Biochemical studies of carbohydrate blood group antigens

Carbohydrate phenotype in relation to cellular glycosyltransferases

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ABSTRACT

The possibility to alter the cell surface carbohydrate expression by insertion or deletion of glycosyltransferase genes is a powerful technique to study the biological function of selected carbohydrate antigens. However, shifting the equilibrium between competing glycosyltransferases might lead to unexpected phenotypic effects, such as accumulation of other carbohydrate antigens and exposure of antigen structures that normally are cryptic or present in minute amounts on the cell surface.

The present work explores the relationship of glycosyltransferase repertoire (genotype) and the resulting cell membrane glycolandscape (phenotype) from a glycosphingolipid perspective using two model systems where glycosyltransferase genes have been deleted or introduced. In addition to contributing to basal glycobiology, the experimental models have the potential to serve clinical purposes in the xenotransplantation and microbial adhesion fields. Glycosylation is not template driven, explaining why the cell membrane carbohydrate expression cannot be predicted from genotyping, but rather requires phenotyping. For phenotyping in this sense, antibody/lectin recognition of cell membrane antigens is insufficient necessitating structural determination.

Neutral and acidic glycolipids from tissues and cells were isolated by means of organic solvent extraction and repeated chromatography steps. Individual glycolipid components were purified by high performance liquid chromatography. The antigenic properties of the glycolipids were examined for reactivity with mono- and polyclonal antibodies, lectins and sera on thin layer chromatography plates. Structural elucidation was conducted by the combined use of mass spectrometry and proton nuclear magnetic resonance spectroscopy.

Our studies report an indisputable correlation between glycosyltransferase gene setup and the resulting glycolandscape phenotype. The study is also indicative of the renowned complexity of glycosylation, *e.g.* the glycosyltransferase dependence on the underlying protein/lipid backbone and the species-, individual- and organ-specific glycosyltransferase activity. In addition, we have identified several novel glycosphingolipids in pig tissues for which function and importance remain to be elucidated.

Key words: glycosphingolipid, glycosyltransferase, genetic modification, carbohydrate, mass spectrometry, proton NMR spectroscopy, xenotransplantation, GalT-KO pig

LIST OF PUBLICATIONS

I **Diswall M**, Ångström J, Schuurman H-J, Dor FJ, Rydberg L, Breimer ME. (2007) Studies on glycolipid antigens in small intestine and pancreas of alpha1,3galactosyltransferase knockout miniature swine. *Transplantation 84(10): 1348-56*

II **Diswall M**, Ångström J, Schuurman H-J, Dor FJ, Rydberg L, Breimer ME. (2008) Glycolipid studies in small intestine and pancreas of alpha1,3galactosyltransferase knockout miniature swine: alpha1,3GALT-KO animals lack alpha-GAL antigens and contain novel bloodgroup H compounds. *Transplantation Proceedings 40(2): 543-6*

III **Diswall M**, Ångström J, Karlsson H, Phelps C, Ayares D, Teneberg S, Breimer ME. Studies of alpha1,3-galactosyltransferase knock-out pig glycolipids and their reactivity with human and baboon antibodies. *Manuscript*

IV Löfling J, **Diswall M**, Eriksson S, Borén T, Breimer ME, Holgersson J. (2008) Studies of Lewis antigens and *H. pylori* adhesion in CHO cell lines engineered to express Lewis b determinants. *Glycobiology 18(7): 494-501*

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ABBREVIATIONS

AVR	Acute vascular rejection
CBA	Chromatogram binding assay
CHO	Chinese hamster ovary
ER	Endoplasmic reticulum
FucT	Fucosyltransferase
Gal	α1,3-galactosyl
GalT	Galactosyltransferase
Gb3	Globotriaosylceramide
GlcCer	Glucosylceramide
GalCer	Galactosylceramide
GSL	Glycosphingolipid
H. pylori	*Helicobacter pylori*
H type 1 / type 2	Core type 1 / 2 blood group H compound
HAR	Hyperacute rejection
HD	Hanganutziu-Deicher
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
iGb3	Isoglobotriaosylceramide
KO	Knockout
LacCer	Lactosylceramide
LacNAc	N-acetyllactosamine
LC-ESI/MS	Liquid chromatography/ mass spectrometry
Le^a	Lewis a
Le^b	Lewis b
Le^x	Lewis x
Le^y	Lewis y
NeuAc	N-acetylneuraminic acid
NeuGc	N-glycolylneuraminic acid
nLc4	Neolactotetraosylceramide
NMR	Nuclear magnetic resonance
PGC	Polyglycosylceramide
SiaT	Sialyltransferase
WT	Wildtype

Symbolic representations of monosaccharides mentioned in the thesis

⬤	Galactose (Gal)	⬤	Mannose (Man)
⬜	*N*-Acetylgalactosamine (GalNAc)	✦	Xylose (Xyl)
⬤	Glucose (Glc)	◆	*N*-Acetylneuraminic acid (Neu5Ac)
⬛	*N*-Acetylglucosamine (GlcNAc)	▲	Fucose (Fuc)
◨	Glucosamine (GlcN)		

THE EUKARYOTIC CELL MEMBRANE

All cells are bounded by a semipermeable lipid bilayer that constitutes the interface between the interior of the cell (endoplasmic side) and the surrounding fluid (exoplasmic side). As depicted in Figure 1, membrane matrix lipids consist mainly of choline phospholipids (outer layer) and amine phospholipids (inner layer). Cholesterol and membrane proteins are interspersed in the bilayer and the exoplasmic side is covered by a dense layer of glycans. This so-called *glycocalyx* or *glycolandscape* is composed of carbohydrates bound to lipids or proteins. Until the 1970´s phospholipids and membrane proteins were believed to be randomly distributed in the cell membrane according to Singer-Nicolson *fluid mosaic model* [1]. Simons and van Meer studied lipid sorting in epithelial cells and observed that certain lipids such as glycolipids were enriched in domains of the plasma membrane [2]. Later Simons and co-authors presented the *lipid raft* model which describes specialized microdomains in the exoplasmic leaflet enriched in cholesterol, glycolipids and selected transmembrane proteins [3].

Cell membrane carbohydrates

The major forms of membrane-associated glycans found in the plasma membrane in eukaryotic cells are proteoglycans, glycoproteins and glycolipids (outlined in Figure 1). *Proteoglycans* are heavily glycosylated proteins composed of a core protein with one or more covalently attached glycosaminoglycan chains. The long, linear glycan chains are negatively charged under physiological conditions due to the presence of sulfate and uronic acid groups and occur mainly in the extracellular matrices of connective tissues but can also be membrane bound [4]. The oligosaccharide unit of *glycoproteins* can be attached to the cell membrane via a protein or phosphatidylinositol-containing glycolipids. The most common types of protein-linked glycans are the N-glycans where the oligosaccharide is covalently attached to the amide nitrogen of asparagine [5] and the O-glycans where the carbohydrate is linked to the oxygen of the side chain of asparagine or threonine in the peptide chain [6].

Glycolipids are of the glycoglycerolipid or the glycosphingolipid (GSL) type. The former type is comprised of a glycan chain covalently linked to one or more glycerol hydroxyl groups whereas in the latter the glycan is covalently attached to the primary hydroxyl group of ceramide. GSLs are the focus of this thesis and their structures are described in more detail below.

Glycan chains are assembled from simple monosaccharides linked to one another via glycosidic linkages to any one of several positions on another monosaccharide in the chain. Glycosidic linkages exist in either α- or β-configurations. Altogether, a glycan chain of merely three monosaccharides can in theory adopt nearly 28,000 combinations; however, the number of biologically relevant glycan conformations is much smaller.
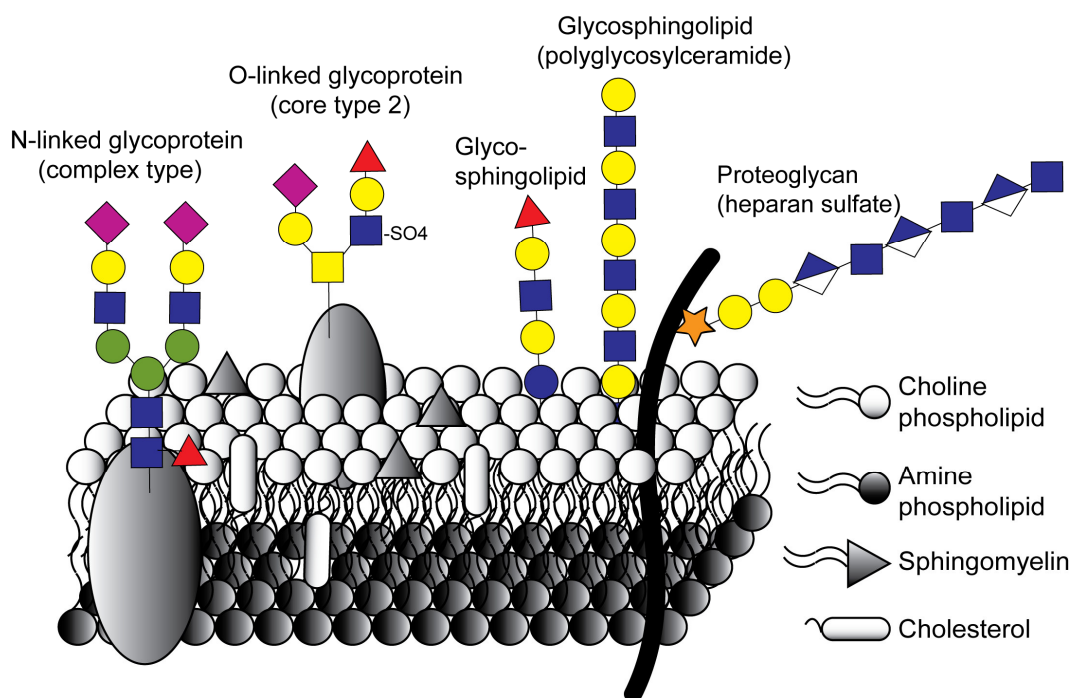
Figure 1. Schematic model of a mammalian plasma membrane. Symbolic presentation of monosaccharides is as recommended by the Consortium for Functional Glycomics (see page 6).

Aspects on functions and roles of glycoconjugates

Carbohydrate-protein interactions are characterized by low affinity interactions, often with K_d values in the μM to mM range. The low affinity is compensated for by multivalent interactions of multiple glycan binding proteins with their ligands, which increases the effective avidity, reviewed in [7]. The low affinity, multivalency and avidity of carbohydrate-protein interactions is the basis of the complex and highly regulated interplay between glycans and proteins and are considered to contribute to the fine-tuning of biological processes, as compared to protein-protein interactions which rather function as "on and off" switches.

The biological roles of glycans range from being seemingly disposable to absolutely essential and can be classified into two broad categories; i) structural and modulatory properties and ii) specific recognition of glycans by surrounding molecules. The second category may be subdivided in intrinsic and extrinsic interactions. Intrinsic functions include interactions of carbohydrates with glycan binding proteins (*i.e.* lectins) in cell-cell interactions, recognition of extracellular molecules or interactions between glycans on the same cell. One of the best characterized examples is the binding of P selectin on endothelial cells to sialyl Lewis x (Le^x) on P-selectin glycoprotein ligand 1 (PSGL-1) during recruitment of leukocytes to sites of infection and inflammation [8]. Another striking example is the Notch signalling receptor, whose activation by ligand binding is modulated by modification of O-glycans [9].

Extrinsic interactions include the recognition of glycan receptors by microbial adhesins, agglutinins and toxins [10]. Examples include the GSL globotriaosylceramide (Gb3) as receptor for shiga- and verotoxin [11], the GM1 ganglioside as receptor for choleratoxin [12], the blood group P1 antigen and other Galα4Gal-structures as receptors for pyelonephritic strains of *E. coli* [13] and the fucosylated blood group antigens H type 1, Lewis b (Le^b) and sialylated dimeric Le^x as receptors for *Helicobacter pylori* (*H. pylori*). The carbohydrate binding of *H. pylori* is further addressed in *paper IV*.

Glycan biosynthesis

The stepwise synthesis of glycans is mediated by the action of enzymatically active *glycosyltransferases*. Thus the glycosyltransferases are the primary gene products and the glycans are the secondary gene products. Most glycosylation takes place in the endoplasmic reticulum (ER) and Golgi apparatus, but nuclear and cytoplasmic glycosylation also occurs. GSL and glycoproteins often share many similarities in the terminal carbohydrate sequences, whereas core structures close to the protein or lipid carrier vary [14]. N-glycan synthesis is initiated in the ER by assembly of the oligosaccharide on a lipid carrier. The oligosaccharide is transferred to the polypeptide chain ("*en bloc"*) and is therafter processed and remodelled in the ER. Further glycosylation occurs in the Golgi apparatus. O-glycans are formed by stepwise addition of glycans in the Golgi apparatus. The ceramide used in GSL biosynthesis is synthesized on the cytoplasmic surface of ER and is thereafter transferred by specific membrane transporters ("flippases") to the ER lumen where the active center of galactosylceramide (GalCer) synthase is localized [15]. Further glycosylation occurs in Golgi apparatus. The structural similarities between terminal sequences of glycoproteins and GSLs indicate that the terminal glycan sequences are synthesized by common glycosyltransferases [14].

Glycosyltransferases

Most Golgi-localized glycosyltransferases are type II membrane proteins comprising a short N-terminal cytoplasmic tail, a transmembrane domain, a stem region and a large luminal enzymatically active domain [16]. More than 240 human glycosyltranferases have been cloned to date and are spread across more than 60 families, classified on the basis of their specificity for the donor sugar they transfer and the linkage they create. It was previously believed that one glycosyltransferase could merely synthesize one glycosidic linkage, but there is now increasing evidence of several glycosyltransferases creating the same glycosidic linkage on the same precursor but with different efficiency, and conversely of glycosyltranferases being able to catalyze several different glycosidic linkages [17]. Many factors contribute to structural determination of glycoconjugates at particular glycosylation sites, such as localization

and regulation of glycosyltransferases, precursor competition, mechanisms of catalysis and specificity for donor and acceptor sugars [18].

Glycosyltransferase localization

The view that glycosyltransferases acting early in glycosylation are localized in the *cis* or *medial* Golgi compartments whereas enzymes acting later in the pathway are found in the *trans* Golgi cisternae and *trans* Golgi network is tempting but hugely simplified. Most glycosyltransferases are distributed in overlapping gradients in the Golgi apparatus and use multiple mechanisms to adopt and maintain their sub-Golgi localization [19]. The mechanisms determining localization have been studied by purification, cloning and characterization of Golgi enzymes [20], analysis of glycosyltransferase mutants [21, 22] and using specific inhibitors of the glycosylation pathways.

Glycosyltransferases characterized to date share little or no amino acid sequence identity arguing that a common Golgi retention sequence is unlikely. The transmembrane region as well as the flanking domains of type II Golgi resident glycosyltransferases have been identified to maintain Golgi retention in some cases [23-25]. The *bilayer thickness hypothesis* proposes that glycosyltransferases localize in Golgi compartmental regions with a membrane thickness to match the length of their transmembrane domains, irrespective of amino acid composition [26]. The model relies on the observation that cholesterol increases the width of a lipid bilayer, and that cholesterol concentration increases in the *cis* to *trans* direction of the Golgi stack. The highest concentration is observed in the plasma bilayer and plasma membrane proteins also appear to have longer transmembrane regions than Golgi enzymes [27, 28]. However, numerous studies have shown that the length of the transmembrane domain does not fully account for Golgi localization and retention [29].

In the *oligomerization/kin recognition model* glycosyltranferases are suggested to form homo- or heterooligomers through interactions between their transmembrane and luminal sequences upon arrival at the appropriate compartment. This prevents them

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from entering vesicles for anterograde or retrograde transport [30] and also increases the efficiency of the stepwise assembly of monosaccharides. The model is supported by the finding that some transferase pairs that catalyze sequential reactions co-localize in specific Golgi compartments and co-precipitate from cell extracts. Also, experiments replacing the cytoplasmic tails of *medial*-Golgi resident transferases with an ER-retention signal have shown that the chimeric transferase was localized in the ER and formed a stable complex with ER-localized wildtype (WT) enzyme [31]. Oligomerization is suggested to occur amongst medial but not late acting Golgi transferases [32, 33]. Merely the formation of high molecular weight complexes does not prevent movement through the secretory pathway since *e.g.* collagen appear to be readily transported through Golgi [34].

Importantly, models describing sub-Golgi localization of glycosyltransferases are not mutually exclusive but rather believed to act in concert with other, known as well as unknown, mechanisms. It should be emphasized that observations made with one enzyme or cell type does not necessarily apply to other enzymes or cell types, or the situation *in vivo*. Fusion of the glycosyltranferase to a reporter protein or studies on chimeric proteins may also affect the localization and expression level of the enzyme.

GLYCOSPHINGOLIPIDS

The work in the present thesis focuses on studies of the carbohydrate moiety of GSLs. However, many of the concepts discussed also apply to protein-bound carbohydrates.

GSL structure and presentation in lipid rafts

Johann Thudichum is considered the father of sphingolipid research for his pioneering work during the second half of the 19th century. Among other achievements he isolated the monoglycosylceramide GalCer from human brain [35]. These novel molecular players were named sphingolipids after the Egyptian Sphinx because of the difficulty of isolation and their, at that time, unknown functional roles.
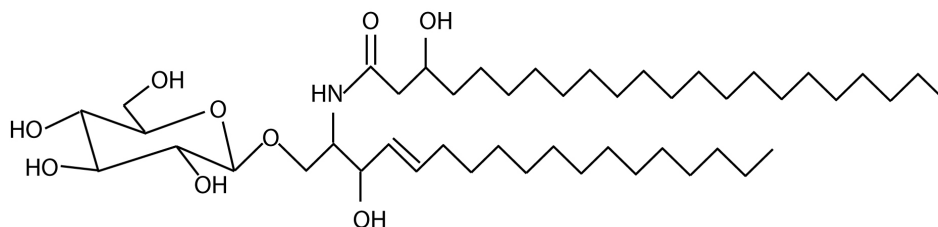
Figure 2. Glucosylceramide with d18:1 sphingosine and h22:0 fatty acid.

The amphipatic GSLs comprise a polar glycan head group linked to a non-polar ceramide and are generally localized in the exoplasmic leaflet of the plasma membrane bilayer with the hydrophobic ceramide embedded in the membrane and the hydrophilic glycan protruding away from the cell into the extracellular space [36]. The ceramide consists of a long chain base amide-linked to a fatty acid (Figure 2). Hence, there are three levels of variability of a GSL: the glycan, the long chain base and the fatty acid. All three variables may differ between GSLs from different species, cells and tissues. In mammals the d18:1 long chain base (sphingosine) is the most common and the fatty acids typically consist of 18-25 carbons.

A correlation has been established between the degree of hydroxylation of the ceramide and the chemical and physical stress the plasma membrane is exposed to [37]. Extensive hydrogen bond formation confers a stabilizing effect on the membrane and is proposed to account for the formation of lateral microdomains containing *e.g.* sphingolipids, cholesterol and specialized membrane proteins [38]. These microdomains serve as organizing centers for the assembly of signaling molecules, influence membrane fluidity and trafficking, and regulate neurotransmission [39]. About a decade ago the concept of a particular form of microdomains termed glycosynapses was developed [40]. In these microdomains GSLs are clustered with signal transducers, adhesion receptors or growth factors. Functional roles of these are indicated as carbohydrate-dependent adhesion creates a signal that is transmitted to the nucleus, reviewed in [41].

Nomenclature

GSLs are commonly divided into neutral and acidic GSLs. Acidic GSLs are further subdivided into gangliosides (sialic acid containing GSLs) and sulfatides (sulfate ester conjugated GSLs). Moreover, GSLs are classified on the basis of differences in their carbohydrate core structures. The most commonly occurring cores, or roots, in mammalian GSLs are summarized in Table 1. Core chains can be further elongated and generate a wide array of different carbohydrate structures such as the histo-blood group antigens. In this thesis the nomenclature, systemic names and abbreviations recommended by IUPAC/IUBMB (http://www.chem.qmul.ac.uk) are obeyed and symbolic presentation of monosaccharides employed in figures is as recommended by the Consortium for functional glycomics (http://www.functionalglycomics.org), outlined on page 6.

Table 1. *Root (core) structures of common mammalian GSLs.*

Root	Symbol	Root structure
1a) Lacto	Lc	Galβ3GlcNAcβ3Galβ4Glcβ1-Cer
b) Neolacto	nLc	Galβ4GlcNAcβ3Galβ4Glcβ1-Cer
2a) Ganglio	Gg	Galβ3GalNAcβ4Galβ4Glcβ1-Cer
b) Isoganglio	iGg	Galβ4GalNAcβ4Galβ4Glcβ1-Cer
3a) Globo	Gb	GalNAcβ3Galα4Galβ4Glcβ1-Cer
b) Isoglobo	iGb	GalNAcβ3Galα3Galβ4Glcβ1-Cer
4. Gala	Ga	Galα4Galβ1-Cer
5. Muco	Mc	Galβ4Galβ4Glcβ1-Cer

Carbohydrate blood groups

The glycan core structures in Table 1 can be further elongated by addition of monosaccharides in various combinations. Among other elaborations, the specific carbohydrate sequences representing the *blood group systems* are created (Table 2). Karl Landsteiner´s observation in the beginning of the 20th century that sera from some individuals agglutinated red blood cells from other individuals [42] eventually led to the classification of the ABO blood group system [43, 44]. The term *histo-blood group antigens* was introduced by Clausen and Hakomori and refers to carbohydrate antigens of both glycoprotein and GSL nature that are present in most cells and tissues [45]. Antibodies directed towards antigens lacking on an individuals own cells are believed to form early during childhood as an immunologic response to *e.g.* gastrointestinal bacteria expressing these carbohydrate epitopes, and is the basis for the self/non-self discrimination of the immune system.

Table 2. *Carbohydrate blood group systems discussed in this thesis. The systems are of both glycoprotein and GSL nature, except the P blood group antigens (marked with asterisk) which have only been found on GSLs.*

Antigen	Structure
ABH system	
H	Fucα2Galβ-R
A	GalNAcα3Fucα2Galβ-R
B	Galα3Fucα2Galβ-R
Lewis system	
Le^a	Galβ3(Fucα4)GlcNAcβ-R
Le^b	Fucα2Galβ3(Fucα4)GlcNAcβ-R
Le^x	Galβ4(Fucα3)GlcNAcβ-R
Le^y	Fucα2Galβ4(Fucα3)GlcNAcβ-R
P system*	
P^k	Galα4Galβ4Glcβ1-Cer
P	GalNAcβ3Galα4Galβ4Glcβ1-Cer
P1	Galα4Galβ4GlcNAcβ3Galβ4Glcβ1-Cer
Ii system	
i	Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ-R
I	Galβ4GlcNAcβ6(Galβ4GlcNAcβ3)Galβ-R
Galα3	Galα3Galβ4GlcNAcβ3-R

Distribution of GSLs

GSLs are ubiquitously distributed among all eukaryotic species and are also found in some bacteria. The expression of GSLs varies considerably between *e.g.* different species, individuals, strains and organs.

Species and strain variation

Numerous comparative studies have shown that GSL expression varies both quantitatively and qualitatively between different species [46, 47]. GSLs isolated from the same tissue, such as small intestine [48] and erythrocytes [49] from different species show considerably different GSL patterns. Interestingly GSL composition also varies between different strains of the same species [50-52]. The species variation is the basis for the immunological barrier leading to graft destruction in xenotransplantation (*papers I-III*).

Individual, organ and cell distribution

The polymorphism of glycosyltransferase genes partly explains the varying glycan expression in different individuals of the same species [45, 53]. Histo-blood group antigens of GSL nature are expressed in most mammalian epithelial cells with a distinct distribution between specific cell types and tissues, reviewed by Ravn and Dabelsteen [54]. The main factors responsible for variation are the blood group ABH, *Lewis* (*Le*) and *Secretor* (*Se*) gene status of the individual, the epithelial cell type and the cellular differentiation status. The type 1 GSLs (lacto-series) are synthesized in ectodermal tissues such as small intestinal mucosa and glandular epithelium [47]. Type 2 GSL chain GSLs (neolacto-series) are expressed in tissues of ectodermal and mesodermal origin such as skin and erythrocytes and in some endodermal tissues. Erythrocytes are also one of the richest source in type 4 carbohydrate chains (globo-series), also found in *e.g.* kidney, small intestine, liver and heart [55].

A remarkable example of the differential distribution of GSLs within the same organ is white and grey matter of the brain. Myelin-rich white matter is abundant in GalCer and sulfatide whereas grey matter contains a higher amount of gangliosides [56].

Furthermore, the apical and basolateral regions of polarized cell types such as epithelial cells differ in GSL composition [57, 58]. The differential distribution of GSLs in different organs necessitates characterization of multiple organs in an experimental model (*papers I-III*).

Developmental and pathological variation

Malignant transformation of cells is linked to distinct alterations of glycan profiles [59, 60]. Some GSLs are abundantly expressed in normal cells and reduced or deleted in transformed cells [61] and conversely, some GSLs are increased in certain types of tumors such as sialyl Le^a in gastrointestinal and pancreatic tumors [62], sialyl Le^x in gastrointestinal, breast and lung tumors [63] and the dimeric "Le^a-on-Le^a" and "sialyl Le^x-on -Le^x" GSLs in *e.g.* colorectal cancer [64-67]. Interestingly, blood group A-like antigens have been isolated from stomach cancer tumors of blood group O and B individuals [68] and the xenogeneic Forssman and N-glycolylneuraminic acid (NeuGc) antigens have been identified in various human tumors [69]. Some of these antigens are of oncofetal nature, meaning that their synthesis is repressed during development and de-repressed in tumors by as yet unknown mechanisms [70].

GLYCAN PHENOTYPE IN RELATION TO GLYCOSYLTRANSFERASE GENOTYPE

"It takes all the running you can do, to keep in the same place. If you want to go somewhere else, you must run twice as fast as that." declared The Red Queen in Lewis Carroll's Through the Looking-Glass [71]. The necessity for the individual to change in order to keep up with a changing environment is a central theme in glycobiology. The glycan repertoire balances between the intrinsic requirements for carbohydrates that are essential for cellular functions, and the microorganismal abuse of these for binding and invading host cells [72]. This may explain the remarkable structural variations of glycans in biology, which contribute to species diversity and perhaps even to the development of novel species. Approaches for understanding the biological functions of glycans include inhibition of specified steps in the glycosylation pathways. The consequences of such manipulations cannot however, be predicted *a priori*. There are many examples of secondary glycosylation effects resulting from genetic modifications of glycosyltransferases. The examples presented below focus on the GSL phenotypic effects, although similar alterations in glycoprotein repertoire are also likely to occur.

GSLs are proposed to be essential for the intercellular coordination of multicellular organisms, but disposable at the single cell level. This is based on the observations that i) cultured mouse cells completely deficient in glucosylceramide (GlcCer) synthase activity showed normal growth and survival [73] and ii) the knockout of the gene for GlcCer synthase in mice resulted in embryonic lethality during gastrulation between embryonic day 6.5 and 7.5 [74]. Further insight into the function of GlcCer synthase in brain development was obtained when neural cell type-restricted disruption of the GlcCer synthase was performed. The modification did not affect embryonic development, mice died however, at about 3 weeks of age, which indicated that GSLs are essential for postnatal brain maturation [75].

Naturally occurring glycosyltransferase variations

There are several examples of glycosyltransferases compensating for one another when a specific transferase is defect or lacking. Selected examples are briefly outlined below.

The ABO and Lewis blood group systems account for some of the most well-established glycosyltransferase mutations and provide numerous examples of enzymes competing for common precursors. A striking example is the elongation of the blood group H precursor by the *Le* gene encoded α1,3/4FucT to form the Le^b hexasaccharide in blood group O individuals. In blood group A and B individuals α1,3GalNAcT and α1,3GalT, respectively, compete with α1,3/4-FucT for the H precursor causing a weak or absent Le^b expression despite the presence of *Le* and *Se* genes. α1,3/4FucT can utilize the blood group A and B compounds to synthesize ALe^b and BLe^b epitopes, but α1,3GalNAcT and α1,3GalT cannot act on Le^b [76-79].

Another example from transfusion biology is individuals of the rare p phenotype, which lack the α1,4GalT (P^k tranferase) that normally produces Gb3. Affected individuals are thus unable to synthesize Gb3, globoside or downstream biosynthetic determinants [80]. Accumulation of lactosylceramide (LacCer), as well as increased levels of neolactotetraosylceramide (nLc4) and Le^x pentaglycosylceramide is indicative of a compensatory effect resulting from the metabolic block of the globo-series biosynthetic pathway.

The first report of a disruption of ganglioside biosynthesis associated with human disease was the loss-of-function mutation of GM3 synthase in infantile-onset symptomatic epilepsy syndrome [81]. Affected individuals lacked GM3 and downstream biosynthetic GSLs completely, whilst the precursor LacCer and GSLs of the globo- and neolacto-series had accumulated. In analogy with this finding the reduced activity of GM3 synthase observed in a human melanoma cell line also resulted in accumulation of LacCer [82].

Genetic modifications of glycosyltransferases

GalCer synthase knockout mice were as expected unable to synthesize GalCer and sulfatide. Increased levels of GlcCer were observed in the myelin sheath, partly compensating for the loss of GalCer and sulfatide [83, 84]. The myelin had a grossly normal ultrastructural appearance but alterations of the paranodal and periaxonal myelin domains resulted in electrophysiological deficits in the central and peripheral nervous systems from about postnatal day 12-16 and mice developed severe tremor and hindlimb paralysis [85]. Similarly, a GM3 synthase knockout mouse strain completely lacked GM3 and downstram gangliosides whilst gangliosides of the competing ganglioside series (o-series) had accumulated. The mice suffered from enhanced insulin sensitivity but had an otherwise normal phenotype [86].

A transgenic mouse model expressing Le^b was established to study the molecular events following Le^b mediated adhesion of *H. pylori* to gastric epithelium [87, 88]. Three novel GSL structures were isolated: Le^b-like hexaglycosylceramide on ganglio core, isoglobotriaosylceramide (iGb3) with an internal blood group B determinant, and elongated fucosyl-gangliotetraosylceramide [88]. The synthesis of the latter two GSLs was not believed to be due to the genetic modification *per se* as no α3- or α4-linked fucoses were present in these compounds, but may, however, reflect an overall change in the GSL pattern upon disturbance of the glycosyltransferase repertoire.

Genotype-phenotype correlations are difficult to establish and impossible to predict

Transgenic incorporation or knockout of glycosyltransferase genes in cells or organisms give mixed results depending on the glycosyltransferase targeted and the cell type used. In general, modification of glycosyltransferases that act early in the ER/Golgi pathway and are necessary for biosynthesis of core structures, will lead to more severe abnormalities or embryonic lethality compared to when glycosyltransferases acting later in the biosynthesis, and are involved in synthesis of

terminal epitopes, are modified. The *genetic background*, meaning all other genes in an organism, differs between species and individuals and provides an explanation for the differential effect of genetic modifications in two different organisms, such as the two independent GM2/GD2 synthase knockout mouse strains of which one suffered from lethal audiogenic seizures [89], whereas mice from the other strain had a milder phenotype [90].

EXPERIMENTAL SYSTEMS USED IN THE PRESENT WORK

Much of our understanding of mammalian glycobiology has been obtained and extrapolated from studies of less complex organisms. In the present work we have used two model systems as experimental windows where we have studied the effect of genetic modifications of glycosyltransferase genes on the glycolandscape. In addition to contributing to basal glycobiology, the experimental models have potential to serve clinical purposes in the *xenotransplantation (papers I-III)* and *microbial adhesion* (*paper IV*) fields.

Genetically modified pig used in xenotransplantation

Clinical transplantation of organs between individuals of the same species (allotransplantation) is at present the effective therapy to cure patients with end-stage organ failure. However, the demand for organs from patients on the transplant waiting lists are not by far met by the supply of donated organs. Therefore xenotransplantation, meaning transplantation of organs between different species, is explored as a potential alternative to allotransplantation [91].

General aspects on xenotransplantation

Pig is considered the most suitable donor in xenotransplantation for several reasons. It is easy to maintain and breed, reproduces in large litters, organs are appropriate in size and ethical objections are less for pig than for nonhuman primates such as chimpanzee.

The type of rejection a graft encounters depends on whether the transplantation is *concordant* or *discordant*. Concordant transplantation refers to transplantation between phylogenetically related species such as nonhuman primates and humans and the rejection response is similar to that observed in allotransplatation. Discordant transplantation refers to non-related species such as pigs and humans and gives rise to

a rapid and violent response, termed *hyperacute rejection* (HAR) due to preformed antibodies that bind to antigens present in the transplanted organ [92]. HAR occurring in xenorejection has many similarities with HAR encountered in ABO incompatible allotransplantation [93].

The Galα3 antigen/antibody barrier

The initiating event of HAR is the binding of human preformed antibodies to the carbohydrate epitope Galα3Galβ4GlcNAc-R (Galα3 antigen, Galili antigen) in pig tissues. α1,3GalT primarily converts N-acetyllactosamine (LacNAc) but is also able act on other core chain types such as Gb3 [57, 94] and Le^x [95]. Galα3 expression on GSLs and glycoproteins appears to be ubiquitous in pigs [96] and the highest expression levels are reported in vascular endothelium, kidney proximal tubules, bronchioli and alveoli [97].

 The Galα3 antigen is expressed by New World primates and many nonprimate mammals, but is absent from cells and tissues of Old World primates including humans due to an inactivation of the gene encoding the α1,3GalT [98]. In agreement with Landsteiner´s principle [42] species lacking the Galα3 epitope express natural anti-Gal antibodies in serum [99], likely because of immunization through exposure to gastrointestinal microbial antigens similar or identical to the Galα3 epitope.

Anti-Gal antibodies are produced by B-cells and are of IgM, IgG and IgA type (IgM>IgG>IgA), constituting approximately 4% and 1% of the total human IgM and IgG pools, respectively [100]. Upon binding of anti-Gal Abs to Galα3 antigens on pig graft endothelium complement activation is initiated through the classical pathway (Figure 3).
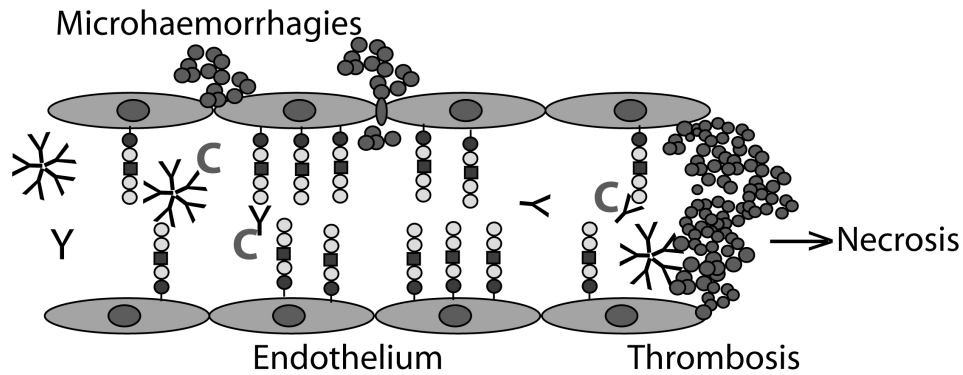
Figure 3. *HAR is initiated by recipient preformed antibodies (mainly IgM and IgG) directed against Galα3 antigens on pig endothelial cells. Antibody binding activates complement and mediates cell injury, microthrombosis and subsequent graft necrosis.*

Strategies to overcome HAR

Many different attempts have been tried in order to realize pig-to-human xenotransplantation, either by altering the recipient immune response or by modifying the donor. Methods for removing anti-Gal antibodies include depletion of total plasma proteins by plasmapheresis [101, 102], removal of IgG and IgM by non-specific antibody adsorbents [103] or specific removal of anti-Gal Ig by immunoadsorption on Galα3Gal-affinity columns [104]. Also, injecting the patient with soluble saccharides containing the Galα3Gal epitope to neutralize anti-Gal antibodies have been tried [105, 106] as well as injecting soluble complement inhibitors such as cobra venom factor or soluble complement receptor type 1 to inhibit the receptor complement cascade [107].

A strategy to reduce Galα3 antigens on pig cells was transgenic expression of human α1,2FucT to compete with α1,3GalT for their common substrate LacNAc. The strategy was successful in mice and resulted in a 90% reduction of Galα3 on the cell surface [108-110] whilst the reduction in pig was only about 50% and cells were still subject to lysis in cytotoxicity assays [111]. Transgenic mice and cultured porcine cells were produced that expressed human α-galactosidase in order to cleave terminal Galα3 antigens, however only a partial reduction was reported. Co-expression of α-

galactosidase and human α1,2FucT resulted in a 99% reduction of terminal Galα3 *in vitro* but this technology was never developed to whole animal experiments [112]. Since the complement system plays an essential role in HAR pigs expressing human complement regulators such as decay accelerating factor (DAF, CD55) or membrane co-factor protein (MCP, CD46) [113] or protectin (CD59) [114] have been produced. Although grafts from these pigs did not undergo HAR they were not protected from later rejection mechanisms such as acute vascular rejection (AVR) [115].

α1,3GalT-KO pig

The gene encoding α1,3GalT was successfully deleted in a mouse model as early as 1996 [116]. Mice developed cataracts but were otherwise healthy. Since Galili reported that pig cells express about 500 times the number of Galα3 epitopes than does the mouse it was doubted whether the latter would develop properly without expression of Galα3 antigens [117]. The nuclear transfer technique was not available for large animals such as pigs until fairly recently when two different groups reported the production of healthy α1,3GalT knockout (KO) pigs [118, 119]. Expectations were high when the first pre-clinical α1,3GalT-KO pig-to-nonprimate transplantation trials were performed. The initial experience showed that HAR was overcome. Kidney co-transplanted with thymus had a survival time of up to 83 days [120] and heterotopic cardiac transplantation had a survival time of up to 179 days [121]. However, survival times are only slightly pro-longed compared to the 113 days reported in the CD46 transgenic pig heart to baboon setting where Galα3-positive pigs were used [122, 123].

At present it is not clear whether the thrombotic microangiopathy seen in rejected α1,3GalT-KO pig tissues is due to the immune system or to molecular incompatibilities between the pig and primate coagulation system [121, 124]. Preformed or elicited antibodies were not detected and the mixed lymphocyte reaction remained unresponsive. Nevertheless, focal Ig and complement deposition were observed in the grafted hearts suggesting that an immune response was involved. This indicates that antigens other than Galα3 contribute to organ rejection and the umbrella

term *non-Gal antigens* is used for these as yet unknown protein and/or carbohydrate antigens.

The quest for the elusive non-Gal antigens from a carbohydrate perspective

The priority in the xenotransplantation field in the "post Galα3 era" is focused on identifying non-Gal antigens that may be targets for preformed or induced human antibodies. As HAR is overcome the present goal is identification of xenoantigens involved in later stages of rejection. A number of non-Gal glycans have been proposed, such as iGb3, Forssman, LacNAc and NeuGc-containing carbohydrates. However, Buhler *et al.* reported that terminal Galα, GalNAcα, Galβ and N-Hex residues were not involved in anti-non-Gal antibody binding since porcine erythrocytes treated with the respective exoglycosidases in the presence of soluble Galα3 trisaccharide did not affect baboon serum antibody binding [125]. Potential non-Gal antigens and their possible involvement in antibody recognition are addressed in *papers I-III*.

A Le^b expressing CHO cell line as a model for *H. pylori* infection

The cloning of the majority of known glycosyltransferases and the ability to express these in different cells have made engineering of specific glycans in well-characterized cell lines possible. Chinese hamster ovary (CHO) cells are often preferred for production of recombinant therapeutic glycoproteins and in glycosylation engineering. CHO cells have been widely employed to study mammalian carbohydrate functions and are useful when characterizing cell membrane expression of novel glycosyltransferase genes. These cells have a well established glycan repertoire and a relatively limited number of endogenous glycosyltransferases. They do not express any α2,6-SiaTs [126], α1,2- or α1,3-FucTs [127] or other tranferases of the blood group family [128, 129]. O-glycans are merely core 1 based [130, 131], and N-glycans are predominantly complex-type with only type 2 core [132]. The GSL repertoire in CHO cells is dominated by GM3, LacCer and low levels of GlcCer.

H. pylori has evolved to recognize specific carbohydrates on host cells [10]. The gram-negative, spiral-shaped bacterium was first isolated from human stomach mucosa by Warren and Marshall in 1984 [133]. Today *H. pylori* infection is considered the world´s most common infection, affecting about 50% of the human population. Most often the infection is asymptomatic, however about 10% of the affected individuals develop symptoms such as gastritis, gastric and duodenal ulcers and stomach cancer [134, 135]. *H. pylori* survives low pH, and has thereby created a niche in the hostile environment of the stomach [136]. Binding of *H. pylori* to host cells is mediated by bacterial membrane proteins called adhesins. Two adhesins have been described in *H. pylori*; SabA [137] and BabA [138]. SabA binds to sialylated dimeric Le^x structures [137] and BabA is specific for the fucosylated blood group antigens H type 1 and Le^b on *e.g.* gastrointestinal epithelial cells [138, 139].

A cell line expressing Le^b may serve a useful *in vitro* model for molecular and cell biological studies of *e.g.* Le^b-mediated *H. pylori* adhesion to host cells. Synthesis of the Le^b epitope requires the concerted action of several glycosyltransferases. H type 1

is synthesized from the type 1 precursor by the action of α1,2FucT (encoded by the *Se* gene). Thereafter α1,3/4FucT (encoded by the *Le* gene) adds a second fucose thus forming the Le^b epitope. Since CHO cells rapidly reach confluence, have a well-defined glycosyltransferase repertoire and are well-characterized from a regulatory point of view, they were the model system of choice for producing the Le^b epitope for studies of BabA positive *H. pylori (paper IV)*.

OBJECTIVES

Altering the cell surface carbohydrate antigen expression by genetic modification of glycosyltransferase genes can be used to study the biological function of selected carbohydrate antigens. However, disrupting the balance between competing glycosyltransferases may lead to unexpected phenotypic effects, such as increased levels of other carbohydrate antigens and exposure of antigen structures that are normally cryptic on the cell surface. The *general aim* of this thesis was to correlate the carbohydrate phenotype with the cellular glycosyltransferases using a knockout pig model and transfected CHO cells.

The *specific questions* we aimed to address were:

Does the $\alpha 1,3$GalT-KO in pig

i) eliminate all Galα3-terminated GSL antigens?

ii) produce new, compensatory GSL antigens?

iii) affect the human and baboon immune response towards porcine xenografts?

Does transfection of CHO cells

i) mediate synthesis of lipid- and protein-linked Lewis antigens?

ii) produce cells that may be used as *in vitro* tools for *H. pylori* infection studies?

METHODOLOGICAL CONSIDERATIONS

Tissue and cell specimens

Small intestine and pancreas were obtained from α1,3GalT-KO and WT miniature swine [119] whereas heart and kidney were obtained from α1,3GalT-KO pigs from a different strain [118]. Knockout of the gene encoding α1,3GalT (*GGTA1*) was in both strains accomplished by means of nuclear transfer which resulted in pigs where one allele of the locus was knocked out. A second round of nuclear transfer eliminated both alleles of *GGTA1*.

CHO-K1 cells were transfected with cDNA encoding β1,3GlcNAcT-VI, β1,3GalT-V, α1,2FucT-II and α1,3/4FucT-III. Two Le^b positive clones, 1C5 and 2C2, were studied.

Isolation and purification of GSLs

The methodological approach to isolate and structurally characterize GSLs is summarized in Figure 4. Experimental details are described in the respective articles and in references cited therein and will not be reiterated in this section.
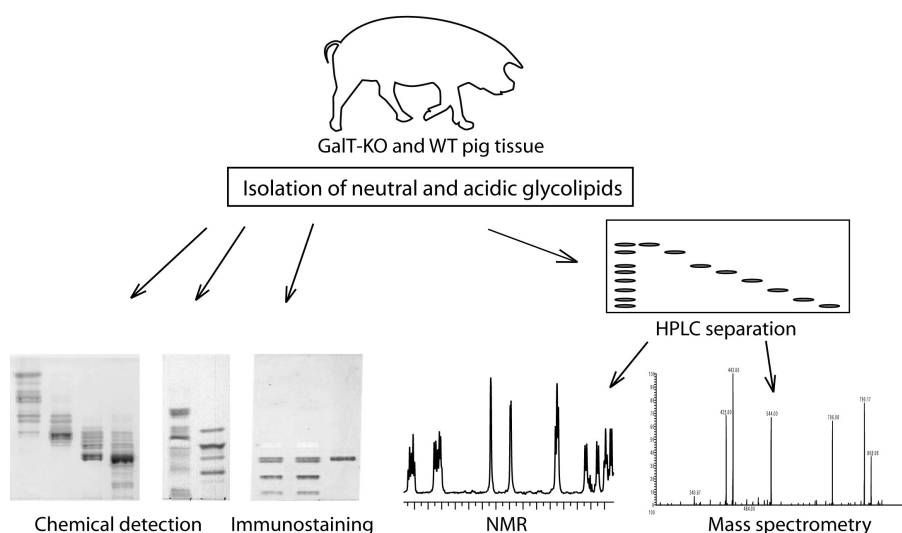
Figure 4. GSLs were isolated from tissues or cells and visualized with chemical reagents, antibodies, lectins and sera. Individual GSL components were purified by HPLC and analyzed by proton NMR spectroscopy and mass spectrometry.

Isolation procedure

An advantage in studying GSLs is the one-to-one ratio of glycan and ceramide as opposed to glycoproteins where several glycans normally are linked to one peptide sequence which make interpretation complex.

Numerous protocols for isolation of GSLs have been described [140]. In this thesis the method developed by Karlsson was employed [141]. Total lipids extracted from tissue contain a cocktail of phospholipids, sphingomyelin, GSLs and non-lipid contaminants. Mild alkaline methanolysis cleaves labile ester bonds of phospholipids and triglycerides. Some GSLs containing O-acyl groups are alkali labile and may also be lost [141]. GSLs are purified by repeated silicic acid column chromatography of both native and acetylated derivatives. The acetylation procedure shifts the chromatographic properties of neutral GSLs into the nonpolar interval whilst sphingomyelin remains in the polar interval thus enabling separation of these. Ion exchange chromatography separates neutral GSLs from acidic GSLs (sulfatides and gangliosides) and also removes N-acetylated derivatives of alkali stable phospholipids. In this work we separated the neutral GSLs into sub-fractions with approximately 1, 2-3 and ≥3 sugars (slow-moving fraction) to enrich blood group active GSLs.

Polyglycosylceramides (PGCs, >30 sugars) are not extracted using the above preparation procedure due to their highly polar properties. Miller-Podraza and co-authors developed a method where tissues or cells are peracetylated prior to organic solvent extraction [142]. PGCs have been shown to express a similar repertoire of terminal epitopes as shorter GSLs does [143, 144] and were therefore not included in our studies. PGCs in the membrane may more likely bind to antibodies and lectins, but for our experimental purposes shorter GSLs are more convenient to handle.

When isolating GSLs from limited amounts of biological material, such as the cultured CHO cells in *paper IV*, a condensed protocol with fewer chromatography steps is used in order to minimize loss of material.

High Performance Liquid Chromatography

When sufficient amount of GSLs is available these may be purified by high performance liquid chromatography (HPLC) in order to resolve individual GSL species. The liquid phase gradient is tailored depending on the polarity of the GSLs to be separated. The chromatographic properties of GSLs in a mixture may be so similar that it is not possible to separate them in native form (illustrated in *paper I; figure 2*). Acetylation of the GSLs influences the relative polarity and hence the elution rate on the HPLC column and may therefore be helpful to resolve individual GSL components [145].

Characterization of GSLs

Structural determination of GSLs in the present thesis means elucidation of type, number and sequence of monosaccharides and anomericity of glycosidic bonds. For this purpose a combination of binding studies, mass spectrometry and proton NMR spectroscopy is preferably used. The structures in the present thesis are in some cases tentative due to limited amounts of material.

Thin Layer Chromatography

High performance thin layer chromatography (HPTLC) can be used to monitor each step of the isolation procedure. HPTLC plates are coated with a superfine layer of silica gel particles and offer an exceptional resolution of individual GSL compounds. The chromatographic separation is mainly determined by the polarity, *i.e.* carbohydrate chain length and to some extent also the degree of hydroxylation and number of hydrocarbons of the ceramide [146]. Neutral GSLs are elegantly resolved using chloroform, methanol and water mixtures, whereas a solvent containing aqueous salts such as KCl or $CaCl_2$ is preferably used for the separation of gangliosides. A single band on a chromatogram usually contains more than one component wherefore it is helpful to use several different solvent systems.

Chromatographed GSLs can be chemically detected using orcinol or anisaldehyde [141]. Anisaldehyde stains all carbohydrates in characteristic greenish colors, where the shade of green reflects the particular carbohydrate structure. Gangliosides may be detected using the Svennerholm resorcinol-hydrochloride reagent that specifically stains gangliosides in a characteristic purple shade [147]. The resorcinol reagent can also be used to quantify gangliosides in microtiter wells [148].

Chromatogram Binding Assay

Binding of antibodies, lectins, bacteria and viruses directly to chromatographed GSLs using the chromatogram binding assay (CBA) [141, 149] provides information of the glycan chain length as well as the binding epitope [141]. Chemical staining of the GSLs in parallel with immunostaining yields valuable information on the concentration and purity of individual GSL components.

Monoclonal antibodies that appear to be specific in agglutination or immunohistochemical assays often show a high degree of unspecificity when used in CBA. A recent study showed that that more than half of 27 antibodies tested by glycan microarray profiling were cross-reactive with other glycans [150]. Therefore, a panel of different antibodies with well-known specificity is useful when assessing GSL reactivity. The temperature and pH dependence should also be evaluated for each antibody as these factors may affect antibody binding and specificity. Structural information should preferably be established in combination with biophysical methods such as mass spectrometry and NMR spectroscopy (see below).

Presentation of GSLs on silicic acid-coated plates does not reflect the actual presentation in biological membranes. The elusive coating of the plates with polyisobutylmethacrylate is believed to "invert" the GSL so that the carbohydrate points away from the silica gel and becomes accessible for binding; however the actual details are not known. The use of model membranes in biosensor-based methods such as surface plasmon resonance (SPR) has revealed that the composition and fluidity of the lipid membrane is a critical parameter in protein-GSL recognition, reviewed in

[151]. SPR has a higher demand for absolutely pure GSLs whereas the chromatography step in CBA enables separation of individual components in a mixture. Loading of GSLs on HPTLC plates results in a high concentration of epitopes in a small area, far from the presentation in lipid rafts.

Mass spectrometry

Gothenburg has a long tradition of mass spectrometry starting with the pioneering work by profs. Stina and Einar Stenhagen [152]. Development of mass spectrometry techniques for GSL analysis revolutionized the field [153]. The first mass spectrum of a GSL was obtained using electron ionization mass spectrometry of trimethylsilylated GlcCer [154]. Subsequently, permethylated [155] and permethylated/reduced [156] derivatives increased the stability and volatility of the molecules.

Today a multitude of techniques for mass spectrometry analysis of native or derivatized GSLs is available. Techniques differ in ionization, separation and detection of the analyzed component. Generally, mass spectrometry provides information on molecular weight, number and type of monosaccharides, sequence and branching of saccharide units and ceramide type.

In *paper III* oligosaccharides were enzymatically released from the ceramide prior to mass spectrometry analysis. The enzyme *Rhodococcus sp* cleaves the linkage between the saccharide and ceramide of acidic and neutral GSLs, producing intact oligosaccharides and free ceramides [157]. The enzyme cannot, however, cleave monoglycosylceramides, glycoglycerolipids or N- or O-glycans. The efficiency with which the enzyme hydrolyzes glycosidic linkages varies depending on GSL series, explaining why the technique is not suitable for quantitative estimations of individual GSLs. Analysis of released oligosaccharides circumvents the complexity of solubilization and ionization of the amphiphatic GSLs, but information on the ceramide is lost. On-line liquid chromatography (LC) separation prior to mass spectrometry analysis adds information of the oligosaccharide *e.g.* regarding the core chain type.
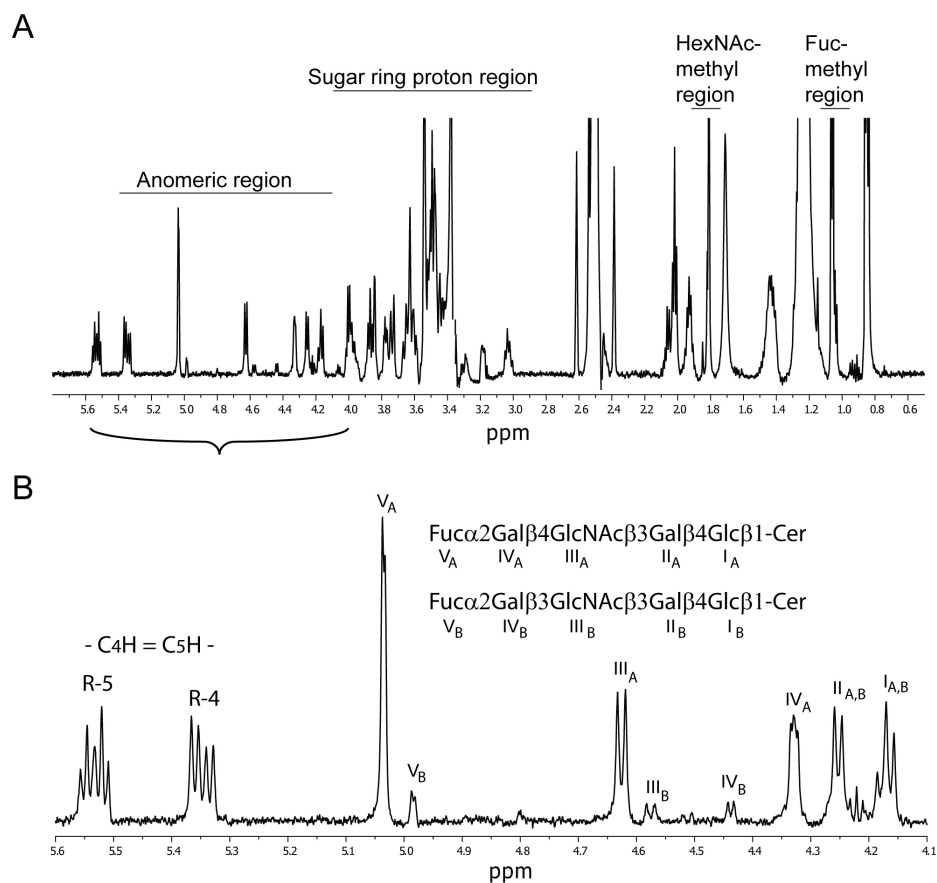
Proton NMR spectroscopy

In the late 1970´s Falk *et al.* described a method to analyze permethylated and reduced GSLs dissolved in $CDCl_3$ by proton NMR spectroscopy [158-160]. Shortly thereafter Dabrowski *et al.* reported the analysis of native GSLs using DMSO and D_2O [161, 162]. The latter method is less time-consuming and non-destructive, *i.e.* sample can be recovered and used for further experiments.

The NMR spectroscopy phenomenon is based on the fact that atomic nuclei in a strong magnetic field absorb radiation at characteristic frequencies. Interactions between the intrinsic magnetic field of a particular nucleus and the extrinsic, applied, magnetic field determines the chemical shift of each signal. NMR spectroscopy provides information on monosaccharide composition, sequence, conformation and anomericity of glycosidic bonds. Information is also obtained regarding ceramide composition, *e.g.* on the degree of hydroxylation and presence of double bonds. NMR spectroscopy from a glycoconjugate perspective is described in detail in [163].

The number of spin systems, *i.e.* the different sugar residues, can be revealed by 1D NMR. For resolving the connectivity of the individual spin systems 2D NMR is useful. Numerous 2D NMR techniques are available including correlated spectroscopy (COSY) and nuclear overhauser effect spectroscopy (NOSEY). In this thesis we have used a combination of 1D and COSY NMR experiments (*papers I-III*).

Establishment of the oligosaccharide is mainly derived from the anomeric region of the spectrum (4.1-5.4 ppm), where the anomeric proton (H-1) of each monosaccharide gives rise to a signal at a specific chemical shift (Figure 5). The chemical shift gives information on *e.g.* type of monosaccharide and glycosidic linkage. Supplementary information from the other ring protons, whose shifts and spatial arrangements often may be deduced from 2D ^{1}H-^{1}H NMR experiments such as COSY and NOESY, are frequently required. Integration of the H-1 signals establishes the number of monosaccharides in the sequence. The anomeric signal is in the form of a doublet due to coupling with the H-2 proton, where the anomericity (α or β linkage to the next

sugar) can be deduced from the splitting (J-coupling) of the signal. α-anomers have a smaller $J_{1,2}$ coupling constant (1-4 Hz) than β-anomers (6-10 Hz). H-5 proton resonances, *e.g.* from fucose and other ring protons may shift into the anomeric region, whereas resonances from ring protons and methyl groups from hexosamine and fucose are found upfield of the anomeric region. Resonance regions are outlined in Figure 5. The elucidation of spectra is greatly facilitated by comparison with reference spectra of well-known structures. Since GSLs in a DMSO/D_2O mixture form temperature-dependent micelles, reference spectra must be recorded under the same conditions in to allow comparative interpretation [164].

Figure 5. A) Full 1D proton NMR spectrum of H5 type 2 (90%) and H5 type 1 (10%). B) Anomeric resonances of H5 type 2 (A) and H5 type 1 (B) denoted with roman numerals I-V. Note the difference in J1,2 of α-anomers and β-anomers. R-4 and R-5 refer to the-C4H=C5H- protons of the sphingosine base.

RESULTS AND DISCUSSION

The four papers presented in this thesis all focus on carbohydrate expression from a GSL perspective in relation to a defined change in glycosyltransferase gene setup. In this section results are briefly summarized and discussed whereas details of interpretation are given in the respective articles.

α1,3GalT-KO effects

Papers I-III investigate if α1,3GalT-KO pig expresses residual Galα3 antigens and whether the genetic modification also results in other alterations of the GSL pattern. As discussed throughout the thesis the GSL pattern differs markedly between different strains, individuals and organs. We have therefore examined small intestine, pancreas, kidney and heart. Pigs have a blood group AO system [165], comparable to the human ABO system, and in this work we investigated organs of both blood groups.

Lack of Galα3 GSL antigens

When our project was initiated the first data from clinical trials using α1,3GalT-KO pig donors had recently been obtained [120, 121, 166]. Results showed some prolongation of survival or these organs in nonhuman primates, but were not the success aimed for. An early study reported that α1,3GalT-KO mice and pig fetal fibroblasts still expressed low levels of the Galα3 antigen [167, 168]. It was therefore proposed that residual Galα3 antigens remained also in α1,3GalT-KO pigs thereby compromizing graft survival.

Immunostaining

Immunostaining of WT organs identified Galα3-positive neutral fractions migrating in the 5, 7/9 and >12 sugars region (small intestine and pancreas), 5 and 7/9 sugar regions (heart) and 5, 6, 7/9 and >12 sugar regions (kidney), in accordance with previous studies [96, 169, 170]. This is illustrated for the heart and kidney GSLs in Figure 6. The figure also shows the complete absence of Galα3 determinants in α1,3GalT-KO tissues.
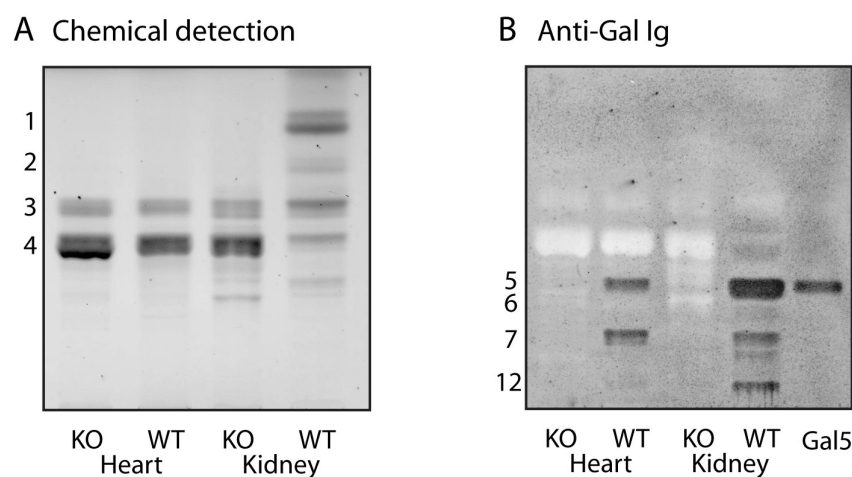
Figure 6. *α1,3GalT-KO and WT pig heart and kidney GSLs chemically detected with anisaldehyde (A) and immunostained with human affinity-purified anti-Gal Ig (B). α3Gal-positive bands were detected in the 5 and 7 sugar regions of WT heart and in the 5, 6, 7/9 and >12 sugar regions of WT kidney. Purified Galα3nLc4 (Gal5) was used as a reference. Numbers to the left denote number of sugars in the glycan chain.*

One Galα3-positive compound only was seen amongst acidic GSLs. This compound was detected in the WT kidney and probably corresponded to the $VI^3NeuAcVI'^3\alpha Gal$-iso-nLc8Cer or $VI^3NeuAcVI'^3\alpha GalV'^3\alpha Fuc$-iso-nLc8Cer previously reported by Bouhours and co-authors [171].

Structural determination by mass spectrometry and proton NMR spectroscopy

In order to confirm the absence of Galα3 antigens in KO tissues and to establish their structures in WT tissues we analyzed GSLs by means of mass spectrometry and proton NMR spectroscopy. In *paper III*, LC-ESI/MS was used for rapid and sensitive screening of the heart and kidney GSLs. Determination of GSLs from these organs are

of utmost importance since heart and kidney have been evaluated in preclinical α1,3GalT-KO pig-to-baboon transplantation trials [120-122, 124]. In WT tissues we showed that the pentaglycosylceramide detected by immunostaining (Figure 6) corresponds to the Galα3nLc4 compound described in several studies before [96, 169, 170, 172]. We also identified a hexasaccharide in kidney, most probably corresponding to Galα3Le^x [95] and a heptasaccharide in kidney and heart most probably corresponding to Galα3nLc6 [173].

LC-ESI/MS did not detect oligosaccharides exceeding 8 sugars in length, although immunostaining revealed that Galα3 antigens with >12 sugars were present in WT kidney. This may be due to irreversible binding of long-chain saccharides to the LC column or due to inability of the enzyme to cleave these structures.

In *papers I and II* we analyzed small intestine and pancreas. When these experiments were performed we did not have access to mass spectrometry, but the amount of WT and α1,3GalT-KO small intestinal GSLs allowed HPLC purification and subsequent analysis using proton NMR spectroscopy. Similarly to kidney and heart the Galα3-positive pentasaccharide in small intestine corresponded to Galα3nLc4 [169, 170].

Small intestine from pig and other mammals has previously been shown to contain large amounts of long-chain blood group active GSLs [174-178]. The intricacy of small intestinal GSLs is also reflected by their specific anatomic localization [57, 58, 179]. When we analyzed GSLs in the 7/9 sugar region a complex mixture of branched compounds carrying blood group H and α3Gal determinants was revealed.

Two different Galα3-antigens were identified (*paper II*). One of these was a linear nona- or heptaglycosylceramide with a Galα3Galβ4GlcNAcβ3 trisaccharide linked to nLc6 or nLc4. From an NMR point of view it was not possible to establish the number of lactosamine disaccharide units, but since it co-eluted in the same HPLC fraction as 9 and 10 sugar structures it was believed to be a nonaglycosylceramide. However the

detection of Galα3nLc6 in the kidney and heart *(paper III)* argues in favor of the heptaglycosylceramide annotation. The second Galα3 antigen in the 7/9 sugar region was a branched decaglycosylceramide with Galα3Galβ4GlcNAcβ3/6 branches on a type 2 tetrasaccharide core [180], shown in Figure 7A.

Analysis of the GSLs migrating in the >12 sugar region revealed a complex mixture of at least three different overlapping triantennary pentadecaglycosylceramides in WT and α1,3GalT-KO tissues with the general structure X-Galβ3/4GlcNAcβ3(Y-Galβ3/4GlcNAcβ6)Galβ4GlcNAcβ3(Z-Galβ3/4GlcNAcβ6)-nLc4 [181]. X, Y and Z were shown to be Fucα2, Galα3 or Galα4 (Figure 7B). The decaglycosylceramide and the pentadecaglycosylceramide have previously been isolated from rabbit erythrocytes [180, 181], but to our knowledge, this is the first time they are reported in pig tissues.
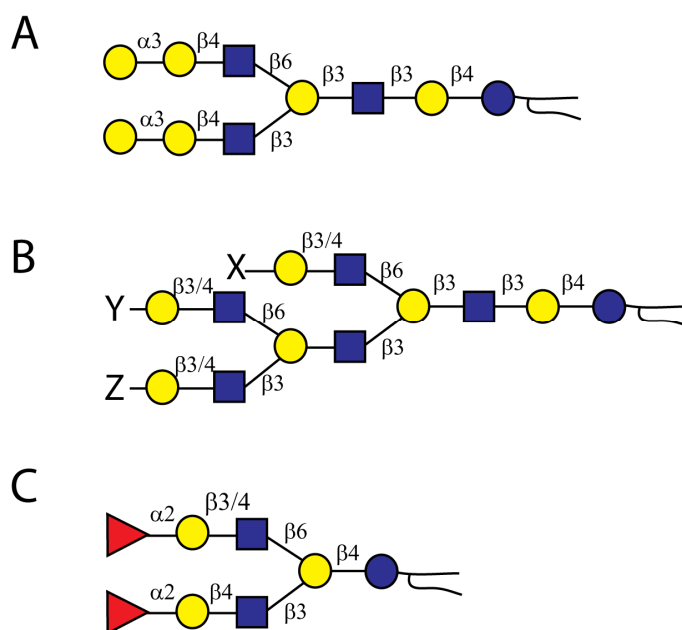
Figure 7. A) decaglycosylceramide identified in WT small intestine. B) pentadecaglycosylceramide identified in α1,3GalT-KO and WT small intestine. X, Y and Z correspond to Fucα2, Galα3 or Galα4. The Galα3 epitope was not detected in the α1,3GalT-KO tissue. C) two novel octaglycosylceramides with a type 1 or type 2 branch from α1,3GalT-KO pig small intestine. Compounds A) and B) have previously been reported in rabbit erythrocytes with X,Y and Z as Galα3 [180, 181].

It should be emphasized that Galα3antigens were not detected in any of the α1,3GalT-KO pig fractions, in agreement with preclinical pig-to-nonprimate trials where HAR was completely overcome.

Production of compensatory GSLs

Our hypothesis was that the metabolic block of the α1,3GalT would lead to i) accumulation of the immediate LacNAc precursor, ii) formation of repetitive LacNAc units or iii) that the biosynthesis would be driven towards competing pathways. These alternatives are briefly outlined below.

Precursor accumulation

Normally α1,3GalT converts the LacNAc precursor. In the absence of compensatory glycosyltransferases LacNAc units of varying lengths may accumulate. α1,3GalT-KO and WT heart and kidney all expressed the uncapped precursor nLc4 compound. Although the LC-ESI/MS technique used here has limitations regarding quantification of individual components, it can be used for interrelational estimations. The amount of nLc4 in GalT-KO heart and kidney was 4 times the level of nLc4 in corresponding WT tissues. Also, the extended LacNAc compounds nLc6 and nLc8 were tentatively established in α1,3GalT-KO pig kidney and heart. WT heart did not express any nLc6 or nLc8 and WT kidney did only express nLc6. LacNAc is the precursor substance in the blood group Ii system, and is abundantly expressed in human cells and tissues. Nevertheless, autoantibodies called cold agglutinins directed towards LacNAc are produced [182] and it is unclear whether this is of significance in xenorejection.

Competing pathways

Similar to Galα3-determinants, blood group H type 2 compounds are synthesized from the LacNAc precursor, hence α1,2FucT is a biosynthetic competitor of α1,3GalT. Increased levels of long chain H type 2 compounds in α1,3GalT-KO pancreas, heart and kidney compared to the corresponding WT tissues were detected using immunostaining (*papers I and III*).

Proton NMR spectroscopy identified two novel blood group H structure in α1,3GalT-KO small intestine (Figure 7C). These were unusual in that the six-linked blood group H trisaccharide branch (type 1 and 2 core chains, respectively) was attached directly to a lactosyl core instead of a tetrasaccharide core (*Paper II*) [162]. The tetrasaccharide core-based structures were also identified. The novel lactosyl core based structures were not detected in the corresponding WT tissue. The synthesis cannot be explained by a direct effect of the genetic modification; however it may be a general consequence of the disturbed glycosyltransferase balance.

Interestingly, the x_2 antigen (GalNAcβ3nLc4) was identified in kidney and heart of the α1,3GalT-KO pig (*paper III*). WT kidney expressed trace amounts of the x_2 antigen whereas WT heart was completely negative. x_2 is most commonly found on human erythrocytes and has to our knowledge not been reported in pig before [183]. Furthermore, the P1 antigen (Galα4nLc4) was identified in the α1,3GalT-KO kidney but was absent in WT kidney and in heart tissues. The transferases responsible for x_2 and P1 synthesis (β1,3GalNAcT and α1,4GalT) both use nLc4 as substrate and hence compete with α1,3GalT (Figure 8). Interestingly, accumulation of nLc4 [80] as well as the x_2 antigen [184] have been reported in erythrocytes from individuals of the rare human blood group p phenotype, which lack the P^k transferase responsible for the synthesis of Gb3, an intriguing concurrence to the precursor competition in the α1,3GalT-KO pig observed in the present work.

α2,3SiaT and α1,3GalT have been shown to compete for the same acceptor in transfected CHO cells [129]. Surprisingly, the level of gangliosides appeared to be decreased to about half the WT level in the α1,3GalT-KO small intestine and pancreas (Figure 8), reported in *paper I*. If the decrease in gangliosides in these tissues is a characteristic of the α1,3GalT-KO, or due to an inter-individual variation between the pigs can only be speculated upon. NeuGc type gangliosides are regarded as non-Gal antigens and are found in large amounts in pig tissues [185, 186]. The NeuGc epitope is expressed in most mammals, but is absent from cells and tissues of humans due to

an inactivation of the hydroxylase catalyzing the conversion of CMP-NeuAc to CMP-NeuGc, explaining why we produce antibodies directed towards NeuGc [187]. The role of NeuGc in xenotransplantation is unclear; however the opinion in the xenotransplantation field is that it may be the next target for genetic modification.
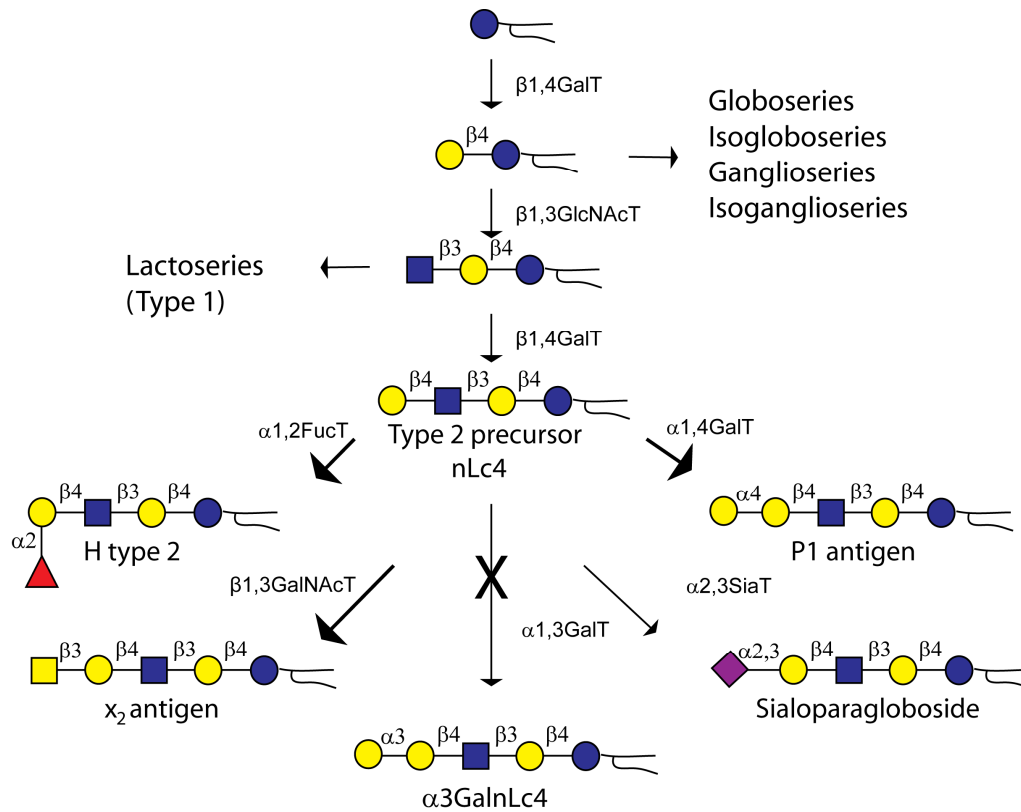
Figure 8. Schematic presentation of the synthesis of Galα3nLc4 antigen and competing biosynthetic pathways. Arrows in bold denote synthesis pathways that were upregulated in the present work.

Other non-Gal GSL candidates

A second enzyme, termed iGb3 synthase is capable of producing the Galα3 linkage and has been suggested to play a role in xenorejection. iGb3 synthase is GSL specific and elongates primarily LacCer but can also act on GalCer and Gb3 [188]. iGb3 is described in various mammals [57, 189-191] occasionally elongated into iGb4 and iGb5. In *paper I* we did not detect iGb3 in α1,3GalT-KO or WT small intestine. However, amongst the tetraglycosylceramides trace amounts of the fucosylated form fucosylα2-iGb3 (Fuc-iGb3), were detected, previously reported in hog stomach mucosa [192]. Neither iGb3 nor Fuc-iGb3 was found in heart and kidney (*paper III*). Fuc-iGb3 is not believed to have any adverse effects in xenotransplantation.

Like iGb3, the Forssman pentasaccharide has only been described in the form of GSL. Forssman is expressed in a tissue-specific and developmentally regulated manner in various mammals [193-196]. Hakomori reported that about 90% of the human population produce antibodies directed against the Forssman antigen [197], explaining why Forssman is regarded a xenoepitope. Nevertheless the Forssman antigen has been found in human tumors and also in small amounts in normal kidney [198]. Forssman GSL has not been detected in pig previously, nor did we detect it in our studies (*papers I and III*). The involvement of Forssman in rejection of xenoorgans is unlikely.

Reactivity with human and baboon serum antibodies

In *paper III* baboon serum samples obtained before and at various time intervals after α1,3GalT-KO pig-to-baboon cardiac grafting [199] were available for our studies. Sera were screened against neutral and acidic heart and kidney GSLs and compared to the binding pattern of human serum. The aim was to identify potential pre-formed and elicited antibodies with antigenic reactivity towards pig GSLs. Human and baboon serum antibodies reacted strongly with Galα3 epitopes in WT organs. The staining reaction was more intense using human serum, indicating a higher prevalence of anti-

Gal antibodies in the human population than in the baboon population. A neutral pentasaccharide was stained in all fractions, most pronounced in the α1,3GalT-KO kidney. As was revealed by structural determination pentasaccharides in this tissue consisted of H5 types 1 and 2, Gb5, FucGg4, Le^x-5, P1 and x$_2$ antigens (Table *1 and 2, paper III*). None of these have any reported antigenic activity against human antibodies.

Reactivity towards NeuGc antigens in acidic GSLs was detected with human serum, but not baboon serum as they, contrary to humans, express the NeuGc antigen. Baboon serum antibodies, on the other hand, reacted with several acidic α1,3GalT-KO compounds that were lacking or present in lower amounts in the corresponding WT fractions (*Figure 5, paper III*). These compounds were present in very small amount, and have therefore not yet been structurally determined. The difference in reactivity pattern of baboon and human serum antibodies indicate the limitations of non-human primates as preclinical human models.

The inhibition of HAR is the main accomplishment with the α1,3GalT-KO pig from a clinical point of view. The pig is considered an appropriate genetic background for additional modifications, such as incorporation of human complement regulators. The hope is that the inhibition of HAR and AVR will lead to accommodation, *i.e* the long term graft survival despite circulating xenoreactive antibodies [200].

Glycosyltranferase core chain dependence

In *Paper IV* the lipid/protein core dependence of Lewis antigen producing glycosyltransferases was compared. We aimed to engineer cells that produce Le^b and support adhesion of BabA-positive *H. pylori*, and thus may be used as an *in vitro* model for detailed host/microbe binding studies. Glycosyltransferases were selected primarily to mediate Le^b expression on core 3 O-glycans since this is the most likely configuration of the natural Le^b receptor in stomach mucosa and also because glycan expression on O-glycans in mucin domains is expected to yield a higher density of the

epitope than expression on N-glycans or GSLs [201]. From a glycobiological perspective we aimed to compare the Le^b expression on GSLs, N- and O-glycans, and observe the expression of Le^a, Le^x and Le^y, produced via competing biosynthesis pathways.

Lewis antigen production differs on GSL and protein cores

The glycosyltransferases examined had clearly different specificity for different core chains (Figure 9). Interestingly α1,3/4FucT-III was shown to act on the type 1 precursor to produce the Le^a epitope on N- and O-glycans but not on a GSL backbone. On the other hand, the enzyme acted on the H type 1 precursor to produce Le^b epitopes on all backbones and on H type 2 to produce Le^y on GSLs and N-glycans. Possible explantations for this may be separate biosynthesis pathways of GSLs and proteins in the ER/Golgi pathway, or substrate-dependent competition between α1,3/4FucT-III and α1,2FucT-II. It has been reported that α1,3FucT-IV selectively acts on GSLs whilst α1,3FucT-VII preferentially acts on glycoproteins [202], but besides this limited studies have been performed comparing glycosyltransferase substrate specificity for different core structures.

The observation of Le^x GSL in both transfected clones and parental cells was surprising. Parental CHO cells have been reported to lack α1,3FucT activity, thus being unable to add fucose in α3-linkage [203]. However the CHO cell genome contains at least two silent α1,3FucT genes that can be activated upon *e.g.* mutagenesis or transfection [127]. Some of the most well known examples are the LEC11 and LEC12 mutants which both express Le^x, sialyl Le^x and VIM-2 determinants due to an activating event of α1,3FucT-VI and α1,3FucT-IX, respectively [204]. The Le^x activity was observed in the >12 sugar region and is, to our knowledge, the most complex GSL detected in CHO cells. Le^x activity was not observed on glycoproteins. This may explain why it has previously not been reported since limited attention has been paid to CHO cell GSLs compared to glycoproteins. Unfortunately the limited amount of GSL material did not permit further structural studies by mass spectrometry or proton NMR spectroscopy.

Binding to H. pylori

Both clones examined supported adhesion of BabA-positive *H. pylori*, and may therefore serve as an *in vitro* model for molecular and cell biological studies on host/microbe relationship. Also, since Lewis antigen expression differed between GSLs, N- and O-glycans, the two Le^b expressing clones may be useful in studies to dissect the role of carrier glycans for *H. pylori* binding.
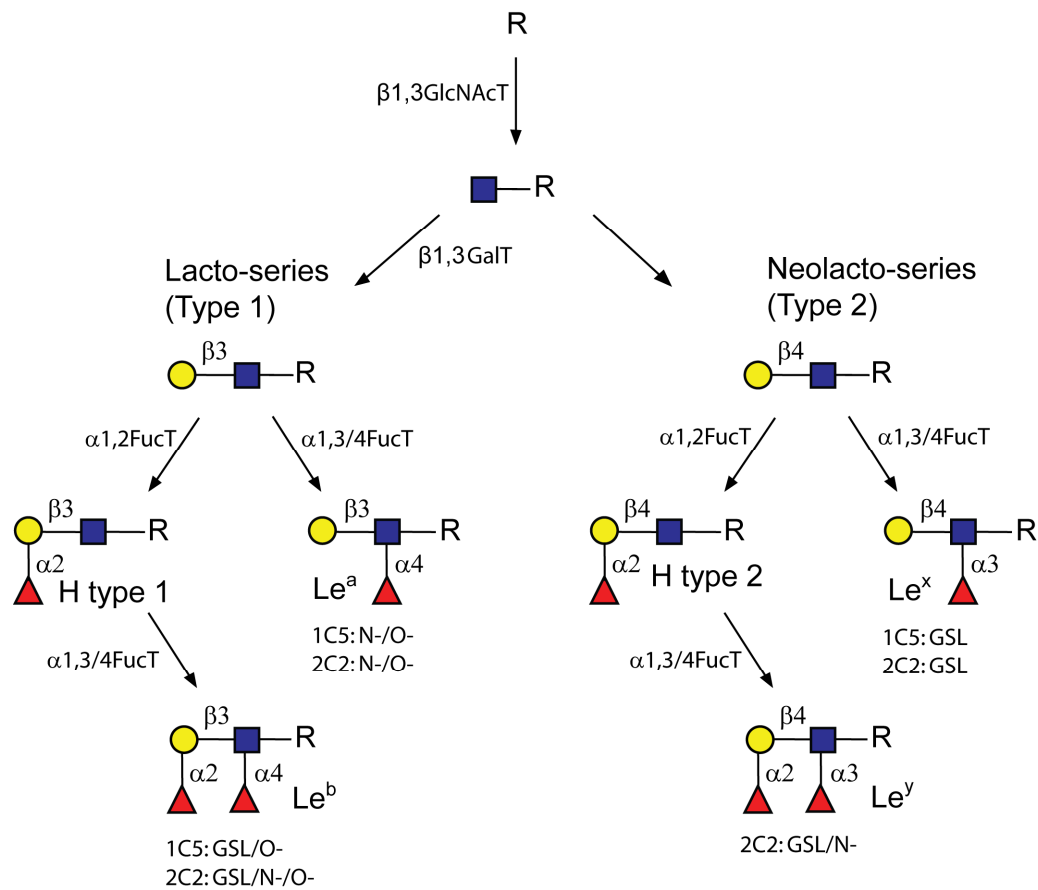
Figure 9. *Schematic presentation of Lewis antigen biosynthesis. Transfected glycosyltransferase are denoted above the arrows. The occurrence of each epitope on GSLs, N-and O-glycans in clones 1C5 and 2C2 is denoted below the glycan symbols.*

SUMMARY AND FUTURE PERSPECTIVES

Lack of Galα3 determinants in α1,3GalT-KO tissues is a direct effect of the genetic modification, whereas the changed levels of blood group H compounds, sialylated structures and the P1 and x_2 antigens rather are secondary effects due to the altered glycosyltransferase repertoire (*papers I-III*). Limited data is available regarding localization of, and competition between, specific glycosyltransferases. However, α1,2FucT is localized in the *medial* Golgi [205] and α1,3GalT in the *trans* Golgi [129, 206]. The growing oligosaccharide chain will therefore first encounter α1,2FucT and become inaccessible to subsequent α3-galactosylation. Consequently, knockout of α1,3GalT should not affect the level of fucosylation. Since blood group H compounds were increased, it supports the current belief that glycosyltransferases localize in overlapping gradients in the ER/Golgi pathway which makes it impossible to predict the effect of glycosyltransferase modifications on the glycolandscape.

The lack of terminal Galα3 epitopes and upregulation of the x_2 and P1 antigens makes it tempting to propose that α1,3GalT-KO pig tissues have adopted a "humanized" glycosylation pattern. However, *de novo* synthesis of foreign GSLs due to a knockout modification is unlikely. It is more likely that pigs normally synthesize minute amounts of these antigens which are increased upon α1,3GalT elimination.

GSLs and glycoproteins share many similarities in terminal carbohydrate sequences whereas the core structures linked to the protein or lipid carriers differ. Therefore the terminal carbohydrate determinants may be synthesized by common glycosyltransferases [14]. Few studies comparing lipid and protein bound blood group expression in cells or tissues have been reported. Expression of terminal Lewis epitopes in glycosyltransferase modified CHO cells differed quantitatively and qualitatively in the same cell clones depending on whether the glycan was protein or lipid core based (*paper IV*). Consequently it would be highly interesting to analyze

protein bound glycans from α1,3GalT-KO pig in order to obtain a more complete view of the cell membrane carbohydrate expression.

Small intestine is a rich source of complex blood group active GSLs, especially fucolipids. The abundance of GSLs and their subcellular distribution in epithelial cells renders small intestine an interesting and relevant tissue for detailed studies of glycosyltransferase activity on different core chain types. Interestingly, it has been shown that the majority of the small intestinal blood group active glycoproteins is of type 2 origin whereas the major GSL counterparts have type 1 core chains [207]. We have initiated a study where we compare small intestinal GSL from WT, α1,3GalT-KO and a combined α1,3GalT-KO/α1,2FucT transgenic pig with the aim to further explore the relation between cell membrane phenotype and cellular glycosyltransferases.

As discussed throughout this thesis, many pathophysiological conditions such as cancer, inflammation and infection are associated with aberrant glycosylation. A deeper comprehension of the correlation between expression of lipid and protein-linked cell membrane carbohydrates and the activities of related glycosyltransferases may prove valuable in diagnosis and development of potential therapies.

ACKNOWLEDGEMENTS

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