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Advanced mass spectrometry of glycosphingolipids (GSLs) takes the central stage in this thesis. Investigations focus on characterization of glycosphingolipid metabolism in health and disease with emphasis to the detection and accurate quantitation of known and so far unknown GSLs and closely regulated metabolites. Inherited defects in lysosomal degradation of GSLs, in particular the glycosphingolipidoses Gaucher disease (GD) and Fabry disease (FD), relatively common lysosomal storage disorders, are key topics of examination.

The thesis provides an introductory background on the field of research (chapter 1) and contains three different sections describing conducted experimental work. The first section (chapters 2-7) consists of studies reporting on the discovery of excessive occurrence of glycosphingoid bases in lysosomal storage diseases, the development of methods for their accurate quantitation in biological samples with UPLC-ESI-MS/MS and the use of these methods in diagnosis and disease monitoring. The great value of identical ^{13}C-encoded (glyco)sphingolipids and their bases as internal standards in mass spectrometric quantitation of these lipids in biological materials is described. The second section (chapters 8 and 9) introduces clinical aspects and challenges of GD and FD and provides examples of the practical value of lipid analyses in the GD and FD clinic. The third section (chapters 10-15) concerns the pathophysiology of lysosomal disorders in glycosphingolipid metabolism and related fundamental investigations. Evidence is put forward for the existence of adaptive metabolism of GSLs during deficiencies in regular lysosomal breakdown. Finally, newly discovered dimensions to GSL metabolism based on transglycosylation reactions are described. Enclosed are three addenda containing related work on GSLs with own contributions.

Chapter 1 introduces GSLs regarding nature and composition. Their synthesis and degradation is described. An overview is presented of inherited disorders in the regular lysosomal breakdown of GSLs resulting in lysosomal storage diseases. The known physiological roles of GSLs are addressed, including their presumed contribution to insulin resistance during obesity. In view of the own experimental work, attention is paid to the analysis of GSLs with emphasis to mass spectrometric methods.

Chapter 2 describes the discovery of globotriaosylsphingosine (lysoGb3) during the investigation of plasma of symptomatic FD patients and materials of α-galactosidase A deficient mice. Reversed-phase chromatography by HPLC allowed detection of using O-phthalaldehyde derivatized lysoGb3, a hitherto unknown lipid. Plasma lysoGb3 was found to be more than 100-fold elevated in symptomatic male FD patients with classic disease manifestations. In most symptomatic female FD heterozygotes plasma lysoGb3 was observed to be more modestly increased above normal values. No clear increases in plasma lysoGb3 were noted in cases with presumed atypical manifestation of FD. Reductions in plasma lysoGb3 were observed in FD patients receiving enzyme replacement therapy (ERT). The occurrence of massively lysoGb3 was recapitulated in tissues of α-galactosidase A deficient mice. It was concluded that quantitative measurements of lysoGb3 holds great promise in assisting diagnosis of FD and monitoring response to therapeutic intervention with ERT.

Chapter 3 reports the development and application of an improved method for quantitation of lysoGb3 in complex biological samples. The procedure is based on the extraction of lipid from material of choice followed by UPLC-ESI-MS/MS detection of lysoGb3 and its analogue lysoGb3-diene. For improved quantitation an identical ^{13}C encoded lysoGb3 was synthesized to be used as spiked internal

standard. The new procedure offers major improvements in sensitivity and accuracy of lysoGb3 quantitation: elevations in lysoGb3 and lyso-ene-Gb3 in FD materials can be accurately determined, even allowing reliable distinction between most classically affected FD heterozygotes and normal females. It is concluded that new method is highly suitable for use in diagnosis of FD and laboratory evaluation of patients prior and during therapy.

Chapter 4 describes the development of an improved UPLC-ESI-MS/MS method for more sensitive and accurate quantification of glucosylsphingosine (GlcSph) with identical ^{13}C isotope encoded standard. This improvement is essential for the detection of the minute amounts of GlcSph in urine. Increased levels of urinary GlcSph in untreated GD patients were found to decrease with ERT, coinciding with corrections in plasma markers of storage cells (chitotriosidase and CCL18) and plasma GlcSph. The new method also revealed the remarkable structural heterogeneity in urinary GlcSph, in analogy to observations for lysoGb3 in urine of FD patients. In contrast to the situation in plasma, GlcSph molecules with a regular sphingoid base are minor component of total GlcSph in urine. Other hydroxylated isoforms of GlcSph are prominent in urine, suggesting active modification of the sphingoid base in the kidney.

Chapter 5 reports on the quantification of sulfatides and lysosulfatides in tissues and body fluids by mass spectrometry. Sulfatides (3-O-sulfogalactosylceramide; SM4) and their base lysosulfatide (3-O-sulfogalactosylsphingosine) are increased in Metachromatic leukodystrophy (MLD), an inherited lysosomal storage disorder caused by a deficiency of arylsulfatase A. Sensitive UPLC-ESI-MS/MS procedures were developed for quantitation of sulfatides and lysosulfatides in urine and plasma from MLD patients and plasma and tissues from an MLD mouse model. It was concluded that it is unlikely that the determination of sulfatides or lysosulfatides in plasma will be useful for the clinic as biomarkers.

Chapter 6 documents a new UPLC-ESI-MS/MS procedure for quantification of sphingosine-1-phosphate (S1P). The zwitterion S1P is notoriously difficult to quantify and its plasma levels are influenced by blood collection and plasma preparation. The study compared the use of ^{13}C isotope encoded natural S1P and C17-S1P as internal standards. To investigate potential S1P abnormalities in FD, plasma and tissues identically collected from FD mice and matched control animals were analyzed. Only an increase in S1P was detected in kidney of FD mice.

Chapter 7 presents work regarding multiplex UPLC-ESI-MS/MS quantitation of major (glyco) sphingoid bases in plasma specimens. Following a single extraction, (glyco)sphingoid bases in the upper water-phase sphinganine, sphingosine, glucosylsphingosine, lactosylsphingsosine, lysoGb3 and lyso-sphingomyelin can be simultaneously quantified by mass spectrometry with a series of identical internal ^{13}C-encoded standards. The approach is presently further developed into a simultaneous method allowing quantitation of (glyco)sphingolipids in the same sample. De-acylation of (glyco) sphingolipids to their bases, either microwave-assisted or by enzymatic digestion, allows subsequent multiplex quantitation.

Chapter 8 presents a review on GD and FD, the two most common glycosphingolipidoses, as an introduction to the second section of experimental work. GD is caused by inherited glucocerebrosidase (GBA1) deficiency causing impaired degradation of glucosylceramide (GlcCer) in lysosomes; FD is due to inherited α-galactosidase A (GLA) deficiency causing impaired breakdown of globotriaosylceramide

(Gb3) in lysosomes. Whilst biochemically closely related, the clinical manifestations of GD and FD are entirely different. In GD patients tissue macrophages characteristically accumulate GlcCer, whereas in male FD patients Gb3 storage occurs in several cell types. Therefore, ERT for GD and FD use different targeting strategies. ERT of GD uses infusions with recombinant GBA1 with mannose-terminated N-linked glycans to promote selective uptake by macrophages. ERTs of FD employ recombinant GLAs containing N-linked glycans with mannose-6-phosphate recognition signals to allowing receptor-mediated uptake by all cells. The challenges in diagnosis of GD and FD also are different. GD is a recessive disease, whereas FD is transmitted X-linked. Female FD heterozygotes may also develop disease, although in attenuated form compared to male hemizygotes. GD heterozygotes have an increased risk for α-synucleinopathies. Biochemical confirmation of diagnosis of carriership by enzymatic activity measurement is a challenge for GD heterozygotes and FD hemizygotes. Conclusive confirmation of carriership relies on genotyping. GD and FD diagnosis is furthermore complicated by the occurrence of mutations of unknown significance in the disease genes GBA1 and GLA, often associated with relative high residual enzyme activities. Biomarkers, circulating surrogate markers of disease, can assist diagnosis. For GD, several plasma protein biomarkers stemming from pathological tissue macrophages have been discovered such as chitotriosidase, CCL18 and sGPNMB. For FD comparable plasma protein biomarkers are lacking. Detection of lipid abnormalities as disease markers for GD and FD holds great promise. In particular clear increases in tissues, plasma and urine of glycosphingoid bases have been identified: globotriaosylsphingosine (lysoGb3) in FD and glucosylsphingosine (GlcSph) in GD. Accurate quantitation of these glycosphingoid bases with UPLC-ESI-MS/MS allows their application as reliable biomarkers in confirmation of diagnosis and monitoring of disease.

Chapter 9 presents a summary of practical applications of lipid analyses for the clinic as illustrated by a number of publications enclosed in the addendum II [ref 1-4]. In brief, relapses in plasma and urinary (lyso)Gb3 reductions in response to formation of antibodies against therapeutic enzyme (recombinant α-galactosidase A) in male Fabry disease patients were documented [1]. Increases in plasma lysoGb3 were observed in FD patients switching involuntary to agalsidase alpha or a reduced agalsidase beta dose as the result of the unfortunate shortage of agalsidase beta [2]. Beneficial reductions in plasma GlcSph, comparable to those of the biomarker chitotriosidase, were detected in GD patients receiving ERT and substrate reduction therapies (SRTs) with *Miglustat* and *Eliglustat*. Corrections with ERT and Eliglustat treatment were comparable and more pronounced than changes with Miglustat treatment [3]. Finally, corrections in tissue GlcCer and GlcSph were documented in response to gene therapy of a GD mouse model, coinciding with improvements in hematological abnormalities, splenomegaly and presence of storage cells in bone marrow [4].

Chapter 10 offers an introduction to glycosphingolipid metabolism in lysosomal diseases with emphasis to arguments for the existence of adaptive metabolism in glycosphingolipidoses like GD and FD. This theoretical concept is substantiated by experimental data in the subsequent chapters. Attention is focused in the chapter to the increasing evidence for toxicity of excessive glycosphingoid bases formed in glycosphingolipidoses. Excessive lysoGb3 has been shown to be toxic for podocytes and nociceptive neurons in sensory nerves of FD patients, offering an explanation for the characteristic albuminuria and peripheral neuropathy. Excessive GlcSph in GD patients is proposed to act as autoantigen inducing B-cell proliferation and increased risk for multiple myeloma. Discussed is also the potential toxic role of excessive GlcCer metabolism by the cytosolic β-glucosidase GBA2 during GBA1 deficiency.

Chapter 11 describes the identification of lysosomal acid ceramidase as the responsible enzyme for formation of glycosphingoid bases during a lysosomal glycosidase deficiency. Absence of acid ceramidase as in Farber disease prevents formation of GlcSph following inhibition of lysosomal GBA1. Likewise, the inhibition of the enzymatic activity of acid ceramidase prohibits formation GlcSph in cells with inactivated GBA1. In the investigation use is made of feeding cells with ^{13}C-encoded lysoGb3 to monitor *in vivo* sphingolipid metabolism, further substantiating the role of acid ceramidase in formation of glycosphingoid bases. The broad substrate specificity of acid ceramidase during intralysosomal glycosphingolipid stress is further illustrated by the observed increased formation of both GlcSph and lysoGb3 upon inactivation of GBA1. This phenomenon is speculated to underlie the slight increases in lysoGb3 in plasma of symptomatic GD patients, reaching concentrations observed for some individuals with presumed atypical FD.

Chapter 12 reports abnormalities in glycosphingolipids in mouse models of FD (α-galactosidase A-deficiency), LIMP-2 deficiency (secondary GBA1 deficiency), Krabbe disease (galactocerebrosidase deficiency) and severe and attenuated Niemann Pick type C (NPC) disease (lysosomal cholesterol accumulation and secondary GBA1-deficiency). In all cases, elevated plasma levels of glycosphingoid bases corresponding to the primary accumulating GSL were identified by UPLC-ESI-MS/MS. Investigation of plasma of NPC patients pointed out increases in plasma GlcSph which may assist biochemical confirmation of diagnosis. The study illustrates the value of accurately quantified glycosphingoid bases in monitoring disease in glycosphingolipidoses.

Chapter 13 focusses on mice deficient in LIMP-2, the transporter of newly formed GBA1 from the endoplasmic reticulum to lysosomes. Deficiency of LIMP-2 in man causes Action Myoclonus Renal Failure syndrome (AMRF). LIMP-2 mice were found to show reductions in GBA1 protein and activity in various tissues, but to a variable extent. In leukocytes of LIMP-2 deficient mice, as in AMRF patients, GBA1 activity is relatively high. It is hypothesized that this may explain the absence of macrophage pathology in AMRF that is characteristic in GD patients suffering from GBA1 deficiency in all cell types. Analyses of lipids with UPLC-ESI-MS/MS revealed that most tissues of LIMP-2 deficient mice store little GlcCer. In contrast, GlcSph levels are clearly increased, pointing to a conversion of accumulating GlcCer to GlcSph via acid ceramidase.

Chapter 14 reports on a hitherto poorly recognized glycolipid in vertebrates: glucosyl-β-cholesterol (GlcChol). An UPLC-ESI-MS/MS method using ^{13}C-encoded GlcChol as internal standard was developed to demonstrate the natural occurrence of this glycolipid in several mouse tissues and human plasma. It is demonstrated that GlcChol is not formed by the UDP-glucose dependent glucosylceramide synthase (GCS). GlcChol is generated via transglucosylation with GlcCer as glucose donor. Both cellular β-glucosidase GBA1 and GBA2 are able to (reversibly) catalyze this transglucosylation, *in vitro* and *in vivo* in cells. GBA1 is located inside lysosomes, whereas GBA2 is bound to cytoplasmic face of membranes. GlcCer is present, as sole GSL, at the cytoplasmic and luminal face of specific membranes. The metabolism of GlcChol was studied in cultured cells, revealing that at normal conditions GBA2 is responsible for formation of GlcChol and GBA1 for its degradation. Consistent with these findings, tissues of GBA2-deficient mice show severely reduced GlcChol levels, whereas glucocerebrosidase-deficient mice show increased tissue glucosylated sterol. Increased formation of GlcChol occurs in fibroblasts from NPC patients and liver of NPC mice show marked elevations in GlcChol. Exposure of cultured cells to the agent U18666A, causing lysosomal cholesterol accumulation, induces increased GlcChol formation. This is prohibited by inactivation of GBA1. In GD patients the plasma level of

GlcChol is increased. GlcCer, required as donor in transglucosylation, is provided by the action of GCS. Consequently, inhibition of GCS reduces plasma GlcChol in Eliglustat-treated GD patients. In conclusion, the investigation revealed an entirely novel metabolic function for GlcCer, being a donor of glucose in transglucosylation reactions catalyzed by β-glucosidases. A search for additional glucosylated metabolites by transglucosylation is undertaken.

Chapter 15 describes a remarkable additional activity of GBA1 resulting in further yet unknown glycolipids. It is shown that GBA1 is able to cleave fluorogenic β-xylosides. Moreover it is able to transfer xylose groups from substrate to cholesterol. This GBA1-catalyzed 'transxylosylation' in the presence of cholesterol as renders mono-xylosyl-cholesterol (XylChol) and even forming di- and tri-xylosyl-cholesterol (Xyl_2Chol and Xyl_3Chol). Generation of xylosyl-cholesterol occurs in intact cells when exposed to 4-methylumbelliferyl-β-D-xylose, inhibitable by specific inhibitors of GBA1. Again this can be promoted by exposure of cells to U18666A. The two other cellular β-glucosidases, GBA2 and GBA3, do not show β-xylosidase activity and are unable to trans-xylosylate. UPLC-ESI-MS/MS revealed the presence XylChol in liver. Further analysis of the occurrence of additional xylosylated structures generated through transxylosylation is warranted. In conclusion, additional glycosylated lipids appear to exist and mass spectrometry will play a crucial role in their identification.

Addendum I is a published report of the synthesis of glycosphingolipids with ^{13}C atoms incorporated into either the sphingosine or the palmitate moiety, or both [5]. The availability of these lipids is of great value since they assist as internal standards the accurate quantification of corresponding natural GSLs in complex biological materials such as urine, plasma and lysates of cells and tissues. Moreover, they may be used in studies on *in vivo* metabolism of lipids in different conditions, e.g. during introduced deficiency of a specific glycosidase (see chapter 12 for an example).
Addendum II contains a series of published papers highlighting the practical use of lipid analyses in the clinic [1-4]. A brief summary of these is presented in chapter 9.
Addendum III is a published paper describing the adaptive response in non-lysosomal GBA2 activity to the reduced activity of lysosomal GBA1 in mice with NPC disease [6]. It demonstrates that protection of motor neuron loss is offered by genetic reduction of GBA2 protein and specific pharmacological inhibition of GBA2 activity.

References:

1 Rombach SM, Aerts JM, Poorthuis BJ, Groener JE, Donker-Koopman W, Hendriks E, Mirzaian M, Kuiper S, Wijburg FA, Hollak CE, Linthorst GE. Long-term effect of antibodies against infused alpha-galactosidase A in Fabry disease on plasma and urinary (lyso)Gb3 reduction and treatment outcome. PLoS One. 2012;7(10):e47805.

2 Smid BE, Rombach SM, Aerts JM, Kuiper S, Mirzaian M, Overkleeft HS, Poorthuis BJ, Hollak CE, Groener JE, Linthorst GE. Consequences of a global enzyme shortage of agalsidase beta in adult Dutch Fabry patients. Orphanet J Rare Dis. 2011 Oct 31;6:69.

3 Smid BE, Ferraz MJ, Verhoek M, Mirzaian M, Wisse P, Overkleeft HS, Hollak CE, Aerts JM. Biochemical response to substrate reduction therapy versus enzyme replacement therapy in Gaucher disease type 1 patients. Orphanet J Rare Dis. 2016 Mar 24;11:28.

4 Dahl M, Doyle A, Olsson K, Månsson JE, Marques AR, Mirzaian M, Aerts JM, Ehinger M, Rothe M, Modlich U, Schambach A, Karlsson S. Lentiviral gene therapy using cellular promoters cures type 1 Gaucher disease in mice. Mol Ther. 2015 May;23(5):835-44.

5 Wisse, P., Gold, H., Mirzaian, M., Ferraz, M.J., Lutteke, G., Van Den Berg, R.J.B.H.N., Van Den Elst, H., Lugtenburg, J., Van Der Marel, G.A., Aerts, J.M.F.G., Codée, J.D.C., Overkleeft, H.S. Synthesis of a panel of carbon-13-labelled (glyco)sphingolipids (2015) European Journal of Organic Chemistry, 2015 (12), pp. 2661-2677.

6 Marques AR, Aten J, Ottenhoff R, van Roomen CP, Herrera Moro D, Claessen N, Vinueza Veloz MF, Zhou K, Lin Z, Mirzaian M, Boot RG, De Zeeuw CI, Overkleeft HS, Yildiz Y, Aerts JM. Reducing GBA2 Activity Ameliorates Neuropathology in Niemann-Pick Type C Mice. PLoS One. 2015 Aug 14;10(8):e0135889.