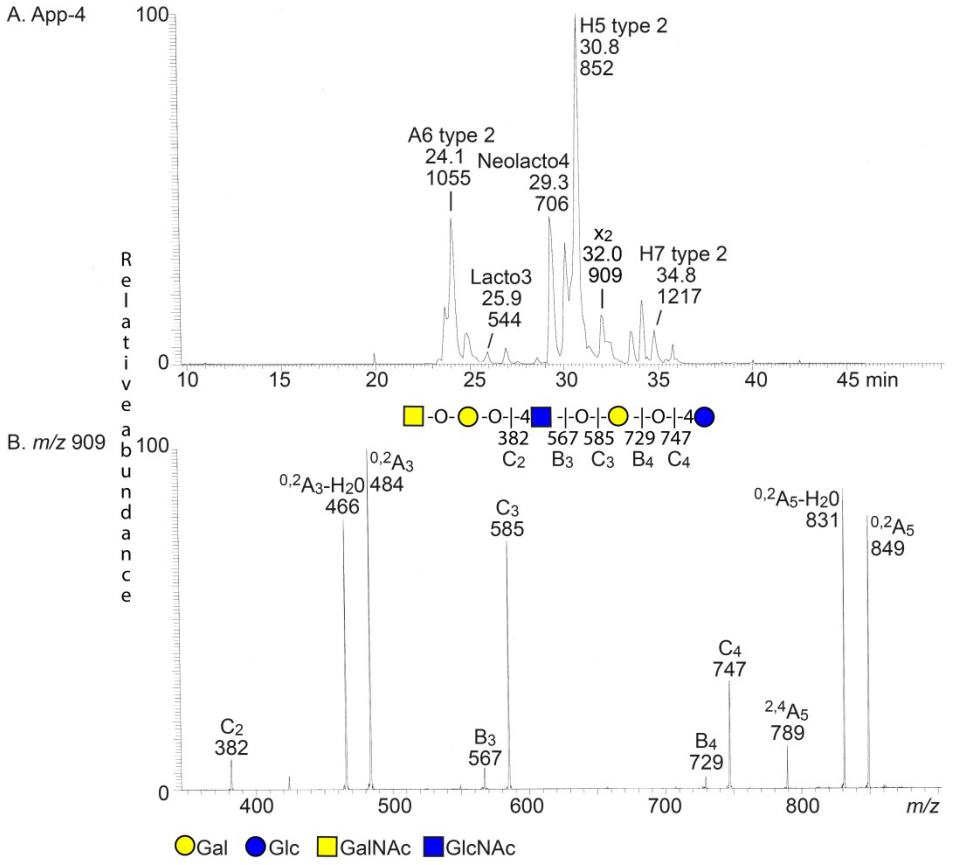

Figure 12: *A)* Base peak chromatogram of glycosphingolipid-derived oligosaccharides of App erythrocytes, obtained after digestion by *Rhodococcus* endoglycoceramidase II. *B)* MS^2 spectrum of the $[M-H^+]^-$ ion at *m/z* 909, retention time 33.7 min. The spectrum displayed C-type fragment ions at *m/z* 382, 585 and 747 representing a pentasaccharide with (HexNAc, Hex)-HexNAc-Hex-Hex sequence. A ^{0,2}A-type-crossring fragment was found at *m/z* 484, and a $^{0,2}A_3$-H_2O ion at *m/z* 466, which revealed a 4-substituted internal HexNAc. Another ^{0,2}A-ion was found at *m/z* 849, with the associated $^{0,2}A_5$-H_2O ion at *m/z* 831, which demonstrated a 4-substituted Glc of the lactose unit at the reducing end. From these data the oligosaccharide was tentatively identified as the x_2 pentasaccharide (GalNAcβ3Galβ4GlcNAcβ3Galβ4Glc).

Other oligosaccharide ions found in the base peak chromatogram at *m/z* 544, 706, 852, 1055, 1217 and 1420, were characterized as lactotri, *neo*lactotetra, H type 2 penta, A type 2 hexa, H type 2 hepta and A type 2 octa saccharides, respectively (Figure 12A).

The base peak chromatogram of the AP_1^k oligosaccharides was similar to App, with molecular ions of lactotri, *neo*lactotetra, H type 2 penta, A type 2 hexa, H type 2 hepta, and A type 2 octa saccharides. However, here a molecular ion at *m/z* 503, identifying a globo tri saccharide was also present, while no molecular ion at *m/z* 909 was found.

The native acid glycosphingolipid fractions from App erythrocytes were analyzed by LC-ESI/MS, with a hydrophilic interaction liquid chromatography column prior to MS. The base peak chromatogram of the first fraction was dominated by the a $[M-2H^+]^{2-}$ ion at *m/z* 813, and MS^2 identified a ganglioside with NeuAc-Hex-HexNAc-Hex-Hex sequence and d18:1-24:1 ceramide. Thus, sialyl-*neo*lactotetraosylceramide was tentatively identified. A $[M-2H^+]^{2-}$ ion at *m/z* 914 was also found in the base peak chromatogram. MS^2 of this ion revealed a NeuAc-HexNAc-Hex-4HexNAc-Hex-Hex carbohydrate sequence and d18:1-24:1 ceramide, indicating a sialyl-x_2 ganglioside. Sialyl-x_2 has previously been found in human erythrocytes [120], and α2,3-sialylation of the terminal GalNAc of x_2 by ST3Gal II has been reported [121].

A $[M-2H^+]^{2-}$ ion at *m/z* 959 was the major ion in the base peak chromatogram of the second acid fraction. MS^2 of this ion indicated a ganglioside with NeuAc-NeuAc-Hex-HexNAc-Hex-Hex sequence and d18:1-24:1 ceramide, such as disialyl-*neo*lactotetraosylceramide ganglioside. A minor $[M-2H^+]^{2-}$ ion at *m/z* 1060 was also found in the base chromatogram. MS^2 identified this as a ganglioside with NeuAc-NeuAc-HexNAc-Hex-HexNAc-Hex-Hex sequence with d18:1-24:1 ceramide, thus proposing a disialyl-x_2 ganglioside.

After showing the presence of x_2 pentaosylceramide among the non-acid glycosphingolipids of App erythrocytes, and the sialyl- and disialyl-x_2 gangliosides among the acid glycosphingolipids, the binding of P_1^k plasma and TH2 antibody (anti-x_2) were tested. The P_1^k plasma and the anti-x_2 TH2 antibody both bound in the pentaosylceramide region of the App non-acid fraction, indicating that the P_1^k plasma had anti-x_2 reactivity. The P_1^k plasma also bound to two gangliosides in the acid fractions from App, most likely to sialyl-x_2 ganglioside and disialyl-x_2 gangliosides. The TH2 antibody did not bind to the acid fractions, demonstrating that this antibody does not tolerate a substitution of sialic acid to the x_2 epitope. Furthermore, no binding of TH2 antibody to the non-acid glycosphingolipids of AP_1^k erythrocytes was observed.

The megakaryoblastic leukemia cell line MEG-01 was transfected with *B3GALNT1* ORF and one additional cell line was mock transfected. These cell lines were used for glycosphingolipid extraction and partial purification, and the crude fractions obtained, containing both acid and non-acid glycosphingolipids, were tested for TH2 binding. Here, the TH2 antibody bound to the glycosphingolipids from *B3GALNT1* transfected MEG-01 cells, while there was no binding to the glycosphingolipids from mock transfected MEG-01 cells (Figure 13). Similar results were seen when tested on flow cytometry with the TH2 antibody.

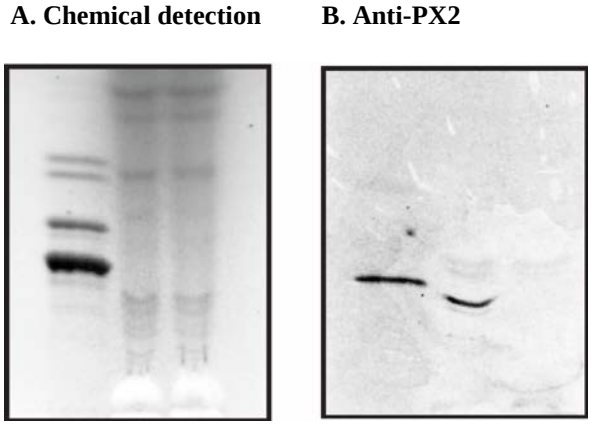

Figure 13: Thin-layer chromatography of glycosphingolipids from *B3GALNT1* and mock transfected MEG-01 cells. *A)* Chemical detection with anisaldehyde. *B)* Autoradiogram obtained by the anti-x_2 antibody TH2. *Lane 1*, non-acid glycosphingolipids from human blood group AB erythrocytes (40 µg); *lane 2*, acid and non-acid glycosphingolipids of *B3GALNT1* transfected cells, (10 µl/150 µl); *lane 3*, acid and non-acid glycosphingolipids of mock transfected cells, (10 µl/150 µl).

LC-MS analyses of the glycosphingolipid-derived oligosaccharide from *Rhodococcus* endoglycoceramidase II digestion of the *B3GALNT1* and mock transfected MEG-01 preparations showed that both cell types had predominantly glycosphingolipids with type 2 core chains, such as Le^x penta- and heptaosylceramide, *neo*lactotetra- and hexaosylceramide. An increased intensity of the ion at *m/z* 909 (indicating the x_2 pentasaccharide) was seen in the base peak chromatogram from the *B3GALNT1* transfected cells (Figure 14, upper chart), indicating an increased production of the x_2 pentaosylceramide upon *B3GALNT1* transfection.

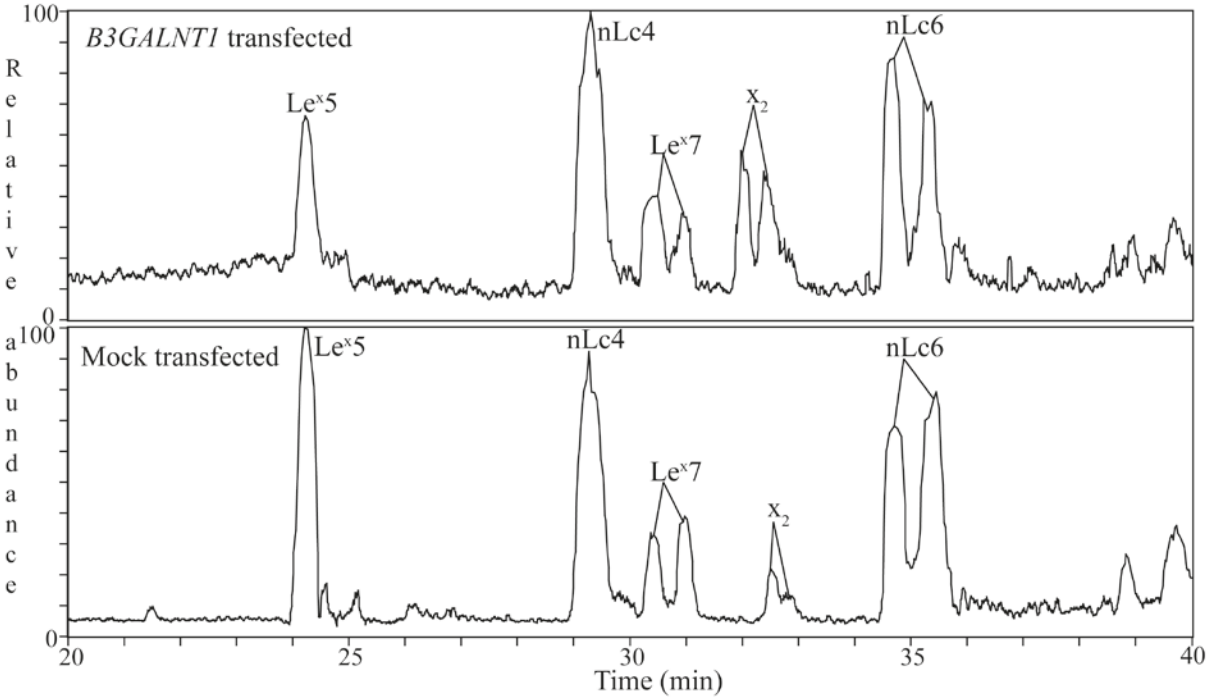

Figure 14: Base peak chromatogram from LC-ESI-MS of oligosaccharides obtained from *B3GALNT1* and mock transfected MEG-01 cells. An increased intensity of the ion at *m/z* 909 (indicating the x_2 pentasaccharide) was seen in the base peak chromatogram from the *B3GALNT1* transfected cells (upper chart).

The glycosphingolipid characterization and binding experiments were supported by data from flow cytometry using the TH2 antibody. Thus, flow cytometry experiments showed that the

B3GALNT1 transcripts were suppressed by *B3GALNT1* siRNA, while overexpression of *B3GALNT1* increased x_2 and P antigen levels.

Taken together the data from this study show that the x_2 glycosphingolipid is lacking in individuals with mutated P synthase (β1,3GalNAc-T1), and these individuals have anti-x_2 antibodies in plasma. We conclude that x_2 fulfils blood group criteria and is synthesized by β1,3GalNAc-T1. It has therefore been given a new name, PX2, and promoted to join P in the GLOB blood group system.

These results will increase the general understanding of *B3GALNT1* and GLOB blood group system. Furthermore it is of importance in transfusion medicine since a new blood selection principle for P^k individuals is recommended, suggesting that, if available, P_1^k and P_2^k blood should be used for transfusion to P^k individuals. This is however not easy due to the rarity of P^k phenotype blood [122]. As a second choice, p blood may be used despite the PX2 mismatch, since P antigen response is generally stronger.

6. Future perspectives

Data on adhesin binding specificities and glycan expression are of importance for understanding the mechanisms, pathogen susceptibilities and possible interventions for treatment. Our intention is that these studies can be used as additional knowledge for further studies in the glycobiology field.

The study of *H. pylori* BabA shows that the BabA binding specificity is more complex than previously known. We are currently investigating the glycosphingolipid binding specificity of a *H. pylori* strain expressing a BabA variant that is unable to bind Le^b, but still mediates adherence of the bacterium to the human gastric epithelium. Further investigations should target more *H. pylori* strains to see if more variants of BabA binding can be found.

The studies of glycosphingolipid recognition of *V. cholerae* revealed three different glycosphingolipid binding patterns. Here, a natural continuation is to try to identify the corresponding adhesins. Currently we are examining the toxin-coregulated pilus as a potential candidate. Furthermore, the function of Galα3Galα3Gal binding is interesting since it should have no function in the infection stage, but might have a role in other interactions as *e.g.* biofilm formation.

The studies of glycosphingolipids in hESC revealed a complex expression that can be used for further investigations, such as examination of differentiation dependent changes in glycosphingolipid expression. The glycosphingolipid expression in hiPSC (human induced pluripotent stem cells) is currently being investigated and compared to hESC. It would also be interesting to compare glycosphingolipid expression in cancer stem cells, which is suggested to be a sub-population of some tumors. Since there are many similarities between stem cells and cancer stem cells, such as both can divide indefinitely and gives rise to heterogeneous cell populations, it is possible that there are several resemblances in glycosphingolipid expression.

The x_2 antigen has been added to the GLOB blood group system and its genetic background confirmed. This gives additional tools in identifying the rare P associated blood groups, especially with the x_2 specific antibody TH2. Whether the sialylated and disialylated x_2 glycosphingolipids can be added to the GLOB blood group system should be further explored.

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8. References

1. Fuster, M.M. and J.D. Esko, *The sweet and sour of cancer: glycans as novel therapeutic targets.* Nat Rev Cancer, 2005. **5**(7): p. 526-42.
2. Chester, M.A., *IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN). Nomenclature of glycolipids--recommendations 1997.* Eur J Biochem, 1998. **257**(2): p. 293-8.
3. in *Essentials of Glycobiology*, A. Varki, et al., Editors. 2009, Cold Spring Harbor Laboratory Press: Cold Spring Harbor (NY).
4. Degroote, S., J. Wolthoorn, and G. van Meer, *The cell biology of glycosphingolipids.* Semin Cell Dev Biol, 2004. **15**(4): p. 375-87.
5. Karlsson, K.-A., *On the chemistry and occurrence of sphingolipid long-chain bases.* Chemistry and Physics of Lipids, 1970. **5**(1): p. 6-43.
6. Sonnino, S. and V. Chigorno, *Ganglioside molecular species containing C18- and C20-sphingosine in mammalian nervous tissues and neuronal cell cultures.* Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes, 2000. **1469**(2): p. 63-77.
7. Prinetti, A., N. Loberto, V. Chigorno, and S. Sonnino, *Glycosphingolipid behaviour in complex membranes.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2009. **1788**(1): p. 184-193.
8. Hakomori, S., *Glycosynapses: Microdomains controlling carbohydrate-dependent cell adhesion and signaling.* Anais da Academia Brasileira de Ciencias, 2004. **76**(3): p. 553-572.
9. Breimer, M.E., G.C. Hansson, K.A. Karlsson, and H. Leffler, *Blood group type glycosphingolipids from the small intestine of different animals analysed by mass spectrometry and thin-layer chromatography. A note on species diversity.* J Biochem, 1981. **90**(3): p. 589-609.
10. Clausen, H. and S. Hakomori, *ABH and related histo-blood group antigens; immunochemical differences in carrier isotypes and their distribution.* Vox Sang, 1989. **56**(1): p. 1-20.
11. Ravn, V. and E. Dabelsteen, *Tissue distribution of histo-blood group antigens.* APMIS, 2000. **108**(1): p. 1-28.
12. Futerman, A.H. and R.E. Pagano, *Determination of the intracellular sites and topology of glucosylceramide synthesis in rat liver.* Biochem J, 1991. **280 (Pt 2)**: p. 295-302.
13. Jeckel, D., A. Karrenbauer, K.N. Burger, G. van Meer, and F. Wieland, *Glucosylceramide is synthesized at the cytosolic surface of various Golgi subfractions.* J Cell Biol, 1992. **117**(2): p. 259-67.
14. Castro, B.M., M. Prieto, and L.C. Silva, *Ceramide: a simple sphingolipid with unique biophysical properties.* Prog Lipid Res, 2014. **54**: p. 53-67.
15. Lannert, H., G. Bünning, D. Jeckel, and F.T. Wieland, *Lactosylceramide is synthesized in the lumen of the Golgi apparatus.* FEBS Letters, 1994. **342**(1): p. 91-96.
16. Tettamanti, G., *Ganglioside/glycosphingolipid turnover: new concepts.* Glycoconj J, 2004. **20**(5): p. 301-17.
17. Sprong, H., B. Kruithof, R. Leijendekker, J.W. Slot, G. van Meer, and P. van der Sluijs, *UDP-galactose:ceramide galactosyltransferase is a class I integral membrane protein of the endoplasmic reticulum.* J Biol Chem, 1998. **273**(40): p. 25880-8.
18. Christiansen, D., J. Milland, E. Mouhtouris, H. Vaughan, D.G. Pellicci, M.J. McConville, D.I. Godfrey, and M.S. Sandrin, *Humans lack iGb3 due to the absence of*

functional iGb3-synthase: implications for NKT cell development and transplantation. PLoS Biol, 2008. **6**(7): p. e172.
19. Allende, M.L. and R.L. Proia, *Simplifying complexity: genetically resculpting glycosphingolipid synthesis pathways in mice to reveal function.* Glycoconj J, 2014.
20. Sandhoff, K. and T. Kolter, *Biosynthesis and degradation of mammalian glycosphingolipids.* Philosophical Transactions of the Royal Society of London B: Biological Sciences, 2003. **358**(1433): p. 847-861.
21. Henry, S., R. Oriol, and B. Samuelsson, *Lewis histo-blood group system and associated secretory phenotypes.* Vox Sang, 1995. **69**(3): p. 166-82.
22. Oriol, R., J. Le Pendu, and R. Mollicone, *Genetics of ABO, H, Lewis, X and related antigens.* Vox Sang, 1986. **51**(3): p. 161-71.
23. Koscielak, J., *The hypothesis on function of glycosphingolipids and ABO blood groups revisited.* Neurochem Res, 2012. **37**(6): p. 1170-84.
24. Bishop, J.R. and P. Gagneux, *Evolution of carbohydrate antigens—microbial forces shaping host glycomes?* Glycobiology, 2007. **17**(5): p. 23R-34R.
25. Yu, R.K., Y.T. Tsai, T. Ariga, and M. Yanagisawa, *Structures, biosynthesis, and functions of gangliosides--an overview.* J Oleo Sci, 2011. **60**(10): p. 537-44.
26. Schnaar, R.L., R. Gerardy-Schahn, and H. Hildebrandt, *Sialic acids in the brain: gangliosides and polysialic acid in nervous system development, stability, disease, and regeneration.* Physiol Rev, 2014. **94**(2): p. 461-518.
27. Pieters, R.J., *Carbohydrate mediated bacterial adhesion.* Adv Exp Med Biol, 2011. **715**: p. 227-40.
28. Hanada, K., *Sphingolipids in infectious diseases.* Jpn J Infect Dis, 2005. **58**(3): p. 131-48.
29. Gaastra, W. and A.M. Svennerholm, *Colonization factors of human enterotoxigenic Escherichia coli (ETEC).* Trends Microbiol, 1996. **4**(11): p. 444-52.
30. Pang, S.S., S.T. Nguyen, A.J. Perry, C.J. Day, S. Panjikar, J. Tiralongo, J.C. Whisstock, and T. Kwok, *The three-dimensional structure of the extracellular adhesion domain of the sialic acid-binding adhesin SabA from Helicobacter pylori.* J Biol Chem, 2014. **289**(10): p. 6332-40.
31. Holmgren, J., I. Lonnroth, and L. Svennerholm, *Fixation and inactivation of cholera toxin by GM1 ganglioside.* Scand J Infect Dis, 1973. **5**(1): p. 77-8.
32. Griffitts, J.S., S.M. Haslam, T. Yang, S.F. Garczynski, B. Mulloy, H. Morris, P.S. Cremer, A. Dell, M.J. Adang, and R.V. Aroian, *Glycolipids as receptors for Bacillus thuringiensis crystal toxin.* Science, 2005. **307**(5711): p. 922-5.
33. Holmgren, J., H. Elwing, P. Fredman, and L. Svennerholm, *Polystyrene-Adsorbed Gangliosides for Investigation of the Structure of the Tetanus-Toxin Receptor.* European Journal of Biochemistry, 1980. **106**(2): p. 371-379.
34. Teneberg, S., T.R. Hirst, J. Angstrom, and K.A. Karlsson, *Comparison of the glycolipid-binding specificities of cholera toxin and porcine Escherichia coli heat-labile enterotoxin: identification of a receptor-active non-ganglioside glycolipid for the heat-labile toxin in infant rabbit small intestine.* Glycoconj J, 1994. **11**(6): p. 533-40.
35. Teneberg, S., I. Lönnroth, J.F.T. López, U. Galili, M.Ö. Halvarsson, J. Ångström, and K.-A. Karlsson, *Molecular mimicry in the recognition of glycosphingolipids by Galα3Galβ4GlcNAcβ-binding Clostridium difficile toxin A, human natural anti α-galactosyl IgG and the monoclonal antibody Gal-13: characterization of a binding-active human glycosphingolipid, non-identical with the animal receptor.* Glycobiology, 1996. **6**(6): p. 599-609.

36. Jacewicz, M., H. Clausen, E. Nudelman, A. Donohue-Rolfe, and G.T. Keusch, *Pathogenesis of shigella diarrhea. XI. Isolation of a shigella toxin-binding glycolipid from rabbit jejunum and HeLa cells and its identification as globotriaosylceramide.* J Exp Med, 1986. **163**(6): p. 1391-404.
37. Robin Warren, J. and B. Marshall, *Unidentified curved bacilli on gastric epithelium in active cronic gastritis.* The Lancet, 1983. **321**(8336): p. 1273-1275.
38. Fischer, W., S. Prassl, and R. Haas, *Virulence mechanisms and persistence strategies of the human gastric pathogen Helicobacter pylori.* Curr Top Microbiol Immunol, 2009. **337**: p. 129-71.
39. Bruce, M.G. and H.I. Maaroos, *Epidemiology of Helicobacter pylori Infection.* Helicobacter, 2008. **13**: p. 1-6.
40. Noach, L.A., T.M. Rolf, and G.N. Tytgat, *Electron microscopic study of association between Helicobacter pylori and gastric and duodenal mucosa.* J Clin Pathol, 1994. **47**(8): p. 699-704.
41. Rossez, Y., P. Gosset, I.G. Boneca, A. Magalhães, C. Ecobichon, C.A. Reis, C. Cieniewski-Bernard, M. Joncquel Chevalier Curt, R. Léonard, E. Maes, B. Sperandio, C. Slomianny, P.J. Sansonetti, J.-C. Michalski, and C. Robbe-Masselot, *The LacdiNAc-Specific Adhesin LabA Mediates Adhesion of Helicobacter pylori to Human Gastric Mucosa.* Journal of Infectious Diseases, 2014. **210**(8): p. 1286-1295.
42. Boren, T., P. Falk, K. Roth, G. Larson, and S. Normark, *Attachment of Helicobacter pylori to human gastric epithelium mediated by blood group antigens.* Science, 1993. **262**(5141): p. 1892-1895.
43. Ilver, D., A. Arnqvist, J. Ögren, I.-M. Frick, D. Kersulyte, E.T. Incecik, D.E. Berg, A. Covacci, L. Engstrand, and T. Borén, *Helicobacter pylori Adhesin Binding Fucosylated Histo-Blood Group Antigens Revealed by Retagging.* Science, 1998. **279**(5349): p. 373-377.
44. Rad, R., M. Gerhard, R. Lang, M. Schöniger, T. Rösch, W. Schepp, I. Becker, H. Wagner, and C. Prinz, *The Helicobacter pylori Blood Group Antigen-Binding Adhesin Facilitates Bacterial Colonization and Augments a Nonspecific Immune Response.* The Journal of Immunology, 2002. **168**(6): p. 3033-3041.
45. Aspholm, M., F.O. Olfat, J. Norden, B. Sonden, C. Lundberg, R. Sjostrom, S. Altraja, S. Odenbreit, R. Haas, T. Wadstrom, L. Engstrand, C. Semino-Mora, H. Liu, A. Dubois, S. Teneberg, A. Arnqvist, and T. Boren, *SabA is the H. pylori hemagglutinin and is polymorphic in binding to sialylated glycans.* PLoS Pathog, 2006. **2**(10): p. e110.
46. Aspholm-Hurtig, M., G. Dailide, M. Lahmann, A. Kalia, D. Ilver, N. Roche, S. Vikström, R. Sjöström, S. Lindén, A. Bäckström, C. Lundberg, A. Arnqvist, J. Mahdavi, U.J. Nilsson, B. Velapatiño, R.H. Gilman, M. Gerhard, T. Alarcon, M. López-Brea, T. Nakazawa, J.G. Fox, P. Correa, M.G. Dominguez-Bello, G.I. Perez-Perez, M.J. Blaser, S. Normark, I. Carlstedt, S. Oscarson, S. Teneberg, D.E. Berg, and T. Borén, *Functional Adaptation of BabA, the H. pylori ABO Blood Group Antigen Binding Adhesin.* Science, 2004. **305**(5683): p. 519-522.
47. Nair, G.B., F. Qadri, J. Holmgren, A.M. Svennerholm, A. Safa, N.A. Bhuiyan, Q.S. Ahmad, S.M. Faruque, A.S. Faruque, Y. Takeda, and D.A. Sack, *Cholera due to altered El Tor strains of Vibrio cholerae O1 in Bangladesh.* J Clin Microbiol, 2006. **44**(11): p. 4211-3.
48. Dutta, P., D. Sur, and S.K. Bhattacharya, *Management of Cholera,* in *Epidemiological and Molecular Aspects on Cholera*, T. Ramamurthy and S.K. Bhattacharya, Editors. 2011, Springer New York. p. 341-353.

49. Ramamurthy, T. and S.K. Bhattacharya, *General Introduction*, in *Epidemiological and Molecular Aspects on Cholera*, T. Ramamurthy and S.K. Bhattacharya, Editors. 2011, Springer New York. p. 1-4.
50. Stroeher, U.H., L.E. Karageorgos, R. Morona, and P.A. Manning, *Serotype conversion in Vibrio cholerae O1.* Proc Natl Acad Sci U S A, 1992. **89**(7): p. 2566-70.
51. Eidels, L., R.L. Proia, and D.A. Hart, *Membrane receptors for bacterial toxins.* Microbiological Reviews, 1983. **47**(4): p. 596-620.
52. Holmgren, J., I. Lonnroth, and L. Svennerholm, *Tissue receptor for cholera exotoxin: postulated structure from studies with GM1 ganglioside and related glycolipids.* Infect Immun, 1973. **8**(2): p. 208-14.
53. Kirn, T.J., B.A. Jude, and R.K. Taylor, *A colonization factor links Vibrio cholerae environmental survival and human infection.* Nature, 2005. **438**(7069): p. 863-6.
54. Bhowmick, R., A. Ghosal, B. Das, H. Koley, D.R. Saha, S. Ganguly, R.K. Nandy, R.K. Bhadra, and N.S. Chatterjee, *Intestinal adherence of Vibrio cholerae involves a coordinated interaction between colonization factor GbpA and mucin.* Infect Immun, 2008. **76**(11): p. 4968-77.
55. Thomson, J.A., J. Itskovitz-Eldor, S.S. Shapiro, M.A. Waknitz, J.J. Swiergiel, V.S. Marshall, and J.M. Jones, *Embryonic stem cell lines derived from human blastocysts.* Science, 1998. **282**(5391): p. 1145-7.
56. Reya, T., S.J. Morrison, M.F. Clarke, and I.L. Weissman, *Stem cells, cancer, and cancer stem cells.* Nature, 2001. **414**(6859): p. 105-111.
57. Kannagi, R., N.A. Cochran, F. Ishigami, S. Hakomori, P.W. Andrews, B.B. Knowles, and D. Solter, *Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells.* The EMBO Journal, 1983. **2**(12): p. 2355-2361.
58. Badcock, G., C. Pigott, J. Goepel, and P.W. Andrews, *The Human Embryonal Carcinoma Marker Antigen TRA-1-60 Is a Sialylated Keratan Sulfate Proteoglycan.* Cancer Research, 1999. **59**(18): p. 4715-4719.
59. Yang, Z., J. Bergstrom, and K.A. Karlsson, *Glycoproteins with Gal alpha 4Gal are absent from human erythrocyte membranes, indicating that glycolipids are the sole carriers of blood group P activities.* J Biol Chem, 1994. **269**(20): p. 14620-4.
60. Liang, Y.J., H.H. Kuo, C.H. Lin, Y.Y. Chen, B.C. Yang, Y.Y. Cheng, A.L. Yu, K.H. Khoo, and J. Yu, *Switching of the core structures of glycosphingolipids from globo- and lacto- to ganglio-series upon human embryonic stem cell differentiation.* Proc Natl Acad Sci U S A, 2010. **107**(52): p. 22564-9.
61. Liang, Y.J., B.C. Yang, J.M. Chen, Y.H. Lin, C.L. Huang, Y.Y. Cheng, C.Y. Hsu, K.H. Khoo, C.N. Shen, and J. Yu, *Changes in glycosphingolipid composition during differentiation of human embryonic stem cells to ectodermal or endodermal lineages.* Stem Cells, 2011. **29**(12): p. 1995-2004.
62. ISBT. 2015; Available from: http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/blood-group-terminology/.
63. Thuresson, B., J.S. Westman, and M.L. Olsson, *Identification of a novel A4GALT exon reveals the genetic basis of the P1/P2 histo-blood groups.* Blood, 2010. **117**(2): p. 678-687.
64. Lisowska, E. and M. Duk, *Polyagglutination NOR: new glycosphingolipid antigens recognized by a new type of common human anti-α-galactosyl antibodies.* Archives of Biochemistry and Biophysics, 2004. **426**(2): p. 142-147.
65. Suchanowska, A., R. Kaczmarek, M. Duk, J. Lukasiewicz, D. Smolarek, E. Majorczyk, E. Jaskiewicz, A. Laskowska, K. Wasniowska, M. Grodecka, E. Lisowska, and M. Czerwinski, *A single point mutation in the gene encoding Gb3/CD77 synthase*

causes a rare inherited polyagglutination syndrome. J Biol Chem, 2012. **287**(45): p. 38220-30.
66. Fletcher, K.S., E.G. Bremer, and G.A. Schwarting, *P blood group regulation of glycosphingolipid levels in human erythrocytes.* J Biol Chem, 1979. **254**(22): p. 11196-8.
67. Hellberg, Å., J. Poole, and M.L. Olsson, *Molecular Basis of the Globoside-deficient Pk Blood Group Phenotype: Identification of Four Inactivating Mutations in the UDP-N-Acetylgalactosamine: Globotriaosylceramide 3-β-N-Acetylgalactosaminyltransferase Gene.* Journal of Biological Chemistry, 2002. **277**(33): p. 29455-29459.
68. Storry, J.R., L. Castilho, G. Daniels, W.A. Flegel, G. Garratty, C.L. Francis, J.M. Moulds, J.J. Moulds, M.L. Olsson, J. Poole, M.E. Reid, P. Rouger, E. van der Schoot, M. Scott, E. Smart, Y. Tani, L.C. Yu, S. Wendel, C. Westhoff, V. Yahalom, and T. Zelinski, *International Society of Blood Transfusion Working Party on red cell immunogenetics and blood group terminology: Berlin report.* Vox Sanguinis, 2011. **101**(1): p. 77-82.
69. Daniels, G., *P blood groups*, in *Human blood groups*. 2002, Blackwell Science: Oxford. p. 175-194.
70. Westman, J.S., Å. Hellberg, T. Peyrard, H. Hustinx, B. Thuresson, and M.L. Olsson, *P1/P2 genotyping of known and novel null alleles in the P1PK and GLOB histo-blood group systems.* Transfusion, 2013. **53**(11pt2): p. 2928-2939.
71. Karlsson, K.A., *Preparation of total nonacid glycolipids for overlay analysis of receptors for bacteria and viruses and for other studies.* Methods Enzymol, 1987. **138**: p. 212-20.
72. Müthing, J., *TLC in Structure and Recognition Studies of Glycosphingolipids*, in *Glycoanalysis Protocols*, E. Hounsell, Editor. 1998, Humana Press. p. 183-195.
73. Svennerholm, L., *The quantitative estimation of cerebrosides in nervous tissue.* J Neurochem, 1956. **1**(1): p. 42-53.
74. Waldi, D., in *Dünnschicht-Chromatographie*, E. Stahl, Editor. 1962, Springer-Verlag: Berlin. p. 496-515.
75. Svennerholm, L., *Quantitive estimation of sialic acids: II. A colorimetric resorcinol-hydrochloric acid method.* Biochimica et Biophysica Acta, 1957. **24**(0): p. 604-611.
76. Fagerberg, D., J. Angstrom, A. Halim, A. Hultberg, L. Rakhimova, L. Hammarstrom, T. Boren, and S. Teneberg, *Novel Leb-like Helicobacter pylori-binding glycosphingolipid created by the expression of human alpha-1,3/4-fucosyltransferase in FVB/N mouse stomach.* Glycobiology, 2009. **19**(2): p. 182-91.
77. Teneberg, S., J. Angstrom, P.A. Jovall, and K.A. Karlsson, *Characterization of binding of Gal beta 4GlcNAc-specific lectins from Erythrina cristagalli and Erythrina corallodendron to glycosphinogolipids. Detection, isolation, and characterization of a novel glycosphinglipid of bovine buttermilk.* J Biol Chem, 1994. **269**(11): p. 8554-63.
78. Müthing, J., *High-resolution thin-layer chromatography of gangliosides.* Journal of Chromatography A, 1996. **720**(1–2): p. 3-25.
79. Brockhaus, M., J.L. Magnani, M. Blaszczyk, Z. Steplewski, H. Koprowski, K.A. Karlsson, G. Larson, and V. Ginsburg, *Monoclonal antibodies directed against the human Leb blood group antigen.* J Biol Chem, 1981. **256**(24): p. 13223-5.
80. Hansson, G.C., K.A. Karlsson, G. Larson, J.M. McKibbin, M. Blaszczyk, M. Herlyn, Z. Steplewski, and H. Koprowski, *Mouse monoclonal antibodies against human cancer cell lines with specificities for blood group and related antigens. Characterization by antibody binding to glycosphingolipids in a chromatogram binding assay.* J Biol Chem, 1983. **258**(7): p. 4091-7.

81. Mason, D.Y. and R. Sammons, *Alkaline phosphatase and peroxidase for double immunoenzymatic labelling of cellular constituents.* J Clin Pathol, 1978. **31**(5): p. 454-60.
82. Fenn, J.B., M. Mann, C.K. Meng, S.F. Wong, and C.M. Whitehouse, *Electrospray ionization for mass spectrometry of large biomolecules.* Science, 1989. **246**(4926): p. 64-71.
83. Hillenkamp, F. and M. Karas, *Mass spectrometry of peptides and proteins by matrix-assisted ultraviolet laser desorption/ionization.* Methods Enzymol, 1990. **193**: p. 280-95.
84. El-Aneed, A., A. Cohen, and J. Banoub, *Mass Spectrometry, Review of the Basics: Electrospray, MALDI, and Commonly Used Mass Analyzers.* Applied Spectroscopy Reviews, 2009. **44**(3): p. 210-230.
85. Taylor, G., *Disintegration of Water Drops in an Electric Field.* Proceedings of the Royal Society of London A: Mathematical, Physical and Engineering Sciences, 1964. **280**(1382): p. 383-397.
86. Wilm, M., *Principles of Electrospray Ionization.* Molecular & Cellular Proteomics, 2011. **10**(7).
87. Thermo, e.c., *Finnigan LTQ Hardware Manual*. 2005, Technical Publications: San Jose.
88. Chai, W., V. Piskarev, and A.M. Lawson, *Negative-ion electrospray mass spectrometry of neutral underivatized oligosaccharides.* Anal Chem, 2001. **73**(3): p. 651-7.
89. Todd, J.F.J., *Recommendations for nomenclature and symbolism for mass spectroscopy (including an appendix of terms used in vacuum technology). (Recommendations 1991)*, in *Pure and Applied Chemistry*. 1991. p. 1541.
90. Hakomori, S.-I., *A Rapid Permethylation of Glycolipid, and Polysaccharide Catalyzed by Methylsulfinyl Carbanion in Dimethyl Sulfoxide.* Journal of Biochemistry, 1964. **55**(2): p. 205-208.
91. Karlsson, K.-A., *Carbohydrate composition and sequence analysis of cell surface components by mass spectrometry. Characterization of the major monosialoganglioside of brain.* FEBS Letters, 1973. **32**(2): p. 317-320.
92. Arita, M., M. Iwamori, T. Higuchi, and Y. Nagai, *Negative Ion Fast Atom Bombardment Mass Spectrometry of Gangliosides and Asialo Gangliosides: A Useful Method for the Structural Elucidation of Gangliosides and Related Neutral Glycosphingolipids.* Journal of Biochemistry, 1983. **94**(1): p. 249-256.
93. Ii, T., Y. Ohashi, and Y. Nagai, *Structural elucidation of underivatized gangliosides by electrospray-ionization tandem mass spectrometry (ESIMS/MS).* Carbohydrate Research, 1995. **273**(1): p. 27-40.
94. Meisen, I., M. Mormann, and J. Müthing, *Thin-layer chromatography, overlay technique and mass spectrometry: A versatile triad advancing glycosphingolipidomics.* Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 2011. **1811**(11): p. 875-896.
95. Muthing, J. and U. Distler, *Advances on the compositional analysis of glycosphingolipids combining thin-layer chromatography with mass spectrometry.* Mass Spectrom Rev, 2010. **29**(3): p. 425-79.
96. Karlsson, H., A. Halim, and S. Teneberg, *Differentiation of glycosphingolipid-derived glycan structural isomers by liquid chromatography/mass spectrometry.* Glycobiology, 2010. **20**(9): p. 1103-16.

97. Levery, S.B., *Glycosphingolipid Structural Analysis and Glycosphingolipidomics*, in *Mass Spectrometry: Modified Proteins and Glycoconjugates*, B. A.L., Editor. 2005, Elsevier Academic Press: San Diego. p. 300-368.
98. Ito, M., Y. Ikegami, and T. Yamagata, *Activator proteins for glycosphingolipid hydrolysis by endoglycoceramidases. Elucidation of biological functions of cell-surface glycosphingolipids in situ by endoglycoceramidases made possible using these activator proteins.* J Biol Chem, 1991. **266**(12): p. 7919-26.
99. Zhou, B., S.C. Li, R.A. Laine, R.T. Huang, and Y.T. Li, *Isolation and characterization of ceramide glycanase from the leech, Macrobdella decora.* Journal of Biological Chemistry, 1989. **264**(21): p. 12272-12277.
100. Berg, J., J. Tymoczko, and L. Stryer, *Exploring proteins*, in *Biochemistry*. 2002, W. H. Freeman. p. 77-116.
101. Dabrowski, J., P. Hanfland, and H. Egge, *Structural analysis of glycosphinoglipids by high-resolution 1H nuclear magnetic resonance spectroscopy.* Biochemistry, 1980. **19**(24): p. 5652-8.
102. Gasa, S., T. Mitsuyama, and A. Makita, *Proton nuclear magnetic resonance of neutral and acidic glycosphingolipids.* Journal of lipid research, 1983. **24**(2): p. 174-82.
103. Kriwacki, R. and T.P. Pitner, *Current Aspects of Practical Two-Dimensional (2D) Nuclear Magnetic Resonance (NMR) Spectroscopy: Applications to Structure Elucidation.* Pharmaceutical Research, 1989. **6**(7): p. 531-554.
104. Macey, M.G., *Principles of Flow Cytometry*, in *Flow Cytometry: Principles and Applications*, M.G. Macey, Editor. 2007, Humana Press Inc: Totowa. p. 1-15.
105. Jaye, D.L., R.A. Bray, H.M. Gebel, W.A.C. Harris, and E.K. Waller, *Translational Applications of Flow Cytometry in Clinical Practice.* The Journal of Immunology, 2012. **188**(10): p. 4715-4719.
106. Nyholm, P.G., B.E. Samuelsson, M. Breimer, and I. Pascher, *Conformational analysis of blood group A-active glycosphingolipids using HSEA-calculations. The possible significance of the core oligosaccharide chain for the presentation and recognition of the A-determinant.* J Mol Recognit, 1989. **2**(3): p. 103-13.
107. Imberty, A., E. Mikros, J. Koca, R. Mollicone, R. Oriol, and S. Perez, *Computer simulation of histo-blood group oligosaccharides: energy maps of all constituting disaccharides and potential energy surfaces of 14 ABH and Lewis carbohydrate antigens.* Glycoconj J, 1995. **12**(3): p. 331-49.
108. Fujimoto, S., O. Olaniyi Ojo, A. Arnqvist, J.Y. Wu, S. Odenbreit, R. Haas, D.Y. Graham, and Y. Yamaoka, *Helicobacter pylori BabA expression, gastric mucosal injury, and clinical outcome.* Clin Gastroenterol Hepatol, 2007. **5**(1): p. 49-58.
109. Marionneau, S., A. Cailleau-Thomas, J. Rocher, B. Le Moullac-Vaidye, N. Ruvoen, M. Clement, and J. Le Pendu, *ABH and Lewis histo-blood group antigens, a model for the meaning of oligosaccharide diversity in the face of a changing world.* Biochimie, 2001. **83**(7): p. 565-73.
110. Chang, W.W., C.H. Lee, P. Lee, J. Lin, C.W. Hsu, J.T. Hung, J.J. Lin, J.C. Yu, L.E. Shao, J. Yu, C.H. Wong, and A.L. Yu, *Expression of Globo H and SSEA3 in breast cancer stem cells and the involvement of fucosyl transferases 1 and 2 in Globo H synthesis.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(33): p. 11667-72.
111. Keusch, J.J., S.M. Manzella, K.A. Nyame, R.D. Cummings, and J.U. Baenziger, *Expression cloning of a new member of the ABO blood group glycosyltransferases, iGb3 synthase, that directs the synthesis of isoglobo-glycosphingolipids.* The Journal of biological chemistry, 2000. **275**(33): p. 25308-14.

112. Gastinel, L.N., C. Bignon, A.K. Misra, O. Hindsgaul, J.H. Shaper, and D.H. Joziasse, *Bovine alpha1,3-galactosyltransferase catalytic domain structure and its relationship with ABO histo-blood group and glycosphingolipid glycosyltransferases.* EMBO J, 2001. **20**(4): p. 638-49.
113. Karlsson, K.A., *Animal glycosphingolipids as membrane attachment sites for bacteria.* Annu Rev Biochem, 1989. **58**: p. 309-50.
114. Breimer, M.E., G.C. Hansson, K.A. Karlsson, G. Larson, and H. Leffler, *Glycosphingolipid composition of epithelial cells isolated along the villus axis of small intestine of a single human individual.* Glycobiology, 2012. **22**(12): p. 1721-30.
115. Wong, E., G. Vaaje-Kolstad, A. Ghosh, R. Hurtado-Guerrero, P.V. Konarev, A.F. Ibrahim, D.I. Svergun, V.G. Eijsink, N.S. Chatterjee, and D.M. van Aalten, *The Vibrio cholerae colonization factor GbpA possesses a modular structure that governs binding to different host surfaces.* PLoS Pathog, 2012. **8**(1): p. e1002373.
116. Buszewski, B. and S. Noga, *Hydrophilic interaction liquid chromatography (HILIC)—a powerful separation technique.* Analytical and Bioanalytical Chemistry, 2012. **402**(1): p. 231-247.
117. Wuhrer, M., A.R. de Boer, and A.M. Deelder, *Structural glycomics using hydrophilic interaction chromatography (HILIC) with mass spectrometry.* Mass Spectrom Rev, 2009. **28**(2): p. 192-206.
118. Thorn, J.J., S.B. Levery, M.E.K. Salyan, M.R. Stroud, B. Cedergren, B. Nilsson, S. Hakomori, and H. Clausen, *Structural characterization of x2 glycosphingolipid, its extended form, and its sialosyl derivatives: accumulation associated with the rare blood group p phenotype.* Biochemistry, 1992. **31**(28): p. 6509-6517.
119. Kannagi, R., M.N. Fukuda, and S. Hakomori, *A new glycolipid antigen isolated from human erythrocyte membranes reacting with antibodies directed to globo-N-tetraosylceramide (globoside).* J Biol Chem, 1982. **257**(8): p. 4438-42.
120. Watanabe, K. and S. Hakomori, *Gangliosides of human erythrocytes. A novel ganglioside with a unique N-acetylneuraminosyl-(2 leads to 3)-N-acetylgalactosamine structure.* Biochemistry, 1979. **18**(24): p. 5502-4.
121. Toivonen, S., O. Aitio, and O. Renkonen, *alpha 2,3-Sialylation of terminal GalNAc beta 1-3Gal determinants by ST3Gal II reveals the multifunctionality of the enzyme. The resulting Neu5Ac alpha 2-3GalNAc linkage is resistant to sialidases from Newcastle disease virus and Streptococcus pneumoniae.* J Biol Chem, 2001. **276**(40): p. 37141-8.
122. Nakajima, H. and T. Yokota, *Two Japanese Families with Pk Members.* Vox Sanguinis, 1977. **32**(1): p. 56-58.