Chapter 1

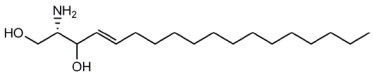
General introduction & Scope of thesis

Sphingolipids

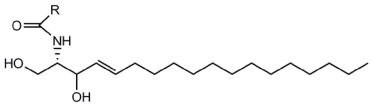
Cellular membranes contain various classes of lipids. Best known are the (phospho)glycerolipids and the sterol cholesterol. The third class of membrane lipids, named sphingolipids, was discovered at the end of the 19th century by the German chemist Johannes Ludwig Thudichum when analyzing the chemical composition of the human brain. He noted the abundant presence of lipids composed of fatty acid, amino acid and sugar elements. Inspired by the multi-faced nature of the compounds and Greek mythology, he coined the term sphingolipids for these structures [1,2].

Sphingolipids occur in all mammalian cells where they reside largely at the cell surface. Their chemical structure is remarkably heterogeneous (see scheme 1). All sphingolipids share a characteristic lipid part named ceramide, consisting of a sphingosine group to which is linked via an amide bond a fatty acid [3]. Attached to the C1 hydroxyl of the sphingosine moiety may be a phosphate (ceramide-1-phosphate, Cer-1-P), a phosphorylcholine (sphingomyelin, SM) or one to multiple carbohydrates (glycosphingolipids, GSL). Most common are glycosphingolipids in which the first sugar attached to ceramide is a glucose linked via a β-glycosidic bond. This glycosphingolipid is named glucosylceramide (glucocerebroside, GlcCer). Alternatively, a β-galactosyl group may be directly linked to ceramide (galactosylceramide, GalCer). Further sugars may be attached to GlcCer and GalCer as well as sulfate groups, resulting in complex glycan structures [4,5]. Due to the many variations in the sphingosine, fatty acid and glycan moieties, there is an enormous structural diversity of GSLs [3].

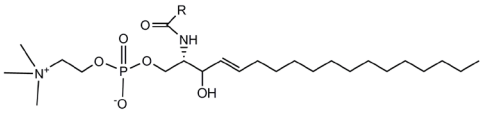
Sphingosine



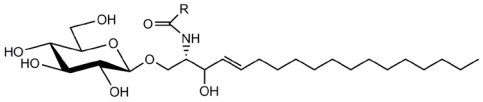
Ceramide



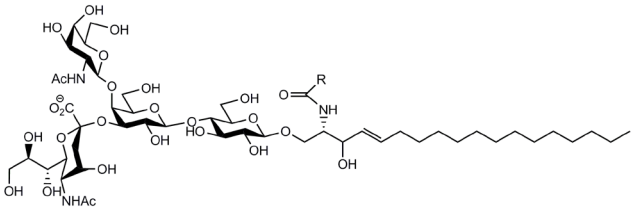
Sphingomyelin



Glucosylceramide



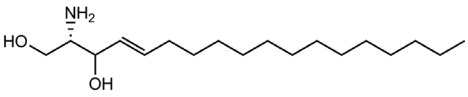
GM2 Ganglioside



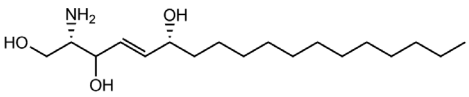
Scheme 1: Outline of sphingolipids

The sphingosine group forms the characteristic backbone of sphingolipids. Of note is the occurrence of heterogeneity in the sphingosine moiety. In mammalian cells the major sphingosine is C18 sphingoid base (d18:0, d18:1, and t18:0) [6], but additional double bonds may occur as well as longer and shorter sphingosine backbones [7,8]. Very recently the existence has also been recognized of deoxy-sphingosines lacking the C1 hydroxyl [9,10]. In other non-mammalian species additional variants of sphingosine exist. For example, in plants the dienes in sphingosines are detected at 4E:8E; the double bonds are also seen in the phytosphingosine-type compounds that are common backbones of plant sphingolipids [6]. Insects have predominantly C16 and C14 sphingoid bases such as 4E-d14:1 [11-13]

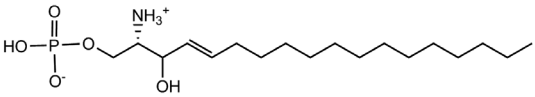
Sphingosine



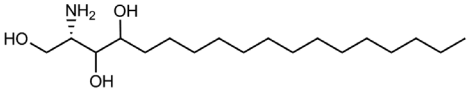
Hydroxylated Sphingosine



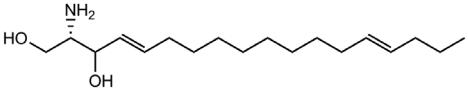
Sphingosine -1-Phosphate



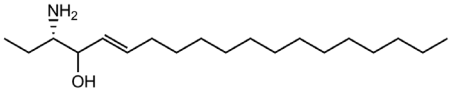
Phytosphingosine



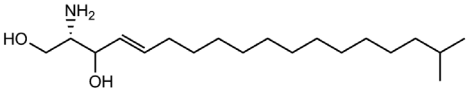
Sphingadiene (4E:14E)



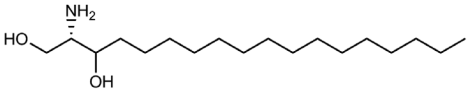
1-DeoxySphingosine



Sphingosine (branch methylated)



Sphinganine

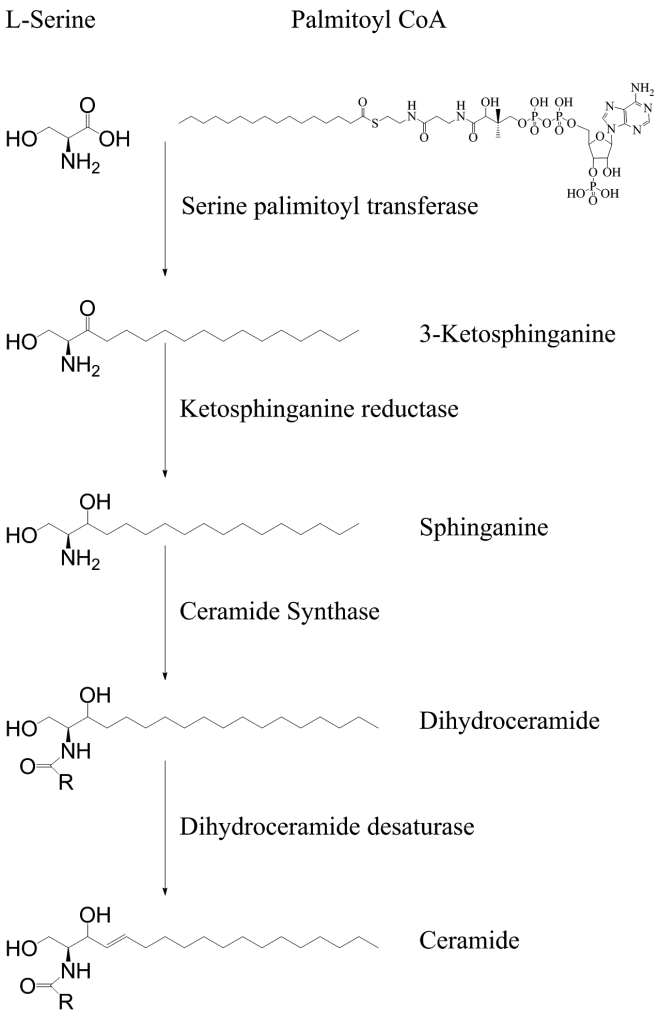


Scheme 2: Variations in the sphingosine backbone

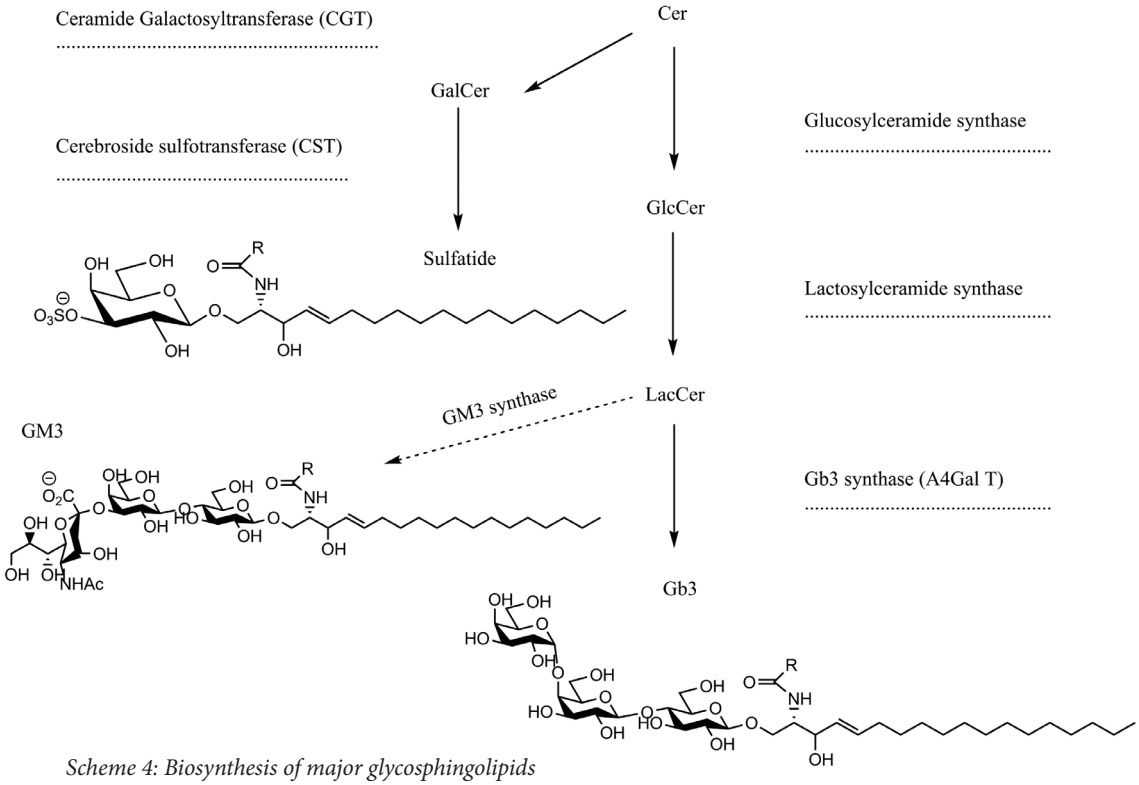
Biosynthesis of GSLs

The de novo synthesis of sphingolipids starts at the endoplasmic reticulum (ER) with two building blocks: the amino acid serine and the CoA-activated fatty acid palmitate [4], see scheme 3. The enzyme serine palmitoyltransferase (SPT) generates keto-sphinganine through condensation of serine and palmitoyl-CoA. The predominance of 18 carbon sphingoid bases in mammalian sphingolipids stems from the substrate preference of SPT [3]. Next, a specific reductase transfer keto-sphinganine to sphinganine. This sphingoid base is subsequently N-acylated by ceramide synthases (CERS). The

generated dihydroceramides are next rapidly converted to ceramides by dihydroceramide desaturase (DES) [3,4]. Subsequently, the newly formed Cer may acquire in the ER a galactose residue linked to the C1 of the sphingosine moiety to yield galactosylceramide (GalCer). Alternatively, Cer is transported by the protein CERT to the cytosolic leaflet of cis-Golgi membranes [14]. There the enzyme glucosylceramide synthase (GCS) can generate glucosylceramide (GlcCer) using UDP-Glc as sugar donor [15]. The newly formed GlcCer is in part translocated from the cytosolic leaflet to the luminal leaflet of the Golgi membrane via an unknown mechanism. Inside the Golgi apparatus, GlcCer can be further modified by stepwise addition of further sugars through the sequential action of specific glycosyltransferases, yielding complex GSLs such as gangliosides and globosides [3,4]. Sulfation of specific GSLs may also take place by sulfotransferases [3]. The various biosynthetic reactions in/at the smooth ER and Golgi apparatus cause the impressive structural heterogeneity of GSLs [16]. See scheme 4.



Scheme 3: Biosynthesis of ceramide

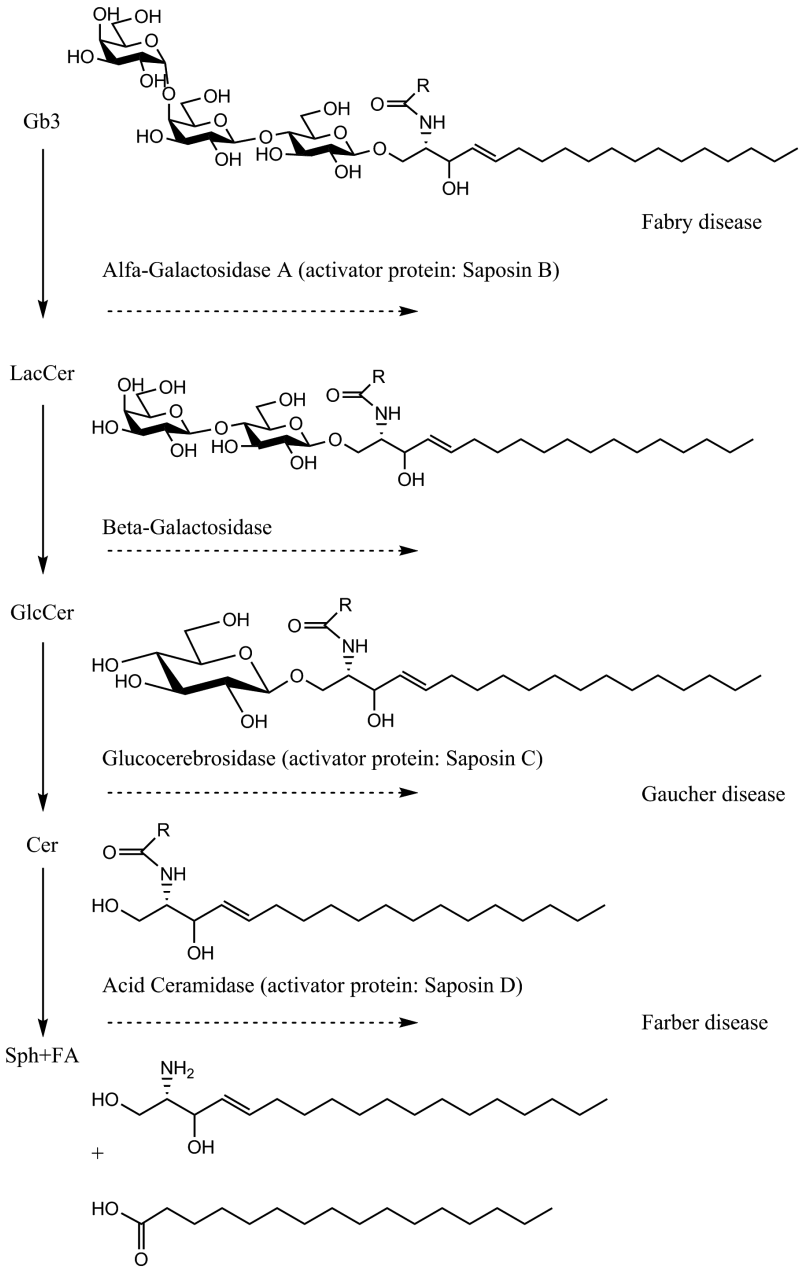


Scheme 4: Biosynthesis of major glycosphingolipids

Degradation of GSLs

From the Golgi apparatus, newly formed GSLs reach the outer leaflet of the plasma membrane. They may leave cells from there by transfer to nascent HDL particles, however most GSLs molecules stay at the plasma membrane and are ultimately internalized. Through endocytosis, GSLs end up in multi-vesicular bodies inside late endosomes. Fusion with lysosomes results in transfer to the lumen of the highly acidic organelle where degradation to building blocks occurs. The intralysosomal breakdown of complex GSLs is more or less a reverse of the biosynthetic pathway: sulfate groups are moved by sulfatases and terminal sugar moieties by corresponding glycosidases in a stepwise manner, often assisted by specific accessory proteins like GM2 activator protein and saposins A-D [17-19]. See scheme 5. The released free sugars from GSLs by glycosidases are exported from lysosomes. The acidity of lysosomes (pH 4.0 - 5.0) overlaps with the pH optimum of the glycosidases involved in GSL degradation. As penultimate step in GSL degradation, ceramide is generated from GalCer by galactocerebrosidase (GALC) or GlcCer by glucocerebrosidase (GBA1). Finally, the lipid ceramide is cleaved by acid ceramidase (AC) to yield free fatty acid and sphingosine ((2S,3R,4E)-2-aminooctadec-4-ene-1,3-diol). Like free fatty acids, sphingosine is subsequently exported from lysosomes to the cytosol where it can be immediately re-used in the salvage pathway to generate new Cer molecules through the action of CERS enzymes [20]. Alternatively, sphingosine can be modified in the cytosol to sphingosine-1-phosphate (S1P) via sphingosine kinases (SK1 and SK2), whereafter S1P lysase (SPL) degrades it to phosphatidylethanolamine and 2-trans-hexadecenal [21].

Of note, the simplest GSL, glucosylceramide (GlcCer), is formed at the cytosolic leaflet of the Golgi membrane. It can be also degraded in the cytosolic leaflet of membranes by the enzyme GBA2 [22-24]. Very recently it has been recognized that GBA2 is able to transfer the released glucose group from GlcCer to cholesterol, generating cholesterol-beta-glucoside [25].



Scheme 5: Degradation of glycosphingolipids

Lysosomal storage disorders

Failure of lysosomal enzymes to degrade glycosphingolipids causes their accumulation in lysosomes. Usually such failure has a genetic cause. Mutations in genes encoding lysosomal glycosidases or activator proteins form the basis for a considerable number of discrete inherited lysosomal storage diseases (LSDs) [26-32]. Those LSDs in which the degradation of glycosphingolipids is primarily deficient are generally named glycosphingolipidoses. An overview of inherited glycosphingolipidoses is presented in table 1. Like other types of LSDs, the clinical expression of most glycosphingolipidoses may vary markedly among patients, ranging from very severe at young age to a relative benign course of disease [32]. Clear genotype-phenotype correlations only occur in some glycosphingolipidoses, suggesting that other factors next to mutations in the glycosidase may influence development of symptoms in patients with a glycosphingolipidosis [32,33].

A relatively common glycosphingolipidosis studied in this thesis is Gaucher disease (GD) (MIM#230800) [32]. This recessively inherited disorder is caused by deficient activity of lysosomal acid β-glucosidase (GBA, glucocerebrosidase, EC.3.2.1.45). The enzyme, encoded by the *GBA* gene (locus 1q21), is responsible for intralysosomal breakdown of GlcCer. Storage of GlcCer in GD patients is most prominent in macrophages that transform into characteristic Gaucher cells. Characteristic signs of GD are hepatosplenomegaly, hematological symptoms such as anemia, leukopenia and thrombocytopenia and skeletal deterioration. More severely affected patients also develop neurological complications (type 2 and 3 GD) [32]. Complete absence of GBA activity results in the collodion baby variant of GD, characterized by impaired barrier function of the skin [34]. Contrary to other lysosomal glycosidases, newly formed GBA1 is not transported via mannose-6-phosphate receptors to lysosomes [35], but as a complex with the lysosomal integral membrane protein type 2 (LIMP2) [36]. Mutations in *SCARB2*, the gene encoding LIMP2, cause Action Myoclonus-Renal Failure syndrome (AMRF) (MIM#602257) [37]. In most cells of AMRF patients GBA1 activity is very low, but not in macrophages. Because of this, the clinical manifestation of AMRF is completely different from that of GD [38].

Other glycosphingolipidoses investigated in this thesis are Fabry disease (FD), Krabbe disease (KD) and Niemann Pick disease type C (NPC). FD (MIM# 301500) is due to inherited defects in lysosomal acid α-galactosidase (α-galactosidase A), encoded by the GALA gene. The enzyme is responsible for intralysosomal degradation of globotriaosylceramide (Gb3, ceramide trihexoside) [32]. The GALA gene is located at Xq22, and consequently Fabry disease is an X-linked disorder that manifests in males. Characteristic signs are skin angiokeratomata, inability to sweat and extreme pains in the extremities at young age. Later in life FD patients may develop renal disease, left ventricular hypertrophy and strokes. Some female Fabry heterozygotes may also develop an attenuated form of disease without the characteristic early disease signs of Fabry males and renal complications. Atypical variants of FD are more recently recognized. In these individuals, showing significant residual GALA activity, only one the late-onset symptoms usually develops [29]. KD (globoid-cell leukodystrophy; MIM# 245200) results from mutations in the *GALC* gene encoding the enzyme galactocerebrosidase (galactosylceramidase, E.C. 3.2.1.46) [39]. This recessive disorder is characterized by severe neurological disease at young age. NPC (MIM# 257220) is not directly caused by a primary deficiency in a lysosomal glycosidase, but by defects in either the *NPC1* or *NPC2* gene encoding proteins mediating efflux of cholesterol from lysosomes [40]. Characteristically in cells of NPC patients, cholesterol accumulates in lysosomes in combination with various sphingolipids. The latter is likely due to a generalized dysfunction of lysosomes following sterol accumulation.

In recent decades considerable attention has been focused on developing treatments for glycosphingolipidoses. A frontrunner in this respect has been GD [32]. Allogeneic bone marrow transplantation

is known to be curative in non-neuronopathic GD patients, however it is little employed due to limited availability of matched donors and the invasive nature and associated risks of transplantation. Gene therapy for GD based on introduction of genetically corrected autologous hematopoietic stem cells is a promising avenue but still at an experimental stage [41]. A successful treatment of non-neuropathic GD patients is offered by so-called enzyme replacement therapy (ERT). Chronic intravenous administration of macrophage-targeted recombinant human GBA1 results in reduction of pathological Gaucher cells in peripheral tissues, followed by major improvement in organomegaly and hematological [32]. ERT is extremely costly and individualized enzyme dosing regimens are warranted. More recently, impressive clinical responses have also been observed for non-neuropathic GD patients upon pharmacological inhibition of GlcCer biosynthesis (so-called substrate reduction therapy; SRT) [32,42,43]. SRT of GD makes use of small compound inhibitors of glucosylceramide synthase (Zavesca, Eliglustat) that are orally administered [44-46]. Finally, small compounds are developed to be used in pharmacological chaperone therapy [32]. This still experimental approach envisions that substrate mimics might promote folding and stability of a mutated GBA enzyme and thus increase degradative capacity in lysosomes of GD patients.

The variable disease expression in patients with similar defects in one particular glycosidase fuels speculations about other factors and mechanisms contributing to the onset and progression of symptoms in the individual patient. It seems that cells can use alternative metabolic pathways to cope with the primary lysosomal defect (for a more detailed discussion of this topic see chapter 8). Such mechanisms might involve the processing of accumulating substrate(s) by other enzymes. Indeed, in some glycosphingolipidoses deacylated forms of the primary accumulating GSL are increased [47-50]. In the case of Gaucher disease, compensatory extra-lysosomal degradation of GlcCer by the enzyme GBA2 may occur [33,51]. Although these metabolic adaptations contribute to amelioration of the lysosomal defect, they also may lead to harmful side effects. An example of this seems to be loss of motor coordination associated with excessive GBA activity [52].

Table 1: Glycosphingolipidoses

Disease	Deficient Hydrolases	Primary Storage Products	Major Organs Involved	Locus Gene
Gaucher (GD)	β-Glucocerebrosidase	Glucosylceramide + Glucosylsphingosine		1q21 GBA
Infantile type 2			CNS, spleen, liver, bone marrow	
Juvenile type 3			CNS, spleen, liver, bone marrow	
Adult type 1			Spleen, liver, bone marrow	
Fabry (FD)	α-Galactosidase	Globotriaosylceramide + Globotriaosylsphingosine	Kidney, brain and blood vessels of skin	Xq22 GLA
Schindler disease	A-*N*-acetylgalactosaminidase	Sialylated and asialopeptides and oligosaccharides	CNS, PNS	22q13 NAGA
Type 1 infantile-onset *Type 2 adult-onset (Kanzaki disease)* *Type 3 intermediate*				

Metachromatic leukodystrophy (MLD)		Sulfatide		
Late Infantile form	Arylsulfatase A		CNS, liver, kidney, gallbladder	22q13 ARSA
Late-onset form	Arylsulfatase A		CNS, liver, kidney, gallbladder	22q13 ARSA
Multiple sulfatase deficiency	At least 7 lysosomal sulfatases and a microsomal sulfatase		CNS, visceral organs, and skeleton	3p26 SUMF1
Niemann-Pick disease (NPD)				
Types A and B	Sphingomyelinase	Sphingomyelin	CNS, liver, spleen, bone marrow	11p15 SPMPD1
Type C1	Proteins required for lipid transport through late endosome	Unesterified cholesterol and sphingolipids	CNS, liver, spleen	18q11 NPC1
Type C2	Proteins required for lipid transport through late endosome	Unesterified cholesterol and sphingolipids	CNS, liver, spleen	14q24 NPC2
G_{M1}-gangliosidosis	β-galactosidase	G_{M1}-ganglioside, oligosaccharides, keratin sulfate	CNS, skeleton, viscera	3p21 GLB1
G_{M2}-gangliosidosis				
Tay-Sachs disease, A variant	β-hexosaminidase A	G_{M2}-ganglioside	CNS	15q23 HEXA
Sandhoff disease	β-hexosaminidases A and B	G_{M2}-ganglioside, oligosaccharides	CNS	5q13 HEXB
AB variant	Deficiency of G_{M2}-activator protein	G_{M2}-ganglioside	CNS	5q33 GM2A
Galactosialidosis (Goldberg Syndrome)	Protective protein/ cathepsin A, resulting in deficiency of β-galactosidase and α-neuraminidase	Glycolipids and oligosaccharides	CNS, spleen, liver, skeleton	20q13 CTSA
Globoid cell leukodystrophy (Krabbe disease)	Galactocerebrosidase, β-galactosidase	Galactosylceramide and galactosylsphingosine	CNS	14q31 GALC
Farber granulomatosis	Ceramidase	Ceramide	Subcutaneous nodules, joints, larynx, liver, lung, heart	8p22 ASAH
Wolman disease	Acid lipase/cholesterol esterase	Triglycerides, cholesteryl esters	Liver, spleen, adrenal	10q24 LIPA

Roles of GSLs in health and disease

A physiological role for GSLs has been postulated for various kind of processes, ranging from regulation of cell differentiation and apoptosis [53], functioning of the central nervous system [54,55], to generation of the skin barrier [56]. GSLs and cholesterol in membranes form spontaneously semi-ordered microdomains [57,58]. These "lipid rafts" are supposed to act as platforms in which glycosylphosphatidylinositol (GPI)-anchored proteins and other proteins specifically reside. Through the assembly of specific components, lipid rafts are thought to be essential in specific signaling pathways and neurotransmission [57,58]. In addition, GSLs are believed to mediate cell-cell adhesion and communication [54].

Abnormalities in GSLs have been implicated in pathophysiological processes. As discussed above, lysosomal accumulation of glycosphingolipids in glycosphingolipidoses is considered to be pathological and thus underlie various disease manifestations in the patients. Excessive GSLs, particularly gangliosides such as GM3 have been implicated in insulin resistance, a major aspect of the Metabolic Syndrome [59]. Pharmacological agents (hydrophobic iminosugars) modulating GlcCer metabolism have been found to exert major beneficial effects in obese rodents such as improved glycemic control, correction of hepatosteatosis, and prevention for atherosclerosis [4,60-70]. Of note, the same iminosugars have also been found to protect LSD mice for motor neuron degeneration [52,71,72]. A pathological role for excessive glycosphingolipids in the brain is further indicated by the observed increased risk for Parkinsonism and Lewy-body dementia in GD patients and even GD carriers [73]. Excessive glycosphingoid bases are also increasingly considered as pathogenic factors. Examples for this are the toxic effects of galactosylsphingosine in KD patients [74,75], the putative pathogenic role of lysoGb3 in kidney fibrosis and peripheral neuropathy in FD patients [76-78], and the role of excessive glucosylsphingosine in promoting multiple myeloma in Gaucher disease patients [79,80].

Analysis of GSLs

Since the discovery of the glycosphingolipids, several methods and approaches were developed for identification and quantification of glycosphingolipids. The oldest method for determination of GSLs which is still widely used is thin layer chromatography (TLC) or the more advanced high performance TLC (HPTLC). Thin-layer chromatography of lipids is usually performed on a sheet of glass coated with a thin layer of silica gel as adsorbent material (the stationary phase). After sample application on the plate, a solvent mixture (the mobile phase) is drawn up the plate via capillary action. The mobile phase has different properties from the stationary phase favoring separation of compounds based on differences in mobility. After the TLC separation, the GSLs are visualized by charring after sulfuric acid or cupric acetate spray (destructive method), staining with Primuline or Rhodamine spray (non-destructive method) or by specific reagents for carbohydrate moieties [81-84]. Lipids may be located on TLC plates first by non-specific methods, for example Primuline spray combined with the relative retention factor (Rf) of the spots on the plates, but an additional reagent is needed to visualize presence of sugar in glycolipids. In contrast to detection with Primuline which is non-destructive, the detection of sugars is a destructive method and no fatty acid composition can be determined. Specific sugar detection involves treatment with either orcinol, naphthyl ethylenediamine or 5-hydroxy-1-tetralone in a strong acid medium can be used after a Primuline spray [85]. In recent times, glycosphingolipids tagged with a fluorophore are often used in experiments. Examples of fluorescent tags are NBD and BODIPY [86,87]. Following TLC separation, NBD- or BODIPY-tagged lipids can be conveniently visualized by fluorescence scanning.

Another classical method for determination of GSLs is high performance liquid chromatography (HPLC) where separation of lipids is reached with normal phase or reverse phase liquid column chromatography and the separated lipids are detected by UV, light-scattering (Evaporative Light Scattering Detector, ELSD), or fluorescence detection [88-90]. The commonly used agent for fluorescence detection is orthophthalaldehyde (OPA). OPA is used in combination with 2-mercaptoethanol and ethanol, in a high pH borate buffer (pH 9-10.5). The OPA reagent reacts with primary amine ($R\text{-}NH_2$) from sphingosine base. The primary amine is accessible in free bases of sphingolipids or can be generated through deacylation of GSLs by microwave-assisted alkaline deacylation [90] or by enzymatic deacylation using sphingolipid ceramide N-deacylase (SCDase) [91]. Fluorenylmethyloxycarbonyl chloride (FMOC-Cl) is also used for labelling of GSLs [92].

A third, less widely applied, method is based on immunochemical detection of GSLs using specific antibodies. The method is also named (far)eastern blotting or TLC blotting. Following TLC separation, lipids are visualized with antibodies using a methodology similar to the one used with protein detection during western blotting. Individual lipids separated on an HPTLC plate are transferred to the polyvinylidene difluoride (PVDF) membrane quantitatively. Lipids can be specifically visualized by immunodetection with (monoclonal) antibodies. Lipids can be isolated from the lipid-blotted membrane by a single-step elution with small amounts of methanol. Treatment of lipids of potential interest with specific lipid-metabolizing enzymes is also possible. Alternatively, separated lipids on the PVDF membrane can be analyzed using matrix-assisted laser desorption/ionization quadrupole ion trap time-of-flight mass spectrometry (TLC-Blot/MALDI-TOF MS) [93,94].

A fourth applied method is nuclear magnetic resonance (NMR) for structure identification of the GSLs of interest. Investigated in this way among others is the structure of the carbohydrate portion, including the configuration and composition of sugar moieties, the sequence and linkage sites of the oligosaccharide chain [95,96].

Currently, the most preferred method for GSLs measurement is mass spectrometry (MS). Mass involves the conversion of components in the sample into gaseous ions, with or without fragmentation, which are then separated and characterized by their mass to charge ratios (m/z) and relative abundances. This method is particularly reliable because of its capability to identify and quantify a broad range of GSL molecules with a high degree of accuracy [97-100]. Various ionization methods are applied, including electron ionization (EI) [101,102], fast atom bombardment (FAB) [103], matrix-assisted laser-desorption ionization (MALDI) [104], electrospray ionization (ESI) [105], and the combination method of high performance liquid chromatography mass spectrometry and tandem mass spectrometry (LC-MS/MS). LC-MS/MS is the preferred technology for analyses of small samples, rendering the essential structural specificity, sensitivity, quantitative accuracy, and in addition high-throughput capacity [100].

Quantitative MS

For quantitation of compounds of interest (analytes) in the sample matrix, different quantification methods are used. The most reliable and accurate method is the use of suitable internal standards. Addition ('spiking') of a known amount of a chosen compound, which is different from the analyte, to the samples to be measured is widely used in quantitative LC-MS. Such internal standards are used for correction of numerous effects, such as extraction errors, instrument drift, variable sample injection volume, matrix effect, and ion suppression or ion enhancement. For internal calibration of measurements, isotopically labelled internal standards are often used in the case of LC-MS. For this it is necessary to obtain or synthesize an analogue of the analyte with a different isotopic composition from the naturally occurring analyte. In most LC-MS applications of this technique the isotope standards are based on enrichment with ^{13}C, ^{15}N or ^{2}H [106]. Another option is the use of internal standard with odd number of carbon, for example C17 sphinganine base, as these are more often commercially available and less expensive compared to isotope internal standards [90]. Obviously these are not exact analogues of the analyte of interest.

	Analyses and detection techniques	
(HP)TLC	Staining (non-specific, carbohydrate), fluorescence	
HPLC/UPLC	UV, ELSD, Fluorescence	
NMR	e.g., ^{1}H NMR	
MS	*Ionization Technologies of MS:* Electron ionization (EI), Chemical ionization (CI), Fast Atom Bombardment (FAB), Matrix-assisted laser desorption/ionization (MALDI), Electrospray ionization (ESI), Atmospheric pressure chemical ionization (APCI), Atmospheric pressure laser ionization (APPI), Desorption electrospray ionization (DESI)	*Mass Analyzers of MS:* Quadrupole (Q), Time-of-flight (TOF), Fourier transform ion cyclotron resonance (FTICR), Orbitrap (OT)
Tandem MS	e.g., QTOF-MS, ESI	

Scheme 6: Methods of Glycosphingolipids analysis

Lipidomics and imaging

Lipidomics of GSLs aims to identify and quantify all lipid species in order to get insight in their presence and to understand how they respond to a biological change. The term "shotgun lipidomics" describes the methodology where lipids are identified and quantified by direct infusion of crude extraction ionized by ESI-MS or MALDI without a previous chromatographic separation [107,108]. This method has been shown to be suitable for highly abundant lipids, but it may also lead to the impression that some compounds are not present when they actually are, due to ion suppression effects. To overcome these limitations, the additional purification and separation needs to be processed prior to analysis. For example, solid phase extraction (SPE) clean-up to separate sulfatides from phospholipids with Silica Gel column [109] or a mild alkaline hydrolysis can be executed to eliminate the highly abundant glycerophospholipids [110]. Another disadvantage is that "shotgun lipidomics" is not able to distinguish isomeric species (e.g., GlcCer and GalCer). Modifications of the shotgun method that use a pre-MS fractionation approach such as LC significantly improve its reliability [111]. Other recent methods in lipidomics are so-called lipid profiling focusing on known metabolites of interest [112,113] and the use of two-dimensional mass spectrometry to increase detection of lipids [114,115].

With conventional techniques in GSL lipidomics, sample preparation involves extraction: resulting in a loss of crucial information about the real life context, such as localization of various GSLs. Direct tissue analysis (in-situ) by imaging mass spectrometry aims to address these specific questions. Imaging mass spectrometry has been shown to allow detection of specific GSLs in tissues in situ, for example in heart, kidney and brain. Both the MALDI and secondary ion mass spectrometry (SIMS) TOF techniques have been used to directly analyze tissues [116,117] and cells [118] for their lipid content and localization [119]. For example, Snel and Fuller have reported on the use of imaging mass spectrometry for determination of glucosylceramides species in spleen sections from a conditional knockout mouse model of type 1 Gaucher disease [120].

*Glycosphingolipid analyses in this thesis employing LC-MS/MS with electron spray ionizatio*n

Quantification of GSLs and their bases is essential in the research on lysosomal glycosphingolipidoses. The main focus of the conducted thesis research was to develop new accurate methods for lipid measurements in a complex biological matrix such as plasma, urine, cells and tissues. In the course of the investigations, quantitative LC-MS using isotope encoded internal standards became the method of choice for determination of glycosphingoid base concentrations in biological samples. This method offered optimal accuracy and quantification. The approach can in principle also be applied for quantitative measurement of GSLs, following their deacylation by microwave-assisted alkaline deacylation (or SCDase-assisted enzymatic deacylation). A similar approach for quantification of neutral GSLs was earlier combined with HPLC-based detection: lipids were deacylated followed by OPA derivatization and chromatography [90]. This thesis describes the development of new analytic methods for various GSLs and their bases allowing unprecedented quantification. The broad applicability of the new methods in fundamental research as well as diagnosis and disease monitoring is illustrated.

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Scope of thesis

The aims of the conducted and here presented thesis research were many-fold.
In the first place, novel sensitive procedures had to be developed to allow quantitative detection of known glycosphingolipids and sphingoid bases. The investigations on this theme are described in Section I.
First, a method for quantification of plasma globotriaosylsphingosine (lysoGb3) in Fabry disease patients and normal subjects was developed [**chapter 2, ref 1**]. Next, improved quantification was accomplished based on LC-MS/MS detection with use of an internal, ^{13}C isotope encoded, standard [**chapter 3, ref 2, addendum I. ref 3**]. Subsequently, an improved LC-MS/MS method for sensitive and accurate quantification of glucosylsphingosine (GlcSph) with ^{13}C isotope encoded identical standard is described as well as differences in GlcSph isoforms in urine and plasma [**chapter 4, ref 4**]. Likewise, an improved LC-MS/MS method for detection of sulfatide is presented [**chapter 5, ref 5**]. Subsequently, a new LC-MS/MS procedure for quantification of sphingosine-1-phosphate (S1P), comparing ^{13}C isotope encoded natural S1P and C17-S1P as internal standards is described [**chapter 6, ref 6**]. **Chapter 7** is presenting multiplex LC-MS/MS quantification of major glycosphingoid bases in human plasma [**ref 7**]. This improved procedure uses a single extraction to separate lipids and bases, deacylation of lipids and quantification of various endogenous and generated sphingoid bases using LC-MS/MS and a series of appropriate internal ^{13}C-isotope encoded standards.

The second theme of the thesis forms the application of the developed lipid analyses for diagnostic purposes and for monitoring of disease progression and correction by therapeutic intervention.
These investigations are presented in section II that starts with a review on the lysosomal disorders Gaucher disease and Fabry disease [**chapter 8, ref 8**]. The practical value of lipid analyses is presented by a number of publications shown in the addendum II [**ref 9-12**]. Section II provides a summary of these investigations [**chapter 9**].

The third research theme concerns fundamental investigations on glycosphingolipid metabolism in health and disease. The section starts with a hypothesis-review arguing for the existence of adaptive metabolism in glycosphingolipidoses like Gaucher disease and Fabry disease [**chapter 10**]. One adaptive pathway involves the conversion of neutral glycosphingolipids to corresponding sphingoid bases by lysosomal acid ceramidase [**chapter 11, ref 13**]. The characteristic elevation of glycosphingoid bases in glycosphingolipidoses is illustrated by an investigation of several mouse LSD models [**chapter 12, ref 14**]. In a separate study biochemical abnormalities in glucocerebrosidase (GBA1)-deficient mice lacking the protein LIMP2 were examined and prominent abnormalities in glucosylsphingosine were again encountered [**chapter 13**]. Another adaptive response to reduced activity of GBA1 is the increase in protein and activity of the cytosolic glucosylceramidase GBA2. An investigation with Niemann Pick type C with impaired GBA1 revealed the beneficial effect of reduction of GBA2 protein and activity was observed [**see addendum III, ref 15**]. The final experimental investigations concern the discovery of novel glycolipids. A study on a poorly recognized glucosylated metabolite, cholesterol-β-glucoside (GlcChol), is described. It demonstrates that *in vitro* and *in vivo* the β-glucosidases GBA1 and GBA2 are both able to reversibly transfer glucose moieties from glucosylceramide to cholesterol generating cholesterol-beta-glucoside. A newly developed UPLC-ESI-MS/MS method using $^{13}C_6$-labelled GlcChol as internal standard allowed the demonstration of natural occurrence of GlcChol in mouse tissues and human plasma [**chapter 14, ref 16**]. The versatility of retaining β-glucosidases in catalysis is further illustrated by the demonstration of xylosylation of cholesterol by GBA1 *in vitro* and *in vivo*. UPLC-ESI-MS/MS rendered indications for the presence of in xylosylated cholesterol in liver of

mice suffering from Niemann Pick type C disease and associated lysosomal cholesterol accumulation [**chapter 15**].

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