# Analytical chemistry and biochemistry of glycosphingolipids : new developments and insights

Mirzaian, M.

# General discussion and perspectives for future research

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Topic of this thesis are the so-called glycosphingolipidoses, a number of inherited diseases in man caused by defects in lysosomal degradation of specific glycosphingolipids (GSLs). Most common among the glycosphingolipidoses are Gaucher disease (GD), inherited deficiency of glucocerebrosidase (GBA1) causing intralysosomal storage of glucosylceramide (GlcCer), and Fabry disease (FD), inherited deficiency of α-galactosidase A (α-GAL or GLA) causing intralysosomal storage of globotriaosylceramide (Gb3), (chapter 8), [1]. Other glycosphingolipidoses studied in this thesis are metachromatic leukodystrophy (MLD), inherited deficiency of arylsulfatase A (ARSA) causing intralysosomal accumulation of sulfatide (3-O-sulfogalactosylceramide) (chapter 5, [2]); Krabbe disease, inherited deficiency of galactocerebrosidase (GALC) causing intralysosomal storage of galactosylceramide (GalCer) and Niemann Pick type C, an inherited deficiency in export of cholesterol from lysosomes leading to secondary deficiency of sphingomyelinase and glucocerebrosidase activities and subsequent intralysosomal increases in sphingomyelin (SM) and GlcCer (chapter 12, [3-5]). Next to the characteristic abnormalities in GSLs, each of these diseases also presents increased levels of corresponding sphingoid bases, (chapters 2-9 and 11-13, [1-3,5-10]). These sphingoid bases are formed by de-acylation of GSLs and are sometimes named lyso-structures. The accurate quantitation of GSLs and their sphingoid bases is of great importance in research on metabolism of these lipids as well as for the diagnosis and clinical management of patients suffering from defects in lysosomal GSL breakdown.

The major aim of this thesis work was to develop improved methods for the quantification of GSLs and sphingolipids (SLs) like ceramide, dihydroceramide and SM as well as their bases in complex biological samples such as plasma, urine, cells and tissues. This goal was reached by the use of UPLC-ESI-MS/MS, often in combination with the use of isotope encoded standards (synthesized by Patrick Wisse or Henrik Gold, Bio-Organic Synthesis, Leiden Institute of Chemistry) (see Addendum I [7,11]). Developed were improved UPLC-ESI-MS/MS procedures for accurate quantification of several (glyco)sphingoid bases: sphinganine, sphingosine, sphingosin-1-phosphate (S1P), glycosylsphingosines, lactosylsphingsosine (lysoLac), globotriaosylsphingosine (lysoGb3), lysosulfatide and phosphorylcholine-sphingosine (lysosphingomyelin, lysoSM). In all cases use was made of an identical isotope-encoded standard. Exceptions were for the quantification of lysosulfatide the use of commercial N-acetyl-sulfatide from Matreya (State College, USA) as internal standard, for quantification of lysoSM the use of commercial phosphorylcholine-sphingosine (C17 base) from Avanti Polar Lipids (Alabaster, USA) as internal standard and for quantification of lysoLac the use of $^{13}$C-encoded lysoGb3 as internal standard. In general, identical, isotope encoded standards allow quantification and at the same time correction of extraction errors, instrument drift, variable sample injection volume, matrix effect, and ion suppression/enhancement. Because of their commercial availability, often odd carbon number lipids, for example C17 sphinganine, are used as internal standards. As demonstrated in a series of published investigations in this thesis, the use of identical isotope- encoded standards gives superior detection and quantification. As the natural and isotope labelled compounds are identical, no correction for the sample matrix (ion suppression and in some cases ion enhancement) is required. In addition, the use of identical internal standards allows compensation for losses during extraction, ionization efficiency and mass spectrometric performance, improves throughput and delivers more reliable data. For example, comparative analysis of $^{13}$C-encoded natural S1P and C17-S1P as internal standard showed the superiority of the former regarding background, especially in samples like cell lysates with low concentration S1P, (chapter 6, [9]).

The development of MS-based lipidomic approaches can be classified into two main directions, namely the non-targeted lipidomics and targeted lipidomics. The non-targeted approaches aim to contribute global information about lipids composition present in tissue, cells and body fluids. These approaches are useful when a general overview of the involved lipids is required. In contrast, the targeted lipidomic approaches focus on identification and quantification of specific lipid of interest, which are more applicable in addressing specific biological questions. The full scan MS profiling of the lipids from prepared biological samples are performed by a non-targeted lipidomics acquired for example by shotgun approaches. This approach can be further developed by using tandem mass spectrometry (MS/MS) for confirmation of peak of interest, such as product ion scan (daughter scan), neutral loss scan (NL) and multiple reaction monitoring (MRM) [12]. By purpose the analytical procedures described in this thesis focused on quantitative determination of sphingoid bases using UPLC-ESI-MS/MS, in some research topics combined with shotgun lipidomics (direct infusion) (see for example [2]). Not pursued was the regular approach of profiling and characterizing of lipid species using high resolution mass spectrometry, for example liquid chromatography coupled to high resolution quadrupole time of flight mass spectrometry [13], liquid chromatography-Fourier transform mass spectrometry, and ion trap tandem mass spectrometry [14]. The motivation for our choice to focus on sphingoid bases stems from the fact that GSLs are intrinsically heterogeneous in composition due to their variable fatty acyl composition. There is a lack of standards for all the different isoforms of various GSLs and SLs, hampering their absolute quantification. In the case of sphingoid bases however it is feasible to synthesize and apply identical isotope-encoded standards to reach absolute quantitation as shown in this thesis. It is possible to de-acylate (glyco)sphingolipids to their base structures which can subsequently be accurately quantified. In this way a reliable number for the total sum of a GSL/SL in a sample can be obtained. De-acylation of sphingolipids in a sample can be accomplished by exposure to microwaves at appropriate conditions or enzymatic digestion using a commercial ceramide N-deacylase [15-18]. Our approach to first convert sphingolipids to their bases has another practical advantage. Sphingoid bases can usually be well separated with HPLC/UPLC procedures, but this is not always the case with the heterogeneous (glyco)sphingolipids. Particular isoforms of (glyco)sphingolipids may be poorly separated from other lipids and missed because of this heterogeneity. In principle, information on the structure of any lipid in a complex mixture can be obtained by electron spray mass spectrometry and analysis of *m/z* of parent and daughter fragments [19].

Although de-acylation of (glyco)sphingolipids offers the possibility to obtain quantitative data on the total amount of a GSL in a sample, information on fatty acyl composition of the GSL is by virtue lacking. In the direct future the newly developed methods should be combined with commonly employed semi-quantitative profiling of GSL isoforms in biological samples (non-targeted scanning with direct infusion, see chapter 5). Information on isoforms, even when semi-quantitative, is of interest since the fatty acyl composition of GSLs/SLs may be relevant. For example, it has been shown that fatty acyl composition of GSLs influences their interaction with cholesterol during formation of semi-ordered lipid domains (lipid rafts) at the cell membrane [20]. These lipid rafts act as platforms in physiological relevant signaling processes [21]. It has been shown for the ganglioside GM1 that fatty acyl saturation and chain length influence participation in lipid rafts [22]. Another example is provided by the finding that a direct and highly specific interaction of exclusively sphingomyelin with a C18 fatty acyl with the transmembrane domain (TMD) of the COPI machinery protein p24 [23].

Accurate diagnosis of disease and sensitive laboratory tests for monitoring disease progression/correction are obviously of great clinical relevance. The analytical methods described in this thesis have

significantly contributed to the quality of diagnosis and disease monitoring during treatment of FD and GD patients. For example, we developed an accurate and very sensitive method for quantitation of lysoGb3 and lyso-ene-Gb3 in plasma and urine samples of FD patients [7]. This method has become vital in confirming diagnosis of FD [10,24]. Because of the high sensitivity of this method for quantification of lysoGb3 in plasma of healthy individuals, it is particularly useful in the analysis of lysoGb3 concentrations in plasma of patients with an atypical clinical presentation of FD [25]. Caution however is needed by our observation that patients receiving lysosomotropic agents as drugs and even GD patients may show plasma lysoGb3 levels overlapping with those seen in some atypical FD males [10,24]. Moreover, the method is very useful in monitoring the effect of costly enzyme replacement therapies in FD patients [26,27]. The UPLC-ESI-MS/MS method was used to determine in FD patients the effect of switching therapeutic enzyme or enzyme dose during the unfortunate shortage of agalsidase beta [26]. Enzyme replacement therapy (ERT) with αGal A is known to cause formation of antibodies (AB) against the therapeutic enzyme in most males with classic FD due their lack of endogenous αGal A [28]. Anti-agalsidase ABs are neutralizing (inhibiting enzyme activity) and consequently negatively influence globotriaosylceramide (Gb3) reduction [27]. The impact of anti-agalsidase AB on plasma and urinary Gb3 and plasma lysoGb3 corrections and clinical outcome in FD patients on ERT was investigated with the newly developed LC-MS/MS procedure, clearly indicating a negative effect of the presence of antibodies [28]. A comparative study has been also made of the biochemical response in type 1 GD patients to substrate reduction therapy versus enzyme replacement using $^{13}C_5$-GlcSph as an internal standard for monitoring the level of GlcSph as a biomarker of GD [29]. The efficacy of gene therapy in a GD mouse model was substantiated by documenting corrections of excessive GlcSph [30]. Finally, the developed LC-MS/MS method for measurement of the lysosulfatides and sulfatides will find applications in research on MLD. The sensitive new method is able to quantify a wide range of sulfatide concentrations and is able to quantify total sulfatide content and levels of individual molecular species of sulfatides in tissues, cells, and body fluids. Instead of the traditional methods like TLC or HPLC, which need large volumes or even 24 h urine collections, this method only needs 0.5 ml of a representative urine sample suffices for the analysis of MLD and normal urines [2].

The UPLC-ESI-MS/MS methods described in this thesis are an important step towards high throughput measurement of GSLs. Firstly, a multiplex analysis for (glyco)sphingoid bases can be used (chapter 7), as very recently also reported by Polo and co-workers [31]. All (glyco)sphingoid bases can be extracted using modified acidic Bligh & Dyer, partitioning in the upper water phase. An exception in this respect is sphingosine-1-phosphate that requires specific extraction conditions (chapter 6, [9]). In principle the same procedure works for measurement of GSLs. The GSLs end up in the lower (chloroform) phase with the same extraction. Next, they may be de-acylated by exposure to microwaves or enzymatic digestion with commercial ceramide N-deacylase. The resulting (glyco)sphingoid bases are next analyzed as described above (see chapter 7 for an example). To increase throughput further, especially for large numbers of clinical samples, adjustments to the LC-MS/MS apparatus can be made like the use of injector towers. Transfer of the method to a 96 well plate platform is also an option. The biggest challenge in order to achieve a higher throughput is to reduce the time consuming extraction methods. Extensive sample clean up remains necessary to reduce the background and artifacts at the same time results in loss of compounds of interest and is labor intensive. At present, the field of lipidomics, and glycosphingolipidomics, is still in full development witnessing great improvements in mass spectrometry as well as annotation software [1,32-36]. Discovery studies often employ global lipidomic approaches in combination with advanced bioinformatics [37]. Shotgun lipidomics using direct infusion without chromatographic separation is despite its limitations becoming popular again because of simplicity, ease of management, and relatively instruments [38]. In validation studies, targeted quantitative methods are still generally used.

Almost all the techniques used for identification and quantitation of GSLs involve extraction and removal from their biological environment which leads to loss of crucial information. Direct tissue analysis (*in situ*) and imaging mass spectrometry are promising and powerful techniques to get information about the (sub)cellular topology of lipids, the questions not answered by the conventional analytical techniques. The techniques used for MS-based imaging are matrix-assisted laser desorption ionization (MALDI) imaging, secondary ion MS (SIMS) and desorption electrospray ionization (DESI). In the case of the MALDI imaging, tissue slices fixed in suitable matrices are irradiated by nitrogen- or infrared laser beams while the mass spectrum is recorded. SIMS is a method in which a surface is bombarded with a focused high energy ion beam resulting in sputtering. The secondary ions are collected and analyzed by MS. In both techniques the ionization beam is gradually moved across the surface of interest generating discrete mass spectra for a given location. Within the x-y plane of a sample specific m/z values are plotted and distinct colors are assigned to each distinct m/z value to create a molecular image. The presence of specific molecules in discrete regions of the sample is in this manner visualized. SIMS gives greater spatial resolution due to a more tightly focused high-energy beam. As a disadvantage, the high-energy ion beam results in extensive fragmentation of molecules sputtered from the surface. This makes identification of the original intact molecular species impossible. In contrast to this, MALDI with a lower spatial resolution due to the larger laser spot size, may generate intact molecular ions [39-41]. DESI is a more recent method. Here, ambient ionization is used requiring limited sample preparation and allowing more simple analysis [41,42]. DESI is the combination of electrospray (ESI) and desorption ionization (DI). An electrically charged mist is placed at close distance. The electrospray mist causes formation of splashed droplets carrying ionized analytes. Next, the ions move through air to the atmospheric pressure interface to which is connected the mass spectrometer [42]. Examples of application of these imaging techniques are investigations on brain slices by MALDI, revealing the local presence of sphingomyelin, sulfatides, and gangliosides [43]. Another example is the *in situ* detection by MALDI-FTICR of ceramides and other sphingolipids in human lung cancer tissues [44]. Brain tissue slices probed by SIMS-TOF showed the distribution of cholesterol and galactosylceramide in rat cerebellar white matter [45]. For the imaging and analysis of rat spinal cord cross sections DESI mass spectrometry has been used. Glycerophospholipids, sphingolipids (in white matter) and fatty acids (in grey matter) were detected [46]. Finally, the pioneering work regarding *in situ* detection of Gb3 by Kuchar and co-workers has to be mentioned [47]. These researchers studied the distribution of Gb3 in kidney of α-Gal A knockout and normal mice. They detected twenty isoforms of five sphingolipid groups (including Gb3 next to ceramides, ceramide monohexosides, and sphingomyelins) with MALDI mass spectrometry imaging (MSI), and compared the data with immunohistochemical (IHC) staining of Gb3. Comparable findings were made, but the resolution of spatial distribution of lipids was far better with MSI than IHC [47]. The imaging techniques hold great promise for future research on pathophysiological processes in lysosomal glycosphingolipid storage disorders.

The new LC-MS/MS methods for quantitative measurement of (lyso)GSLs have meanwhile been amply used in fundamental research. They assisted studies leading to the discovery of the crucial role of acid ceramidase in the intralysosomal formation of glycosphingoid bases from accumulating glycosphingolipids (chapter 11), [10]. In this work cells were fed with isotope-encoded lyso-Gb3 and its metabolism in time was followed using LC-MS/MS. The same principle of following metabolism of fed isotope-encoded sphingolipids or sphingoid bases can be applied much more broadly. For example, preliminary work at the department of Medical Biochemistry at Leiden University indicates that both $^{13}$C-sphingosine and $^{13}$C-sphinganine when fed to cells are nicely incorporated in (glyco)sphingolipids

(see addendum I for synthesis of isotope-labeled lipids). Again LC-MS/MS is essential to follow the metabolism of fed isotope-encoded sphingosine backbones into various (glyco)lipids.

Until recently glycosphingolipids like GlcCer and phosphatidylglucoside (PtdGlc) were considered to be the sole glycolipids in mammalian cells [48]. Recently, the tissue-wide presence of a new vertebrate glycolipid was documented by us, being glucosylated cholesterol (GlcChol) [49]. Earlier the existence of GlcChol had been observed in cultured fibroblasts and gastric tissue [50-52]. To demonstrate the natural occurrence of GlcChol in mouse tissues and human plasma (chapter 14) a new LC-MS/MS method using $^{13}C_6$-labelled GlcChol as internal standard was required [49]. Indications were already obtained from *in vitro* experiments by Akiyama and co-workers that GlcChol synthesis was not taking place by UDP-glucose dependent glucosylceramide synthase but more likely transglucosylation [53,54]. Our study clarified the metabolism of GlcChol [49]. It was shown to be mediated by the β-glucosidase GBA2, a membrane bound enzyme with its catalytic pocket at the cytoplasmic membrane face. GBA2 transfers glucose from the donor GlcCer to cholesterol to yield GlcChol. This transglucosylation is reversible. GlcChol can be degraded in cells by the lysosomal β-glucosidase GBA1. However, at a high concentration of cholesterol in lysosomes even GBA1 starts to generate GlcChol via transglucosylation. Such a condition is found in Niemann Pick type c disease where the export of cholesterol from lysosomes is deficient. Consequently, GlcChol levels are clearly elevated in liver of NPC mice and modestly in plasma of NPC patients [49]. Formation of GlcChol can be induced in cultured cells by their exposure to the compound U18666A, causing lysosomal cholesterol accumulation [49]. Importantly, it was also observed that inhibition of GlcCer synthesis with Eliglustat or inhibition of GBA2 with Miglustat (N-butyldeoxynojirimycin) decreases plasma GlcChol in GD patients. The same effects of GCS and GBA2 inhibitors on GlcChol were observed with cultured cells [49]. The physiological role of GlcChol is not clear at the moment. Glucosylation of cholesterol clearly changes the physico-chemical properties of the sterol. It has been speculated that GlcChol plays a role in the protective response to heat shock [50,51]. GlcChol was reported to be rapidly induced by heat shock even before the activation of heat shock transcription factor 1 (HSF1) leading to the expression of heat shock protein 70 (HSP70). The addition of GlcChol to cultured human fibroblasts cells in turn caused HSF1 activation and HSP70 production [51]. These intriguing findings have not yet been reproduced by other researchers.

Recently it has been noted by us that not only cholesterol may serve as acceptor in transglucosylation reactions catalyzed by GBA1 and GBA2, but also diacylglycerol and potentially monoacylglycerol. These findings suggest that in particular GBA2 is well positioned to transfer glucose groups among the three major lipid classes: sphingolipids, sterols and glycerophospholipids. If this is correct, GBA2 would serve a key role in maintaining local membrane composition. The properties of cellular membranes, and their functions, are influenced by their lipid constituents. In particular the relative amounts of different lipid classes have great effects on the physical characteristics of lipid bilayers [55-57]. In view of the above, it is not so surprising that intricate interplay between sterols and sphingolipids has been proposed [58]. Transglucosylation reactions may be an important part of this interplay. Of note, the transfer of head groups among lipid classes is not new. In mammalian cells, the enzyme sphingomyelin synthase 2 (SMS2) can transfer phosphorylcholine from phosphatidylcholine (PC) to ceramide to yield SM and diacylglycerol [59,60].
Moreover, indications have been obtained from *in vitro* experiments that GBA1 can transfer glucose from a donor (4-methylumbelliferyl-β-glucoside or glucosylceramide) to retinol and 7-dehydrocholesterol. More candidate acceptors are now tested. In other words, multiple important metabolites might

undergo transglucosylation by β-glucosidases with GlcCer as donor, changing their properties in the process and potentially the related biology. The upcoming challenge is to identify these candidate glucosylated metabolites by LC-ESI-MS/MS. Finally, a non-targeted screen for candidate glucosylated metabolites has been conceived. The approach has been coined 'reverse metabolomics'. It builds on the knowledge that GBA1 tolerates a modification at the C6 of glucose in substrates [61]. This was observed for β-glucosyl configured cyclophellitol-epoxide activity-based probes with an installed fluorophore at C6 [62]. Proof of concept for this reverse metabolomics approach has already been obtained with GBA1 and 6-deoxy-6-azidoglucoside with β-linked umbelliferyl as leaving group and cholesterol as acceptor. The bioorthogonal handle will allow retrieval of the glucosylated metabolites following Staudinger ligation or click ligation, after which the metabolites can be identified using mass-spectrometry. Natural occurrence of newly discovered glucosylated metabolites can be studied by UPLC-MS/MS with synthesized isotope-encoded standards as earlier for GlcChol [49].

A completely new dimension to the catalytic versatility of GBA1 has recently become apparent. As described in chapter 15, GBA1 known to able to hydrolyze β-xyloside substrate [63], moreover can transfer the xylose group to the acceptor cholesterol: so-called transxylosylation. Remarkably, not only generating XylChol, but even $Xyl_2$Chol and $Xyl_3$Chol are generated. The other β-glucosidase GBA2 is not able to use β-xylosides as substrate in hydrolysis or transxylosylation. Transxylosylase activity has also been demonstrated in cells exposed to 4-methylumbelliferyl-β-xyloside, being promoted by parallel incubation with the agent U18666A. This *in vivo* activity is completely inhibited by the inactivation of GBA1 with an irreversible inhibitor. Importantly, evidence has been obtained by LC-ESI-MS/MS for the presence of XylChol in liver and brain of Niemann Pick type c mice. These findings point out again that there are still hitherto unknown glycosylated metabolites to be discovered and that LC-MS/MS will play a key role in this.

**Conclusions**

This thesis demonstrates that glycosphingoid bases have great potential as biomarkers of glycosphingolipidoses. Their detection with UPLC-ESI-MS/MS in combination with isotope encoded identical internal standards improves further glycosphingolipidomics. The use of the new methods allows for better knowledge on and clinical management of lysosomal storage diseases. More specific, the presented methods provide important tools for diagnosis and disease monitoring of FD, GD, MLD and NPC patients (this thesis). The methodology will also assist fundamental investigations on sphingolipid metabolism and biology. Moreover, it should find good use in drug and biomarker development for glycosphingolipidoses and disease conditions with an acquired disturbance in glycosphingolipid metabolism such as the metabolic syndrome [1,64,65]. The analytical work presented here also contributed in an essential manner to a completely new insight in the role GlcCer in cells. This glycosphingolipid is not only a structural membrane lipid and precursor of complex GSLs (such as gangliosides and globosides) [66], but it also serves as donor of glucose in transglucosylation reactions of other metabolites [49]. Through this mechanism GlcCer seems to fulfill a central role in regulation of membrane composition as well as regulation of other metabolites and their related biology. This new insight might explain why GD presents clinically not as simple monogenetic disorder, but more like a network disease involving various metabolic pathways and manifestations in different organs.

In January 1882, Philippe C.E. Gaucher described in his doctorate at the University of Paris, titled *De l'epithelioma primitif de la rate, hypertrophie idiopathique de la rate sans leucemie*, a 32-year old female patient whose spleen and liver were enlarged and showing abnormal deposits in macrophages

[67]. Soon it was appreciated that the patient represented a distinct inheritable disease entity that was subsequently named Gaucher's disease. The correct structure of the storage lipid in GD as GlcCer (glucocerebroside) was described by Aghion in his PhD dissertation titled *La Maladie de Gaucher dans l'Enfance* [68]. In 1884, Johannes Ludwig Thudichum published a monograph titled *A Treatise on the Chemical Constitution of Brain* [69]. He firstly described the existence of numerous distinct lipid molecules built upon sphingoid base backbones for which he coined the name "sphingosin" in "commemoration of the many enigmas which it has presented to the inquirer" [69, p 149]. He closed his treatise [69, p. 259-260] with the prescient conclusion: "It is probable that by the aid of chemistry many derangements of the brain and mind, which are at present obscure, will become accurately definable and amenable to precise treatment, and what is now an object of anxious empiricism will become one for the proud exercise of exact science." GSLs and related storage disorders like GD, topics of this thesis, are presently again receiving considerable interest. As research in these fields widens and deepens it seems that several lipid classes interplay with GSLs resulting in a growing recognition for the need of fundamental research into these relationships.

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