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# Glycosphingolipids and lysosomal storage disorders as illustrated by gaucher disease

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**Abstract**

Glycosphingolipids are important building blocks of the outer leaflet of the cell membrane. They are continuously recycled, involving fragmentation inside lysosomes by glycosidases. Inherited defects in degradation cause lysosomal glycosphingolipid storage disorders. The relatively common glycosphingolipidosis Gaucher disease is highlighted here to discuss new insights in the molecular basis and pathophysiology of glycosphingolipidoses reached by fundamental research increasingly using chemical biology tools. We discuss improvements in the detection of glycosphingolipid metabolites by mass spectrometry and review new developments in laboratory diagnosis and disease monitoring as well as therapeutic interventions.

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**Keywords**

Glycosphingolipids, Lysosome, Gaucher disease, Glucocerebrosidase, Glucosylsphingosine.

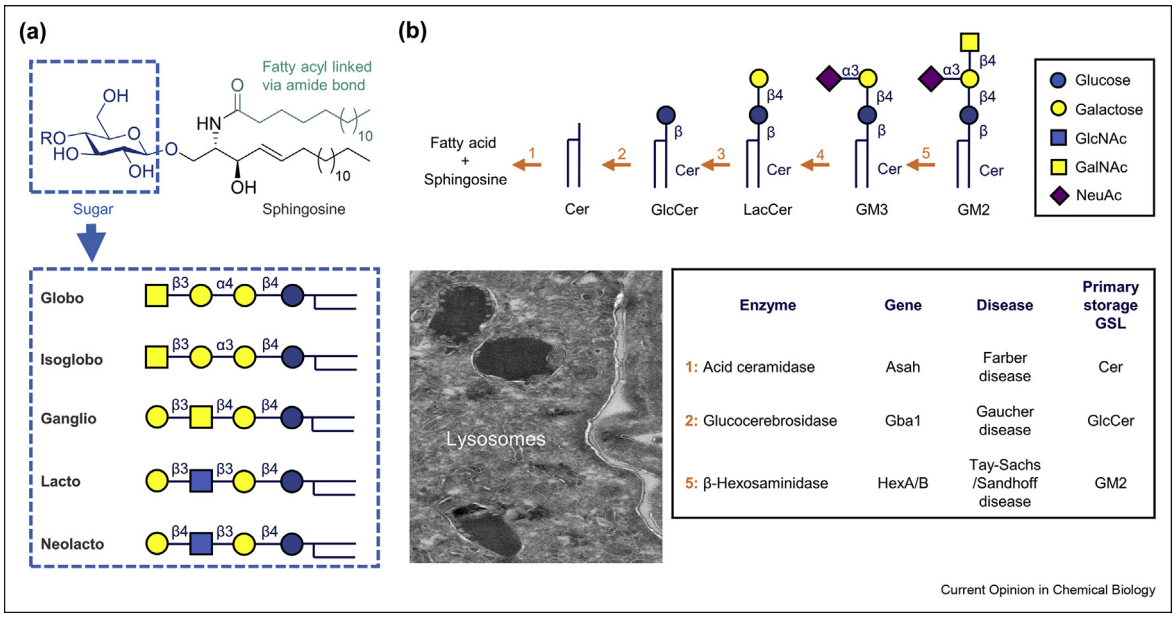
## Introduction

In 1884, Thudichum [1] described the presence of an entirely new class of lipids in the brain, now known as glycosphingolipids (GSLs) (Figure 1a). He named the unique backbone of these lipids sphingosine 'in commemoration of the many enigmas which it presented to the inquirer' [1]. The multifaced sphingolipids share the presence of sphingoid base backbones as structural feature. In vertebrates, d18 sphingosine, (2*S*,3*R*,4*E*)2aminooctadec-4-ene-1,3-diol is the major form. *N*-acylation of sphingosine renders ceramides (*N*-acylsphingosines). In the case of GSLs, a monosaccharide (glucose or galactose) is attached to ceramide (Cer). Additional sugars may be linked to glucosylceramide (GlcCer) and galactosylceramide, resulting in the major ganglioseries, globoseries, and neolactoseries of GSLs in vertebrates. The remarkable structural diversity and complex nomenclature of GSLs has been excellently reviewed by Merrill [2].

## Functions of GSLs

GSLs are primarily located in the outer leaflet of the plasma membrane of cells. GSLs also play essential roles outside cells, for example, in the noncellular layer and in myelin insulating axons of neuronal cells [3]. GSLs at the surface of cells, with their glycans exposed to the extracellular space, fulfill diverse functions (reviewed in the study by Schnaar et al.[4]). On the surface of erythrocytes, and many epithelial or endothelial cells in tissues, the glycan-type ABO blood group antigens, of prime importance in transfusion medicine, are not only expressed by membrane glycoproteins but also by GSLs [5]. Lack of specific gangliosides in the brain may cause spastic paraplegia, likely resulting from disturbed interaction of proteins in the innermost myelin membrane with specific gangliosides on axons [6]. Roles for GSLs in inflammation are observed, for example, in the E-selectin–mediated binding of leukocytes to endothelial cells before invading tissue [7]. The GSL globotriaosylceramide (Gb3, a.k.a. CD77 or P(k) blood group antigen) binds verotoxin and the human immunodeficiency virus adhesin gp120 and plays important roles in verotoxin-induced hemolytic uremic syndrome and human immunodeficiency virus infection [4,8,9]. GSLs are also implicated in regulation of T-cells [10]. For example, the major histocompatibility complex class I molecule (CD1d) of dendritic cells presents glycolipid antigens via T-cell receptor recognition to activate natural killer T (NKT) cells. NKT cells are activated by nonmammalian Gal-α-ceramide and iGb3Cer (Galα1-3Galβ1-4GlcβCer). More recently,

**Figure 1**




**Glycosphingolipids (GSLs) and some corresponding lysosomal storage disorders. (a)**. Structure of GSLs; **(b)**. Lysosomal GSL (ganglioside) degradation and corresponding diseases. Of note, inherited lactosylceramidosis (lysosomal storage of LacCer) due to β-galactosidase deficiency do not occur. This is most likely explained by the existence of two distinct lysosomal β-galactosidase encoded by the *GLB1* and *GALC* genes. Deficiency of Neu1 (neuraminidase 1) does not cause prominent GM3 accumulation in all cell types because of the fact that the ganglioside is degraded by the Neu3 and Neu4 isoenzymes [102,103]. Neu1 deficiency causes accumulation of sialylated glycopeptides and oligosaccharides [103].

excessive GlcCer has also been proposed to act as an NKT activator [11].

In the cell membrane, semiordered domains spontaneously form through interactions of GSL and cholesterol molecules. In these lipid rafts, specific proteins preferentially reside, often capable of mediating signaling events [3,12,13]. Local GSLs may influence such processes. For example, mice lacking the enzyme responsible for synthesis of the ganglioside GM3 show improved glucose tolerance and insulin sensitivity [14]. Likewise, lowering of ganglioside biosynthesis via pharmacological inhibition of the synthesis of GlcCer, the precursor of GM3, improves markedly insulin sensitivity in obese rodents [15]. A modulating role for GM3 in insulin signaling is considered [16,17], but it has also been reported that accumulation of the gangliosides GM1 and GM2 upon overexpression of the sialidase Neu3 in mice might cause impaired insulin receptor phosphorylation which leads to insulin insensitivity [18]. Along the same line, different types of T cells appear to require distinct gangliosides for their activation, a requirement that might offer therapeutic targets for specific immune diseases such as asthma [19].

## Metabolism of GSLs

The life cycle of GSL molecules involves various subcellular compartments where specific modifications occur [3]. Briefly, at the endoplasmic reticulum, the enzyme serine palmitoyltransferase generates from serine and palmitoyl-CoA the building block ketosphinganine. This is next transformed to sphinganine that becomes N-acylated by any of a set of ceramide synthases (CerS 1−6) with different acyl-CoA length preference. The dihydroceramides are converted to ceramides by the enzyme dihydroceramide desaturase 1. Then, ceramide transfer protein transfers Cer molecules to the cytosolic leaflet cis-Golgi membranes. Here, GlcCer is generated by the enzyme GlcCer synthase (; UGCT) using UDP-glucose as sugar donor [20]. Some of the GlcCer is metabolized again to Cer by the nonlysosomal glucosylceramidase (GBA2), a cytosol-faced β-glucosidase [21], but most GlcCer enters the Golgi apparatus where it is modified via stepwise addition of further sugars by glycosyltransferases, yielding various types of GSLs (Figure 1a).

In some cells, newly formed Cer already enters the lumen of the endoplasmic reticulum (ER) where it is converted to galactosylceramide. Sulfation of GSLs may occur, contributing to the vast structural diversity of GSL. After their processing in the Golgi apparatus, GSLs reach the plasma membrane to fulfill various functions. GSLs are subsequently internalized via endocytosis, becoming part of multivesicular bodies within late endosomes. Then, their degradation inside

lysosomes occurs. Likewise, exogenous GSLs such as components of phagocytosed debris and senescent cells or endocytosed lipoproteins, are degraded in lysosomes following endocytosis. Lysosomal GSL degradation implies stepwise removal of terminal sugar moieties from GSLs by sequential action of glycosidases, assisted by specific accessory proteins (GM2 activator protein and saposins A-D) [22]. The final lipid product of this fragmentation, Cer, is split by the lysosomal acid ceramidase into free fatty acid and sphingosine. After export to the cytosol, sphingosine may be reused in the salvage pathway to generate again Cer molecules. Alternatively, it is metabolized by sphingosine kinases (SK1 and SK2) to sphingosine-1-phosphate [3].

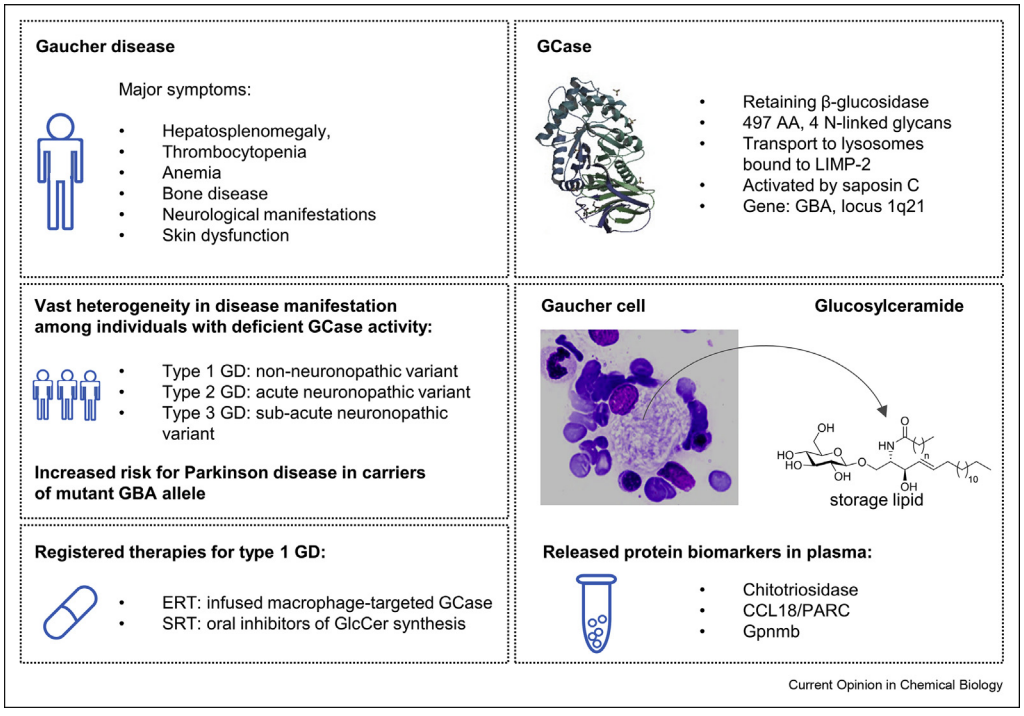
## Lysosomal GSL storage disorders: gaucher disease

Inherited defects in lysosomal enzymes fragmenting GSLs cause lysosomal GSL storage disorders (glycosphingolipidoses) [23,24]. Impaired fragmentation of a substrate in cells of patients suffering from a glycosphingolipidosis causes its ongoing accumulation, often in typical storage deposits inside lysosomes (Figure 1b). Gaucher disease (GD), a prototype lysosomal storage disease, is highlighted here to illustrate the features of glycosphingolipidoses and review new developments in diagnosis, therapy and fundamental research.

### Gaucher disease

GD is named after the clinician Ernest Gaucher who published the first case report [25] (Figure 2). GD occurs panethnic but is relatively common among Ashkenazi Jews with a birth prevalence of 1 in 800 [26]. GD patients suffer from mutations in the *GBA* gene encoding the lysosomal acid β-glucosidase (EC. 3.2.1.45), known as glucocerebrosidase (GCase) [27]. The 497 amino acid glycoprotein removes the glucose group from GlcCer, the penultimate step in lysosomal breakdown of most GSLs. Characteristically, deficiency of GCase in GD patients results in prominent GlcCer accumulation in tissue macrophages called Gaucher cells [26,28]. The clinical manifestation of GCase deficiency is very heterogeneous, ranging from lethal neonatal complications to an almost asymptomatic course. In the most prevalent manifestation of GD among Caucasians, type 1 or the non-neuronopathic variant, the major symptoms are enlargement of spleen and liver, infiltration of the bone marrow cells by storage cells, thrombocytopenia, coagulation abnormalities, anemia, and bone disease. Rarer are lung involvement, pulmonary hypertension, and renal and cardiac involvement [27]. Two further phenotypic variants are type 2 GD, the acute neuronopathic variant, and type 3 GD, consisting of subacute neuronopathic forms with neurological manifestations at later age [26]. The most severe manifestation of GD, the so-called collodion

**Figure 2**




**Gaucher disease.** Clinical features, involved lysosomal β-glucosidase, storage cell biomarkers and therapies.

baby, involves lethal skin permeability. The stratum corneum, the outermost layer of the skin, has been shown to contain relatively large amounts of GCase [29]. In recent years, it has been recognized that carriers of a mutant *GBA* gene are at increased risk, about 20-fold, for developing Parkinson disease [30].

### Causes for disease heterogeneity

Numerous mutations in the *GBA* gene have been identified in patients with GD [1]. Some are associated with a benign disease course, for example, the amino acid substitution N370S [28]. Some homozygotes for N370S *GBA* may stay virtually asymptomatic although others develop fulminant disease. Other *GBA* mutations, such as the 84GG deletion and amino acid substitution L444P, are associated with more severe disease. For example, homozygosity for L444P *GBA* results in type 3 GD, albeit with remarkable variability in severity [28]. Intriguingly, the *GBA* genotype of a GD patient predicts relatively poorly the actual clinical presentation. For several *GBA* genotypes considerable variability in disease severity is documented, even among monozygotic twins [28]. It seems likely that modifier genes, and possibly epigenetics and external factors, modify expression of symptoms. A genome-wide association study has identified the transmembrane protein CLN8 (ceroid-lipofuscinosis, neuronal 8), recycling between the ER and Golgi apparatus, as possible modifier [31]. It has recently been reported that CLN8 is involved in trafficking lysosomal enzymes between ER and Golgi apparatus [32]. Other proteins directly influence the life cycle and activity of GCase. Saposin C is the lysosomal activator protein of GCase and patients with a defective saposin C develop symptoms similar to patients with GD [33]. Newly formed enzyme does not undergo prominent phosphorylation of mannose molecules in its N-linked glycans and is transported independently of mannose-6-phosphate receptors to lysosomes [34]. The GCase transport is mediated by the lysosomal membrane protein 2 (LIMP-2) encoded by the *SCARB2* (scavenger receptor class B, 2) gene [35]. A mutation in the *SCARB2* gene has been reported to be a GD modifier [36]. Other proposed GD modifiers are polymorphisms in the gene encoding GlcCer synthase [37]. Along the same line, microRNAs have been identified that either upregulate or downregulate GCase as well as one downregulating LIMP-2. Conceivably, microRNAs could impact on GCase activity and act as GD modifiers [38].

### Gaucher cells and plasma biomarkers

The accumulation of GlcCer in cells of GD occurs almost exclusively in macrophages in the spleen, liver, bone marrow, lymph nodes, and lung [28]. These Gaucher cells have a characteristic morphology and are metabolically active, alternatively activated, macrophages [39]. Storage lesions in GD spleens consist of a core of mature Gaucher cells surrounded by proinflammatory macrophages, explaining the complexity of cytokine, chemokine, and protease abnormalities in the spleens and plasma of patients with GD [28]. For type 1 patients with GD, low-grade inflammation and low-grade activation of both coagulation and the complement cascade has been reported [40,41]. Gaucher cells overexpress and secrete proteins into the circulation and some of these are presently used as biomarkers. The first identified biomarker is chitotriosidase, named after its artificial substrate 4-methylumbelliferyl-chitotrioside [31,42,43]. The enzyme's activity is on average about 1000-fold elevated in plasma of symptomatic type 1 patients withGD. Immunohistochemistry and *in situ* hybridization have revealed that chitotriosidase is massively produced by Gaucher cells. Plasma chitotriosidase level does not reflect the total body burden of Gaucher cells. Quantification of chitotriosidase levels by enzyme assay is complicated by apparent substrate inhibition, which prohibits the use of saturating substrate concentrations. The inhibition of enzyme activity at excess substrate concentration is because of transglycosylation of substrate molecules [44]. An improved substrate, 4′-deoxy-chitobiose-4-methylumbelliferone, offers a more sensitive and convenient assay because it cannot serve as an acceptor in transglycosylation [44] (Figure 4a). Moreover, it also allows reliable measurement of activity of polymorphic G102S chitotriosidase [43]. Another very common abnormality in the *CHIT1* gene is a 24 base pair duplication that excludes synthesis of active chitinase [40]. An alternative circulating marker of Gaucher cells is the chemokine CCL18/PARC (chemokine (C—C motif) ligand 18; pulmonary and activation-regulated chemokine) [45]. Plasma specimens of untreated symptomatic type 1 patients with GD show 20-fold to 50-fold elevated levels of CCL18/PARC. Similar to chitotriosidase, the chemokine is secreted by Gaucher cells [28]. Using liquid chromatography-mass spectrometry in data-independent analysis mode proteomics, abundant proteins in laser dissected Gaucher cells from GD spleens were identified, including glycoprotein nonmetastatic melanoma protein B (gpNMB) [46]. A soluble fragment of gpNMB is found to be elevated over 50-fold in plasma of patients with type 1 GD. A recent investigation confirms the value of soluble gpNMB as plasma marker of Gaucher cells [47]. Increased gpNMB levels have also been identified in cerebrospinal fluid and brain of patients with type 3 GD [48].

### Adaptations in GlcCer metabolism during GCase deficiency

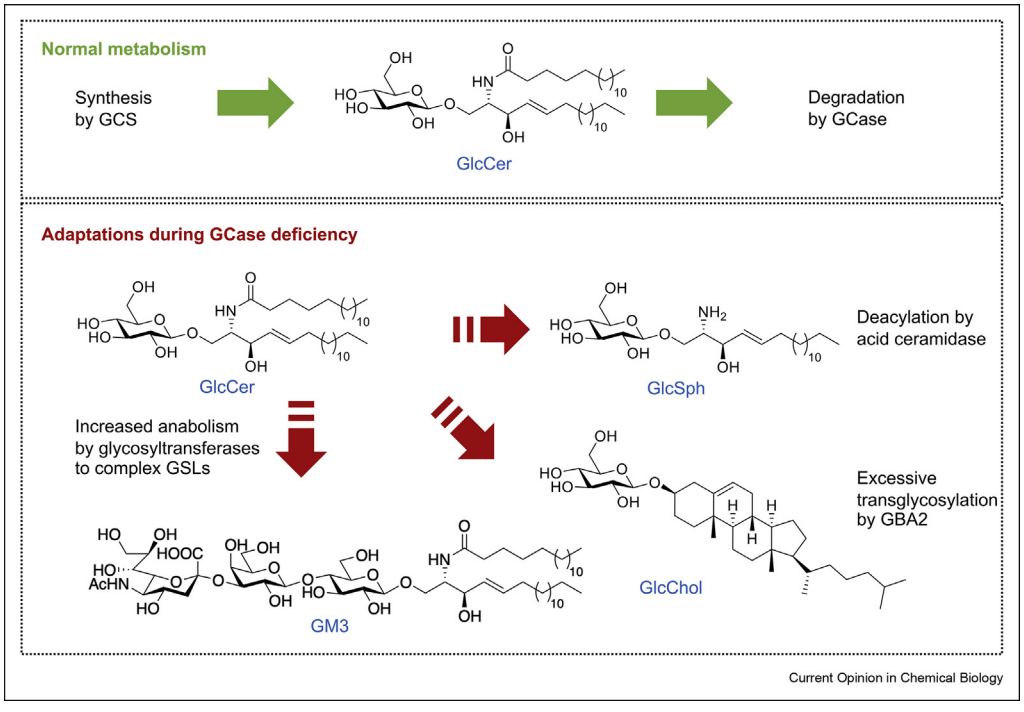
Important metabolic adaptations occur during GCase deficiency (Figure 3). First, increased anabolism of GlcCer to gangliosides takes place as indicated by the increased ganglioside GM3 in plasma and spleen of patients with GD [49]. This adaptation appears

physiologically relevant; elevated concentrations of GM3 contribute to insulin resistance and patients with GD may show insulin insensitivity, without overt hyperglycaemia [50].

Another adaptation involves the cytosol-faced retaining β-glucosidase GBA2. The activity of this enzyme is increased during GCase deficiency, resulting in increased formation of the proapoptotic Cer from GlcCer. Reducing GBA2 activity, genetically or by means of small compound inhibitors, has remarkable beneficial effects in Niemann-Pick type C (NPC) mice with a defect in the lysosomal protein NPC1 mediating efflux of cholesterol from lysosomes [51]. Daily oral treatment of NPC mice with as little as 25 μg of the nanomolar GBA2 inhibitor *N*-adamantanemethyloxypentyl-1-deoxynojirimycin ameliorates the neuropathic course of disease and prolongs lifespan significantly [51,52]. A comparable neuroprotective effect of the iminosugar *N*-adamantanemethyloxypentyl-1-deoxynojirimycin has also been reported for mice with Sandhoff disease, another neuropathic glycosphingolipidosis [53]. Recent investigations have revealed that GBA2 acts as transglycosylase and can efficiently transfer glucose from GlcCer to cholesterol, generating glycosyl-β-cholesterol (GlcChol) in the process [54]. The possible pathogenic effect of excessive glucosylated metabolites warrants further investigation. Of note, *N*-butyldeoxynojirimycin (miglustat), a very potent GBA2 inhibitor, exerts positive effects in patients with NPC and is registered as therapeutic agent for this condition.

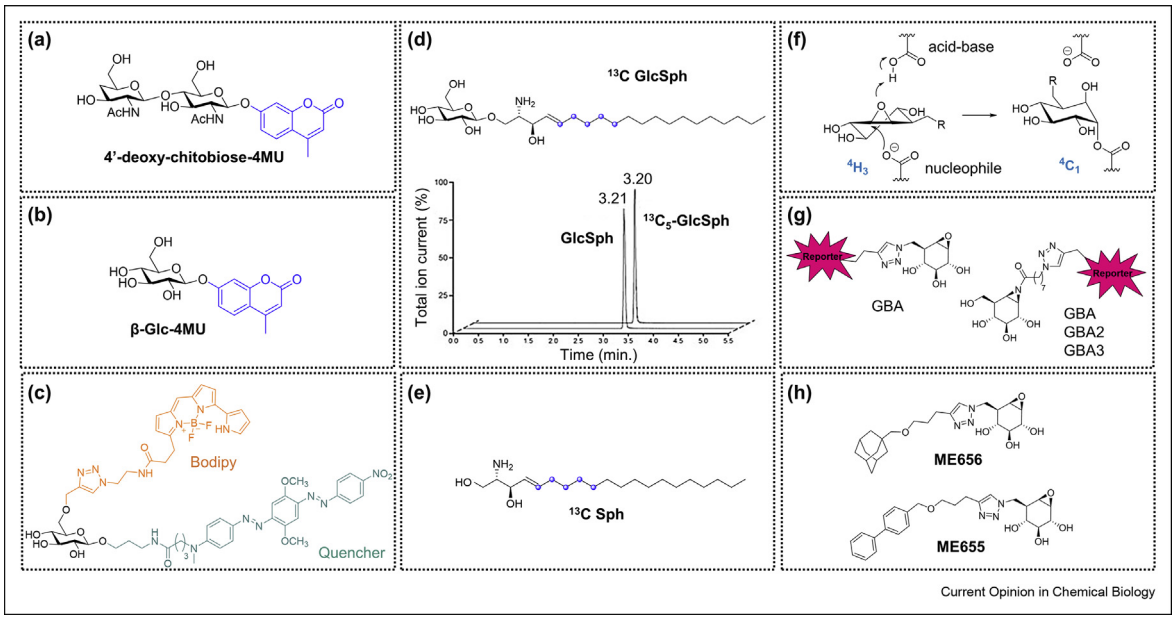
An important third adaptation during GCase deficiency deserves notice. We demonstrated that accumulating GlcCer in lysosomes is actively converted by lysosomal acid ceramidase to its sphingoid base, glucosylsphingosine (GlcSph) [55]. GlcSph is sometimes also referred to as lyso-GL1 or lyso-GB1. Elevated levels of GlcSph in the brain and spleen of patients with GD were earlier observed [23,25]. The quantitation of GlcSph in biological samples was significantly improved by *ortho*-phthaldialdehyde derivatization and high performance liquid chromatography [56]. Further improvement was reached by the development of a liquid chromatography–tandem mass spectrometry method using an identical (13)C-encoded GlcSph standard (Figure 4d). With sensitive methods in place, we detected an average 200-fold increases in GlcSph level in the plasma of symptomatic type 1 patients with GD [57]. A clear increase in GlcSph isoforms is also detectable in urine of patients with GD [58]. Pharmacological inhibition of GCase in cultured cells as and in zebrafish results in a rapid increase in GlcSph [59]. Within a few years after the discovery, the

**Figure 3**




**GlcCer metabolism: normal and during GCase deficiency.** Lysosomal degradation of GlcCer by GCase in cells of healthy individual (top). Adaptations in GlcCer metabolism during GCase deficiency as in GD (bottom). GD, Gaucher disease

**Figure 4**




**Research tools to study Gaucher disease.** (**a**). Improved fluorogenic substrate for chitotriosidase activity measurement (4′-deoxy-chitobiose-4MU = 4′-deoxy-chitobiose-4-methylumbelliferone).; (**b**). Fluorogenic substrate for measuring GCase activity *in vitro*; (**c**). Fluorescence-quenched substrate for measuring GCase activity *in vivo*; (**d**). LC-MS/MS quantitation of GlcSph with isotope-encoded standard; (**e**). Isotope-containing sphingosine as a precursor for monitoring *in vivo* GSL metabolism; (**f**). Reaction mechanism of covalent binding of ABP to catalytic nucleophile; (**g**). Activity-based probes; (**h**). Specific inhibitors of GCase *in vivo*. ABP, activity-based probes; GSL, glycosphingolipids; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

measurement of elevated plasma GlcSph has come into use for confirmation of GD diagnosis.

## Pathophysiology of gaucher disease

There is compelling evidence for a direct role of Gaucher cells in GD pathology. Their presence in spleen, liver, and bone marrow is associated with splenomegaly, hepatomegaly, and hematological abnormalities, respectively [23,25]. The same is observed in a GD mouse model with induced GCase deficiency in white blood cells [60]. Excessive GlcSph is considered to be pathogenic in patients with GD. It is thought to contribute to the common osteopenia (reduced bone mineral density) in patients with GD by impairing osteoblasts [61]. More recently, it has been reported to promote α-synuclein (α-syn) aggregation, a hallmark of Parkinson disease [62]. In addition, evidence has been presented for a role of GlcSph in the common gammopathies in patients with GD that can evolve into multiple myeloma, a relatively common blood cancer in patients with GD [63]. Antigenicity of GlcCer and GlcSph has been postulated to also lead to complement cascade activation promoting local tissue inflammation and destruction [64]. The diminished cerebral microvascular density in a neuronopathic GD mouse has been attributed to GlcSph based on the observed ability of the sphingoid base to interfere with endothelial cytokinesis *in vitro* [65]. Earlier *in vitro* experiments have suggested that GlcSph might cause lysis of red blood cells, impair cell fission during cytokinesis, damage specific neurons, interfere with growth, and promote inflammation via activation of phospholipase A2 [25]. These findings are in line with signs and symptoms in patients with GD such as occurrence of hemolysis, multinucleated macrophages, neuropathology, growth retardation, and chronic low-grade inflammation. At present, the impact of excessive glucosylated metabolites, such as GlcChol, generated by GBA2 activity during GCase deficiency is unknown. It is conceivable that further glucosylated compounds exist, are abnormal in patients with GD and contribute to specific signs and symptoms.

## Therapies of gaucher disease

The crucial role of lipid-laden macrophages in GD pathology has prompted the development of enzyme replacement therapy (ERT), an approach in which patient's macrophages are supplemented with the lacking enzyme by repeated intravenous infusion [66]. To ensure the desired targeting GCase, initially of placental source and nowadays recombinant, the therapeutic has N-linked glycans with terminal mannose groups to favor uptake via the mannose receptor (or another mannose-accepting lectin) present at the

surface of tissue macrophages. Two weekly ERT of type 1 patients with GD reverses hepatosplenomegaly and corrects hematological abnormalities [66]. Moreover, it ameliorates bone disease and reduces storage cells in the bone marrow as can be visualized with noninvasive magnetic resonance imaging techniques [67]. Unfortunately, ERT does not prevent neurological symptoms because of the inability of the enzyme to pass the blood–brain barrier.

An alternative registered treatment of type 1 GD is substrate reduction therapy (SRT) [68,69]. SRT attempts to balance the synthesis of GlcCer with the diminished capacity of patients with GD to degrade it. In SRT, orally available inhibitors of GlcCer synthase are used. Two drugs (miglustat and eliglustat) are approved for treatment of type 1 patients with GD. The more potent and specific eliglustat causes improvements on a par with ERT but fails to penetrate the brain effectively [70]. The design of brain-permeable inhibitors of GlcCer synthase is actively pursued by industry and academic researchers [71].

The response to treatment of patients with GD is primarily monitored by clinical assessments. However, plasma markers of Gaucher cells (chitotriosidase, CCL18 and GlcSph) are regularly assessed in patients with GD at the major GD clinics. A retrospective evaluation of the outcome of ERT revealed that reductions in plasma chitotriosidase during therapy correlate with corrections in liver and spleen volumes, improvements in hemoglobin, platelet count, and bone marrow composition [72].

Based on the positive outcome of bone marrow transplantation in type 1 patients with GD, genetic modification of hematopoietic stem cells has been, and still is, seriously considered as therapeutic avenue [60]. Various novel treatment options are presently researched for the neuronopathic GD variants with unmet clinical need. Among the drugs considered are so-called chemical chaperones, small compounds interacting with the enzyme that should chaperone the folding of (mutant) GCase in the ER, resulting in increased transport of enzyme to the lysosome. Clinical studies with chaperones have unfortunately not been successful for GD. Worth mentioning are current studies with ambroxol, a weak inhibitor of GCase [73], that also inhibits GBA2 (Aerts, unpublished observations). Impressive reductions in spleen and liver volumes of ambroxol-treated type 1 patients with GD have been documented and improvements in type 3 patients with GD [74,75]. Another compound of interest is arimoclomol, a heat shock protein amplifier. It has been found to improve refolding, maturation, and lysosomal activity of GCase in GD fibroblasts and neuronal cells [76].

## New research and diagnostic tools for gaucher disease

### Enzyme activity measurements

The measurement of GCase activity is of obvious interest in relation to GDand more recently Parkinson disease. Commonly used as substrate to measure GCase activity in cell and tissue extracts is the artificial fluorogenic substrate 4-methylumbelliferyl-βglucoside (Figure 4b). Used substrates for the measurement of GCase activity in cultured cells are fluorescein-diglucoside substrate or C12-NBD (nitrobenzoxadiazole)-GlcCer, but such assays suffer major technical limitations [25]. A breakthrough was recently accomplished by Yadav et al.[77] and Ashmus et al. [78] who designed a fluorescence-quenched substrate for GCase that allows convenient time-dependent monitoring of enzyme activity within cells and localization of activity within lysosomes (Figure 4c).

(13)C-encoded sphingosine [79] offers the possibility to analyze *in vivo* GSL metabolism in cells and small organisms such as zebrafish embryos (Figure 4e). The lipid is rapidly taken up and converted to GlcCer and subsequently further metabolized. The availability of specific inhibitors for key enzymes allows the assessment of their contribution to the metabolism of isotope lipids as followed with liquid chromatography–tandem mass spectrometry.

### Activity-based probes and highly selective GCase suicide inhibitors

A new research toolbox for GCase are fluorescent activity–based probes (ABPs) (Figure 4g). Briefly, cyclophellitol is a known potent suicide inhibitor of GCase (Figure 4f). By attaching a hydrophobic tag at C8 (cyclophellitol numbering, the primary carbon corresponding to C6 in glucose) of cyclophellitol, a more potent and highly specific suicide inhibitor of GCase was generated [80]. As tags commonly (boron-dipyrromethene)-red, (boron-dipyrromethene)-green or Cy5 (2-[5-[1-[6-[(2,5-dioxo-1-pyrrolidinyl)oxy]-6-oxohexyl]-1,3-dihydro-3,3-dimethyl-5-sulfo-2H-indol-2-ylidene]-1,3-pentadien-1-yl]-1-ethyl-3,3-dimethyl-5-sulfo-3H-indolium) are used. The fluorescent cyclophellitol-type ABPs covalently bind to the nucleophile E340 of GCase. These ABPs can conveniently visualize active GCase molecules for diagnostic purposes and to study its subcellular localization [80]. The amphiphilic ABPs cross membranes and allow *in situ* labeling of active GCase molecules in cells, tissues, and entire zebrafish larvae [81]. The probes are actively removed from the brain by permeability glycoproteins. However, the direct intracerebroventricular administration of the ABP allows visualization of active GCase molecules in neurons, microglia, and astrocytes [82]. Labeling of active GCase molecules in the brain offers an exciting

new research tool for neurobiologists in view of the link between GCase and risk for Parkinson disease. Another application for the ABPs is in monitoring the fate of therapeutic enzyme that can be prelabelled with an ABP of choice. Using correlative light electron microscopy and labeling of endogenous GCase and therapeutic enzyme with distinctly fluorescent ABPs, the remarkable efficient delivery of therapeutic enzymes into individual endogenous GCase-containing lysosomes of fibroblasts expressing mannose-receptor could recently be demonstrated [83]. A second class of GCase ABPs exists of cyclophellitol-β-aziridine structures, again tagged with a fluorophore [84]. These broad-specific ABPs covalently bind to nucleophiles in several β-glucosidases, including GCase, the enzymes GBA2 and GBA3 (cytosolic β-glucosidase) and the β-glucosidase pocket III of lactase/phlorizin hydrolase [85].

Building on the scaffold of the ABPs, and crystallographic findings, novel entirely specific GCase suicide inhibitors have been designed (Figure 4h) [59]. One of these, adamantyl-tagged cyclophellitol (ME656), penetrates cells and brain of zebrafish, inactivating swiftly GCase. The novel inhibitor, and its biphenyl analog, is of great value as a chemical tool to look into consequences of GCase deficiency that is generated on demand. The compound conduritol B-epoxide (CBE), another suicide inhibitor of GCase covalently binding to its catalytic nucleophile, has earlier been used to inactivate GCase and generate GDmodels in mice [86,87]. CBE is not entirely specific for GCase and inhibits with lower affinity also lysosomal α-glucosidase and nonlysosomal β-glucosidase GBA2. Earlier generated ABPs labeling relevant retaining glycosidases have been used to establish *in situ* target engagement of CBE administered to cells and mice [80]. By measuring the *in situ* competition of CBE of the catalytic nucleophile labeling of glycosidases by ABPs, it was demonstrated that the dosing of CBE used to generate Gaucher mice is not accompanied by off-target inactivation of the retaining glycosidases GBA2, α-glucosidase, or β-glucuronidase [81]. Another attractive application for ABPs can be found in the high-throughput screening of compound libraries for inhibitors of a retaining glycosidase for which an ABP is available. With this approach, compound libraries were successfully screened for inhibitors of the enzyme GBA2, resulting in discovery of high-affinity inhibiting agents [88]. Yet another application of ABPs is found in protein identification. Tagging the cyclophellitol warhead with a biotin moiety allows the convenient enrichment of labeled proteins with streptavidin beads that can be followed by protein identification via proteomics [84].

In conclusion, the availability of cell-permeable fluorescent ABPs for β-glucosidases allows unprecedented visualization of active enzyme molecules at the cellular level. In addition, it allows identification of compounds that interact *in situ* with the catalytic pockets of these enzymes in intact cells and organisms.

## Similarities with other lysosomal GSL storage disorders

Albeit clinically distinct diseases, the glycosphingolipidoses show some biochemical similarities. Accumulation of primary storage lipid in lysosomes is accompanied by secondary abnormalities in lysosomes and subsequent induction of lysosome biogenesis. A uniform reaction seems the conversion of the accumulating GSL to its corresponding sphingoid base. In Fabry disease (α-galactosidase deficiency), Krabbe disease (galactocerebrosidase deficiency), GM2 gangliosidosis (β-hexosaminidase deficiency), and Niemann-Pick diseases types A and B (acid sphingomyelinase deficiency) the corresponding sphingoid bases of the accumulating substrates (lysoGb3 [globotriaosylsphingosine], galactosylsphingosine, lysoGM2 and lysoSM [1-phosphocholine-sphingosine], respectively) are formed and their plasma levels are markedly increased, offering diagnostic possibilities [25,89]. The availability of (isotope-encoded) standards of the various sphingoid bases allows multiplex assays for various glycosphingolipidoses [90].

The noted toxic effect by GlcSph in individuals with a GCase deficiency might not be unique. It has already been reported that the elevated sphingoid base in Fabry disease, lysoGb3, is toxic to podocytes and nociceptive neurons and thus might contribute to the peripheral pain and renal complications in patients with Fabry disease [91,92]. Galactosylsphingosine is also considered to be a neurotoxic agent in Krabbe disease [25,93].

At present, only the transglycosylation activities of GCase and GBA2 have been carefully investigated. Other retaining glycosidases involved in glycosphingolipidoses *a priori* might show similar activities and generate unforeseen glycosylated metabolites.

## Future challenges

The vast structural diversity of GSLs in cells, tissues, and bodily fluids poses an analytical challenge. This should become more manageable by increasing availability of appropriate standards and advanced lipidomics. The thorough identification and quantification of GSLs in health and disease is essential to reach a deeper understanding of the mysterious lipids first encountered by Thudichum. The recent discovery of novel compounds interlinked with specific GSLs, such as glucosylated metabolites stemming from transglycosylation with GlcCer as glucose donor, warrants further research. How many of such structures occur and what is their (patho)physiological role? Noninvasive methods to

visualize in the human body key enzymes in GSL metabolism like GCase are of great interest. Appealing in this respect is the approach by Phenix et al. [94] to tag therapeutic GCase with an (18)F-labeled substrate analog that becomes trapped within the active site of the enzyme. Using micro−positron emission tomography, the tissue distribution of injected enzyme could be imaged in mice.

The recently recognized association between Parkinson disease, the most prevalent motor disease and second most common neurodegenerative disorder, and genetic defects in lysosomal enzymes and proteins participating in lysosomal catabolism warrants attention [95]. A chronic disturbance in the lysosomal apparatus causes accumulation of undigested macromolecules and concomitantly impacts on autophagy and exosome exocytosis. The long-lived neuronal cells seem particularly sensitive for such disturbances and neurodegeneration is a common feature among lysosomal storage diseases. The association between abnormal *GBA* alleles and Parkinson disease is intriguing [96]. There exists an intimate relationship between α-syn and GCase, forming a bidirectional loop in synucleinopathies [97]. It has been observed that GlcCer promotes harmful aggregation of α-syn. On top of that, α-syn oligomers reduce the transport of GCase to lysosomes, causing further accumulation of GlcCer [97]. Besides loss-of-GCase function, a pathogenic gain-in-toxic function of mutant forms of GCase is considered [98]. In accordance with this hypothesis, improperly folded GCase overwhelms the folding machinery, causing ER stress and subsequent cell death [98]. A similar mechanism is observed in *Drosophila melanogaster* with a mutant GCase ortholog [99.] Of interest, mice deficient in LIMP-2, the protein transporting GCase to lysosomes, accumulate oligomeric forms of α-syn [100]. This finding indicates that deficiency of GCase in lysosomes itself negatively impacts on α-syn. Recently, a haplodeficiency of *Gba* in mice expressing human α-syn was found to result in Parkinson disease symptoms, resembling carriers of GD[101]. Interestingly, it was observed that GlcSph, the sphingoid base reported to promote α-syn aggregation [62], is increased in brain of the affected animals with haplodeficiency of *Gba* [101]. At present, therapies aiming to boost GCase activity in the brain are being developed, ranging from gene therapy to small compound interventions (section 2.6). The future will learn whether these approaches will be also of value to treat Parkinson disease.

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## Conflict of interest statement

Nothing declared.

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