Charles University in Prague
Faculty of Pharmacy in Hradec Králové
Department of Pharmaceutical Chemistry and Drug Control

STUDIES ON GLYCOSPHINGOLIPIDS AS IMMUNE TARGETS IN BIOPROSTHETIC HEART VALVES

Master Thesis

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I declare that this thesis is my original author's work. All literature and other sources, which I used for elaboration of this thesis, are properly cited in the text and stated in the reference list. This work has never been used to obtain different or the same degree.

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V Praze dne: Petr Jansa

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Abstract

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Title: Studies on glycosphingolipids as immune targets in bioprosthetic heart valves

The treatment of valvular heart disease represents approximately 20% of all cardiac surgery. One alternative of this treatment is replacement of diseased valve with bioprosthetic heart valves (BHV). These BHV are manufactured from divergent human or animal tissues *e.g.* porcine pericardium.

Although this treatment is beneficial for certain groups of patients, it is susceptible to some complications such as rejection of the xenograft or early BHV deterioration. In both of them the immune system is involved and both might result in BHV failure.

This study aims to isolate and characterize important targets of this immune response – glycosphingolipids (GSL). Therefore, 8 non-acidic and 7 acidic GSL from porcine pericardium have been isolated and characterized by mass spectrometry and carbohydrate binding assays.

The acidic GSL from goat erythrocytes have been isolated and characterized. Notable is characterization of a new NeuGc-containing GSL - NeuGc-GT1b ganglioside.

And finally, 60 binding assays examined the presence of antibodies against various GSLs in patient serum collected before, one and six months after BHV treatment surgery. The radioactive iodine-125-labeled anti-human antibodies and autoradiographic visualisation have been used for detection. Results were mostly negative. However, in few samples an increased reactivity Galα3 and NeuGc antigens was found.

This knowledge might contribute to explanations of immune response against xenografts. This might help with further research on strategies preventing early BHV deterioration and, therefore, increase final outcome of BHV treatment of valvular heart disease.

Keywords: glycosphingolipids, bioprosthetic, heart valves, xenotransplantation, Galα3, NeuGc

Abstrakt

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Název: Studie glykosfingolipidů jako imunitních cílů v bioprotetických srdečních chlopních

Léčba vad srdečních chlopní představuje přibližně 20% všech kardiochirurgických operací. Jednou z možností léčby je náhrada vadné chlopně pomocí bioprotetické srdeční chlopně (BSCh). Tyto BSCh jsou zhotovovány z různých lidských nebo zvířecích tkání, např. ze srdečního perikardu.

Ačkoli tato léčba je prospěšná pro určité skupiny pacientů, pojí se k ní komplikace jako odhojení xenotransplantátu, či časné opotřebení BSCh. V obou případech je zapojena reakce imunitního systému a oba mohou vyústit ve ztrátu funkce BSCh.

Tato práce si klade za cíl izolovat a charakterizovat významné cíle imunitní odpovědi – glykosfingolipidy (GSL). Proto bylo z prasečího perikardu izolováno 8 nekyselých a 7 kyselých GSL, které byly charakterizovány pomocí hmotnostní spektrometrie a carbohydrate binding assay.

Z kozích erytrocytů byly izolovány a charakterizovány kyselé GSL. Za zmínku stojí charakterizace nového GSL obsahujícího NeuGc - NeuGc-GT1b gangliosidu.

Nakonec byla pomocí 60 binding assayí zkoumána přítomnost protilátek, proti různým GSL, v séru pacientů, odebraném před, jeden a šest měsíců po léčbě pomocí BSCh. Pro dektekci byly užity protilátky proti lidským protilátkám značené pomocí radioaktivního jódu-125 a autoradiografická vizualizace. Výsledky byly převážně negativní. V několika případech však byla nalezena zvýšená reaktivita vůči Galα3 a NeuGc antigenům.

Tyto poznatky mohou přispět k vysvětlení imunitní odpovědi na xenotransplantáty, což dále může pomoci v dalším výzkumu strategií předcházení časnému opotřebení BSCh a tedy i zvýšit celkovou úspěšnost léčby nemocí chlopenních vad pomocí BSCh.

Klíčová slova: glykosfingolipidy, bioprotetický, srdeční chlopně, xenotransplantace, Galα3, NeuGc

Abbreviations

AHXR	Acute humoral xenograft rejection
ADCC	Antibody dependent cellular cytotoxicity
BHV	Bioprosthetic heart valve
BSCh	Bioprotetická srdeční chlopeň
C:M (x:x)	Chloroform: methanol in ratio x:x
C:M:W	Chloroform: methanol: distilled water
CBA	Carbohydrate binding assay
CMAH	Cytidine monophosphate-*N*-acetylneuraminic acid hydroxylase
DEAE	Diethylaminoethyl cellulose
DiChl	Dichlormethane
ESI	Electrospray ionization
Fuc	Fucose
Gal	Galactose
GalCer	β-linked galactose to ceramide
GalNAc	*N*-Acetylgalactosamine
GalT	α-1,3-galactosyltransferase
Galα3	Galactose-α-1,3-galactose
Glc	Glucose
GlcCer	β-linked glucose to ceramide
GlcNAc	*N*-Acetylglucosamine
GSL	Glycosphingolipid
	Glykosfingolipid
HAR	Hyperacute rejection
Hex	Hexose
HexNAc	*N*-Acetylhexoseamine
-KO	Knocked out *gene* for -
LC-ESI/MS	Liquid chromatography-electrospray ionization/MS
Man	Mannose
MeOH	Methanol
MHV	Mechanical heart valve
MiC	Methanol in chloroform
MS	Mass spectrometry
NeuAc	*N*-Acetylneuraminic acid
NeuGc	N-Glycolylneuraminic acid
NGal-Ab	Antibodies targeted against non-Galα3 antigens
PBS	Phosphate buffered saline
SAC	Silicic acid chromatography

1. Introduction

This thesis is a part of the Translink project, which concerns about assessing the mid- to long-term risk factors and aims to improve outcome of xenotransplantation of animal heart valves to the patients. This kind of treatment using bioprosthetic heart valves is one of the available alternatives for transplantation of human heart valves.

Bioprosthetic heart valves (BHV) are routinely used in the clinical practice to replace diseased heart valves. However, even BHVs suffer from late dysfunction, *i.e.* the valves also deteriorate with time. The xenografts will expose molecules on their surfaces which may provoke immune system of the recipient, which might lead to subsequent graft damage. In humans, there are a number of preformed antibodies directed against carbohydrate antigens. Thus, in addition to the well-known blood group ABO system, humans have performed antibodies directed towards carbohydrates found exclusively in animals *e.g.* the Galα3 epitope and *N*-glycolylneuraminic acid (NeuGc). Here we aim to identify glycosphingolipids on animal tissues used for bioprosthetic heart valves, and evaluate the immune reactions towards these potential antigens in patients after transplantation of bioprosthetic heart valves.

1.1. Carbohydrates at the cell surface

In nature, all kind of cells are covered by surface membrane comprising, except ordinary lipids, also different types of glycoconjugates. From this reason, the surface of every cell is densely covered with layer of sugars, so-called glycocalyx.

Glycoconjugates are macromolecules which consist of one or more monosaccharide or oligosaccharide units (the glycone) linked by covalent bond to non-carbohydrate compound (the aglycone). All kind of cells in nature are covered by different types of glycoconjugates (Varki, et al., 2009)

Such a large diversity of glycoconjugates on the cell surface caused by plurality of monosaccharides units and their derivates, structural linking of saccharides, and also different kinds of core chains plays important role in communication. (Varki, et al., 2009)

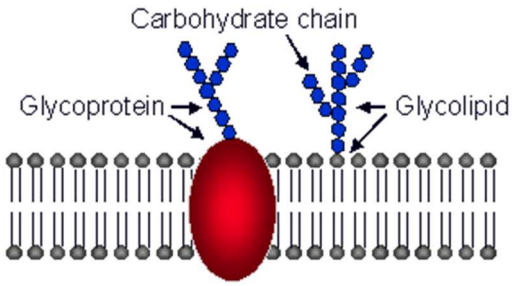


Figure 1: Part of membrane with glycocalyx, adapted from: (Anonymous, 2011)

1.2. Glycoconjugates

In general view, glycoconjugates (or glycans) are extremely various group of chemical compounds. The glycone part of the macromolecule contains the main part of the mass in naturally occurring glycoconjugates.

Glycoconjugates are classified depending on whether the aglycone is protein or lipid and according to the way of binding (O-glycosylation, N- glycosylation, …).

Glycoproteins are mainly the N-linked, where the oligosaccharide chain is bound to the nitrogen atom of amidic group of the side chain of asparagine, or O-linked where the side chain of serine or threonine is used for linking saccharide to its free hydroxyl group. (Varki, et al., 2009)

All N-linked glycans share a core sugar sequence Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ1-Asn-X-Ser/Thr or shorter: $Man_3GlcNAc_2Asn$. Those can be subclassified into three groups according to branching and composition of single branches; oligomannoses, complex and hybrid. (Stanley, et al., 2009)

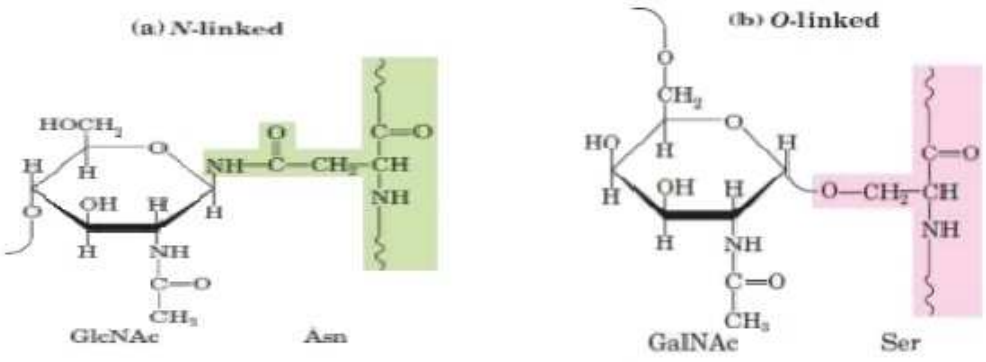


Figure 2: Schematic view of O- and N- linked glycoproteins, modified from (Jabbar, 2014)

O-linked glycans in eukaryotes are linked to the hydroxyl group of serine or threonine with carbohydrate residue of *N*-acetylgalactosamine (GalNAc). The largest group of O-GalNAc glycans is called mucins for their ubiquitous presence in mucous secretions on cell surface and in body fluids. (Brockhausen, et al., 2009)

The simplest mucin glycan, which is *N*-acetylgalactosamine linked to Ser/Thr is called also Tn glycan. The extension by galactose (Gal), sulfate, fucose (Fuc), sialic acids or next GalNAc forms 4 basic core chains, which can be further modified by *i.e.* acetylation or sulfation. By this, there is reached such a great variability giving to O-glycans possibility to act like receptors and antigens in many processes in body like *i.e.* T-lymphocyte activation or sperm-egg interaction. (Brockhausen, et al., 2009)

Proteoglycans are heavily glycosylated macromolecules, where the carbohydrate component represents more than 95% of the molecule mass. Usually there are repeating polysaccharides sequences attached by xylosyl-serine linkage the one central protein. They occur in the extracellular matrix produced by cells of connective tissues, but they can also be attached directly to the cytoplasmic membrane. (Varki, et al., 2009)

Whereas the two previous categories can appear both in the cell membrane and outside the cell, glycolipids are anchored by their nonpolar lipid part only in the cell membrane.

Glycolipids can be either glycerolipids or sphingolipids. In both cases there is only one carbohydrate chain to each lipid (Barone, 2014b)

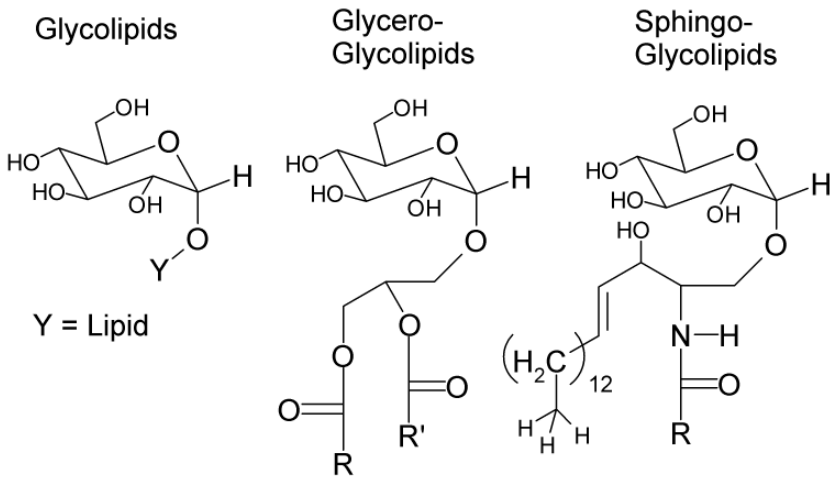


Figure 3: Schematic formulas of glycolipids, adapted from (RicHard-59, 2012)

1.3. Glycosphingolipids

Since the discovery of glycosphingolipids it has been shown that they are important component for many essential functions like: cell adhesion, cell growth, cell regulation, differentiation, intercellular interaction, cell recognition, interaction with signal molecules, microbes and microbial toxins. (Watts, 2003)

The glycosphingolipid (GSLs) comprises of two moieties:

The first is the oligosaccharide chain containing from one to approximately forty monosaccharide units. The variability of its structure is due to variations in types of carbohydrate units, in binding positions, branching, different sequence order and anomeric configuration. More than 350 different carbohydrate chains on glycosphingolipids have been discovered. (Merril, 2011)

The second part is nonpolar and incorporated into the outer leaflet of the cell membrane. It consists of a long chain aliphatic amino-alcohol (sphingoid base) acylated on the nitrogen by a fatty acid. (Watts, 2003)

The most common **amino-alcohols** are sphingosine, sphinganine and phytosphingosine. The fatty acids are mainly in the range from 16 to 24 carbon atoms, and are either hydroxylated or not. Compound where a fatty acid and sphingosine (after Egyptian *Sphinx*, because of the similarity between its difficult enigmas and problematic description of sphingosine structure) are bound together was named ceramide. (Varki, et al., 2009)

The word **ceramide** comes from Latin *cera* (wax) and -amid referring to amidic group. (Wikipedia contributors, 2016). Ceramide structures vary in length, rate of hydroxylation and number of unsaturated bonds of both chains, which leads to great diversity and might influence presentation of glycans on the cell membrane.

β-linked galactose (GalCer) or glucose (GlcCer) is usually bound to ceramide. In higher animals, mainly GlcCer extended with larger carbohydrate chains occur. GalCer is rarely extended. Typical substitution of GlcCer is with a β-linked galactose on the C-4 hydroxyl of glucose – this altogether is called lactosylceramide (Galβ1-4GlcβCer). Further extensions of it then forms a series of core structures which are the base of the GSL nomenclature.

Subfamily series	Structure	Abbreviation
Lacto	GlcNAcβ1-3Galβ1-4GlcβCer	Lc_3Cer
	Galβ1-3GlcNAcβ1-3Galβ1-4GlcβCer	Lc_4Cer
Neolacto	Galβ1-4GlcNAcβ1-3Galβ1-4GlcβCer	nLc_4Cer
	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcβCer	nLc_6Cer
Ganglio	GalNAcβ1-4Galβ1-4GlcβCer	Gg_3Cer
	Galβ1-3GalNAcβ1-4Galβ1-4GlcβCer	Gg_4Cer
Globo	Galα1-4Galβ1-4GlcβCer	Gb_3Cer
	GalNAcβ1-3Galα1-4Galβ1-4GlcβCer	Gb_4Cer

Figure 4: Glycosphingolipidic subfamily series and nomenclature, adapted from (Schnaar, et al., 2009)

In the official **nomenclature** GSLs can be described in more ways. The first option is describing the whole structure by abbreviations for sugars, numbers and Greek letters referring to type of binding to another structure. The second is naming GSL using the abbreviation expressing subfamily series and variant *i.e.* Lc_4Cer. To this core structure name are then added next substituents. (Schnaar, et al., 2009)

The third is used especially for gangliosides in daily use. Common GSLs are named simply like "GM3", where first letter refers subfamily (G = gangliosides), the second letter refers to the number of sialic residues (M = mono) and the number which denotes position on thin-layer chromatography plate, (GM1 is more polar than GM2 or GM3 and occurs closer to the start). (Schnaar, et al., 2009)

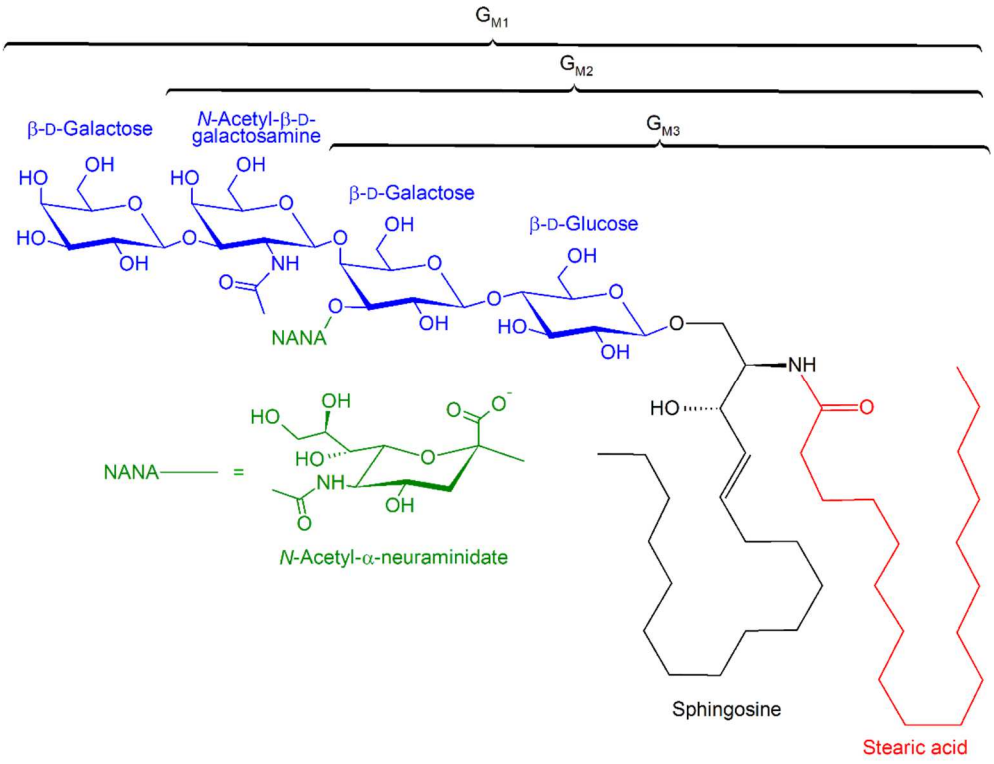


Figure 5: Structure of GM1, GM2 and GM3, adapted from: (Voet, et al., 2008)

1.3.1 Non-acidic glycosphingolipids

Non-acidic (or neutral) glycosphingolipids do not contain charged carbohydrates, neither other ionic groups. The most common in human peripheral tissues are core chains from globo, lacto and neolacto families. The determinants of ABO or Lewis blood group systems are often found on neutral GSL core chains. (Varki, et al., 2009)

1.3.2 Acidic glycosphingolipids

Acidic glycosphingolipids have carbohydrates with sulfate groups or sialic acid. The latter are known as gangliosides, because of its discovery in the "ganglion" or cluster in the brain of patients with from Tay-Sachs disease. (Schnaar, et al., 2009)

Gangliosides (GSLs substituted by sialic acid) have mainly ganglio or neolacto core chains, but lacto and globo core are also possible. The major forms of sialic acid in mammalian GSLs are *N*-glycolylneuraminic acid (NeuGc) and *N*-acetylneuraminic acid (NeuAc). Those two diverge only on C-5 position, where NeuAc has acetyl group and NeuGc a glycolyl group. (Schauer, 1982)

NeuAc is the precursor of NeuGc, and NeuAc is the only form of sialic acid occurring in humans due to a mutation in the human CMP-Neu5Ac hydroxylase (CMAH) gene that occurred over 2 million years ago. (Varki, 2001)

The most common sulfated GSL is sulfatide (SO_3-3GalCer) but a few GSLs based on lacto- ganglio and globo core chains have also been described. (Nagai, 1989)

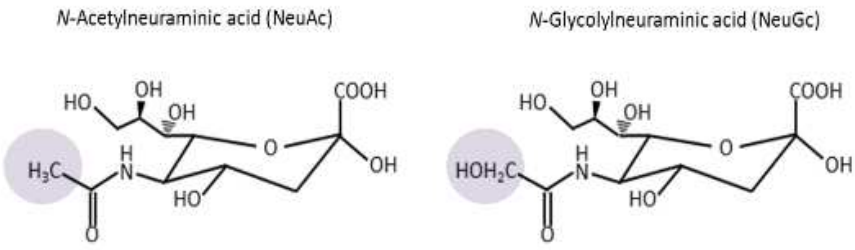


Figure 6: Sialic acids, adapted from: (Barone, 2014b)

1.3.3 Metabolism of GSLs

Biosynthesis

Glycans are secondary gen products, *i.e.* their structure cannot be read directly from DNA. Instead the glycoconjugates are created by enzymes *e.g.* glycotransferases, sulfotransferases, and transporters encoded in the DNA. These enzymes perform posttranslational modifications of proteins and glycosylations of core lipids.

Most of pathways are connected with the luminal side of endoplasmic reticulum and Golgi apparatus. However, some pathways are present also in the nucleus and cytoplasm. (Varki, et al., 2009)

The final outcome of glycosylation is influenced by a number of factors, as *e.g.* distribution of enzymes responsible for start and continuing of glycosylation, their activity, accurate income of precursors and sugars derived to be activated form of nucleotide sugars or balance between biosynthesis and degradation.

The biosynthesis of galactosylceramide, galabiaosylceramide and sulfatide starts on the cytosolic side of endoplasmic reticulum, where the ceramides are assembled. Thereafter they are moved to luminal side of endoplasmic reticulum in order to add galactose by a ceramide galactosyltransferase. For biosynthesis of sulfated GSLs the galactosylceramide is transferred to the luminal Golgi apparatus, where it is modified by sulfotransferase.

More composite glycosphingolipids are made by different process starting with ceramide transfer to cytosolic part of Golgi, where glucose is added by ceramide glucosyltransferase. After that it is moved to the luminal side of Golgi to further glycosylation by sequential work of enzymes attached to the membrane. At the end of biosynthesis, newly synthesized GSLs are relocated by vesicular transport and incorporated into the cell membrane.

Biodegradation

The degradation of GSLs starts with internalization into vesicles. Fusion with endosome by its GSL-enriched areas of membrane may lead to the second invagination of GSLs and to creation of multi-compartment particle. Due to fusion of this secondary endosome with prolysosomes, GSLs are exposed to the hydrolytic enzymes using sphingolipid activator proteins (SAPs) as co-factors. Particles from degradation are then released into the cytoplasm, where they are used for other biosynthesis pathways or further degraded. (Schnaar, et al., 2009)

1.3.4 The diversity of GSLs

A very complex distribution pattern of GSL determinants on the cell, tissue and organ level even within a single individual has been demonstrated by immunohistochemistry. This is due to differences in expression of glycosyltransferases in different cells. (Barone, 2014b)

Different GSLs are found in different species, and this might provoke the immune system and could cause complications after xenotransplantation. For example isoglobo-series GSLs, *N*-glycolylneuraminic acid and GSLs with terminal Galα3 (described below) (are not present in human, although such GSLs are present in dog, horse, rat or pig. (Breimer, 1981)

There is also a diversity among the individuals, where different glycosphingolipids are expressed in different subjects, as *e.g.* GSLs with determinant of the ABO and Lewis blood group systems. (Bjork, 1987)

Even glycosphingolipids in various organs of one individual may diverge. One example is the difference between epithelial cells in the small and large intestine, which mainly have lacto core chains and the pancreas, where globo and lacto core chains are in equal balance.

Diversity among the cells within an organ is demonstrated by human blood, where the main GSLs of erythrocytes are based on the globo core, while the major GSLs in human neutrophils have neolacto core chains and GSLs with globo core chains are completely absent. (Bjork, 1987)

1.4. Xenotransplantation

"Transplantation of cells, tissues and organs between individuals of different species, commonly known as xenotransplantation, has been studied with the aim to solve the critical shortage of human graft sources for clinical transplantation." (Vadori, et al., 2015, p.239)

The recipients' humoral immune response is the dominating cause of graft damage and eventually loss of the transplanted organs, tissues or cells. Moreover, the histopathological features and the kinetics of the appearance of specific antibodies is very similar to the situation occurring after allotransplantation. Thus, it is expected that strategies aimed at avoiding graft damage may be applied to xenotransplantation as well as to allotransplantation in order to extend long-term graft survival. (Vadori et Cozzi, 2015)

Although our knowledge is still incomplete, there are several extracellular materials which are already in use in cardiac, oesophageal, urinary tract and in orthopaedic surgery to restore functions of human organs. In addition, transplantation of whole bioartificial organs, made from xenogeneic scaffolds and repopulated by suitable cells, and using organs, such as heart, liver or kidneys from genetically modified animals, seems to be a promising approach which still needs to be further explored.

1.4.1 The immunological barriers in transplantation

An increasing number of xenotransplantation procedures are performed in wide-spread clinical practice, therefore the increasing evidence of contribution by both parts of immunity, innate and adaptive, is now being revealed. (Vadori et Cozzi, 2015)

1.4.2 Cell mediated reactions

Neutrophils are the most common circulating leukocytes and are also the first cell population responding after transplantation of xenografts (Ezzelarab, et al., 2009). In particular, their activity (chemotaxis and transmigration) rapidly increased after exposition to porcine endothelial cells, compared to the human endothelium after allotranslplantation. This means that neutrophils distinguish between allogeneic and xenogeneic surfaces. In addition, penetration of neutrophils into the target tissue is enhanced by pro-inflammatory cytokines. (Vadori et Cozzi, 2015)

Production of reactive oxygen metabolites (ROMs), IL-1a/b, IL-6 and IL-8 by neutrophils and increased expression of P-selectin and VCAM-1 on the endothelium facilitate infiltration of ***NK cells***. NK cells have direct cytotoxic activity on target cell which lack self-MHC class I molecules and do not provide to NK cell inhibitory signals. It has been reported that NK cells quickly migrate into porcine xenografts perfused ex vivo with human blood. (Vadori et Cozzi, 2015)

Since the Galα3 residues (see below) are probably not included in the increased cytotoxic potential of NK cell on the porcine graft, it has been suggested that NK cells react to unknown glycosylation patterns and that NK cells take part in the antibody-mediated rejection reaction by antibody dependent cellular cytotoxicity (ADCC). (Resch, et al., 2015)

Monocytes and activated macrophages have major role in the cellular part of the immune response against vascularized and either nonvascularized grafts. Macrophages are also regulated by a high number of cytokines. However, in any case, their reactions have to be confirmed by $CD4^+$ T-cells. (T_H lymphocyte). In particular, the phagocytic activity is modulated by ADCC mechanisms or by direct cell-to-cell contact. (Vadori et Cozzi, 2015)

Activated macrophages infiltrate graft tissue very early and persist there until graft rejection. Thus, they are considered as possible contributors to the transplanted graft miscarriage due to rejection. (Ezzelarab, et al., 2009). Immunity response mediated by macrophages has been revealed to occur more often after xenotransplantation in comparison with allotransplantation. (Griesemer, et al., 2014)

The role of Galα3 in increasing monocyte adhesion remains unclear, since macrophage infiltration has been found also in GalT-KO (α-1,3-galactosyltransferase gene knocked out) donor porcine islet grafts. (Thompson, et al., 2011)

INF-γ produced by NK cells and other cytokines stimulate ***T-cell*** activity. (Griesemer, et al., 2014). Experimental evidence suggested that T-cells are the next part of the complex reactions against transplanted xenogeneic graft. T-cells infiltrate the graft, where they are probably in cooperation with macrophages part of immune response leading to rejection of the graft. (Vadori et Cozzi, 2015)

These cells play major part in the rejection process of non-vascularized xenografts. Notable is also that T-cells are the main cause of anti-pig cell cytotoxicity. It has also been shown that Galα3 residues attached to the porcine epithelium stimulates T-cell response by an unexplained mechanism. (Vadori et Cozzi, 2015)

1.4.3 Antibody mediated reaction

Adaptive immunity response, in particular mediated by antibodies, has the most important role in xenograft rejection of solid organs, tissues and even cells. Antibodies are produced by B-cells and their mature form plasma cells in a process that is thought to be mainly T-cell dependent.

Studies of the binding specificities of the antibodies have been done on both human and primates. The majority of them were focused on the well-known xenogeneic antigen epitope Galα3Galβ4GlcNAc-R (Galα3) which is the main target in antibody mediated response of humans against xenograft. This glycodeterminant is present in all animals except Old World monkeys and humans. In addition, around 1% of circulating B-cells in human serum are producing anti-Galα3 antibodies. There are several approaches targeting the B-cells producing anti- Galα3 antibodies and the pathway between them and T-cells.

In this context, anti-CD20 agents, anti-CD19 monoclonal antibodies and drug interrupting communication CD40 and CD154 like have been tested in order to suppress graft rejection. (Vadori et Cozzi, 2015)

All these ways provide significant prolongation of graft survival.

Generally, there are **two divergent kinds** of immune response which may cause rejection of the xenograft:

a) Hyperacute rejection (HAR)

The HAR occurs from a few minutes to hours following the transplantation. The rejection is caused by antibodies pre-existing in the body of the recipient. "Immunoglobulins and complement are deposited in the vessel wall, causing severe endothelial injury and fibrin-platelet rich thrombi. Moreover, a rapid accumulation of neutrophils is observed within the capillaries." (Vadori et Cozzi, 2015 p. 240) There is also thrombotic blockage of capillaries and necrosis in arterial walls. This all together may then lead to the ultimate destroying of the graft.

b) Acute humoral xenograft rejection (AHXR)

The AHXR may appear within days after transplantation due to antibodies elicited as a reaction against the xenograft. This reaction is similar to rejection after allotransplantation from an inappropriate donor. "The histological features of AHXR of a solid organ xenograft are IgM, IgG, C4d and C5b-9 deposition, loss of capillary integrity, endothelial cell death and extensive fibrin deposition." (Vadori et Cozzi, 2015, p.241)

Whereas HAR is performed mainly by naturally present IgM antibodies, the AHXR is mediated by elicited IgM and IgG antibodies. The humoral response is also performed by other mechanisms like antibody dependent cell mediated cytotoxicity (ADCC), inflammation and endothelium in a state supporting coagulation and thrombosis. In addition, activation of the classical complement system creating the membrane attack complex has been referred as contributing in early graft damage. (Vadori et Cozzi, 2015)

Pre-existing and elicited antibodies may also be involved in the reaction of the body consequence after transplantation of extracellular matrices like cartilage, pig ligaments or bioprosthetic heart valves. Here the participation of macrophages, activated by IgM and IgG, and neutrophils, seems to be pivotal in the damaging of bioprosthetic heart valves. (Vadori et Cozzi, 2015)

1.4.4 Carbohydrate targets of the human humoral immune response

Epitopes of proteins and carbohydrates act like antigens in the process of humoral immune response. Because of their presence on the cell surface and their already noted structural diversity, especially among species, they represent principal barrier for successful xenotransplantation.

According to some studies, pigs are considered as the most suitable source of xenografts. However, equine and bovine heart valve cusps are also used for BHV. (Vadori et Cozzi, 2015)

a) Galα3- residue

In previous studies it has been shown that the ***Galα3 residue*** (Galα(1,3)Galβ4GlcNAc-R epitope) is a major target of humoral immune response of the body. It is composed of Galα(1,3)Gal dimer bound to *N*-acetyllactosamine and synthesized by enzyme called α-1,3-galactosyltransferase (GalT), which is not expressed in humans and Old World monkeys and apes. The Galα3 is expressed in both cellular material such as porcine endothelium or epithelium and in extracellular matrices. (Breimer, 2011)

This antigen has a function for signalling and inter-cell interaction like A and B blood group antigens. The physiologic purpose of anti-Gal antibodies is protection of against pathogens and malignant cells and the removal of old or abnormal blood cells which all express Galα3 on their surfaces. (Manji, et al., 2012)

Removal Galα3 leads to improvement of biocompatibility and reduction of acute rejection reactions due to reduced titres of both pre-existing and elicited antibodies. (Griesemer, et al., 2014)

b) Non-Galα3 carbohydrate antigens

13% of IgM and 36% of IgG in human serum bind to so-called non-Galα3 epitopes. The studies concerned about xenotransplantation in primates, using as a source GalT-KO pigs, have proved presence of antibodies against non-Galα3 epitopes (NGal-Ab) at time of onset of the AHXR. This led to suggestion of their role in graft damaging via complement and ADCC reaction. (Vadori et Cozzi, 2015)

Pathogenic roles of NGal-Ab has been identified by Lam (2004). Further studies reported HAR, caused by NGal-Ab in GalT-KO pig-to-baboon xenotransplantation. The study by Yeh (2010) used ELISA assay to measure antibody reactivity to glycoconjugates and concluded that NGal-Ab are predominantly targeted to proteins. Further studies supported the postulation that immunogenic polypeptides are the main targets of NGal-Ab. (Byrne, et al., 2015)

The exceptions to this are carbohydrate epitopes with terminal *N*-acetylgalactosamine linked to a sialic acid called N-glycolylneuraminic acids. (Byrne, et al., 2015)

In human serum there are also naturally occurring antibodies against N-glycolylneuraminic acid. Notably, humans do not have the enzyme for synthesis of NeuGc from NeuAc so called CMAH = cytidine monophosphate-*N*-acetylneuraminic acid hydroxylase. NeuGc found in human tissues is derived from milk and meat in the diet. (Vadori et Cozzi, 2015)

1.5. Heart valves

The treatment of valvular heart disease represents approximately 20% of all cardiac surgery worldwide and more than 250 000 heart valves are replaced every year. There are two basic options of valvular heart surgery – repairing and replacing them. Replacement of diseased valve could be done either with bioprosthetic heart valve or with mechanical valve prosthesis. Unfortunately, both ways are facing certain obstacles, divided according to the type of used prosthetic valve; and a decision, which valve type is more suitable for which patient, depends on number of factors. (Manji, et al., 2014)

1.5.1 Mechanical heart valves

MHVs are fabricated of synthetic material such as polymers, metal or carbon. (Manji, et al., 2014)

The greatest advantage of mechanical valve prosthesis is that MHV provides a long-term durability, which helps to avoid repetition of cardiac surgery associated with a higher rate of complications and 2-3 times higher risk of death in comparison with the initial operation. (Manji, et al., 2012)

Despite this huge benefit, they are not ideal for everybody, because implantation of MHV requires lifelong regulated anticoagulant treatment with associated risks. In particular, there are risks of spontaneous bleeding, thrombosis and thromboembolism which all might be fatal. The cumulative annual risk of those complications is 2 % per year. (Thus, a patient undergoing valve replacement in his 25 years has more than 99% percent chance of complication connected with anticoagulant treatment by the age of 75 years). (Manji, et al., 2014)

Due to previous information and the fact that young patients are more likely to experience these complication because of their lower adherence to restrictions in their diet, their more active lifestyle (work, sport) increases risk of injury and lower drug compliance in general. The risk is also increased in young women who are menstruating and during the pregnancy due to bleeding and potential birth defects in the child. (Manji, et al., 2014)

1.5.2 Bioprosthetic heart valves

Bioprosthetic heart valves avoid all these complications connected with lifelong anticoagulant treatment and due to this they are more suitable than MHVs for young people and people in the developing world, where it is not so easy to assure sufficient anticoagulant management. However, BHVs significantly deteriorate with time, which leads to necessary replacement connected with the risks of reoperation. This structural deterioration is age dependent.

The BHV failure rate is less than 10% for patients above 65 in 10 years and for patients under 35 in only 5 years. Thus, those who could most benefit from BHV implantation are not ideal candidates. This is why in the most cases MHV are chosen as a treatment for young people despite anticoagulant treatment. (Manji, et al., 2012)

1.5.3 BHV material

BHVs are made of biologic tissues of various origins. Those derived from human tissues are called homografts and those from animal tissues are referred to heterografts or also xenografts. Tissues are removed from the hearth and then cleaned, sized and fixed in different ways and pressures.

Homografts are of cadaveric origin or autografts (patient's own valve taken from one side (pulmonary) and transplanted to the aortic side. To the place on pulmonary side is then usually implanted cadaveric homograft).

Xenografts are made from different animal tissues such as porcine aortic valve, calf (bovine), equine or porcine pericardium. (Singhal, et al., 2013)

1.5.4 BHV deterioration

There are many causes and factors influencing the reason and the speed of the function loss in BHVs. The changes would be broadly divided into two groups:

Changes related to the host

a. **The age of the host**

Children and young adults suffer from higher risk of valve failure due to more competent immune system than elder adults.

b. **Host reaction to the tissue**

Except cellular reaction and damage caused by macrophages and mononuclear cells, the immune process mediated by humoral immunity plays important roles either in early reaction or in long-term valve deterioration. Side of implantation (mitral BHV suffers from more frequent failure due to overgrowth of the tissue), comorbid condition *e.g.* hyperparathyroidism mediates mineral metabolism and could contribute to early calcification) or pannus (normal tissue healing reaction after surgery, which can cause stenosis of valve) also plays role in the reactions to BHVs.

Changes related to the device

a. **Cellular reaction** – one month after transplantation almost all BHVs show mononuclear cell infiltrate with or without thrombus on their surface.

b. **Paravalvular leak** – it causes symptoms similar to the valve failure and could be connected also with hemolysis or low grade jaundice.

c. **Valve thrombosis** – the highest probability of valve thrombosis is during the first three months after surgery, and it occurs at the mitral site more often. Embolism caused by this is rare.

d. **Infective endocarditis** – infection usually starts around the sewing ring leading to abscesses, tears and cusp destruction. Inflammation as a response on the presence common gram-negative bacilli or fungi is the main destroying force in this way.

e. **Tissue degeneration** is usually slow and gradually progressive leading to cusp tears due to pressure changes and collagen degeneration.

f. **Calcification** – This is considered to be the main cause of BHV failure.

(Singhal, et al., 2013) (Siddiqui, et al., 2009)

1.5.5 Calcification

Calcic deposits are present within three years after transplantation. It is generally accepted that it is due to interactions between phospholipids, free aldehyde groups and other components of the valve with calcium ions. It can lead to stenosis and then to regurgitation or incompetence due to cusp tears. (Singhal, et al., 2013)

Young people and children suffer from significantly higher rates of BHV failure caused by calcification due to their higher immune competence and more rapid metabolism. The manufacturers have already developed a several anti-calcification chemical processes (alpha-oleic acid, Tween-80) to avoid this problem in elder people. (Manji, et al., 2014) Notably, some of these anti-mineralization treatments (AOA, ethanol) prevent only experimental cusp calcification, but not calcification in the aortic wall. (Siddiqui, et al., 2009)

The mechanisms of valve deterioration are not fully understood, there are several studies that suggest a connection between inflammation, thrombogenicity, coagulation and calcification.

Inflammation-calcification relationship

It has been shown that treatment by glutaraldehyde has a protective effect against humoral immune reactions, but increase cellular responses. Furthermore, it has been shown that glutaraldehyde is not responsible for calcification and a role of a graft specific antibody causing calcification has been suggested. (Manji, et al., 2006)

Moreover, animal studies have revealed that destruction of valve is significantly higher in xenogeneic group than in allogeneic control group and that this inflammation reaction of the recipient's body could be decreased by treatment with steroids. In addition, there was a linear relationship between calcification and inflammation, because the extent of calcification correlated with cellular infiltrate. (Manji, et al., 2006) The potential linkage may be through cytokine osteopontin produced by macrophages and T-Cells (Nau, et al., 1997)

1.5.6 Overcoming of obstacles

The clinical replacement of diseased heart valve by porcine BHV underwent a big advancement from the first attempts in the early 1960s to the presence. The initial problem was to preclude a hyperacute rejection initiated by naturally occurring antibodies to the antigens on porcine endothelial cells. This reaction leads to complement deposition and to activation of the endothelium that results in interstitial haemorrhage, graft thrombosis, edema, ischemia and organ failure. The major target of these antibodies is galacatose-α1,3-galactose (Galα3) present in all mammals except humans, Old World monkeys and apes. (Manji, et al., 2012)

a) Due to Galα3 antigen

In order to prevent interaction of anti-Galα3 antibodies with the graft, various methods were invented such as removal of anti-Galα3 antibodies, complement inhibition, removal of Galα3 antigens by the enzyme α-galactosidase, and development of α-1,3-galactosyltransferase gene-knockout (GalT-KO) pigs.. The absence of Galα3 expression reduces the humoral response as well as cellular immune response, and GalT-KO pericardium has a lower grade of calcification than wild type porcine pericardium. (Manji, et al., 2014)

However, other antibody-mediated rejections (initiated by NGal-Ab), rejection caused by cells (NK cells, lymphocytes and macrophages) and coagulant disorders are still remaining barriers in xenotransplantation.

b) Due to Non-Galα3 antigens

Human serum also contains antibodies directed against N-glycolylneuraminic acid (NeuGc). These antibodies are possibly involved in reactions after xeno and either allotransplantation. (Byrne, et al., 2015)

The presence of the NeuGc has been shown in porcine heart, pericardium and aortic and pulmonary valves. Therefore genetic engineered pigs lacking both the GalT and the CMAH have been produced. (Vadori et Cozzi, 2015)

2. Aims

The present study aims to isolate and characterize glycosphingolipids from porcine pericardium in order to define glycosphingolipid antigens present in this tissue and compare it to glycosphingolipids of porcine heart valve cusps. Isolated glycosphingolipids are characterized by mass spectrometry and binding of carbohydrate recognizing ligand in solid-phase binding assay. Furthermore, binding assays are done using serum from patients with BHV in order to define the antibody response of patients after prosthetic heart valve transplantation.

3. Methods and materials

3.1. Materials

Source of the samples

Porcine pericardium was acquired from the local slaughterhouse. The pericardial tissue was surgically dissected and stored at -80 °C.

Serum was collected before surgery and one and six months after the transplantation from 10 patients undergoing transplantation of bioprosthetic heart valves. The serum was stored at -80 °C.

Other material

For extraction and separation common laboratory glass and machines were used *e.g.* separation columns, flasks and tubes, water bath and tube gas evaporator and the analytical scales (d = 0.1 mg, Sartorius, Germany).

Solvents: Dichloromethane (HPLC solvent; Fisons Scientific Equipment), chloroform (redistilled), methanol (redistilled) (VWR Chemicals), filtered water.

Stationary phase: Silica gel 60 (0.040-0.063 mm; Merck KGaA), Iatrobeads (Iatron laboratories), DEAE cellulose (diethylaminoethyl cellulose) (Whatman)

Dialysis tubes: (Spectra/Por Dialysis Membrane; Spectrum Laboratories, Inc.)

TLC plates: HPTLC silica gel 60 (Glass plates 20×10 cm, Merck KGaA), HPTLC silica gel 60 (Aluminium sheets 20×20 cm, Merck KGaA)

Detection reagents for TLC:

Resorcinol reagents:

10 ml of resorcinol solution (1 g of the resorcinol (benzene-1,3-diol, Fluka AG, Germany) in 50 ml of H_2O, 80 ml of HCl (Hydrochloric acid fuming 37% pro analysis, Merck, Germany), 0.25 ml of 0.1M $CuSO_4$ (250 mg $CuSO_4$ in 10 ml H_2O).

Anisaldehyde reagents:

Anisaldehyde (4-methoxybenzaldehyde, Merck, Germany) in amount of 1 ml, 98 ml of acetic acid (Acetic acid (glacial) 100%, Emsure, Merck, Germany) and 2 ml of sulfuric acid 95-97% (Sulfuric acid for analysis, Merck, Germany).

Carbohydrate binding assay ligands:

In the process of chromatogram binding assays following carbohydrate binding ligands were used:

Ligand	Clone/Designation	Manufacturer/Reference	Isotype	Dilution
Anti-Galα4Gal	*E coli* 291-15	1	-	-
G. simplicifolia IB4	-	Advanced Targeting System	-	1:200
Erytrina christagalli lectin	-	Vector Laboratories Inc.	-	1:100
Aleuria aurantia lectin	-	Vector Laboratories	-	1:100
Anti-A MAb	HE193	GeneTex/Abcam	IgM	1:500
Anti-H type 1 MAb	17-206	GeneTex/Abcam	IgM	1:50
Cholera toxin B-subunits	-	List Biological laboratories	-	1:200
Anti-GD1a MAb	GD1a-1-s	DSHB	IgM	1:100
Anti-sialyl-lactotetra MAb *	TR4/SL-50	2	IgM	1:200
Anti-sialyl-neolactotetra MAb *	LM1:1a	3	IgM	1:200

* This antibody was a kind gift from Doctor Maria Blomqvist, Department of Clinical Chemistry and Transfusion Medicine

Table 1: Antibodies and lectins used in Chromatogram Binding Assay (CBA)

TLC References:

Glycosphingolipids previously isolated and characterized by local research group. In particular total neutral GSLs from human erythrocytes blood group AB, acetylated sphingomyelin, calf brain gangliosides, GM3 from pig, sulfatide GSLs from pig, GD1a, GD3, GM1 and Gc-neolactohexaosyl glycosphingolipids.

Human serum for binding assay:

Frozen serum collected from each patient before, one and six month after BHV treatment were thawed and then diluted in ratio 1:9 with blocking solution.

Blocking solution:

Human albumin 1% in PBS/BSA or Carbo-Free Blocking Solution (10× concentrate in H_2O) (Vector laboratories, USA)

Concentrated **p**hosphate **b**uffered **s**aline (PBS):

80 g NaCl, 2 g KCl, 11.5 g $Na_2HPO_4.2H_2O$, KH_2PO_4, distilled water 1000 ml and subsequently diluted *pro re nata* by 10.

Other:

Plastic disposable Petri's dishes, Pasteur pipettes and tubes (Sarstedt, Germany).

Biomax MR Film XAR-5- x-ray films (Eastman Kodak Co.) (Carestream, U.S.A).

LC-ESI/MS and ESI/MS/MS (liquid chromatography-electrospray ionization-tandem mass spectrometry).

3.2. Methods

Glycosphingolipids are a group of chemical compounds where physical-chemical and physiological properties vary a lot. In order to extract and purify glycosphingolipids, their differences in polarity and solubility in solvents has to be taken into account. Notable is that GSLs resist to alkaline degradation, whereas even weak acids may result in cleavage of glycosidic bond.

Hence, various protocols for isolation of GSLs have been developed.

The protocol used in our laboratory was developed by Karlsson (1987) and enables to obtain total acidic and total non-acidic glycosphingolipid fractions free of other lipid components. This procedure has been further modified to be applicable on small amounts of samples.

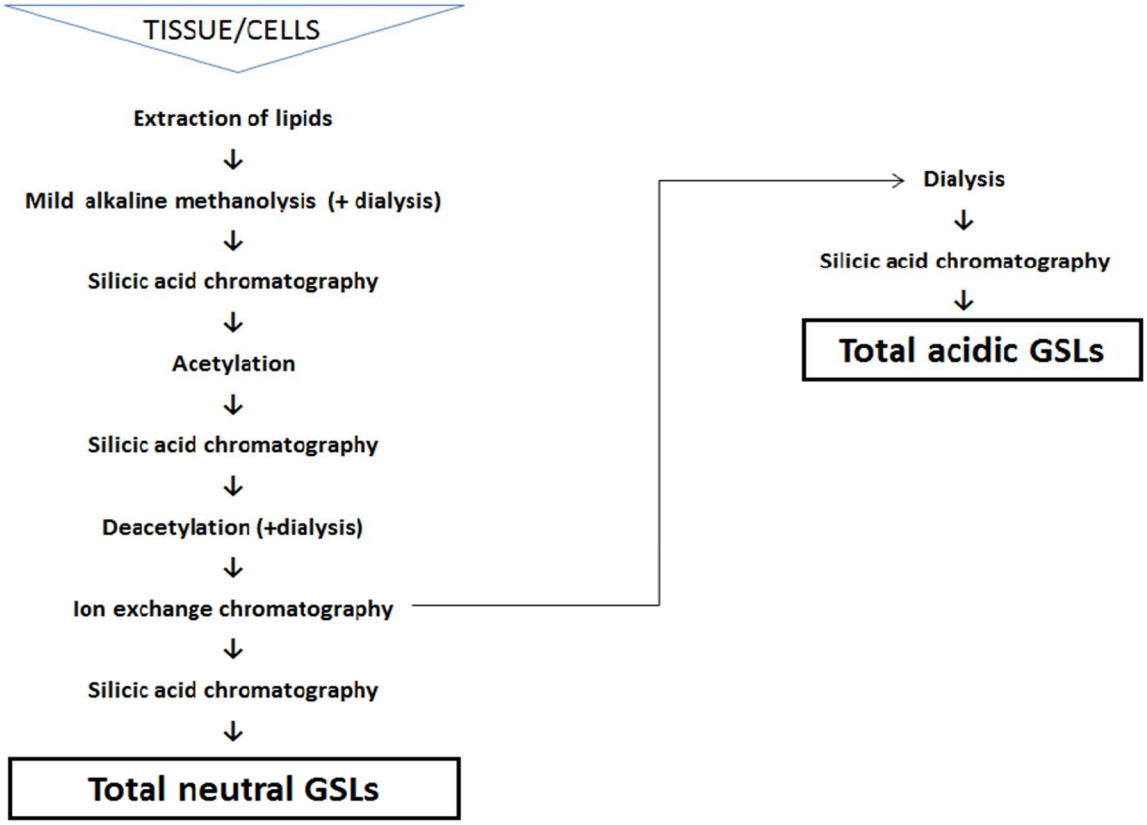
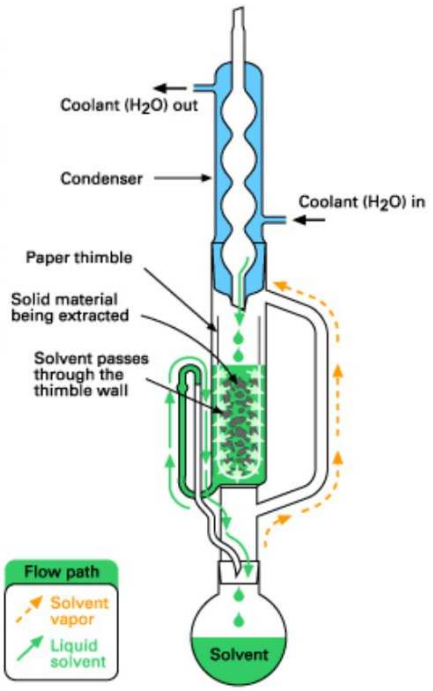


Figure 7: Schematic overview of glycosphingolipid isolation, modified from (Barone, 2010)

3.2.1 Extraction of lipids

Lyophilized porcine pericardium was extracted for 24 hours in a Soxhlet apparatus with mixture of chloroform and methanol (C:M) in 2:1 ratio and for another 24 hours with the mixture of the same solvents but in ratio 1:9 (v/v), the extraction was held in glass apparatus composed of flask, Soxhlet extractor, condenser and cooker. The liquid extracts were pooled and dried and the total lipid profiles were checked by thin layer chromatography.



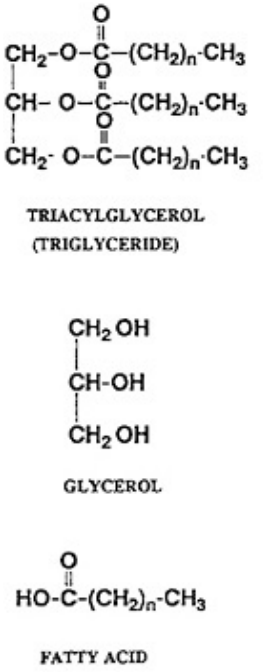
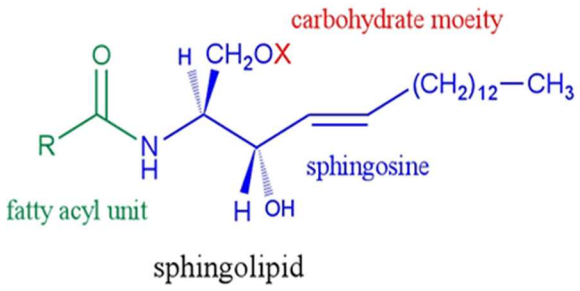


Figure 8: Soxhlet apparatus diagram, adapted from: (Berghe, 2003)

Figure 9: TAG, FA and glycerol, modified from: (Jandacek, 1994)

3.2.2 Mild alkaline hydrolysis

After extraction, mild alkaline hydrolysis was performed in order to cleave ester bonds, this facilitate the separation of glycerophospholipids from GSLs.



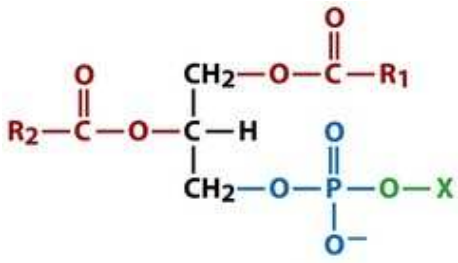


Figure 10: Schematic formula of glycosphingolipid, adapted from: (Anonymous)

Figure 11: Schematic formula of glycerophospholipid, adapted from: (SDSU, 2013)

The glycosphingolipids stayed undamaged because of their structure, where carbohydrate residues are bound to ceramide core chain by etheric linkage (called O-glycosidic), whereas glycerophospholipids and also the rest of triacylglycerides underwent

hydrolysis (or preferentially alkaline methanolysis) in this process. Therefore, glycophospholipids were deacylated (the removal of fatty acids esterified to glycerol), which resulted in fatty acid methyl-esters (the methyl group comes from methanol as the solvent) and polar glycerophosphoryl derivatives. The sphingomyelin, which always occurs in total lipid extracts, is not affected and stays unchanged. In this process possible O-acetylated glycolipids could be degraded. (Karlsson, 1987)

To perform alkaline methanolysis, the solution of 0.2 M KOH in methanol were added to the mixture and incubated for 3 hours in darkness at room temperature.

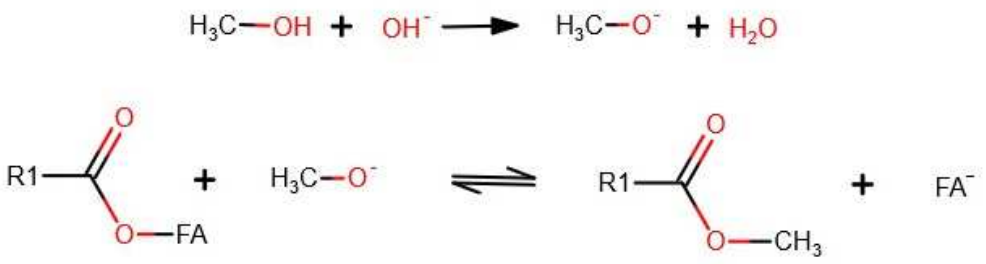


Figure 12: Schematic view of methanolysis; FA is fatty acid residue, R1 is the rest of glycerophospholipide or triacylglyceride

3.2.3 Dialysis

The mixture was moved to a dialysis bag and C:M:W in ratio 8:4:3 was added in order to achieve a two phase system. Dialysis was then left for 3-4 days in a beaker with continuous income of tap water. The mechanism of dialysis is based on diffusion through the semipermeable membrane. Small molecules (like salts) and solvents are able to pass the membrane to the flowing liquid (tap water), but the macromolecules and in general everything larger than the pores cannot and stay.

Although the individual molecular weight of glycosphingolipids is smaller than the pores in dialysis tubing, they are not lost during the dialysis. It is believed that this is caused by formation of micelles and the maintenance of the two-phase environment. (Henry, 1993)

The dialysed extract was evaporated to dryness at 55 °C in water bath.

3.2.4 Silicic acid chromatography

The separation is a foundation stone of isolation. There are many techniques based on divergent mechanism, but most common are those using silica gel and its modifications as a stationary phase. The silicic acid chromatography (SAC) is essential, simple and available technique for separation of compounds from samples.

It is based on interaction of separated compounds with stationary and mobile phase. As stationary phase, silica gel is used. This is a hydrated form of silicic acid, which is an acidic adsorbent with many hydroxyl groups in the molecule. The interaction of molecules in the mobile phase with the silica gel (stationary phase) is mediated by van der Waals forces and hydrogen bonding. (Karlsson, 1987)

Because of the relatively high polarity of the silica gel, polar substances will bind strongly to its surface and a more polar eluent will be required to elute it. Therefore, the first silicic acid chromatography enabled to separate the major part of less polar compounds like cholesterol and fatty acids methyl-esters from glycolipids and sphingomyelin by using a relatively non-polar solvent in the first step.

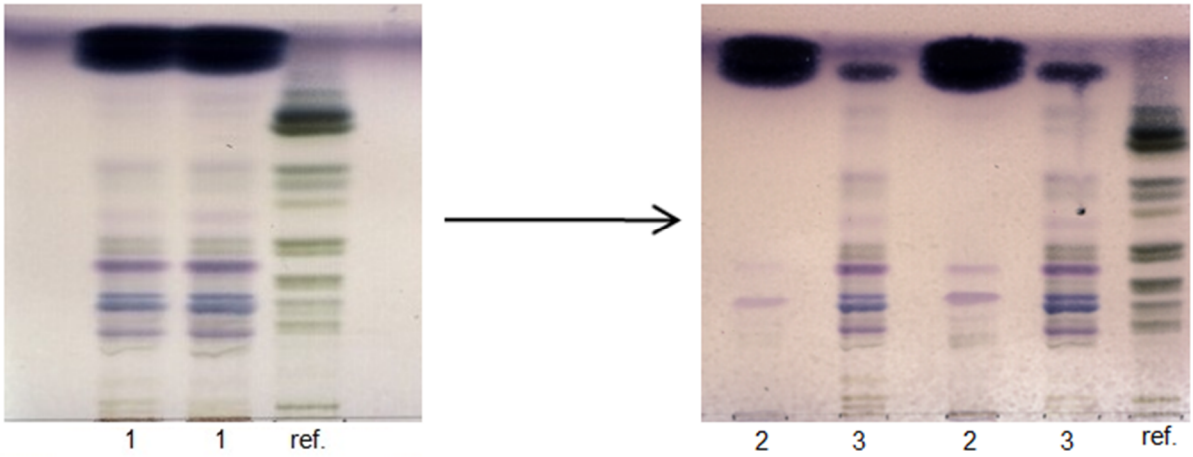


Figure 13: TLC detected by anisaldehyde reagent showing difference before and after separation from fats. Fraction 1 are unseparated lipids, in fraction 2 are cholesterol and fatty acids, fraction 3 are glycolipids and sphingomyelin, modified from (Barone, 2010).

The separation was achieved by using the following solvents; dichloromethane, 1% methanol in chloroform, 2% methanol in chloroform, 75% methanol in chloroform and 100% methanol. Three fractions were collected to the pre-weighted flasks according to close polarity of the solvents and monitored by TLC.

3.2.5 Acetylation and deacetylation

Acetylation was performed in order to remove sphingomyelin from the rest of the sample. The chromatographic properties of sphingomyelin and glycolipids are similar. However, sphingomyelin has only one acetylation site, whereas glycosphingolipids, especially their glycosylated parts, have a lot of them. Therefore acetylation will change the glycolipids from the polar to the non-polar, while sphingomyelin stays in the polar group. As a consequence, the glycolipids can be separated from sphingomyelin. (Karlsson, 1987)

For acetylation a mixture of chloroform, pyridine and acetic anhydride in equal parts was used and incubated over night at a dark place. The reaction was stopped with the addition of methanol during cooling by ice.

Acetylated GSLs were separated from sphingomyelin on a second silicic acid column. Thereafter the GSL-containing fraction was treated with methanol, toluene and 0.2M KOH in methanol in ratio 1:1:2 to revert the GSLs back to their original state. The process was followed by dialysis in order to remove part of reaction waste.

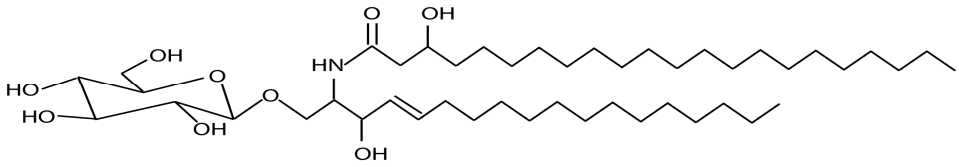


Figure 14: Example of glycosphingolipid (Barone, 2010)

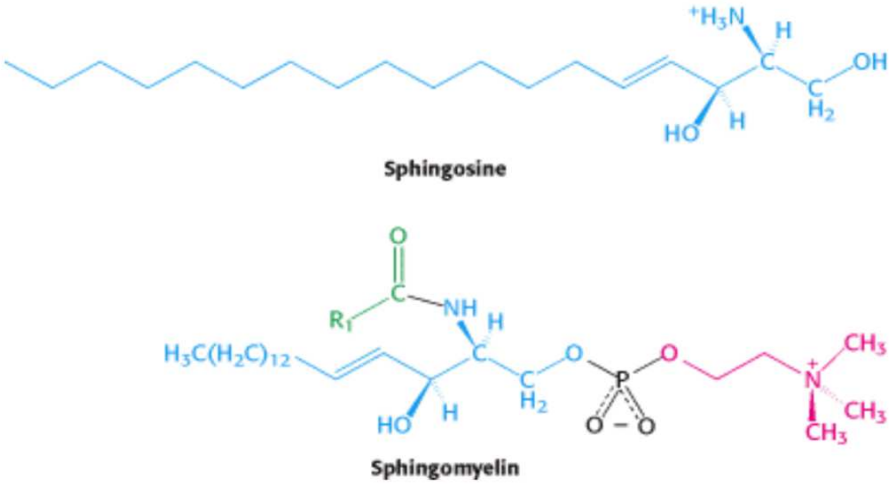


Figure 15: Formula of sphingosine and sphingomyelin, adapted from: (Berg, et al., 2001)

3.2.6 Ion-Exchange Chromatography

The method is based like silicic acid chromatography on separation of compounds according to chromatographic properties in the relationship to stationary and mobile phase. The difference between this kind of chromatography and SAC is that ion chromatography is used for a separation of ions and highly polar molecules. Mechanism of ion exchange chromatography is based on columbic (ionic) interactions between sample and stationary phase, which displays ionic functional groups of the opposite charge. (Weiss, 1995)

Ion exchange chromatography separated acidic GSLs (sulfatides and gangliosides) from non-acidic GSLs. For this purpose, the solid phase DEAE was used. As the mobile phases were used first C:M (2:1) followed by pure methanol to elute less polar compounds including total neutral GSLs and then as the second mobile phase a 5% (w/v) solution of LiCl in methanol to elute the acidic compounds. Subsequent dialysis was performed for four days to remove salts.

Further purifications, mainly by SAC, had to follow to get the product in sufficient purity for analysis. The fractions were further separated by chromatography on Iatrobeads columns.

3.2.7 Drying

Drying is a method aiming to get rid of redundant volume of solvents after each process mentioned above. In addition, drying up all solvent present in the sample enable us to replace it with another one more suitable solvent for storing or sampling for TLC.

For drying, water bath or tube heater were used under a stream of nitrogen (g) into flask or tube.

3.2.8 Thin layer chromatography

Thin layer chromatography can be performed for getting a preliminary overview of GSLs in every step of the preparation. Like in the case of silicic acid column chromatography, the same principles are applied in TLC. Thus, separation of individual compounds depends on their polarity and size of the molecule. In the context of glycolipids, the length of the oligosaccharide chain with its rich hydroxylation and the ceramide play important roles. The position on TLC roughly indicates the number of carbohydrate residues in the glycolipid.

Plates used in high performance thin layer chromatography are covered by uniform superfine silica gel and are manufactured on glass and aluminium plates.

The preliminary identification was enabled by using suitable references, which was evolved in the same chamber by the same solvent system and on the same plate as the samples. Detection was then performed by using chemical staining with anisaldehyde or resorcinol reagent. Anisaldehyde colours all carbohydrates in different shades of green and resorcinol stains only those carbohydrates containing at least one molecule of any sialic acid (*i.e.* gangliosides) in purple.

On each line GSLs were applied and then developed in a chamber with a solvent composed of chloroform-methanol-water in 60:35:8 (v/v/v), dried, and then sprayed with one of the reagents. Then the plate was put into at 200 °C pre-heated oven for some time to facilitate the reaction with reagent.

TLC is used for preliminary detection of GSLs. However, single band on the chromatogram may contain more glycosphingolipids with similar chromatographic properties. The identification is further complicated by the fact that GSLs are homogenous in their carbohydrate part, but may differ from each other in their sphingosine and fatty acid part. This results in multiple banding patterns of the same glycosphingolipid. Principles of TLC method enable to separate glycosphingolipids for further analytical usage.

3.2.9 Chromatogram Binding Assays

This method has been originally invented by Magnani (1980) and has a great importance for characterization of GSLs.

Aluminium sheets covered by silica gel are more suitable for chromatogram binding assays (CBA), due to their higher resistance to layer disruption during many washing steps than silica gel covered glass plates.

Binding assay chromatograms with separated glycosphingolipids were dipped in diethylether/n-hexane (1:5 v/v) containing 0.5% (w/v) polyisobutylmethacrylate (Aldrich) for one minute. After drying, the plate was ready for binding assay of choice. This treatment prevents the destruction of silica gel due to repeated washing steps in the binding assays. The fixation of silica gel by this plastic is sufficient to make the silica gel layer compact, but not too much to obstruct sample-detection agent binding.

Notable is the phenomenon of cross-reactivity in using antibodies, lectins and carbohydrate binding proteins. This and the fact that some carbohydrate epitope may be cryptic make binding assay characterization more difficult. In this context, it leads also to postulate, that the absence of binding does not prove the absence of certain epitope in the sample. (Gahmberg, et al., 1973)

CBA with lectins or specific monoclonal antibodies

The TLC was developed, dried and put into plastic solution for one minute and dried out properly, as mentioned above.

The plate was then put into blocking solution (2% BSA in PBS) in order to prevent unspecific binding. The plate was incubated for 2 hours and after them was the blocking solution decanted and poured out. The plate was then incubated with ^{125}I labelled lectins or with specific monoclonal antibodies (table 1). After 2 hours of incubation the plate was washed 6 times by PBS. In the case of specific antibodies ^{125}I labelled secondary antibodies were applied, incubated and washed as above. Dried chromatograms were autoradiographed for 6-48 hours.

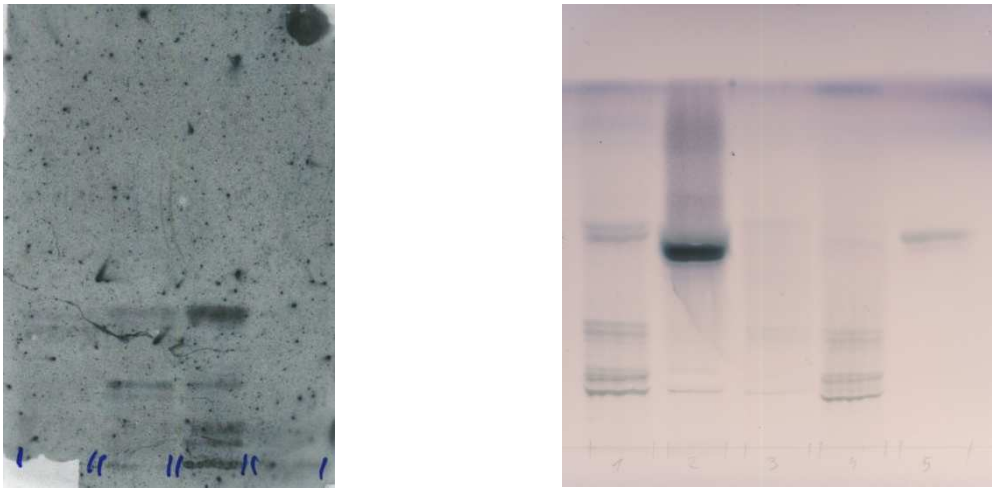


Figure 16: Example of TLC plate detected by autoradiography (left) and by chemical reagent (right)

CBA with the sera of the patients

The main difference between this and previously mentioned kind of binding assay is application of real patient serum containing antibodies instead of specific reagent. The aim is to find out whether the tested serum sample contains any antibodies directed against already characterized carbohydrates or their mixtures.

In this protocol the TLC plates with tested GSLs were prepared, developed and treated in plastic as before. Then 1% albumin or a carbohydrate free blocking solution as a blocking solution was poured on the TLC plate to prevent unspecific binding in further process. The incubation of the blocking solution lasted 2 hours and then it was poured out.

In the next step, the patient serum diluted in ratio 1:10 by the blocking solution was carefully poured on the TLC plate and left to incubate again for 2 hours. Afterwards the TLC plate was washed 6 times with PBS. The secondary anti-human Ig antibodies diluted in an appropriate blocking solution in ratio 1:100 (v/v) were poured on the washed TLC plates. After 2 hours of incubation, 6 times washings followed to get rid of unbound secondary antibodies.

The secondary antibodies marked by radioactive isotope of iodine were directed against primary human antibodies. This visualisation step enabled us to detect them by autoradiography.

Dry TLCs were fixed into metal cassette and the special film was put on them in the dark room. After sufficient time the film was developed and evaluated.

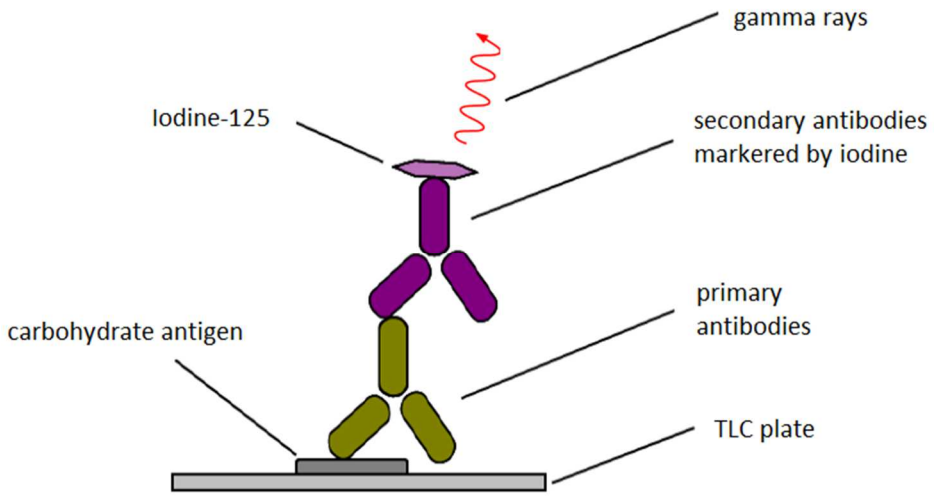


Figure 17: Radioactive iodine marked secondary antibodies, modified from (Barone, 2014b)

3.2.10 Mass spectrometry

Mass spectrometry (MS) is a key analytical technique for characterization of glycosphingolipids. The MS is based on ionization of the molecules in the sample, by both adding and removing charge and subsequent separation of them according to their *m/z* ratio in order to detect them by a detector.

In the context of glycolipid MS provides valuable information about the number of carbohydrate residues, carbohydrate sequence, branching points and type of sugar residue (hexose, pentose, deoxyhexose, sialic acid, hexosamine). (Karlsson, et al., 2010)

It should be noted that mass spectrometry is not "one size fits all" kind of method, thus different compounds may need different methods for ionization and detection. Therefore, more modifications for analysis of glycosphingolipids have been developed.

A previously used method called electron ionization (EI) was used for this analysis of GSLs. To briefly explain, this method used evaporation of a sample by heat on high voltage electrode. This caused a lot of problems due to damage of unstable GSLs; and this is why another solution came with introducing of soft ionization techniques such as Electrospray Ionization (ESI).

ESI works on the mechanism based on the production of small droplets of liquid sample from metal capillary at voltage 3.5 kV and their nebulization. This fine spray from liquid sample is rapidly evaporated by heat and dry nitrogen. The ionized analytes then continue into highly evacuated through a series of small orifices and focusing voltages to detector. (Pitt, 2009)

The **ESI-MS** which is used for characterization of glycosphingolipids in this study does not require high heat in the process of ionization. However, it has a few disadvantages like low sensitivity or no fragment ions creation. This was solved by introduction of nano-ESI which increased sensitivity and mass tandem (MS/MS or MS^2), which enabled fragmentation of ions. (Karlsson, et al., 2010)

In our measurements ***LC-ESI/MS*** was used:

The glycosphingolipids (dissolved in methanol/acetonitrile 75:25, by volume) were separated on a 200 x 0.150 mm column, packed in-house with 5 mM polyamine II particles (YMC Europe GMBH, Dinslaken, Germany), and eluted with a water gradient (A: 100% acetonitrile; B: 10 mM ammonium bicarbonate). Samples were analysed on an LTQ linear quadrupole ion trap mass spectrometer (Thermo Electron) by LC-ESI/MS at -3.5 kV. Full-scan (*m/z* 500-1800, 2 microscans, maximum 100 ms, target value of 30 000) was performed, followed by data dependent MS^2 scans (2 microscans, maximum 100 ms, target value of 10 000) with normalized collision energy of 35%, an isolation window of 2.5 units, an activation q = 0.25, and an activation time of 30 ms.

Manual assignment of glycosphingolipid sequences was done with the assistance of the Glycoworkbench tool (Version 2.1), and by comparison of retention times and MS^2 spectra of reference glycosphingolipids.

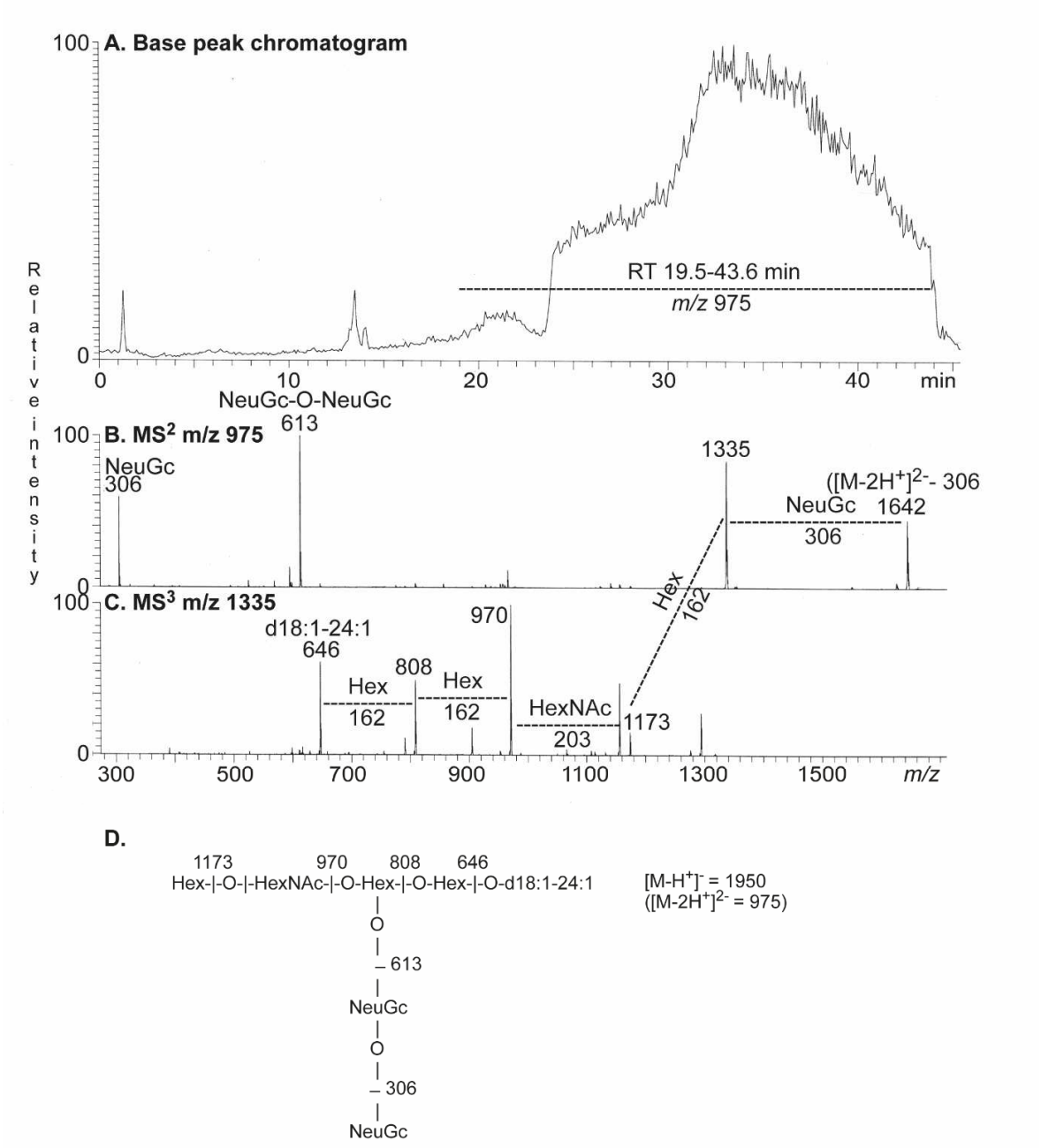
4. Results

4.1. Isolation of NeuGc-containing GSL from goat Erythrocytes

780 mg of total acidic glycosphingolipid fraction from goat erythrocytes was separated first by 2%, 10%, 75% and 100% methanol in chloroform and then repeatedly by C:M:W (60:35:8) on silicic acid (Iatrobeads) columns. Finally, 17 fractions were obtained and characterized by LC-ESI/MS.

4.2. MS characterization of NeuGc-containing GSL from goat erythrocytes

In one of the fractions obtained the NeuGc-GT1b ganglioside was characterized (result fig. 1). This sample was later used as reference in the binding assays. (result fig. 8)



Result figure 1: LC-ESI/MS of an acidic glycosphingolipid fraction isolated from goat erythrocytes.

A. Base peak chromatogram.

B. MS^2 spectrum of the $[M\text{-}2H^+]^{2-}$ ion at *m/z* 975.

C. MS^3 spectrum of the ion at *m/z* 1335.

D. Interpretation formula showing the deduced glycosphingolipid sequence.

4.3. Isolation GSLs from porcine pericardium

108 gram wet weight (72 gram dry weight) porcine pericardium was extracted by C:M (2:1) and C:M (1:9) for 24 hours per solvent in Soxhlet apparatus. After drying it was treated with 0.2M solution of KOH and then dialyzed for 4 days. The 62 gram thereby obtained were repeatedly separated on silicic acid separation column by dichloromethane, 1%, 2% MiC and by 75%, 100% MiC. All fractions containing GSLs were pooled.

1.8 gram of sample was acetylated by mixture of $CHCl_3$, pyridine and acetic anhydride and left over night in dark place. Acetylation was stopped by addition 50 ml MeOH during cooling by ice. Next separation on silica-gel (DiChl; 5%; 7.5%; 75% and 100% MiC) was performed to get rid of acetylated sphingomyelin. Fraction of interest was deacetylated by mixture of MeOH, toluene and 0.2M KOH in MeOH, then dialyzed for 4 days and dried to dryness on the water bath. Ion exchange chromatography with C:M (2:1) and 5% LiCl in MeOH on a DEAE column was performed to divide roughly fractions containing acidic and non-acidic glycosphingolipids.

The non-acidic sample underwent a few further silicic acid chromatographies resulting in 43.0 mg of total non-acidic glycosphingolipid fraction.

The acidic GSL sample was first dialyzed for 4 days and then repeatedly separated on silica gel columns. After pooling three fractions were collected: ganglioside fraction (total amount 5.2 mg), sulfatide fraction (total amount 1.0 mg) and sulfatide/ganglioside mixture (total amount 0.9 mg).

Each separation step was checked by HPTLC.

4.4. Characterization of GSLs from porcine pericardium

The isolated samples were, as described above, analysed on an LTQ linear quadrupole ion trap mass spectrometer (Thermo Electron) by LC-ESI/MS at -3.5 kV. Then manual assignment of glycosphingolipid sequences was done with the Glycoworkbench tool (Version 2.1), and by comparison of retention times and MS^2 spectra of reference glycosphingolipids.

The specific ligand carbohydrate binding assays were performed separately with acidic and with non-acidic glycosphingolipids.

4.5. LC-ESI/MS characterization of non-acidic GSLs

(These results are included in the attachment which is not disclosed to the public.)

Result figure 2: LC-ESI/MS of the oligosaccharides obtained by digestion of the non-acidic glycosphingolipid fraction of porcine pericardium (Tab. 1) with Rhodococcus endoglycoceramidase II.

(These results are included in the attachment which is not disclosed to the public.)

Result figure 3: Interpretation formulas for result figure 2

(These results are included in the attachment which is not disclosed to the public.)

Table 2: Summary of non-acidic glycosphingolipids characterized in porcine pericardium by LC-ESI/MS.

4.6. CBA characterization of non-acidic GSLs

(These results are included in the attachment which is not disclosed to the public.)

Result figure 4: Characterization of the non-acidic glycosphingolipids of porcine pericardium by antibody and lectin binding.

Thin-layer chromatogram after detection with anisaldehyde (A) and autoradiograms obtained by binding of P-fimbriated *E. coli* strain 291-15 (B), *E. cristagalli* lectin (C), *G. simplicifolia* IB4 lectin (D), monoclonal antibodies directed against the blood group H type 1 determinant (E), and monoclonal antibodies directed against the blood group A determinant (E). The lanes were:

4.7. LC-ESI/MS characterization of acidic GSLs

(These results are included in the attachment which is not disclosed to the public.)

Result figure 4: LC-ESI/MS of the gangliosides of porcine pericardium.

Base peak chromatogram from LC-ESI/MS of the acid glycosphingolipid fraction Pa-III from porcine pericardium. The structures of identified gangliosides, and the abbreviations used, are given in Table 3.

(These results are included in the attachment which is not disclosed to the public.)

Table 3: Summary of gangliosides characterized in porcine pericardium by LC-ESI/MS.

(These results are included in the attachment which is not disclosed to the public.)

4.8. CBA characterization of acidic GSLs

(These results are included in the attachment which is not disclosed to the public.)

Result figure 6: Characterization of the acidic glycosphingolipid fraction of porcine pericardium by antibody and toxin binding.

Thin-layer chromatogram after detection with anisaldehyde (A), and autoradiograms obtained by binding of monoclonal antibodies directed against the NeuAcα3-neolacto epitope (B), monoclonal antibodies directed against the GD1a ganglioside (C), and cholera toxin B-subunits (D) were done. The lanes were:

4.9. Carbohydrate binding assay with human serum

In total number of 60 binding assays with the serum of patients before, one month and six months after xenotransplantation of porcine heart valves were performed by described protocol.

(These results are included in the attachment which is not disclosed to the public.)

Result figure 7: Increased binding of human serum to Galα3-terminated glycosphingolipids after bioprosthetic heart valve transplantation.

Thin-layer chromatogram after detection with anisaldehyde (A), and autoradiograms obtained by binding of human serum taken before surgery (B), one month after surgery (C), and six month after surgery (D). The lanes were:

(These results are included in the attachment which is not disclosed to the public)

Result figure 8: Increased binding of human serum to NeuGc-GM3 after bioprosthetic heart valve transplantation

Thin-layer chromatogram after detection with anisaldehyde (A), and resorcinol (B), and autoradiograms obtained by binding of human serum taken before surgery (C), one month after surgery (D), and six month after surgery (E). The lanes were:

(These results are included in the attachment which is not disclosed to the public.)

5. Discussion

This study was designed to contribute by its findings to solve the questions - why do transplanted bioprosthetic heart valves (BHV) deteriorate with time and which mechanisms are involved in this process?

In this study glycosphingolipids from different animal tissues have been isolated, characterized and assessed as potential targets of human immune response after BHV transplantation.

5.1. Summary of GSL findings in porcine pericardium

Isolation

Total acidic and total non-acidic glycosphingolipids from porcine pericardium have been isolated by the preparation method derived by Karlsson (1987).

Chemical and physical-chemical properties were used in order to extract, purify and separate them into groups suitable for analysis. In particular, solubility was used in extraction of raw material by C:M (2:1) and C:M (1:9), polarity in all silicic and ion-exchange chromatographies and chemical reactivity in removal of sphingomyelin.

All of final collected fractions, particularly: total neutral glycosphingolipid fraction (total amount 43.0 mg), ganglioside fraction (total amount 5.2 mg), sulfatide fraction (total amount 1.0 mg) and sulfatide/ganglioside mixture (total amount 0.9 mg).

Characterisation

LC-ESI/MS analysis was facilitated by foregoing digestion of the non-acid GSLs by Rhodococcus endoglycoceramidase II, which breaks linkage between ceramide and oligosaccharide part of the GSL (divides glycone and aglycone). Then manual assignment of GSL sequences was done with the Glycoworkbench tool (Version 2.1), and by comparison of retention times and MS^2 spectra of reference glycosphingolipids. Eight non-acidic GSLs and seven acidic GSLs were characterized by this method (tables 2 and 3).

CBA characterisation of the non-acidic GSLs was performed by monoclonal antibodies (MAb) and by lectins. Notable is that in other lanes were situated GSLs used as references, which have been already isolated and characterized by LC-ESI/MS.

Reactivity of **acidic GSL** from porcine pericardium with MAb directed against NeuAcα3-neolacto epitope, MAb directed against GD1a ganglioside and cholera toxin B-subunits (Ludwig, et al., 1985) is shown in the result figure 6. These results revealed presence of GD1a, NeuAcα-neolacto and NeuAc-GM3 in the sample, where the GD1a was the major one.

The **non-acidic GSL** from porcine pericardium were characterized by specific antibodies and by lectins as it is shown in the result figure 4.

The binding to P-fimbriated *E. coli* confirmed the presence of globotetraosylceramide in the GSL sample from porcine pericardium. This ligand has a specific reaction with Gal(α1,4)Gal antigens. (Roberts, et al., 1994)

In the same manner the binding of the Galα3-recognizing *G. simplicifoila* IB4 lectin (Goldstein, 1983) confirmed the presence of Galα3-neolactopenta- and Galα3-neolactoheptaosylceramide.

The other tested ligands showed no distinct binding to the non-acid GSLs of porcine pericardium.

Context of our findings

Although BHV are routinely used, clinical BHV treatment still suffers from complications such as hyperacute rejection or fast deterioration with time and subsequent loss of function. It has been shown that the major part of negative immunity response against xenograft is targeted to the Galα3 antigen. However other antigens such as NeuGc also have important roles. (Vadori et Cozzi, 2015).

One goal of this study is isolation and characterization of glycosphingolipids, as potential antigens of porcine pericardium, which is one of the materials in use for BHV manufacture.

Studies concerning about isolation and characterization have been already performed with porcine valves (Lee, et al., 2015) and porcine valve cusps. (Barone, et al., 2014a) Comparison with them gives us image about diversity among these tissues in GSL composition *i.e.* absence of NeuGc in porcine cusps (Barone, et al., 2014a). These differences might be those which involve intensity of immune response against material from which xenografts are manufactured.

Our results correlates with findings of other studies (Lee, et al., 2015) and confirms also presence of Galα3 and NeuGc antigens in some porcine tissues, particularly in porcine pericardium. The first two are not biosynthesized in human tissues and therefore they are considered as important immune targets in porcine xenografts.

5.2. Isolation of GSL from goat erythrocytes

Findings

In order to identify as many naturally occurring GSLs as possible, one part of my work is concerned about isolation of GSL from goat erythrocytes. My work represents only the final parts of isolation and analysis.

From the goat erythrocytes was isolated new NeuGc-containing ganglioside - NeuGc-GT1b which was characterized by LC-ESI/MS as described in figure 1. However, there are over 350 already described carbohydrate chains in GSLs (Merril, 2011), the characterization of any new might be in the future useful for studies in xenotransplantation field as well as in studies in other fields *i.e.* transdermal drug delivery systems or cell membrane concerning studies.

5.3. Reactivity with human serum

To sum it up, the most important antigen structures causing complications in xenotransplantation are:

- **Galα3** – Antigen missing in all humans, apes and Old World monkeys due to evolutionary loss of enzyme α-1,3-galactosyltransferase and represented by terminal α-galactose of carbohydrate sequence in many tissues. It is the major barrier in xenotransplantation and several approaches to avoid complications caused by it have been invented.
- **NeuGc** – Antigen is not synthesized in humans due to lacking of enzyme CMAH. It is considered as the main target of immune response against xenografts from Galα3 antigen lacking donors.

In order to investigate which antigens are targets of immune response mediated by antibodies, 60 binding assays with the patient serum have been performed. The sera of the patients have been collected before surgery, one month and six months after transplantation of heart valve made from porcine pericardium and then was tested with different GSLs and their mixtures to find out if there are any antibodies targeting them. This would help to clarify not only which glycosphingolipids are the targets, but also change of targets and intensity in the time.

Testing of non-acidic GSL

30 binding assays with non-acid GSLs and with patients' serum have been done using described protocol and detected by autoradiography. Autoradiography on special films was detecting radioactive rays from ^{125}I-marked antibodies used for detection human antibodies against GSL in the sample.

The results are mostly negative but occasionally an increased binding to antigens with terminal Galα3 is seen. In the cases where this was revealed, increased binding occurred in the samples from bovine pericardium and from rabbit erythrocytes.

Progress in the time has not been clearly observable. However, in a few samples intensity of observed reactivity increased with the time.

Testing of acidic GSL

The GSL from porcine pericardium and reference NeuAc-GM3, NeuGc-GM3 and NeuGc-GD1b gangliosides have been tested with patient serum. The binding was mostly negative, occasionally increased, especially in the samples from porcine pericardium and NeuGc-GM3 ganglioside.

No conclusion in time-relation was found. However, the intensity of strips on the plates mostly decreased with the time.

Context of our findings

Our data collaborates with the previous theory about induced antibody response against Galα3 antigen after xenotransplantation of graft from porcine tissues. (Mangold, et al., 2009)

Also, it is extending findings about porcine BHV (Barone, et al., 2014a) due to tests of occurring GSL with real patients' serum. Notable is that in this study porcine cusps were isolated and those in opposite to porcine pericardium do not contain NeuGc antigen.

The results of binding assay might be changed by the change of blocking solution during the study. The change from 1% human albumin in PBS solution was switched to carbo-free blocking solution. The replacement of blocking solution led to better observability of strips on plates. Carbo-free in the name of new blocking solution means, that it does not contain any carbohydrates potentially leading to unspecific blocking of antibody binding.

It has been already shown that there is a relationship between BHV deterioration especially by process of calcification (Human, et al., 2001) and immune response. This is why the knowledge of reactivity of antigens with antibodies present in human body before and after xenotransplantation is important.

Once the targets of immunity and mechanisms of deterioration are known, several methods could be invented to avoid complications caused by them *i.e.* production GalT-KO pigs (McGregor, et al., 2013), anti-calcification procedure (Choi, et al., 2016), antigen removing protocols such as (Nam, et al., 2012) or glutaraldehyde treatment (Siddiqui, et al., 2009). The suppression of human immune reactivity against xenograft like thymus transplantation or mixed chimerism due to bone marrow transplantation is also a possible way to prevent damage of the graft. (Griesemer, et al., 2014)

5.4. Future research perspective

The study helps to increase the knowledge about glycosphingolipids as possible targets for immune response against graft. In the future, this knowledge would help to clarify mechanisms of BHV deterioration caused by calcification and other mechanisms and in the final impact would help to increase final outcome from xenotransplantation.

As subsequent logical steps based on my results, I would recommend for future researchers to explore next potential carbohydrate and non-carbohydrate antigens because the greater knowledge about them we have, the earlier ideas for avoiding complications connected with them would be thought of.

Based on our results, the usage of pericardial tissue from blood group 0 pigs with knockout of GalT + CMAH for BHV would be one of these complication avoiding ideas.

Also screening of GSLs in next animal tissues would be recommended in order to get the most antigenic suitable source of material for manufacturing of BHV.

Last, but not less important is also to properly investigate the mechanism and antibody contribution to the valve calcification, which seems to be essential process in BHV early deterioration and subsequent loss of function.

6. Conclusion

The current study contributes to the knowledge about glycosphingolipids as antigens in bioprosthetic heart valves.

1. From porcine pericardium 8 non-acidic and 7 acidic GSL have been isolated and characterized by LC/MS-ESI and CBA. Notable is the presence of the Galα3 and the NeuGc antigens in the tissue.
2. All total neutral GSL from goat erythrocytes have been isolated and characterized by LC/MS-ESI. In this sample new GSL containing NeuGc (NeuGc-GT1) has been characterized.
3. Finally, the reactivity of GSLs isolated from porcine pericardium, goat erythrocytes and from other samples have been tested via series of binding assays with the serum of the patients, who patients who underwent treatment with BHV. Results were mostly negative. However in a few samples increased reactivity with Galα3 and NeuGc antigens was revealed.

The knowledge about composition of GSL in various tissues and about their reactivity with human immunity helps to clarify why BHV treatment suffers from early deterioration of BHV due to calcification and other mechanisms and also helps to prevent complications connected with xenotransplantation treatment such as hyperacute reaction.

Also it creates more solid background for development of the methods used for getting rid of these antigens from the xenograft or antibodies against them from the recipient's body. These methods especially development GalT-KO and CMAH-KO (Galα3 and NeuGc free) pigs combined with transgenetic modulation of complement and haemostasis and exploration for next potential antigens should be further researched.

This all together might increase final outcome of BHV treatment of heart valve disease.

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