Studies on glycosphingolipids in regenerative medicine

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Gothenburg 2014

Cover illustration: MS/MS spectrum of sialyl-lactotetraosylceramide from human embryonic stem cells

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ISBN 978-91-628-8919-7

Printed in Gothenburg, Sweden 2014
by Kompendiet

ABSTRACT

Regenerative medicine, including stem cell based therapies and xenotransplantation, is a new and developing field that aims to restore normal function in end stage cell/organ failure. However, in therapeutic settings involving transfer of cells with a different genetic background, these cells will expose the recipient to non-self cell surface antigenic determinants that may evoke an immune response and subsequent graft damage. Thus, the cell surface antigen expression of cells aimed for transplantation has to be defined.

The first part of this study deals with glycosphingolipids of human embryonic stem cells (hESC). The glycosphingolipids were isolated from a relatively high number of cells ($1x10^9$ cells/cell line) allowing separation of the glycosphingolipids into total non-acid and acid fractions, that could be further separated into sub-fractions. These fractions were structurally characterized by mass spectrometry, proton NMR spectroscopy and binding studies with carbohydrate binding ligands. This allowed identification of several glycosphingolipids not previously described in hESC.

In the non-acid glycosphingolipid fractions several novel blood group H, Le^x and Le^y compounds based on neolacto core chains were characterized, in addition to the already identified lacto- and globo-series glycosphingolipids. The acidic glycosphingolipid fractions contained several novel hESC acid glycosphingolipids, like the gangliosides sialyl-lactotetraosylceramide and sialyl-globotetraosylceramide, and the sulfated glycosphingolipids sulfatide, sulf-lactosylceramide and sulf-globopentaosylceramide. The cellular and subcellular distribution of sialyl-lactotetraosylceramide and sulfated glycosphingolipids in hESC and in human induced pluripotent stem cells (hiPSC) was explored by flow cytometry, immunohistochemistry and electron microscopy. A high cell surface expression of sialyl-lactotetra on hESC and hiPSC was demonstrated, whereas the sulfated glycosphingolipids were restricted to intracellular compartments.
During differentiation of hiPSC into hepatocyte-like cells a rapid down-regulation of the sialyl-lactotetra epitope was found. Taken together these data demonstrate that the sialyl-lactotetra carbohydrate sequence is a novel marker for undifferentiated human pluripotent stem cells.

Diseased human heart valves are substituted with either mechanical valves or biological heart valves (BHV) produced from porcine and bovine valves or pericardial tissues. The BHV function deteriorates with time partly due to an

immunological process. The second part of the study aimed at defining carbohydrate antigens of porcine heart valves with a potential of being immune targets for this process. Here, a number of acidic glycosphingolipids (sulfatide and the gangliosides GM3, GM2, GM1, fucosyl-GM1, GD3 and GD1a) and non-acid glycosphingolipids (globotetraosylceramide, H type 2 pentaosylceramide, fucosyl-gangliotetraosylceramide and Galα3neolacto-tetraosylceramide) were characterized. Interestingly, no gangliosides with the non-Gal xenoantigen NeuGc were found. However, the Galα3 epitope is the major xenoantigen, and thus a possible target for antibody mediated immune reactions to xenogeneic bioprosthetic heart valves in humans.

Keywords: Glycosphingolipids, cell surface markers, human embryonic stem cells, porcine heart valves, xenorejection, regenerative medicine

ISBN: 978-91-628-8919-7

SAMMANFATTNING PÅ SVENSKA

Regenerativ medicin är ett relativt nytt forskningsfält som syftar till att återställa funktionen hos sjuka organ i kroppen genom att byta ut döda eller skadade celler och vävnader. Dessa celler och vävnader kan t.ex. produceras från stamceller. Embryonala stamceller är celler från mycket tidiga stadier av fosterutvecklingen och kan utvecklas till kroppens alla olika celler/organ. En annan möjlighet är att använda vävnad och organ från djur som transplanteras in i människa (xenotransplantation). Redan idag används hjärtklaffar från gris för att ersätta skadade klaffar hos människa. I båda fallen kan det överförda biologiska materialet aktivera mottagarens immunsystem och leda till att den nya vävnaden/cellerna förstörs. Denna immunreaktion riktas delvis mot en grupp av molekyler på cellytan vilka utgörs av kolhydrat. I denna avhandling har vi studerat cellytans kolhydrater, i form av glykosfingolipider, från humana embryonala stamceller (hESC) samt från hjärtklaffar från gris.

I studierna från humana embryonala stamceller identifierades ett stort antal glykosfingolipider som inte tidigare påvisats i hESC. Detta utfördes med biokemisk strukturell karaktärisering med masspektrometri, NMR spektroskopi och bindningsstudier med kolhydratbindande ligander. Därmed identifierades glykosfingolipider som inte tidigare påvisats i hESC både neutrala komponenter och glykosfingolipider som innehåller sulfat samt sialinsyra. En av dessa komponenter (sialyl-laktotetra) kunde med cellbiologiska analysmetoder som flödescytometri, immunohistokemi och elektronmikroskopi visas utgöra en specifik markör för omogna stadier av hESC och inducerade humana pluripotenta stamceller (hiPSC). Inducerade PSC är mogna celler som reprogrammerats och därmed återförts till ett omoget cellstadium. När dessa celler mognade ut till leverliknande celler minskade cellytans mängd av sialyl-laktotetra och försvann helt efter två veckor. Dessa fynd talar för att sialyl-laktotetra kan vara en ny markör för pluripotenta stamceller.

Hjärtklaffar från gris som transplanteras till människa har en begränsad överlevnad, speciellt hos unga patienter, och i många fall krävs att dessa byts ut en tid. Den skada som klaffarna utsätts för anses till viss del bero på en immunreaktion mot det inopererade materialet. I denna studie påvisades ett antal glykosfingolipider med terminal Galα3, vilket är ett känt xenoantigen, och kan utgöra måltavla för antikroppsmedierade immunreaktioner mot dessa hjärtklaffar.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Barone, A., Benktander, J., Ångström, Aspegren, A., Björquist, P., Teneberg, S., Breimer, M.E. (2013) **Structural complexity of non-acid glycosphingolipids in human embryonic stem cells grown under feeder-free conditions.** *J. Biol. Chem*. **288,** 10035-10050

II. Barone, A., Säljö, K., Benktander, J., Blomqvist, M., Månsson, J.-E., Johansson, B. R., Mölne, J., Aspegren, A., Björquist, P., Breimer, M. E., Teneberg, S. **Sialyl-lactotetra: a novel cell surface marker of undifferentiated human stem cells.** *Submitted manuscript.*

III. Barone, A., Benktander, J., Teneberg, S., Breimer, M. E. **Characterization of acid and non-acid glycosphingolipids of porcine heart valves as potential immune targets in biological heart valve grafts.** *Submitted manuscript.*

CONTENT

ABBREVIATIONS

BHV	Bioprosthetic heart valve
Gal	Galactose
GalNAc	*N*-Acetylgalactosamine
Glc	Glucose
GlcNAc	*N*-Acetylglucosamine
GSL	Glycosphingolipid
hESC	Human embryonic stem cell
hPSC	Human pluripotent stem cell
iPSC	Induced pluripotent stem cell
ihPSC	induced human pluripotent stem cell
LC-ESI/MS	Liquid chromatography- electrospray ionization/MS
Man	Mannose
MHV	Mechanical heart valve
MS	Mass spectrometry
NeuAc	*N*-Acetylneuraminic acid
NeuGc	*N*-Glycolylneuraminic acid
NMR	Nuclear magnetic resonance
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TLC	Thin-layer chromatography
Xyl	Xylose

1 INTRODUCTION

The relative shortage of human organs/cells available for transplantation is currently the limiting factor for treatment of patients with terminal organ failure. To overcome these obstacles two scientific fields offer alternative solutions; the use of organs from animals (xenotransplantation) [1] or stem cell therapies with human embryonic stem cells (hESC) [2] or human induced pluripotent stem cells (hiPSC) [3], collectively called human pluripotent stem cells (hPSC). Human pluripotent stems cells offer great potential in regenerative medicine, in the field of cell based therapy [4], as well as for stem cell-based, tissue-engineered organs [5]. The term regenerative medicine involves both xenotransplantation and stem cell technology with the aim to replace or regenerate human organs, tissues and cells [6].

Even if transplantation of vascularized organs and cells from animals are currently far from clinical reality, transplantation of bioprosthetic heart valves (BHVs) generated from porcine or bovine tissues is today routinely used in the clinic to replace diseased heart valves. However this is not without complications. The valves deteriorate with time and questions are raised if this might be partly due to an immune response of the grafts [7].

Both for xenografts as well as the transfer of human cells with different genetic background, these cells will expose the recipient to non-self cell surface antigenic determinants that may evoke an immune response and subsequent graft damage. Therefore, these organs/cells need to be thoroughly characterized regarding their cell surface antigens to predict and develop strategies to prevent activation of the recipient's immune system.

The cell surface contains a complex pattern of molecules that are potential antigens for immune recognition. These are mainly of protein (*i.e.* the human leucocyte antigens, HLA) or carbohydrate (*i.e.* the blood group AB(O)H antigens) nature. This thesis focuses on the characterization of potential glycosphingolipid antigens of human embryonic stem cells and bioprosthetic heart valves.

1.1 Mammalian cell membrane glycoconjugates

Every living cell studied so far, including pluripotent stem cells, are covered by a complex network of carbohydrates often referred to as the glycocalyx [8]. This ultrastructure is formed by glycans covalently bound to proteins or lipids, *i.e.* glycoproteins, proteoglycans and glycolipids, collectively called glycoconjugates (outlined in Figure 1) [8].

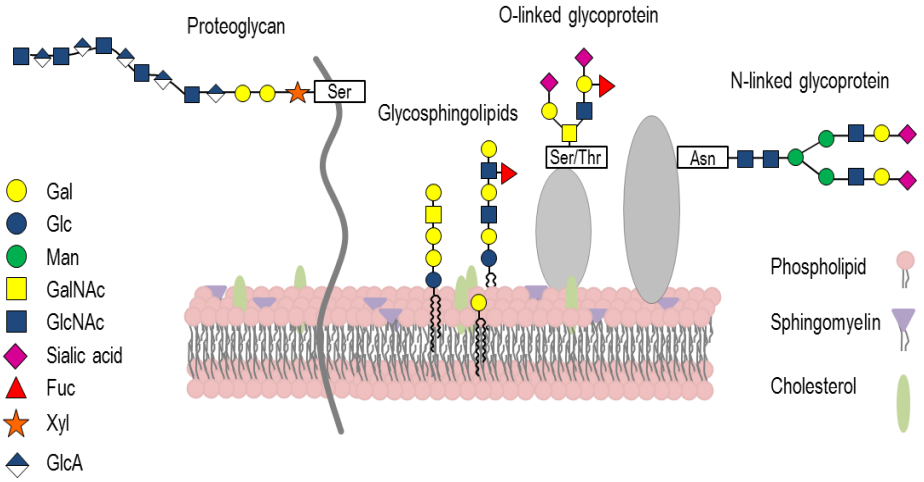


Figure 1.Schematic picture of different glycoconjugates in the mammalian cell membrane. (Modified from Varki A. 1997. FASEB J. 11:248-255.)

Glycoproteins mainly fall into two categories, the *N*-linked, where the oligosaccharide is linked to the side chain amide nitrogen (N) atom of asparagine residues and the *O*-linked glycoproteins where the oligosaccharide is covalently attached to the hydroxyl oxygen (O) at the side chain of serine or threonine residues.

Proteoglycans are heavily glycosylated macromolecules mainly secreted into the extracellular matrix as major components of the connective tissue, but may also be anchored in the cell membrane [9]. To one core protein many linear repeating polysaccharide units are attached via a xylosyl-serine linkage, these carbohydrate units comprise up to 95% of the molecule [10].

Glycolipids have carbohydrates attached to either glycerolipids or sphingolipids, with the lipid part inserted in the membrane. To each lipid there is only one carbohydrate chain attached, in contrast to proteins which may carry several carbohydrate chains.

The glycoconjugates are in contrast to proteins not under direct genetic control. Instead they are the products of several enzymes (glycosyltransferases see below), each encoded for by a specific gene. Therefore, they are secondary gene products (Figure 2).

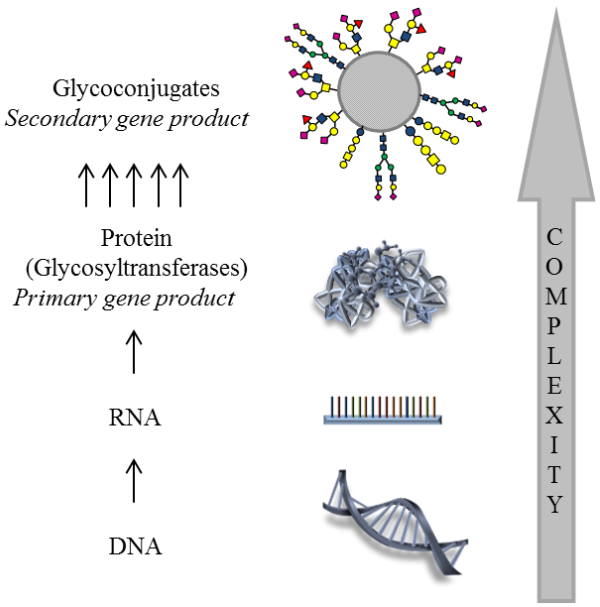
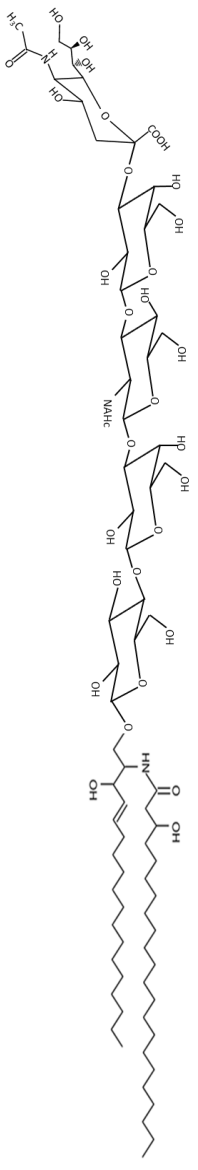


Figure 2. The biosynthesis of glycoconjugates occurs through the action of several different template based glycosyltransferases.

Cell surface glycoconjugates are involved in a variety of biological processes such as in the inflammatory response, where leucocytes are recruited to the site of inflammation and infection. The selectins (P-, E- and L-selectins) bind to sialyl-Le^x, and other ligands, on activated endothelium. This makes the leucocytes slow down (rolling phenomenon) and subsequently make their way between the endothelial cells to the site of injury [11-13]. Cell surface glycoconjugates may also function as receptors for pathogens, *e.g.* specific glycosphingolipids (GSLs) act as receptors for bacteria [14], viruses [15], and toxins [16]. Cell surface glycoconjugates are also implicated in embryogenesis [17], in tumors as tumor associated antigens [18], and determining cell fate such as, proliferation and differentiation [19].

1.2 Glycosphingolipids

Since their discovery over 100 years ago by J. L. W. Thudichum (1829-1901) [20] glycosphingolipids have emerged as an essential component of the mammalian cell membrane. These amphipathic molecules are comprised of two chemically joined components, the hydrophobic ceramide embedded in the outer leaflet of the membrane, and the hydrophilic oligosaccharide chain protruding out from the membrane facing the external milieu (Figure 3) [21].



The oligosaccharide chain may vary enormously due to the number of monosaccharides and their sequential order, variations in binding positions, anomeric configuration and branching [22]. To date over 350 different carbohydrate chains on glycosphingolipids have been identified [23].

The ceramide part adds another level to the complexity of the glycosphingolipid structures. The main long chain bases found in mammals are sphingosine, sphinganine, and phytosphingosine. There are also different fatty acids, ranging mainly from 16 to 24 carbon atoms, with or without hydroxyl groups.

Glycosphingolipids are usually divided into two groups: non-acid (neutral) and acidic. The acidic are grouped into two categories, gangliosides and sulfatides, the former carry one or more sialic acid and the latter carries sulfate groups. Glycosphingolipids are classified into different series on the basis of their different carbohydrate core structures (Table 1).

Figure 3. Schematic drawing of glycosphingolipid sialyl-lactotetraosylceramide (NeuAcα3Galβ3GlcNAcβ3Galβ4Glcβ1Cer) with d18:1 and h22:0 fatty acid

Table 1.Major mammalian glycosphingolipid core structures

Series	Abbreviation	Core structure
Lacto/neolacto:	Lc	Galβ3GlcNAcβ3Galβ4Glcβ1Cer
	nLc	Galβ4GlcNAcβ3Galβ4Glcβ1Cer
Globo/isoglobo:	Gb	GalNAcβ3Galα4Galβ4Glcβ1Cer
	iGb	GalNAcβ3Galα3Galβ4Glcβ1Cer
Ganglio:	Gg	Galβ3GalNAcβ4Galβ4Glcβ1Cer

1.2.1 Non-acid (neutral) glycosphingolipids

The main core chains of the non-acid glycosphingolipids in peripheral human tissues are the globo-, lacto- and neolacto cores. Terminal AB(O)H and/or Lewis blood group determinants are often found on lacto- and neolacto core chains, and also the globo and ganglio chains may carry terminal blood group AB(O)H determinants (Table 2). The blood group P system is based on the globo core chain, where globotriasoylceramide is the P^k antigen and globotetraosylceramide is the P antigen [24].

Table 2.Blood group AB(O)H, $Le^{a/b}$ and $Le^{x/y}$ determinants and their carbohydrate core chain structures

Determinant	Structure	Lacto	Neolacto	Globo	Ganglio
A	GalNAcα3(Fucα2)Galβ	X	X	X	
B	Galα3(Fucα2)Galβ	X	X	X	X
H	Fucα2Galβ	X	X	X	X
Le^a	Galβ3(Fucα4)GlcNAc	X			
Le^b	Fucα2Galβ3(Fucα4)GlcNAc	X			
Le^x	Galβ4(Fucα3)GlcNAc		X		
Le^y	Fucα2Galβ4(Fucα3)GlcNAc		X		

1.2.2 Acidic glycosphingolipids

The main types of acidic glycosphingolipids have carbohydrates substituted with sulfate groups or sialic acids. The most common of the sulfated glycosphingolipids is sulfatide-I (SO_3-GalCer) but sulfated glycosphingolipids based on lactose (sulfatide-II), and based on the ganglio- [25] and globo core chains have also been described [26, 27]. In addition HNK-1 (human natural killer-1), an unusual glycosphingolipid with a sulfoglucuronyl residue on a neolacto core [28], has been found in the brain of varies species ranging from insects to mammals [29].

Sialic acid carrying glycosphingolipids, *i.e*. gangliosides, have mainly ganglio or neolacto core chains. The predominant forms of sialic acids of mammalian glycosphingolipids are *N*-acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc) (Figure 4). NeuAc is the precursor of NeuGc and the two sialic acids differ only at C-5 where NeuAc has an acetyl group and NeuGc a glycolyl group [30].

Due to a mutation in the CMP-Neu5Ac hydroxylase gene that occurred over 2 million years ago [31], NeuAc is the only sialic acid present in human gangliosides [32]. The sialic acids may be α3- or α6-linked, and in di-sialo gangliosides α8-linkages are also present.

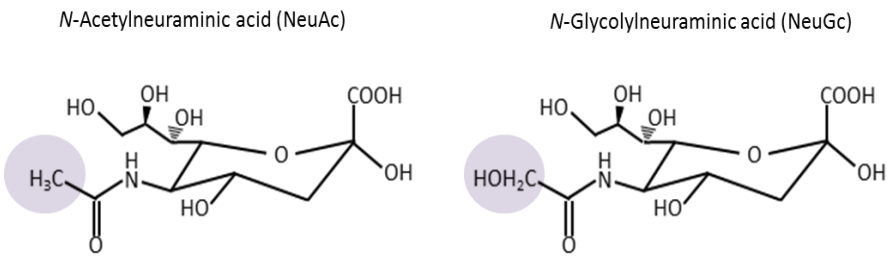


Figure 4. *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid differs only by different groups (marked in violet) on carbon 5.

1.2.3 Glycosphingolipid metabolism

Biosynthesis

The biosynthesis of glycosphingolipids occurs mainly in the endoplasmatic reticulum and Golgi, and to some extent also at the cell membrane (reviewed in [33, 34]). The first step is the assembly of the ceramide on the cytosolic side of the endoplasmatic reticulum. For synthesis of galactosylceramide and sulfatide the ceramide moves into the lumenal side of endoplasmatic reticulum where Gal is added by a ceramide galactosyltransferase. The resulting galactosylceramide is thereafter transferred to the lumenal Golgi where a sulfate group is added by a sulfate transferase.

The synthesis of more complex glycosphingolipids occurs *via* an alternate route. Here the ceramide is first transferred to cytosolic side of the *cis*-Golgi, where glucose is added by a ceramide glucosyltransferase. The glucosylceramide is moved to the lumenal side of the *cis*-Golgi stack, where further glycosylation occurs by the sequential action of a battery of membrane-bound glycosyltransferases. Finally, the newly synthesized glycosphingolipids are transported to the cell membrane by vesicular transport.

The glycosphingolipid expression of a certain cell type is dependent on the individual genetic set-up, and transcriptional regulation, of the glycosyltransferases [35]. However, other factors as *e.g.* the availability of sugar donors, and acceptors, precursor competition and the topological distribution of the glycosyltransferases within Golgi, contribute in the biosynthesis/expression of these molecules.

There are differences in the glycosylation of glycosphingolipid and glycoprotein within a single cell. One such example is the human small intestinal enterocytes [36], where the glycosphingolipids have mainly lacto core chains, while the glycoproteins have predominantly neolacto cores [37]. This indicates that the glycosyltransferases involved in synthesis of the carbohydrate chains of glycosphingolipids and glycoproteins are found in different compartments of the Golgi apparatus.

Degradation

The glycosphingolipids are degraded after endocytosis by a complex machinery of degrading enzymes, negatively charged lipids and activator proteins [38]. Thus, the breakdown starts with internalization of membrane bound glycosphingolipids into vesicles, along the endocytotic pathway, the vesicles fuse with endosomes, and are finally exposed to the hydrolytic

enzymes when late endosomes fuse with lysosomes. Most of the glycosidases acting on the membrane bound glycosphingolipids are soluble enzymes, which explains the requirement for the membrane perturbing and lipid binding sphingolipid activator proteins (SAPs) as co-factors for lysosomal degradation of glycosphingolipids. The products of the degradation (long chain bases, fatty acids and monosaccharides, and also ceramides and short chain glycosphingolipids) are released into the cytosol where they may be used in biosynthetic processes (salvage pathway) or further degraded.

Attempts to calculate the life span of glycosphingolipids have been made (reviewed in [33]), giving values ranging from 2.5 h to 30 h in different cell lines. However, given the complex machineries for glycosphingolipid degradation, including salvage pathways, most of these data are probably overestimations.

1.2.4 Tissue distribution of glycosphingolipids

The localization of carbohydrate antigens within tissues and cells have been characterized by immunohistochemistry [39]. This has revealed a very complex distribution pattern with an organ/cell specific distribution within a single individual. Regarding glycosphingolipid distribution much less is known especially on the cellular level, except for blood cells.

There is a remarkable variability in the distribution of glycosphingolipids which is thought to reflect differences in expression of glycosyltransferases. This variability is found at different levels:

(i) *Species*: Isoglobo-series glycosphingolipids and glycosphingolipids with terminal Galα3, or with *N*-glycolylneuraminic acid, are not present in humans, while these compounds are found in many animal species as *e.g*. dog, horse, rat and pig. In all three cases the genetic background for the absence of these glycans in humans has been described [32, 40, 41]. The diversity between species was also demonstrated in a study where glycosphingolipids from small intestines of *e.g.* cat, guinea-pig, hen, mouse and rabbit were identified [42].

(ii) *Individuals*: also within a given species different glycosphingolipids are present in different individuals. Thus, the expression of glycosphingolipids is related to the ABO, Le and Se phenotype of the individual [36]. The blood group P system is another example of variability among individuals, some

individuals are of the rare phenotype p and as a consequence do not express the globoseries glycosphingolipids [24].

(iii) *Organ:* within a given individual the glycosphingolipids of different organs vary. As reviewed by Holgersson *et al*. [43], the blood group AB(O)H carbohydrate core chains vary in different organs, *e.g.* erythrocytes carry mostly neolacto core chains while kidney is rich in globo cores. Epithelial cells of small and large intestine are abundant in lacto core while the pancreas has both lacto and globo cores in equal amounts.

(iv) *Cells:* also the cells within a given organ/tissue have different glycosphingolipids. One example is human blood where the major glycosphingolipids of the erythrocytes are globoseries compounds, while neolacto is the main core structure in human neutrophils, and no globoseries glycosphingolipids are present. Epithelial cells of the small intestine contain blood group ABO and Lewis glycosphingolipids, while globoseries glycosphingolipids are absent. Instead these are found in the sub-epithelial part of the small intestine [36, 37].

The available data does not allow a full comparison of the differential expression of acid glycosphingolipids in different species, individuals and tissues. One example, however, is sulfatide which is found in the epithelial cells of the small intestine of most species, but is not present in rat, cod or mouse small intestinal epithelium [44]. The human brain gangliosides have been thoroughly characterized [45]. These gangliosides have mainly ganglio core chains. Interestingly, NeuAc is the only sialic found in brain gangliosides of different species [46]. Also the gangliosides in human neutrophils (mainly neolacto core chains) [47-49], and human small intestinal epithelium [50] have been well characterized.

1.3 Human embryonic stem cells aimed for clinical therapy

All mammals originate from one single cell, the zygote. This cell divides and around day five post-fertilization, a blastocyst is formed. The blastocyst comprises two different cell types, the tropohoblast and the epiblast or embryoblast, also known as the inner cell mass. These cells are pluripotent *i.e.* have the built-in capacity to transform into virtually all cells, tissues and organs of the body, and at the same time, they are capable of self-renewal *i.e.* to proliferate indefinitely while keeping their state of pluripotency.

Embryonic stem cells (shown in Figure 5), derived from the inner cell mass of blastocysts, have these characteristics [2]. The first evidence of the existence of this cell type was found by studies on mouse teratocarcinomas some 50 years ago [51]. Since then, several different types of pluripotent stem cells have been described, as *e.g.* mouse embryonic stem cells, human embryonic stem cells (hESC), and induced pluripotent stem cells (iPSC). The iPSC originate from adult somatic cells that have been reversed by different techniques into an immature stem cell like state, with the potential of pluripotency [3]. On the other hand, hESC are generated from surplus human embryos [52]. These two cell types are collectively called human pluripotent stem cells (hPSC). Their capability of self-renewal and pluripotency makes them well suited for use in the laboratory to study different biological processes. Furthermore, the potential of pluripotent stem cells to differentiate into all the three germ layers of the body has raised high expectations in clinical applications.

Different markers are used for the characterization of hPSCs, these include markers at the transcriptional level as well as cell-surface markers [53, 54]. Several widely used cell-surface markers are glycoconjugates [55], as *e.g.* the proteoglycans TRA-1-60 and TRA-1-81 [56]. Other carbohydrate markers are the stage specific embryonic antigens (SSEAs), initially detected by the development of antibodies. The SSEA-1 antibody (MC 480) [57] recognizes the trisaccharide Galβ4(Fucα3)GlcNAc also recognized as the Lewis^x epitope, present on both proteins and lipids [58], while the SSEA-3, and SSEA-4 antibodies recognize the globo-series glycosphingolipids, globopentaosylceramide and sialyl-globopentaosylceramide, respectively [59-61]. There is also an SSEA-5 antibody [62] which recognizes the lacto core determinant (Fucα2Galβ3GlcNAc-) on glycoproteins and glycosphingolipids.

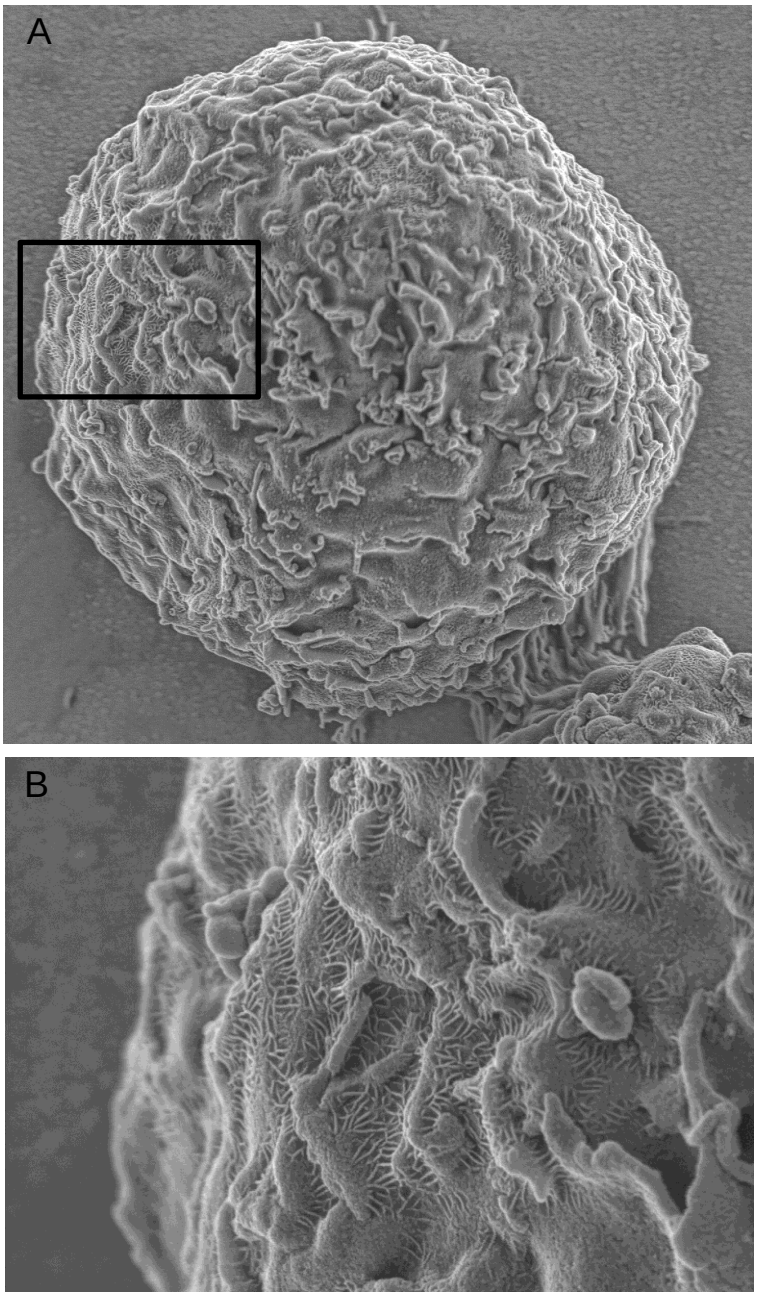


Figure 5. A human embryonic stem cell. The surface of one single cell from hESC line SA181 as revealed by scanning electron microscopy (A). Squared box in A is magnified 30 000 times (B), in order to reveal surface topography.

However, several issues have to be addressed regarding stem cells aimed for clinical therapy such as:

(i) Immune responses:
a feeder-free system is essential. When hESC are cultured on mouse feeder cells, otherwise non-human compounds such as the non-human sialic acid NeuGc may be incorporated [63, 64]. Thus, the ganglioside NeuGc-GM1, which in a recent study of the total cellular glycome of hPSCs, was described as a major ganglioside, was most likely derived from the mouse feeder cells used [65].

(ii) Transfer of potential zoonosis:
the risk of zoonosis transfer in clinical xenotransplantation of vascularized organs [66] is also applicable if hESC are grown using serum and feeder cells of animal origin.

(iii) Tumor development:
if lineage specific stem cells are to be transplanted into recipients, the cells have to be completely devoid of any residual pluripotent cells, since these cells are capable to form teratomas or teratocarcinomas (benign or malign tumors comprising tissues from the three germlayers) [67]. Therefore, monitoring and characterizing the differentiated state of human pluripotent stem cells is crucial in stem cell based therapy.

1.4 Heart valve replacement

Patients with defect heart valve cups and retained myocardial function are treated with replacement of the defect valve. Prosthetic heart valves used are either mechanical heart valves (MHV) or bioprosthetic heart valves (BHV). As the name implies MHVs are made by non-biological material, while BHVs may be derived from humans (homograft) or animals (xenograft), mainly from porcine and bovine origin. Over 250 000 diseased heart valves are surgically replaced world-wide each year, approximately 45 % of these comes from xenogenic tissues [68].

The MHVs have superior durability, but may put the patient at risk by the lifelong anticoagulant therapy needed, with accumulated annual risk of about 2% for serious bleeding event such as a stroke. In contrast, patients treated with BHV do not require anticoagulation but the valve function is reduced

during time due to calcification. This process has been shown to be partly due to an immune reaction in the recipient [69].

If vascularized xenoorgans grafted from pig to humans they will experience a hyperacute rejection (HAR) due to preformed antibodies present in the human recipient [70]. The major part of these antibodies are directed to the Galα3Gal determinant that is present in all animals except humans and Old world monkeys [71], where the enzyme responsible for adding the terminal Gal is mutated and consequently can produce anti-Gal antibodies. The Galα3-glycolsyltransferase has been knocked-out in pigs and these organs do not experience HAR when grafted into non-human primates [72]. However, when this barrier has been overcome a number of other antigen/antibody non-Gal barriers have been identified [73].

One example is *N*-Glycoloyl-neuraminic acids (NeuGc) that are found in all mammals, but not in humans that lack the enzyme responsible for conversion of the *N*-Acetyl-neuraminic acid to the corresponding NeuGc variant, as described above.

The BHVs are treated with different de-cellularization protocols, mainly glutaraldehyde treatment, to make the tissue resistant to damage, remove the xenogeneic antigens to which the recipient immune system will react and to eliminate potential infectious agents such as endogenous retroviral DNA [74]. This treatment has been very successful but still the valves are damaged with time. Recent immunohistochemical studies have identified Gal-antigens in BHV used in the clinic [75] and there are also indications that the implanted valves can induce anti-Gal antibodies in the recipients [76]. Furthermore, knowledge regarding non-Gal antigens in BHV is at present lacking.

2 AIMS

The use of xenogenic BHV for replacement of human heart valves, as well as a future clinical use of hESC, will challenge the recipient's immune system. Carbohydrate cell surface antigens are recognized by the immune system and are known to mediate cell damage in human organ allotransplantation. Our knowledge regarding carbohydrate antigen expression in hESC and porcine BHV is in general incomplete, and regarding glycosphingolipid structures the knowledge is limited.

General aim of this work is therefore to:

- characterize the cell surface carbohydrate antigen determinants that may be target for immune detection of grafted tissues/cells.

by specific focus on the isolation and structurally characterization of non-acid and acid glycosphingolipids in

- human embryonic stem cells grown under feeder and serum free conditions.
- porcine aortic and pulmonary heart valves.

3 METHODS

Cell and tissue specimens

Human Embryonic Stem Cells (hESC), supplied by Cellectis Ltd (former Cellartis Ltd) were generated from surplus *in vitro* fertilized embryos, grown in a serum- and feeder-free system [77]. Cells were harvested using an enzyme-free cell dissociation buffer for isolation of glycosphingolipids, in an attempt to minimize destruction to outer cell membrane components. Approximately $1x10^9$ cells were provided in cell pellets and stored at -80 ^oC.

Porcine heart valves from 20 hearts were obtained from the local slaughter house. The pulmonary and aortic valves were surgically dissected from the vascular wall and stored at -80 ^oC.

3.1 Isolation of glycosphingolipids

As described above, glycosphingolipids are a group of compounds that have great variation in their physiochemical properties, as *e.g.* solubility properties. Compounds with short saccharide chains are easy to dissolve in organic solvents, while those having large saccharide chains can only be dissolved in very polar solvents. Another example is their ability to resist alkaline degradation while even weak acids may result in cleavage of the glycosidic bonds. Therefore various isolation protocols have been developed to purify glycosphingolipids from cells and tissues. The protocol used in our laboratory was developed by Karlsson [78], and is schematically outlined in Figure 6. By this procedure total acid and non-acid glycosphingolipid fractions, free from other lipid components, and having 1 to about 18 carbohydrate residues, are obtained in a reproducible way. This procedure has been further modified during the years to be applied on small amounts of cells/tissues ([79, 80] and Paper III).

Briefly, lipids are extracted from lyophilized tissues with mixtures of chloroform and methanol, thereafter the extracts are pooled and dried. Mild alkaline methanolysis and dialysis followed by silicic acid chromatography removed the majority of the non-polar lipids (mainly cholesterol-esters and free fatty acids). Acid and non-acid glycosphingolipids are separated by ion-exchange chromatography and sphingomyelin is removed after acetylation on a second silicic acid column. Deacetylation reverts the glycosphingolipids

back to their original state and final purification is achieved by ion exchange and silicic acid chromatography. The total acid and non-acid fractions may be further separated into individual species by HPLC or chromatography on Iatrobeads columns [81], eluted with suitable mixtures of chloroform/methanol/water.

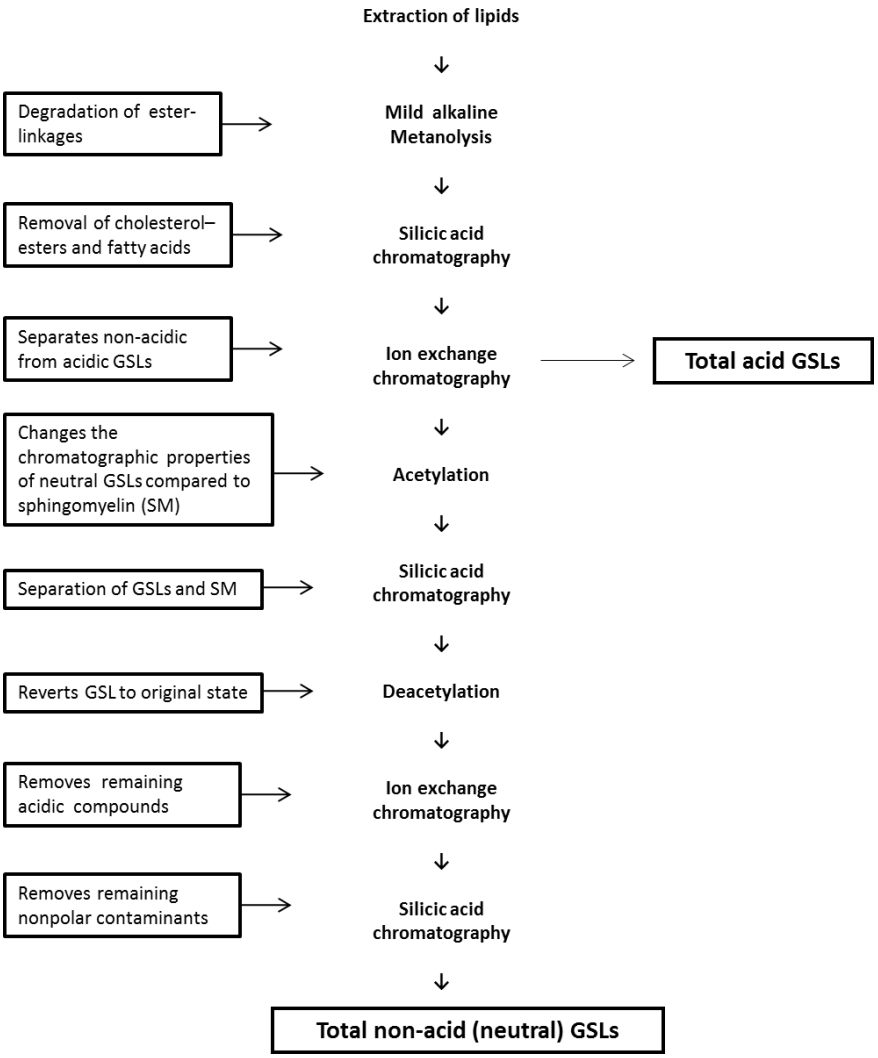


Figure 6. The different steps in the glycosphingolipid isolation process are outlined, ranging from extraction of lipids from cells or tissues total non-acid and acid glycosphingolipid fractions. The main purpose of each step is defined in the boxes to the left.

The isolation process is monitored by thin-layer chromatography (TLC), a simple and rapid technique giving a preliminary overview of the compounds present in a glycosphingolipid preparation. Separation of individual compounds is depending on size and polarity of the carbohydrate chain but is also influenced by the degree of hydroxylation and chain length of the ceramide [82]. For visualization, chemical staining with anisaldehyde, for non-acid or resorcinol for acidic glycosphingolipids is used. Anisaldehyde stains all carbohydrates in shades of green [83] while resorcinol stains only sialic acid with a typical purple color [84]. Thin-layer chromatography with chemical detection thus gives a preliminary view of the glycosphingolipid composition (see *e.g.* Figure 7). However, due to the great heterogeneity of glycosphingolipids, a single band on the chromatogram may contain more than one glycosphingolipid species.

3.2 Structural characterization of glycosphingolipids

In order to characterize the structure of a glycosphingolipid the following characteristics need to be determined:

(i) saccharide composition

(ii) saccharide sequence

(iii) saccharide linkage positions

(iv) anomeric configurations of glycosidic bonds

(v) branching

(vi) ceramide composition (fatty acid and long chain base)

In general several different analytical techniques are needed to achieve this, as *e.g.* a combination of mass spectrometry, proton NMR, degradation studies [85, 86] and carbohydrate binding assays.

3.2.1 Mass spectrometry

Mass spectrometry (MS), a central analytical technique in structural biology, is used for characterization of individual molecules. The basic principle is the production of ions from the analyte, either by loss or gain of charge, followed by separation according to their mass-to-charge ratio (*m/z*) and finally measurements in a detector. Mass spectrometry is not a "one size fits all" method, since different compounds ionize more or less readily.

Trimethylsilylated cerebroside was the first complete glycosphingolipid to be characterized by mass spectrometry, obtained by EI (electron ionization)-MS [87]. Subsequently, increased stability and volatility was gained by introducing analysis of permethylated [88], and permethylated and reduced [89], derivatives. In previous mentioned EI-MS the ions were generated by high-energy electrons. However, the requirement of a gaseous phase sample is a disadvantage since the non-volatile glycosphingolipids had to be vaporized by heating, and this made them unstable. The analyzes of permethylated glycosphingolipids introduced by Karlsson *et al*. (1974) solved this problem.

The vaporizing of the sample in the ion source by heating was no longer needed when the soft ionization techniques, as *e.g.* Fast Atom Bombardment (FAB) – MS, and Electrospray Ionisation (ESI) – MS [90], were introduced. ESI-MS was adopted to analyze large macromolecules. However, there were two disadvantages with ESI-MS: firstly, the sensitivity achieved was low, and secondly, no fragment ions were produced. This was however solved by the introduction of nano-ESI which gave improved sensitivity, and the introduction of mass tandem (MS/MS or MS^2) which allowed fragmentation of ions.

In these studies the non-acid glycosphingolipids were hydrolyzed by endoglycoceramidase II from *Rhodococcus* spp. prior to LC-MS analyses using graphitized carbon columns. This method gives resolution of isomeric oligosaccharides, and the MS^2 analyses give sequence information and allow differentiation of linkage positions by diagnostic cross-ring ^{0,2}A-type fragments [91]. One drawback however, is that the *Rhodococcus* endoglycoceraminidase has a relatively restricted hydrolytic capacity, *i.e.* ganglio- and globo-series glycosphingolipids are relatively resistant to this enzyme [92].

3.2.2 Proton NMR spectroscopy

The use of proton NMR spectroscopy to determine anomerity of the glycosidic bonds in glycolipids was first introduced for per-methylated glycosphingolipid derivatives [93-95]. Prior to this method, anomerity was determined by stepwise exoglycosidase digestion of monosaccharides from the carbohydrate chain [96], which could only be performed on pure glycolipid compounds with sufficient amount of starting material. Proton NMR spectroscopy also give in part supplementary information regarding binding position as well as saccharide type and sequence.

Subsequently, new methods came into play enabling analyses of native glycosphingolipids dissolved in D_2O and DMSO [97, 98]. The development in magnetic strength and software also allowed analysis of glycosphingolipid mixtures. A limitation for the technique has been the need for a large amount of substance compared to other analytical techniques such as mass spectrometry and immunoassays. However, if high amounts of material are available, the 2D (COSY) NMR method may solve all structural aspects of a glycosphingolipid species.

3.3 Binding assays with carbohydrate binding biological ligands.

There are several different biological reagents that may be used to detect, characterize and localize carbohydrate antigens/epitopes on membranes, cells or tissue sections (exemplified in Figure 7). The reagents may be *e.g.* antibodies, lectins, viruses or bacteria. These reagents can be labelled with fluorescent dyes [99], enzymes [100-102], radioactive compounds or gold nanoparticles [103]. Subsequently, the reaction may be visualized directly on the membrane, by light or electron microscopy or by autoradiography. When antibodies are used as primary reagent, usually a secondary antibody labeled as described, is used as detection reagent. By choosing appropriate secondary antibodies it is also possible to detect class (IgM/IgA/IgG) or subclass (*e.g.* IgG1 - IgG4) of antibodies present in sera from humans or experimental animals.

However, it should be noted that absence of binding does not prove the absence of the carbohydrate binding epitope, since certain carbohydrate structures might be cryptic [104]. Furthermore, cross-reactivity is a well-known phenomenon when using carbohydrate binding proteins, such as anti-carbohydrate monoclonal antibodies and lectins [105, 106]. Appropriate positive and negative controls are therefore necessary.

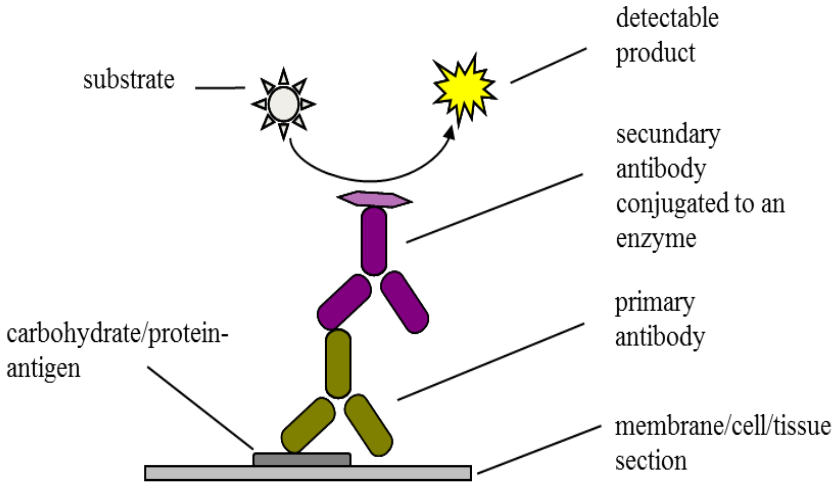


Figure 7. Illustration of the basic principle of binding assays, here exemplified with the use of primary and secondary antibodies and enzyme-conjugated visualization.

3.3.1 Thin-Layer Chromatogram Binding Assay

In 1980, Magnani *et al*. [107] lay the foundation to what has since been a flexible and useful tool to identify and characterize binding active carbohydrates. First adapted to detect binding of cholera toxin to glycosphingolipids the method has since been developed to include *e.g*. binding of lectins [108], antibodies [109] viruses [110] and bacteria [111]. Briefly, two aluminum-backed TLC plates are chromatographed in parallel, one is subjected to chemical staining and one to binding experiments. After visualization the patterns of the two plates are compared, (exemplified in Figure 8). By using structurally characterized reference glycosphingolipids, conclusions may be drawn about binding epitopes. Furthermore, non-specific binding of the glycosphingolipids may be revealed.

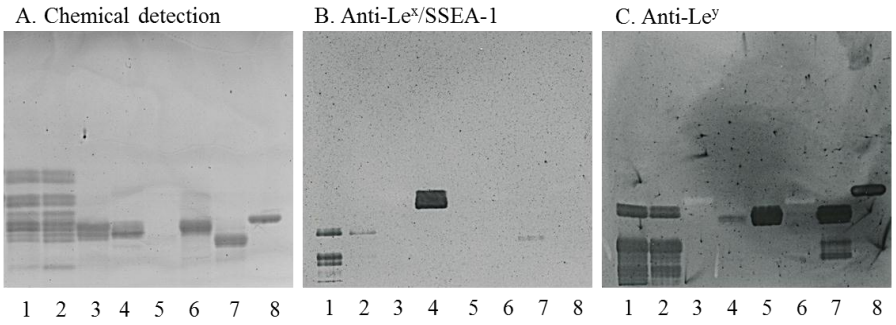


Figure 8. Binding of monoclonal antibodies to slow migrating non-acid glycosphingolipid fractions of human embryonic stem cells. *A-C*, thin-layer chromatograms after staining with anisaldehyde (A), anti-Le^x monoclonal antibody P12 (B) and anti-Le^y monoclonal antibody F3 (C). Lane 1: hESC SA181, lane 2: hESC SA121, lane 3: reference A type 1 hexaglycosylceramide, lane 4: reference Le^x pentaglycosylceramide, lane 5: reference Le^y hexaglycosylceramide, lane 6: reference Le^a pentaglycosylceramide, lane 7: reference Le^b hexaglycosylceramide, lane 8: reference H type 2 pentaglycosylceramide.

3.3.2 Western Blot

Western blot detects and identifies specific proteins present in a sample of cells or tissues [112]. The proteins are first purified from other cell material, and are thereafter separated according to their molecular weight with gel electrophoresis, and then blotted on to a membrane of choice and finally detected with antibodies, lectins or microbial agents [113].

3.3.3 Flow cytometry

Flow cytometry is a technique that rapidly measures individual cells in large heterogeneous samples. Cells may be counted, sorted, identified and quantified by recording the light scattering and fluorescent characteristics of each cell. In general, labeling is achieved by fluorophores attached to antibodies or different cellular components [114]. FITC (fluorescein isothiocyanate) and PE (phycoerythrin) are two commonly used fluorophores having different emission spectrum, which makes them well suited to be used simultaneously in one experiment. Briefly, antibody stained cells in mono-suspension flows in a stream of liquid, through a canal which is radiated with laser. The fluoroscense detected reflects size, complexity and number of cells positive for the antibody in question. The different wavelengths are measured and detected by the flow cytometer and finally analyzed with software

programs. For presentation of the data, histograms and 2-D plots are commonly used. The method is valuable in the clinic for diagnosis of various disorders or in research for detection of *e.g.* biomarkers and antigens on the cell surface. On the down side, the sample used has to be prepared as a single cell suspension before subjected to the flow cytometer [115], this means that information about the architectural structure of tissues is lost.

3.3.4 Immunohistochemistry

While flow cytometry gives information at the single cell level, immunohistochemistry may be viewed as the method of choice for observation of intact tissue. Immunohistochemistry is routinely used in the clinic for diagnosis of *e.g.* tumors and in science to observe and localize the presence and distribution of antigens/markers on intact cells or tissue sections [116]. Proper sample preparation and fixation is crucial to retain tissue architecture and cell morphology and also to avoid alteration of epitopes. The procedure involves fixation, usually with plain formalin or buffered paraformaldehyde, embedding in paraffin and semi-thin sectioning. The sections are then subjected to immunostaining with antibodies, and the result is visualized with, either light (immunoperoxidase) or fluorescence (immunofluorescence) microscopy, depending on the labeling of the antibodies.

3.3.5 Electron microscopy

The electron microscopy (EM) techniques depend on the use of a beam of electrons as the "source of light" [117]. The optical properties of the instruments are achieved by strong magnetic fields and EM provides a resolution that is about three orders of magnitude better than that of standard light microscopy. There are two basic principles in the handling of the electron beam in electron microscopes: (i) a defocussed beam is allowed to transilluminate a section of the sample in order to create a "shadow image" of its contents, *i.e.* **Transmission Electron Microscopy** (TEM); (ii) a sharply focused electron beam is forced to scan over the specimen surface in close parallel lines whereupon various physical interaction phenomena between the incident beam and the matter are recorded and quantified for each x/y position of the beam, *i.e.* **Scanning Electron Microscopy** (SEM) (exemplified in Figure 5). One major application of SEM is to get information on the detailed surface topography of the specimen.

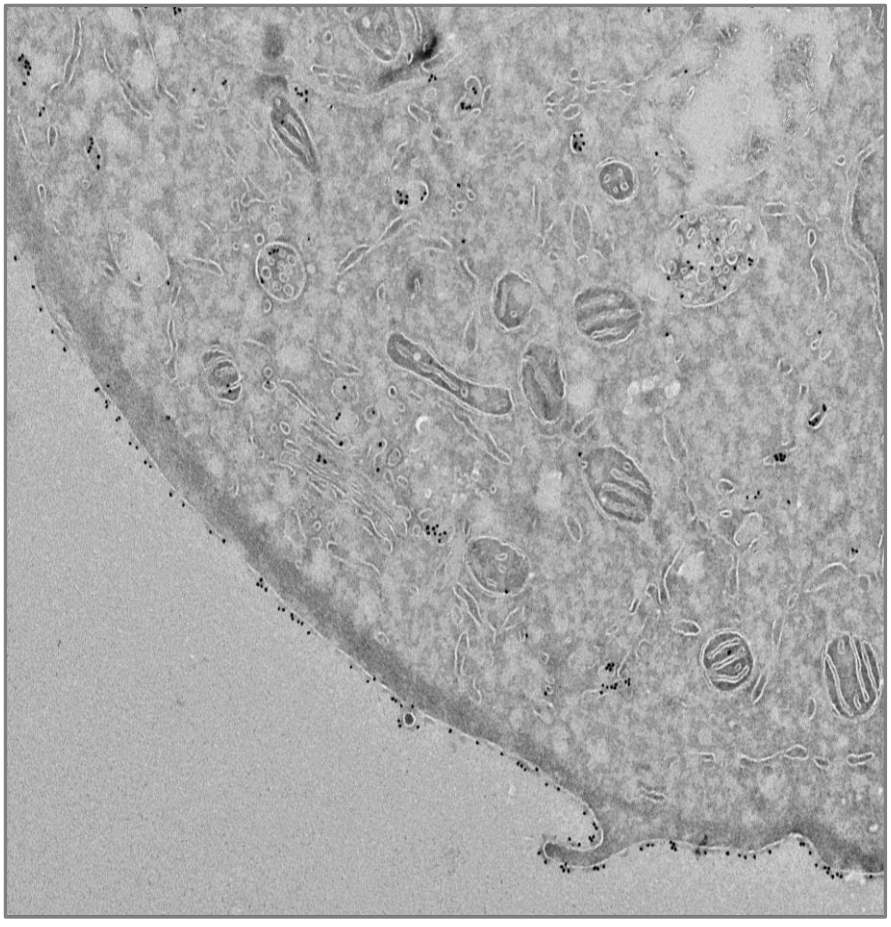


Figure 9. TEM image of hESC line SA181. Incubation with a sialyl-lactotetra antibody revealed an intense staining along the cell surface membrane (gold labelling is visualized as dark round spots) as well as intracellular staining of e.g. mitochondria and Golgi stacks.

The *specimen preparation* is a major challenge in biological EM, where cells and extracellular components originally present in a hydrated environment, should be possible to expose to the highly energetic electron beam in a high-vacuum condition, and still retain a subcellular-molecular architecture. In addition, the specimen must be given image forming properties that interact with the electron beam.

For TEM the key preparative events are fixation, ultrathin sectioning (preceded by dehydration and plastic infiltration, or by freezing), and contrasting (adding heavy elements such as Os, Pb, and U).

Sectioning is performed with an ultramicrotome at a section thickness of 50-60 nm, at room temperature for plastic embedded specimens, or at ≈-110°C for frozen objects (cryo-sectioning). Sections are collected on support grids before examination in the TEM.

For SEM, the specimen must be dried after fixation by a method that overcomes the deleterious effects of surface tension, otherwise surface details will collapse. This preparation step is followed by conductive mounting on a specimen holder and, as a rule, deposition of a thin (a few nm) film of metal in a sputter coater to improve imaging properties.

A methodological variation to be mentioned is immunocytochemistry. The aim is to localize the subcellular distribution of a biomolecule. It is usually performed in TEM. EM sections, preferably cryo-sections of lightly fixed material, are exposed to a dilution of primary antibodies that binds to exposed antigen, followed by a second incubation step secondary antibodies pre-labeled with electron dense gold nanoparticles. Thus, in the TEM image the distribution of gold particles will reveal the locations of the original antigen (exemplified in Figure 9).

4 RESULTS AND DISCUSSION

4.1 Paper I and II

The structural complexity of human embryonic stem cell glycosphingolipids is far greater than earlier identified

At the time the human embryonic stem cell glycosphingolipid studies were initiated, the structural characterization of glycosphingolipids of embryonic stem cells came solely from experiments performed on mouse stem cells [118]. However, recently, two studies on structurally characterized glycosphingolipids in hESC were reported [119, 120]. These previous studies were done on minute amounts of hESC (10^5-10^6 cells). In this study we aimed at performing a more comprehensive analysis of non-acid and acid glycosphingolipids in human embryonic stem cells.

Paper I: Structural characterization of non-acid glycosphingolipids in hESC grown under feeder free conditions.

The total non-acid (neutral) glycosphingolipids were isolated from the two human embryonic stem cell lines (SA121, SA181). We had the advantage to start with large quantities of cells ($1x10^9$cells/cell line), which gave the opportunity to isolate subfractions in order to obtain extended structural information regarding the slow migrating complex compounds. The separation procedure is outlined in Figure 9. By mass spectrometry (exemplified in Figure 10) and proton NMR, in combination with antibody and lectin binding, twelve glycosphingolipids not previously described in hESC were identified (summarized in Table 3).

Thus, in addition to the already identified globo and lacto core chain glycosphingolipids, we identified several glycosphingolipids with neolacto core chains i.e. neolactotetraosylceramide, the H type 2 pentaosylceramide, the Lex pentaosylceramide and heptaosylceramide, and the Ley hexaosylceramide and octaosylceramide.

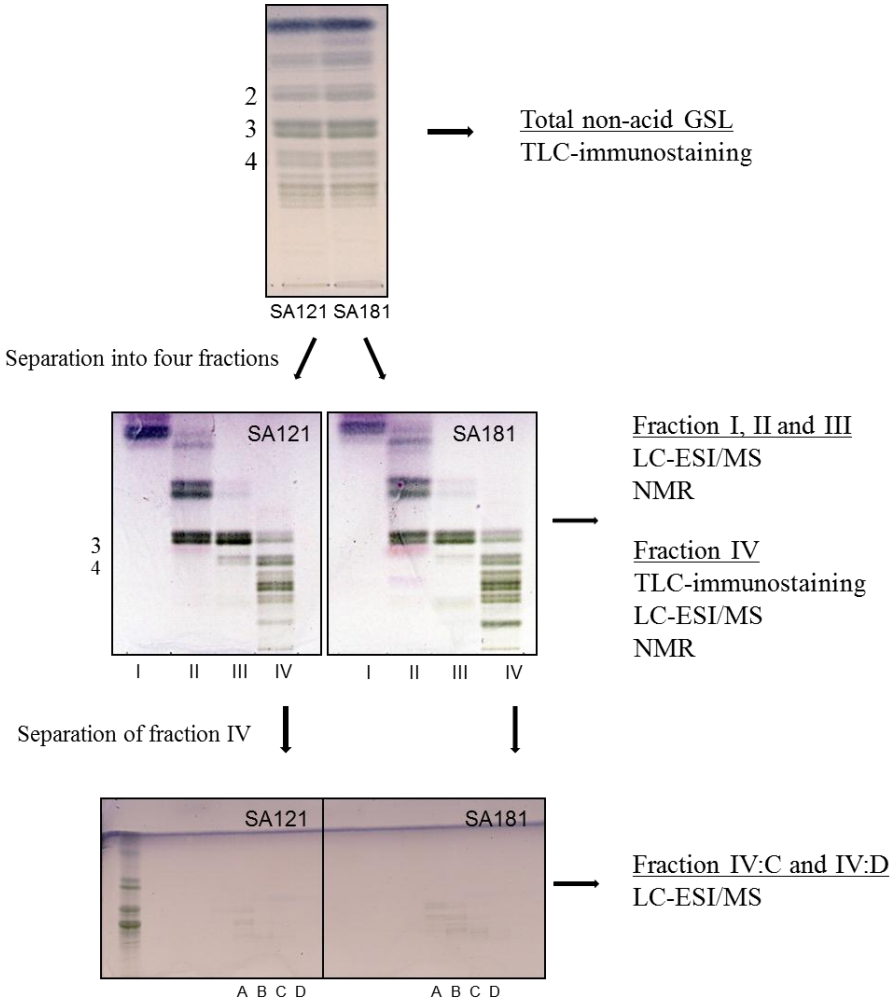


Figure 10. Summary of the characterization of the isolated non-acid glycosphingolipids from hESC lines SA121 and SA181 (paper I). Numbers to the left of the chromatograms denotes approximate number of sugars in the glycan chain. GSL, glycosphingolipid; TLC, thin-layer chromatography; LC-ESI/MS, liquid chromatography-electrospray ionization/ mass spectronomy; NMR, nuclear magnetic resonance.

The presence of Le^x sequences in undifferentiated hESC is a controversial and confusing issue. The anti-SSEA-1 antibody MC480 binds to the Le^x epitope [121] and also recognizes Le^x-terminated hepta-, nona- and decaosylceramides of human erythrocytes [122]. The absence of binding of the MC480 antibody has led to the conclusion that Le^x is not expressed in undifferentiated hESC, while it appears in early differentiation [123].

However, by binding studies using another anti-Le^x antibody (P12) (exemplified in Figure 7B and Figure 11), and by mass spectrometry we identified the Le^x pentaosylceramide and heptaosylceramide in the non-acid glycosphingolipid fractions of undifferentiated hESC. Furthermore, when hESC were analyzed by flow cytometry, the P12 antibody gave distinct cell membrane staining, while the MC480 antibody was negative (K Säljö, unpublished data). Taken together this indicates that the requirements for binding of these two Le^x-recognizing antibodies differ.

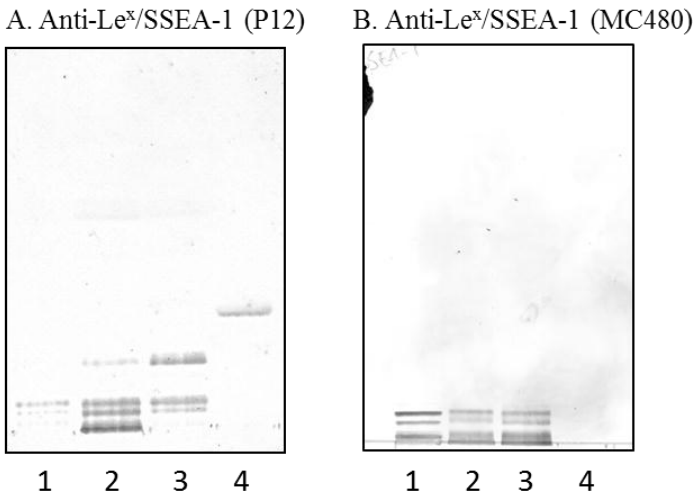


Figure 11. TLC-immunostaining with Le^x-binding antibodies to embryonic stem cell lines SA121-IV and SA181-IV demonstrate binding to slow migrating non-acid glycosphingolipids by the P12 antibody (A) and the MC480 antibody (B). Note the absence of binding by the MC488 antibody to the reference Le^x pentaosylceramide, in contrast to the P12 antibody that gives an intense staining. Lane 1: hESC line SA121-IV, Lane 2: hESC line SA181-IV, Lane 3: reference of total non-acid glycosphingolipids of erythrocytes and lane 4: reference Le^x pentaosylceramide.

Binding assays with the MC480 antibody demonstrate that in the non-acid fractions of hESC SA121 and SA181 only very slow migrating GSLs are recognized (Figure 11). Thus, one possibility is that Le^x terminated GSLs with extended core chains are up-regulated upon differentiation, and are the "real" SSEA-1 markers. This item requires further studies.

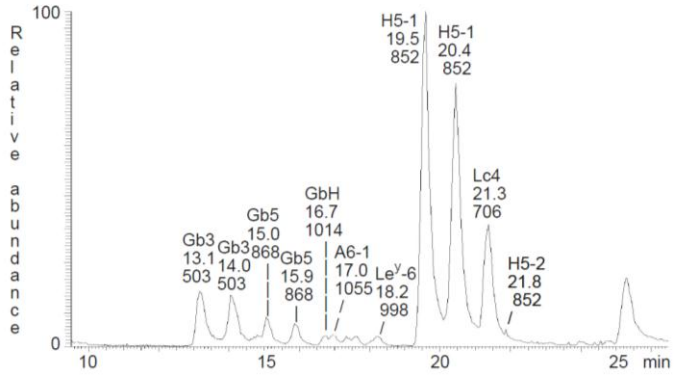


Figure 12. Base peak chromatogram from LC-ESI/MS of the oligosaccharides obtained by digestion of fraction 181-IV from human embryonic stem cells with *Rhodococcus* endoglycoceramidase II. The identification of individual glycosphingolipid-derived oligosaccharides was based on their determined molecular masses and subsequent MS^2 sequencing. Gb3, Galα4Galβ4Glc; Gb5, Galβ3GalNAcβ3Galα4Galβ4Glc; GbH, Fucα2-Galβ3GalNAcβ3Galα4Galβ4Glc; A6-1, GalNAcα3(Fucα2)Galβ3GlcNAcβ3Galβ4Glc; Le^y-6, Fucα2Galβ4-(Fucα3)GlcNAcβ3Galβ4Glc; H5-1, Fucα2Galβ3GlcNAcβ3Galβ4Glc; Lc4, Galβ3GlcNAcβ3Galβ4Glc; H5-2, Fucα2-Galβ4GlcNAcβ3Galβ4Glc

Table 3.Glycosphingolipids characterized in hESC

I. Previously identified in hESC	
Globotetraosylceramide	GalNAcβ3Galα4Galβ4Glcβ1Cer
Globopentaosylceramide/SSEA-3	Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer
Globo H hexaosylceramide	Fucα2Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer
Lactotetraosylceramide	Galβ3GlcNAcβ3Galβ4Glcβ1Cer
H type 1 pentaosylceramide	Fucα2Galβ3GlcNAcβ3Galβ4Glcβ1Cer
II: Novel hESC glycosphingolipids	
Glucosylceramide	Glcβ1Cer
Galactosylceramide	Galβ1Cer
Lactosylceramide	Galβ4Glcβ1Cer
Galabiaosylceramide	Galα4Galβ1Cer
Globotriaosylceramide	Galα4Galβ4Glcβ1Cer
Neolactotetraosylceramide	Galβ4GlcNAcβ3Galβ4Glcβ1Cer
H type 2 pentaosylceramide	Fucα2Galβ4GlcNAcβ3Galβ4Glcβ1Cer
A type 1 hexaosylceramide	GalNAcα3(Fucα2)Galβ3GlcNAcβ3Galβ4Glcβ1Cer
Le^x/SSEA-1 pentaosylceramide	Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer
Le^y hexaosylceramide	Fucα2Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer
Le^x/SSEA-1 heptaosylceramide	Galβ4(Fucα3)GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer
Le^y octaosylceramide	Fucα2Galβ4(Fucα3)GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer

Paper II: Sialyl-lactotetra is a Novel Cell Surface Marker of Undifferentiated Human Pluripotent Stem Cells

In the companion study we examined the total acid glycosphingolipid fractions isolated from the two human embryonic stem cell lines by TLC-immunostaining and LC-ESI/MS (Figure 13). Here, in addition to the hESC gangliosides previously reported [119, 120] several novel hESC compounds were characterized, like the sulfated glycosphingolipids sulfatide, sulf-lactosylceramide and sulf-globopentaosylceramide, and the gangliosides sialyl-globotetraosylceramide and sialyl-lactotetraosylceramide.
Thereafter, the cell surface expression of sialyl-lactotetra (Figure 14) and sulfated glycosphingolipids was examined by flow cytometry, immunohistochemistry and electron microscopy. Flow cytometry demonstrated a high cell surface expression of the sialyl-lactotetra epitope on all hPSC examined, but no expression of sulfated glycosphingolipids. hESC-derived hepatocyte-like and cardiomyocyte-like cells were devoid of sialyl-lactotetra expression.
The high cell surface expression of sialyl-lactotetra on hESC was confirmed by immunohistochemistry, and the sialyl-lactotetra epitope was also found on hiPSC.

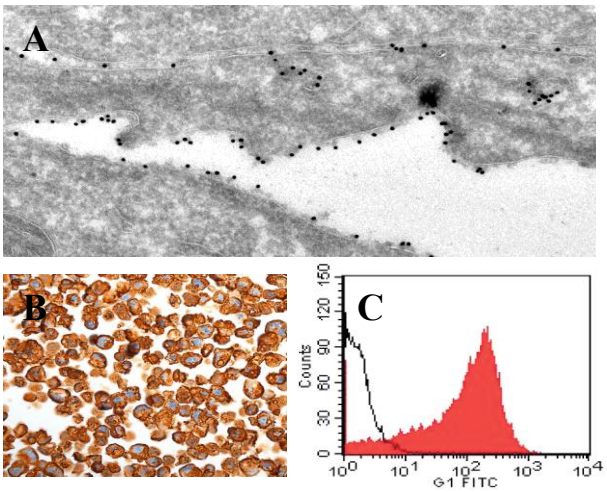


Figure 14. Staining of hESC with the sialyl-lactotetra antibody examined by electron microscopy (A), light microscopy (B) and flow cytometry (C).

In contrast, immunohistochemistry using the anti-sulfatide antibody gave a diffuse intracellular staining but no staining of the cell surface. Electron microscopy was used to further investigate this different subcellular localization of sialyl-lactotetra and sulfatide in hESC.

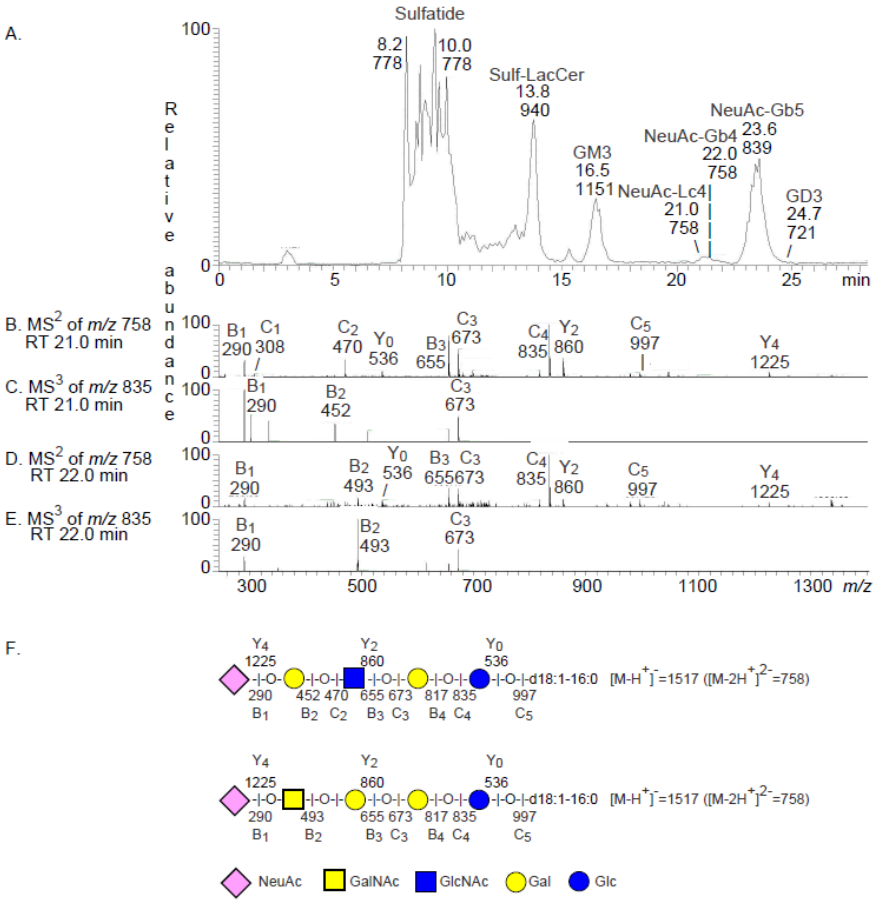


Figure 13. Characterization of sialyl-lactotetraosylceramide and sialyl-globotetraosylceramide by LC-ESI/MS of the total acid glycosphingolipids from hESC. (A) Base peak chromatogram. (B) MS^2 of the molecular ion at *m/z* 758 (retention time 21.0 min) gave a series of B and C ions (B_1 at *m/z* 290, C_2 at *m/z* 470, B_3 at *m/z* 655, C_3 at *m/z* 673, B_4 at *m/z* 817, C_4 at *m/z* 835, and B_5 at *m/z* 979), and a series of Y ions (Y_0 at *m/z* 536, Y_2 at *m/z* 860, and Y_4 at *m/z* 1225), demonstrating a glycosphingolipid with NeuAc-Hex-HexNAc-Hex-Hex carbohydrate sequence and d18:1-16:0 ceramide. (C) MS^3 of the fragment ion at *m/z* 835 (retention time 21.0) min gave a B_2 ion at *m/z* 452 confirming a terminal NeuAc-Hex sequence. (D) MS^2 of the molecular ion at *m/z* 758 (retention time 22.0 min) gave a B_1 ion at *m/z* 290 indicating a terminal NeuAc, which was accompanied by a B_2 ion at *m/z* 493, demonstrating a terminal NeuAc-HexNAc sequence. There was also a series of B and C type ions (B_3 at *m/z* 655, C_3 at *m/z* 673, B_4 at *m/z* 817, C_4 at *m/z* 835, and C_5 at *m/z* 997), and Y type ions at *m/z* 536 (Y_0) and *m/z* 860 (Y_2). Taken together this demonstrated a ganglioside with NeuAc-HexNAc-Hex-Hex-Hex sequence with d18:1-16:0 ceramide, i.e. sialyl-globotetraosylceramide. (E) MS^3 of the fragment ion at *m/z* 835 (retention time 22.0 min) gave a B_2 ion at *m/z* 493, confirming a terminal NeuAc-HexNAc sequence. (F) Interpretation formulae showing the deduced glycosphingolipid sequences.

The anti-sialyl-lactotetra antibody gave a distinct labeling of the cell membrane, along with labeling of mitochondria, endoplasmatic reticulum and Golgi stacks. The latter three compartments were also labeled by the anti-sulfatide antibody, which however, gave no labeling of the cell membrane.
Finally we followed the expression of sialyl-lactotetra during differentiation of hiPSC into hepatocyte-like cells. Here the sialyl-lactotetra expression rapidly decreased, along with reduced expression of established pluripotency markers, and two weeks after the induction of expression, sialyl-lactotetra was virtually absent. Taken together these findings suggest that sialyl-lactotetra is a novel marker of human pluripotent stem cells. This suggestion is strengthened by the fact that the distribution of sialyl-lactotetraosylceramide in normal human tissues is very limited. This ganglioside has been found in human meconium [124], indicating that it is present in the developing fetal gastro-intestinal tract, and in the fetal brain [125].

There are to date very few specific highly sensitive markers of hPSC identified, which severely limits their utility in the analysis and recovery of hPSC [126]. However, it has been suggested that a combination of OCT4, SSEA-3, and TRA-1-60 should be used to validate PSC pluripotency, since these markers are the most sensitive to differentiation signals [127]. Sialyl-lactotetra closely follows the rate of disappearance of TRA-1-60 upon differentiation, and should be added to the currently available panel of stem cell markers.

Prior to clinical applications of differentiated hPSC, these cells need to be thoroughly characterized. Thus, an important application of markers of undifferentiated hPSC is to distinguish residual pluripotent from differentiated cells, to avoid teratoma formation by contaminating undifferentiated hPSC.

In addition to the proposed potential of hPSC in cell-based therapies, these cells serve as valuable models for studying both basic human development and cancer development. Cancer stem cells are subpopulations of tumor cells with stem or progenitor cell characteristics, and these cells have been proposed to maintaining cancers through initiation and propagation of perpetuating tumor growth [128]. Thus, an important next step is to determine whether sialyl-lactotetra can be utilized for isolation of malignant stem cells from different cancers, and eventually be used as a predictor of clinical outcome.

4.2 Paper III

Characterization of acid and non-acid glycosphingolipids of porcine heart valves as potential immune targets in biological heart valve grafts

Due to minute amounts of starting material, an alternative preparative procedure was used. After extraction and mild alkaline methanolysis the total lipid extract was acetylated and subjected to silicic acid column chromatography. The non-polar compounds such as the free fatty acid methyl esthers were eluted with dichloromethan and the acetylated non-acid and acid glycolipids were eluted with 7.5 and 15% methanol in chloroform while acetylated sphingomyeline was eluted with 75% methanol in chloroform. After deacetylation and dialysis the fraction containing glycolipids were separated into total non-acid and acid glycolipid fractions respectively on DEAE ion exchange columns.

LC-ESI/MS and TLC-immunostaining revealed that the non-acid glycosphingolipids were globotetraosylceramide, two blood group H compounds based on neolactotetraosylceramide and gangliotetraosylceramide respectively as well as the Gal xenoantigen Galα3neolactotetraosylceramide. The acidic glycolipids were identified as sulfatide and the gangliosides GM3, GM2, GM1, fucosyl-GM1, GD3 and GD1a as illustrated in Figure. 15.

Surprisingly, all gangliosides contained the *N*-Acetyl-neuraminic acid, *i.e.* we did not find the non-Gal antigen *N*-Glycolyl-neuraminic acid which is a major ganglioside component in pig organs [73]. No difference between glycosphingolipids from porcine aortic and pulmonary valves was found.

The BHV used in the clinic are treated with different de-cellularization procedures, such as glutaraldehyde. Therefore it can be anticipated that many of the glycosphingolipids identified here will be absent in BHV. However, Gal antigens have been identified in commercially used BHV [129-131]. Even if the recipients preformed anti-Gal xenoantibodies do not cause a HAR of the grafted BHVs as in vascularized xenografts [70] they may react with the Gal antigens still remaining in the BHV tissue and contribute to the destruction of valves. In this respect the GalT-KO pig strains might be advantageous compared to wild type pigs currently used [72].

Our data showing that the porcine heart valve ganglioside fraction lack of NeuGc has to be supported by studies of the glycoprotein linked saccharides. If the porcine heart cusps lack NeuGc also in this molecular compartment, the newly produced pig strain transgenic for the CMP-Neu5Ac hydroxylase gene will not add any advantage when used for production of valve tissue of porcine origin.

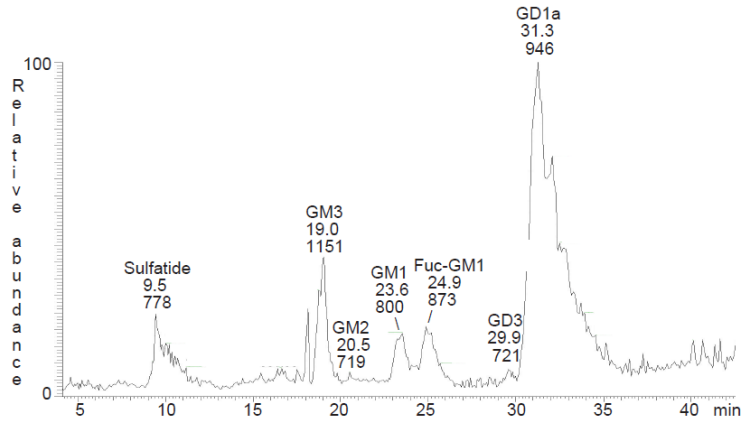


Figure 15. Base peak chromatogram from LC-ESI/MS of the acid glycosphingolipids from porcine aortic heart valves. The identification of individual glycosphingolipids was based on their determined molecular masses and subsequent MS^2 sequencing. Sulfatide, SO_3-3Galβ1Cer; GM3,NeuAcα3Galβ4Glcβ1Cer; GM2,GalNAcβ4-(NeuAcα3)-Galβ4Glcβ1Cer; GM1,Galβ3GalNAcβ4-(NeuAcα3)Galβ4Glcβ1Cer; Fuc-GM1, Fucα2Galβ3GalNAcβ4-(NeuAcα3)-Galβ4Glcβ1Cer; GD3, NeuAcα8NeuAcα3Galβ4Glcβ1Cer; GD1a,NeuAcα3Galβ3GalNAcβ4-(NeuAcα3)-Galβ4Glcβ1Cer.

5 FUTURE PERSPECTIVES

This thesis presents novel data about the glycosylation of cells and tissues (human embryonic stem cells and porcine heart valves) aimed for used in regenerative medicine, and provide a basis for further investigations. The sialyl-lactotetra carbohydrate sequence has been identified as a marker of undifferentiated hPSC, which disappears rapidly from the cells after induction of differentiation into hepatocyte-like cells. An important next step is to investigate whether sialyl-lactotetra may serve as markers for cancer detection, and be used for identification and enrichment of cancer stem cells.

Another item that merits further investigations is the sub-cellular localization of sulfatide during stem cell differentiation. Sulfatide is a marker that is used to follow the differentiation of hPSC into oligodendrocytes. However, our findings demonstrate that sulfatide is produced already in undifferentiated hESC, but is retained within intracellular compartments. The mechanism for mobilization of sulfatide to the cell membrane upon oligodendrocyte differentiation should be further investigated.

Differentiation dependent changes in the expression of other identified hESC glycosphingolipids, as well as their subcellular distribution, should also be evaluated.

Further investigations in the case of BHV is primarily to evaluate the immune reactivity of BHV recipients, by glycosphingolipid binding studies using both identified BHV compounds and reference glycosphingolipids, as NeuGc-containing gangliosides. The glycosylation of BHV glycoproteins should also be investigated.

6 ACKNOWLEDGEMENTS

Numerous people have helped, encouraged and supported me over my PhD-years and for that I am tremendously thankful, some of these are listed below. I could go on forever and name every one of you, but for obvious reasons this is not possible. However, if I failed to thank you, please blame it on my head and not my heart!

Min handledare - Michael Breimer, ett stort tack för att du vågade satsa på och anta mig som doktorand och för ditt stöd, din uppmuntran, ditt förtroende, din generositet och inte minst för ditt innovativa tänkande!

Min bihandledare - Susann Teneberg, när du kom in i bilden medförde du en enorm kunskap och entusiasm, projektet lyfte och tog fart, ditt engagemang sträckte sig långt utöver vad som förväntades av dig, jag är dig evigt tacksam!

Min bihandledare - Johan Mölne, för att du delat med dig av ditt expertiskunnande i patologi och immunohistokemi och för att du aldrig tröttnade när jag kom springande med antikroppar av alla dess slag!

Mina medförfattare – John Benktander, Karin Säljö, Jonas Ångström, Petter Björquist och Anders Aspegren på Cellartis, Maria Blomqvist, Jan-Eric Månsson och Bengt. R. Johansson. Tack för gott samarbete!

”Min grupp” - Marianne – för att du är du! John - vi har kämpat ihop sen dag ett, tack för all hjälp och för att du orkat svara på alla mina frågor om MS. Karin Säljö – för att du stöttat mig och uppmuntrat mig och för att du alltid varit lika entusiastisk inför nya ”fynd” som jag! Miralda, Klara, Filip och Danni, för att ni berikar vår grupp!

GTB-gruppen - Mette och Lola, vad hade jag gjort utan er! Tack, för hjälp och stöd, för ändlösa diskussioner om ditt och datt och för många goda skratt. Christel, tack för all hjälp med stort och smått.

Göran Larssons grupp – tack för att ni ”adopterade” in mig i er grupp under åren på Sahlgrenska.

Everybody at MedKem – the Mucin group, Sara Lindens group, MPE core facilitys and Proteomics, thank you for always being so nice to me! Ett speciellt tack till Malin, Richard och Anna-Carin för all hjälp med WB.

EM-gruppen – Bengt, Yvonne och Kanita, tack för stort kunnande, utmärkta handledning och inte minst för att ni introducerade mig till elektronmikroskopins magiska värld! Ett extra tack till Bengt för de fantastiskt fina EM-bilderna du har hjälpt mig med till denna bok.

Mina underbara vänner som betyder så mycket för mig, tack för att ni finns!

Min fantastiska familj – för att ni alltid finns där för mig och stöttar mig, ni är ovärderliga!

Sono molto felice che ho di nuovo contatto con la mia famiglia italiana!
Baci a tutti voi!

Peter, Emelie och Josefine 

This work was supported by grants from the Sahlgrenska Academy at University of Gothenburg and the Sahlgrenska University Hospital, and by the EU FP7 Collaborative Project "TransLink" (contract # HEALTH-F4-2013-603049).

REFERENCES

1. Griesemer, A., K. Yamada, and M. Sykes, *Xenotransplantation: immunological hurdles and progress toward tolerance.* Immunol Rev, 2014. **258**(1): p. 241-58.
2. Thomson, J.A., et al., *Embryonic stem cell lines derived from human blastocysts.* Science, 1998. **282**(5391): p. 1145-7.
3. Takahashi, K. and S. Yamanaka, *Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors.* Cell, 2006. **126**(4): p. 663-76.
4. Tabar, V. and L. Studer, *Pluripotent stem cells in regenerative medicine: challenges and recent progress.* Nat Rev Genet, 2014. **15**(2): p. 82-92.
5. Elliott, M.J., et al., *Stem-cell-based, tissue engineered tracheal replacement in a child: a 2-year follow-up study.* Lancet, 2012. **380**(9846): p. 994-1000.
6. Mason, C. and P. Dunnill, *A brief definition of regenerative medicine.* Regen Med, 2008. **3**(1): p. 1-5.
7. Manji, R.A., et al., *Porcine bioprosthetic heart valves: The next generation.* Am Heart J, 2012. **164**(2): p. 177-85.
8. Varki, A. and N. Sharon, *Historical Background and Overview*, in *Essentials of Glycobiology*, A. Varki, et al., Editors. 2009: Cold Spring Harbor (NY).
9. Hook, M., L. Kjellen, and S. Johansson, *Cell-surface glycosaminoglycans.* Annu Rev Biochem, 1984. **53**: p. 847-69.
10. Esko, J.D., K. Kimata, and U. Lindahl, *Proteoglycans and Sulfated Glycosaminoglycans*, in *Essentials of Glycobiology*, A. Varki, et al., Editors. 2009: Cold Spring Harbor (NY).
11. Zimmerman, G.A., *Two by two: the pairings of P-selectin and P-selectin glycoprotein ligand 1.* Proc Natl Acad Sci U S A, 2001. **98**(18): p. 10023-4.
12. Carlos, T.M. and J.M. Harlan, *Leukocyte-endothelial adhesion molecules.* Blood, 1994. **84**(7): p. 2068-101.
13. Walz, G., et al., *Recognition by ELAM-1 of the sialyl-Lex determinant on myeloid and tumor cells.* Science, 1990. **250**(4984): p. 1132-5.
14. Rydell, G.E., et al., *QCM-D studies of human norovirus VLPs binding to glycosphingolipids in supported lipid bilayers reveal strain-specific characteristics.* Glycobiology, 2009. **19**(11): p. 1176-84.
15. Leffler, H., et al., *Chemical and clinical studies on the interaction of Escherichia coli with host glycolipid receptors in urinary tract infection.* Scand J Infect Dis Suppl, 1982. **33**: p. 46-51.

16. King, C.A. and W.E. van Heyningen, *Evidence for the complex nature of the ganglioside receptor for cholera toxin.* J Infect Dis, 1975. **131**(6): p. 643-8.
17. Muramatsu, T., *Developmentally regulated expression of cell surface carbohydrates during mouse embryogenesis.* J Cell Biochem, 1988. **36**(1): p. 1-14.
18. Hakomori, S., *Possible functions of tumor-associated carbohydrate antigens.* Curr Opin Immunol, 1991. **3**(5): p. 646-53.
19. Yanagisawa, M. and R.K. Yu, *The expression and functions of glycoconjugates in neural stem cells.* Glycobiology, 2007. **17**(7): p. 57R-74R.
20. J.L.W, T., *A treatise on the chemical constitution of the brain.*1884, London: Baillière, Tindall & Cox.
21. van Meer, G., D.R. Voelker, and G.W. Feigenson, *Membrane lipids: where they are and how they behave.* Nat Rev Mol Cell Biol, 2008. **9**(2): p. 112-24.
22. Hakomori, S.I., *Structure and function of glycosphingolipids and sphingolipids: recollections and future trends.* Biochim Biophys Acta, 2008. **1780**(3): p. 325-46.
23. Merrill, A.H., Jr., *Sphingolipid and glycosphingolipid metabolic pathways in the era of sphingolipidomics.* Chem Rev, 2011. **111**(10): p. 6387-422.
24. Lindstrom, K., et al., *Blood group glycosphingolipid expression in kidney of an individual with the rare blood group A1 Le(a-b+) p phenotype: absence of blood group structures based on the globoseries.* Glycoconj J, 1996. **13**(2): p. 307-13.
25. Leffler, H., G.C. Hansson, and N. Stromberg, *A novel sulfoglycosphingolipid of mouse small intestine, IV3-sulfogangliotetraosylceramide, demonstrated by negative ion fast atom bombardment mass spectrometry.* J Biol Chem, 1986. **261**(3): p. 1440-4.
26. Nagai, K., et al., *Mono-sulfated globotetraosylceramide from human kidney.* J Biochem, 1989. **106**(5): p. 878-86.
27. Nagai, K., et al., *Mono-sulfated globopentaosylceramide from human kidney.* J Biol Chem, 1989. **264**(27): p. 16229-37.
28. Needham, L.K. and R.L. Schnaar, *The HNK-1 reactive sulfoglucuronyl glycolipids are ligands for L-selectin and P-selectin but not E-selectin.* Proc Natl Acad Sci U S A, 1993. **90**(4): p. 1359-63.
29. Mitsumoto, Y., et al., *Cloning and chromosomal mapping of human glucuronyltransferase involved in biosynthesis of the HNK-1 carbohydrate epitope.* Genomics, 2000. **65**(2): p. 166-73.
30. Schauer, R., *Chemistry, metabolism, and biological functions of sialic acids.* Adv Carbohydr Chem Biochem, 1982. **40**: p. 131-234.

31. Varki, A., *Loss of N-glycolylneuraminic acid in humans: Mechanisms, consequences, and implications for hominid evolution.* Am J Phys Anthropol, 2001. **Suppl 33**: p. 54-69.
32. Chou, H.H., et al., *A mutation in human CMP-sialic acid hydroxylase occurred after the Homo-Pan divergence.* Proc Natl Acad Sci U S A, 1998. **95**(20): p. 11751-6.
33. Tettamanti, G., *Ganglioside/glycosphingolipid turnover: new concepts.* Glycoconj J, 2004. **20**(5): p. 301-17.
34. Sandhoff, K. and K. Harzer, *Gangliosides and gangliosidoses: principles of molecular and metabolic pathogenesis.* J Neurosci, 2013. **33**(25): p. 10195-208.
35. Breton, C., J. Mucha, and C. Jeanneau, *Structural and functional features of glycosyltransferases.* Biochimie, 2001. **83**(8): p. 713-8.
36. Bjork, S., et al., *Structures of blood group glycosphingolipids of human small intestine. A relation between the expression of fucolipids of epithelial cells and the ABO, Le and Se phenotype of the donor.* J Biol Chem, 1987. **262**(14): p. 6758-65.
37. Finne, J., et al., *Novel polyfucosylated N-linked glycopeptides with blood group A, H, X, and Y determinants from human small intestinal epithelial cells.* J Biol Chem, 1989. **264**(10): p. 5720-35.
38. Schnaar, R.L., A. Suzuki, and P. Stanley, *Glycosphingolipids*, in *Essentials of Glycobiology*, A. Varki, et al., Editors. 2009: Cold Spring Harbor (NY).
39. Oriol, R., et al., *Genetic regulation of the expression of ABH and Lewis antigens in tissues.* APMIS Suppl, 1992. **27**: p. 28-38.
40. Christiansen, D., et al., *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.
41. Joziasse, D.H., et al., *Bovine alpha 1----3-galactosyltransferase: isolation and characterization of a cDNA clone. Identification of homologous sequences in human genomic DNA.* J Biol Chem, 1989. **264**(24): p. 14290-7.
42. Breimer, M.E., et al., *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.
43. Holgersson, J., M.E. Breimer, and B.E. Samuelsson, *Basic biochemistry of cell surface carbohydrates and aspects of the tissue distribution of histo-blood group ABH and related glycosphingolipids.* APMIS Suppl, 1992. **27**: p. 18-27.
44. Breimer, M.E., et al., *The preparative separation of sialic acid-containing lipids from sulphate group-containing glycolipids from small intestine of different animals. Analysis by thin-layer chromatography and detection of novel species.* J Biochem, 1983. **93**(6): p. 1473-85.

45. Svennerholm, L., *Designation and schematic structure of gangliosides and allied glycosphingolipids.* Prog Brain Res, 1994. **101**: p. XI-XIV.
46. Leela R. L. Davies and A. Varki, *Why Is N-Glycolylneuraminic Acid Rare in the Vertebrate Brain?*, in *Topics in Current Chemistry* 2013.
47. Karlsson, A., et al., *Different glycosphingolipid composition in human neutrophil subcellular compartments.* Glycoconj J, 2001. **18**(3): p. 231-43.
48. Stroud, M.R., et al., *Monosialogangliosides of human myelogenous leukemia HL60 cells and normal human leukocytes. 2. Characterization of E-selectin binding fractions, and structural requirements for physiological binding to E-selectin.* Biochemistry, 1996. **35**(3): p. 770-8.
49. Stroud, M.R., et al., *Monosialogangliosides of human myelogenous leukemia HL60 cells and normal human leukocytes. 1. Separation of E-selectin binding from nonbinding gangliosides, and absence of sialosyl-Le(x) having tetraosyl to octaosyl core.* Biochemistry, 1996. **35**(3): p. 758-69.
50. Breimer, M.E., et al., *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.
51. Evans, M., *Discovering pluripotency: 30 years of mouse embryonic stem cells.* Nat Rev Mol Cell Biol, 2011. **12**(10): p. 680-6.
52. Heins, N., et al., *Derivation, characterization, and differentiation of human embryonic stem cells.* Stem Cells, 2004. **22**(3): p. 367-76.
53. Young, R.A., *Control of the embryonic stem cell state.* Cell, 2011. **144**(6): p. 940-54.
54. Zhao, W., et al., *Embryonic stem cell markers.* Molecules, 2012. **17**(6): p. 6196-236.
55. Fenderson, B.A., E.M. Eddy, and S. Hakomori, *Glycoconjugate expression during embryogenesis and its biological significance.* Bioessays, 1990. **12**(4): p. 173-9.
56. Andrews, P.W., et al., *Three monoclonal antibodies defining distinct differentiation antigens associated with different high molecular weight polypeptides on the surface of human embryonal carcinoma cells.* Hybridoma, 1984. **3**(4): p. 347-61.
57. Solter, D. and B.B. Knowles, *Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1).* Proc Natl Acad Sci U S A, 1978. **75**(11): p. 5565-9.
58. Hennen, E. and A. Faissner, *LewisX: a neural stem cell specific glycan?* Int J Biochem Cell Biol, 2012. **44**(6): p. 830-3.
59. Kannagi, R., et al., *Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells.* EMBO J, 1983. **2**(12): p. 2355-61.

60. Kannagi, R., et al., *New globoseries glycosphingolipids in human teratocarcinoma reactive with the monoclonal antibody directed to a developmentally regulated antigen, stage-specific embryonic antigen 3.* J Biol Chem, 1983. **258**(14): p. 8934-42.
61. Shevinsky, L.H., et al., *Monoclonal antibody to murine embryos defines a stage-specific embryonic antigen expressed on mouse embryos and human teratocarcinoma cells.* Cell, 1982. **30**(3): p. 697-705.
62. Tang, C., et al., *An antibody against SSEA-5 glycan on human pluripotent stem cells enables removal of teratoma-forming cells.* Nat Biotechnol, 2011. **29**(9): p. 829-34.
63. Lanctot, P.M., F.H. Gage, and A.P. Varki, *The glycans of stem cells.* Curr Opin Chem Biol, 2007. **11**(4): p. 373-80.
64. Martin, M.J., et al., *Human embryonic stem cells express an immunogenic nonhuman sialic acid.* Nat Med, 2005. **11**(2): p. 228-32.
65. Fujitani, N., et al., *Total cellular glycomics allows characterizing cells and streamlining the discovery process for cellular biomarkers.* Proc Natl Acad Sci U S A, 2013. **110**(6): p. 2105-10.
66. Fishman, J.A., L. Scobie, and Y. Takeuchi, *Xenotransplantation-associated infectious risk: a WHO consultation.* Xenotransplantation, 2012. **19**(2): p. 72-81.
67. Blum, B. and N. Benvenisty, *The tumorigenicity of human embryonic stem cells.* Adv Cancer Res, 2008. **100**: p. 133-58.
68. Manji, R.A., et al., *Bioprosthetic heart valves of the future.* Xenotransplantation, 2014.
69. Lila, N., et al., *Gal knockout pig pericardium: new source of material for heart valve bioprostheses.* J Heart Lung Transplant, 2010. **29**(5): p. 538-43.
70. Naso, F., et al., *Alpha-Gal detectors in xenotransplantation research: a word of caution.* Xenotransplantation, 2012. **19**(4): p. 215-20.
71. Galili, U., et al., *Man, apes, and Old World monkeys differ from other mammals in the expression of alpha-galactosyl epitopes on nucleated cells.* J Biol Chem, 1988. **263**(33): p. 17755-62.
72. McGregor, C.G., et al., *Cardiac xenotransplantation technology provides materials for improved bioprosthetic heart valves.* J Thorac Cardiovasc Surg, 2011. **141**(1): p. 269-75.
73. Diswall, M., et al., *Structural characterization of alpha1,3-galactosyltransferase knockout pig heart and kidney glycolipids and their reactivity with human and baboon antibodies.* Xenotransplantation, 2010. **17**(1): p. 48-60.
74. Siddiqui, R.F., J.R. Abraham, and J. Butany, *Bioprosthetic heart valves: modes of failure.* Histopathology, 2009. **55**(2): p. 135-44.
75. Konakci, K.Z., et al., *Alpha-Gal on bioprostheses: xenograft immune response in cardiac surgery.* Eur J Clin Invest, 2005. **35**(1): p. 17-23.

76. Park, C.S., et al., *Anti-alpha-Gal antibody response following xenogeneic heart valve implantation in adults.* J Heart Valve Dis, 2013. **22**(2): p. 222-9.
77. Andrews, P.D., et al., *High-content screening of feeder-free human embryonic stem cells to identify pro-survival small molecules.* Biochem J, 2010. **432**(1): p. 21-33.
78. 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.
79. Breimer, M.E., et al., *Glycosphingolipids and the differentiation of intestinal epithelium.* Exp Cell Res, 1981. **135**(1): p. 1-13.
80. Holgersson, J., P.A. Jovall, and M.E. Breimer, *Glycosphingolipids of human large intestine: detailed structural characterization with special reference to blood group compounds and bacterial receptor structures.* J Biochem, 1991. **110**(1): p. 120-31.
81. Ando, S., M. Isobe, and Y. Nagai, *High performance preparative column chromatography of lipids using a new porous silica, Iatrobeads. I. Separation of molecular species of sphingoglycolipids.* Biochim Biophys Acta, 1976. **424**(1): p. 98-105.
82. Schnaar, R.L. and L.K. Needham, *Thin-layer chromatography of glycosphingolipids.* Methods Enzymol, 1994. **230**: p. 371-89.
83. Waldi, D., *[Simple quantitative rapid determination of pregnanediol on thin layer plates for early pregnancy demonstration and for supervision of the menstrual cycle in women].* Klin Wochenschr, 1962. **40**: p. 827-30.
84. Svennerholm, L., *Chromatographic Separation of Human Brain Gangliosides.* J Neurochem, 1963. **10**: p. 613-23.
85. Yang, H.J. and S.I. Hakomori, *A sphingolipid having a novel type of ceramide and lacto-N-fucopentaose 3.* J Biol Chem, 1971. **246**(5): p. 1192-200.
86. Stellner, K., H. Saito, and S.I. Hakomori, *Determination of aminosugar linkages in glycolipids by methylation. Aminosugar linkages of ceramide pentasaccharides of rabbit erythrocytes and of Forssman antigen.* Arch Biochem Biophys, 1973. **155**(2): p. 464-72.
87. Karlsson, K.A., et al., *Mass spectra of trimethylsilyl derivatives of homogeneous cerebrosides (monoglycosylceramides).* Chem Phys Lipids, 1972. **9**(3): p. 230-46.
88. Hakomori, S., *A Rapid Permethylation of Glycolipid, and Polysaccharide Catalyzed by Methylsulfinyl Carbanion in Dimethyl Sulfoxide.* J Biochem, 1964. **55**: p. 205-8.
89. Karlsson, K.A., *Carbohydrate composition and sequence analysis of a derivative of brain disialoganglioside by mass spectrometry, with molecular weight ions at m-e 2245. Potential use in the specific microanalysis of cell surface components.* Biochemistry, 1974. **13**(18): p. 3643-7.

90. Fenn, J.B., et al., *Electrospray ionization for mass spectrometry of large biomolecules.* Science, 1989. **246**(4926): p. 64-71.
91. 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.
92. Ito, M. and T. Yamagata, *Purification and characterization of glycosphingolipid-specific endoglycosidases (endoglycoceramidases) from a mutant strain of Rhodococcus sp. Evidence for three molecular species of endoglycoceramidase with different specificities.* J Biol Chem, 1989. **264**(16): p. 9510-9.
93. Falk, K.E., K.A. Karlsson, and B.E. Samuelsson, *Proton nuclear magnetic resonance analysis of anomeric structure of glycosphingolipids. Lewis-active and Lewis-like substances.* Arch Biochem Biophys, 1979. **192**(1): p. 191-202.
94. Falk, K.E., K.A. Karlsson, and B.E. Samuelsson, *Proton nuclear magnetic resonance analysis of anomeric structure of glycosphingolipids. Blood group ABH-active substances.* Arch Biochem Biophys, 1979. **192**(1): p. 177-90.
95. Falk, K.E., K.A. Karlsson, and B.E. Samuelsson, *Proton nuclear magnetic resonance analysis of anomeric structure of glycosphingolipids. The globo-series (one to five sugars).* Arch Biochem Biophys, 1979. **192**(1): p. 164-76.
96. Hakomori, S.-i., *Chemistry of Glycosphingolipids*, in *Sphingolipid Biochemistry, Handbook of Lipid Research* 1983. p. 1-165.
97. 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.
98. Angstrom, J., et al., *Default biosynthesis pathway for blood group-related glycolipids in human small intestine as defined by structural identification of linear and branched glycosylceramides in a group O Le(a-b-) nonsecretor.* Glycobiology, 2004. **14**(1): p. 1-12.
99. Coons, A.H., *Fluorescent antibodies as histochemical tools.* Fed Proc, 1951. **10**(2): p. 558-9.
100. Nakane, P.K. and G.B. Pierce, Jr., *Enzyme-labeled antibodies: preparation and application for the localization of antigens.* J Histochem Cytochem, 1966. **14**(12): p. 929-31.
101. Avrameas, S. and J. Uriel, *[Method of antigen and antibody labelling with enzymes and its immunodiffusion application]*. C R Acad Sci Hebd Seances Acad Sci D, 1966. **262**(24): p. 2543-5.
102. 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.
103. Faulk, W.P. and G.M. Taylor, *An immunocolloid method for the electron microscope.* Immunochemistry, 1971. **8**(11): p. 1081-3.

104. Gahmberg, C.G. and S.I. Hakomori, *External labeling of cell surface galactose and galactosamine in glycolipid and glycoprotein of human erythrocytes.* J Biol Chem, 1973. **248**(12): p. 4311-7.
105. Manimala, J.C., et al., *High-throughput carbohydrate microarray profiling of 27 antibodies demonstrates widespread specificity problems.* Glycobiology, 2007. **17**(8): p. 17C-23C.
106. Manimala, J.C., et al., *High-throughput carbohydrate microarray analysis of 24 lectins.* Angew Chem Int Ed Engl, 2006. **45**(22): p. 3607-10.
107. Magnani, J.L., D.F. Smith, and V. Ginsburg, *Detection of gangliosides that bind cholera toxin: direct binding of 125I-labeled toxin to thin-layer chromatograms.* Anal Biochem, 1980. **109**(2): p. 399-402.
108. Smith, D.F., *Glycolipid-lectin interactions: detection by direct binding of 125I-lectins to thin layer chromatograms.* Biochem Biophys Res Commun, 1983. **115**(1): p. 360-7.
109. Magnani, J.L., et al., *A monosialoganglioside is a monoclonal antibody-defined antigen of colon carcinoma.* Science, 1981. **212**(4490): p. 55-6.
110. Hansson, G.C., et al., *A novel approach to the study of glycolipid receptors for viruses. Binding of Sendai virus to thin-layer chromatograms.* FEBS Lett, 1984. **170**(1): p. 15-8.
111. Karlsson, K.A. and N. Stromberg, *Overlay and solid-phase analysis of glycolipid receptors for bacteria and viruses.* Methods Enzymol, 1987. **138**: p. 220-32.
112. Burnette, W.N., *"Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A.* Anal Biochem, 1981. **112**(2): p. 195-203.
113. Mahmood, T. and P.C. Yang, *Western blot: technique, theory, and trouble shooting.* N Am J Med Sci, 2012. **4**(9): p. 429-34.
114. Davey, H.M. and D.B. Kell, *Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses.* Microbiol Rev, 1996. **60**(4): p. 641-96.
115. Ormerod, M.G. and P.R. Imrie, *Flow cytometry.* Methods Mol Biol, 1990. **5**: p. 543-58.
116. Ramos-Vara, J.A. and M.A. Miller, *When tissue antigens and antibodies get along: revisiting the technical aspects of immunohistochemistry--the red, brown, and blue technique.* Vet Pathol, 2014. **51**(1): p. 42-87.
117. Bozzola, J. and L.D. Russel, *Electron microscopy: Principles and Techniques for Biologists*1999.
118. Yanagisawa, M., *Stem cell glycolipids.* Neurochem Res, 2011. **36**(9): p. 1623-35.

119. Liang, Y.J., et al., *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.
120. Liang, Y.J., et al., *Changes in glycosphingolipid composition during differentiation of human embryonic stem cells to ectodermal or endodermal lineages.* Stem Cells, 2011. **29**(12): p. 1995-2004.
121. Gooi, H.C., et al., *Stage-specific embryonic antigen involves alpha 1 goes to 3 fucosylated type 2 blood group chains.* Nature, 1981. **292**(5819): p. 156-8.
122. Kannagi, R., et al., *A series of human erythrocyte glycosphingolipids reacting to the monoclonal antibody directed to a developmentally regulated antigen SSEA-1.* J Biol Chem, 1982. **257**(24): p. 14865-74.
123. Draper, J.S., et al., *Surface antigens of human embryonic stem cells: changes upon differentiation in culture.* J Anat, 2002. **200**(Pt 3): p. 249-58.
124. Prieto, P.A. and D.F. Smith, *A new ganglioside in human meconium detected with antiserum against human milk sialyltetrasaccharide a.* Arch Biochem Biophys, 1986. **249**(1): p. 243-53.
125. Svennerholm, L., et al., *Human brain gangliosides: developmental changes from early fetal stage to advanced age.* Biochim Biophys Acta, 1989. **1005**(2): p. 109-17.
126. Cho, H.C., et al., *Surface markers in stem cells and cancer from the perspective of glycomic analysis.* Int J Biol Markers, 2012. **27**(4): p. e344-52.
127. Ramirez, J.M., et al., *Brief report: benchmarking human pluripotent stem cell markers during differentiation into the three germ layers unveils a striking heterogeneity: all markers are not equal.* Stem Cells, 2011. **29**(9): p. 1469-74.
128. Jordan, C.T., M.L. Guzman, and M. Noble, *Cancer stem cells.* N Engl J Med, 2006. **355**(12): p. 1253-61.
129. Naso, F., et al., *First quantification of alpha-Gal epitope in current glutaraldehyde-fixed heart valve bioprostheses.* Xenotransplantation, 2013. **20**(4): p. 252-61.
130. Naso, F., et al., *First quantitative assay of alpha-Gal in soft tissues: presence and distribution of the epitope before and after cell removal from xenogeneic heart valves.* Acta Biomater, 2011. **7**(4): p. 1728-34.
131. Kasimir, M.T., et al., *Presence and elimination of the xenoantigen gal (alpha1, 3) gal in tissue-engineered heart valves.* Tissue Eng, 2005. **11**(7-8): p. 1274-80.