Chapter 11

Lysosomal glycosphingolipid catabolism by acid ceramidase: formation of glycosphingoid bases during deficiency of glycosidases

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Lysosomal glycosphingolipid catabolism by acid ceramidase: formation of glycosphingoid bases during deficiency of glycosidases

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Glycosphingoid bases are elevated in inherited lysosomal storage disorders with deficient activity of glycosphingolipid catabolizing glycosidases. We investigated the molecular basis of the formation of glucosylsphingosine and globotriaosylsphingosine during deficiency of glucocerebrosidase (Gaucher disease) and α-galactosidase A (Fabry disease). Independent genetic and pharmacological evidence is presented pointing to an active role of acid ceramidase in both processes through deacylation of lysosomal glycosphingolipids. The potential pathophysiological relevance of elevated glycosphingoid bases generated through this alternative metabolism in patients suffering from lysosomal glycosidase defects is discussed.

Keywords: acid ceramidase; Fabry disease; Gaucher disease; globotriaosylsphingosine; glucosylsphingosine; glycosphingolipids

Highlights

- Elevated plasma glycosphingoid bases occur in glycosphingolipid storage diseases.
- Acid ceramidase actively forms glycosphingoid bases in Gaucher and Fabry disease.
- Genetic loss or inhibition of acid ceramidase prevents formation of glycosphingoid bases.
- Modest elevations in plasma globotriaosylsphingosine are not specific for Fabry disease.

The lysosomal glucocerebrosidase (GBA1), encoded by the *GBA1* gene, degrades the lipid substrate glucosylceramide (GlcCer) in lysosomes. Marked deficiency of this acid β-glucosidase is the molecular basis of Gaucher disease (GD), a clinically heterogeneous, recessively inherited lysosomal storage disorder (LSD). The hallmark of the common non-neuronopathic type 1 variant of GD (GD1) is the presence of lipid-laden macrophages (Gaucher cells) in tissues such as spleen, liver, bone marrow and lung [1]. Clinical manifestations of GD1 like sple-

Abbreviations
AC, acid ceramidase; CBE, conduritol B-epoxide; DMEM, Dulbecco's Modified Eagle's Medium; GCS, GlcCer synthase; GD, Gaucher disease; FD, Fabry disease.

nomegaly, hepatomegaly, pancytopenia, skeletal disease and pulmonary hypertension are thought to be triggered by the presence of Gaucher cells [1]. These pathological macrophages secrete large amounts of specific proteins such as chitotriosidase and CCL18 [2,3]. Detection of this chitinase and chemokine in plasma of GD1 patients is, therefore, used as biomarker to assess body burden of Gaucher cells. The glycosphingoid base (lyso-GSL) of the primary storage lipid GlcCer, glucosylsphingosine (GlcSph), is also markedly increased in plasma of symptomatic GD1 patients and its potential as a biomarker has been increasingly recognized [4–6]. Accumulation of GlcSph in GD patients was firstly documented in brain of neuronopathic patients several decades ago [7,8]. A sensitive method for accurate quantification of GlcSph in biological samples, employing LC-MS/MS and an isotope-labelled internal standard, has meanwhile been developed [5]. Recent studies suggest that plasma GlcSph levels correlate with disease severity in GD1 patients [4] and its monitoring is considered useful to evaluate disease status and efficacy of therapeutic intervention. In male Fabry patients with classic disease manifestations a reminiscent phenomenon occurs. A massively increased concentration of the deacylated form of the primary storage lipid, the globoside Gb3, is detected in plasma of these patients [9]. This lyso-GSL, globotriaosylsphingosine (lysoGb3), is about hundred fold increased in classic Fabry males [9]. In heterozygous Fabry females with milder symptoms of disease, plasma lysoGb3 is increased but far less prominently [10,11]. Likewise, in Krabbe disease, a devastating LSD caused by deficiency of galactocerebrosidase, elevated levels of galactosylsphingosine have been documented in parallel with accumulation of the primary substrate galactosylceramide [12].

The origin of the excessive plasma GlcSph in GD1 patients, or that of excessive lysoGb3 in Fabry patients, is still elusive. In the case of GlcSph, it has been initially proposed to be formed by *de novo* synthesis in which GlcCer synthase (GCS) conjugates glucose from UDP-glucose to sphingosine [13]. Similar hypothetical formation of lysoGb3 through such pathway would imply sequential conversion of GlcSph to lactosylsphingosine by β-galactosyltransferase BGalT-V or VI and subsequent formation of lysoGb3 by α-galactosyltransferase A4GalT. No evidence for these reactions, however, exists. In view of this, it might be considered that the excessive GlcSph in GD1 patients and lysoGb3 in Fabry patients stems from a simpler alternative pathway, that is, direct intralysosomal deacylation of GlcCer to GlcSph and that of Gb3 to lysoGb3.

A candidate enzyme to catalyse such alternative catabolism of GlcCer and Gb3 is acid ceramidase (AC; *N*-acylsphingosine deacylase; EC 3.5.1.23) encoded by the *ASAH1* gene. The possibility of broadened substrate specificity of AC during lysosomal lipid accumulation was investigated and experimental evidence for this is here presented. The remarkable ability of AC to initiate alternative metabolism of GSLs when regular catabolism fails is discussed in relation to its physiological relevance in patients suffering from deficiencies in lysosomal GSL degradation.

Materials and methods

Plasma collection

Samples were collected prior to therapy from symptomatic GD1 and classical FD patients referred to the Academic Medical Center in Amsterdam. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki. Plasma (EDTA) was stored at −20 °C until further use. Diagnosis of GD1 in patients was confirmed by GBA1 genotyping and demonstration of reduced GBA1 activity in leucocytes or fibroblasts. The diagnosis of classic Fabry disease (FD) was based on clinical manifestations and confirmed by demonstrating deficiency of α-galactosidase A activity in leukocytes and a mutation in the *GLA* gene.

Fabry mice

Male Fabry $Gla^{-/0}$ mice and wild-type (*wt*) littermates were generated by crossing heterozygous $Gla^{\pm}$ female mice with *wt* males. Gla^{tm1Kul} mice (stock number 003535) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mouse pups were genotyped as previously described [14]. The mice were housed at the Institute Animal Core Facility in a temperature- and humidity-controlled room with a 12-h light/dark cycle and given access to food and water *ad libitum*. All animal protocols were approved by the Institutional Animal Welfare Committee of the Academic Medical Centre Amsterdam in the Netherlands. Animals were first anaesthetized with a dose of Hypnorm (0.315 mg mL^{-1} phenyl citrate and 10 mg mL^{-1} fluanisone) and Dormicum (5 mg mL^{-1} midazolam) and then sacrificed by cervical dislocation. Bile was collected from anaesthetized animals as earlier described in detail [15].

Cell culture

Human embryonic kidney 293T (HEK293T) cells and fibroblasts obtained from healthy individuals, FD, GD, Mucolipidosis II (also known as I-cell disease) and Farber disease patients were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Calf Serum, 10 units mL^{-1} penicillin/streptomycin in a 5% CO_2 humidified incubator at 37 °C. HEK293T cells were cul-

tured in plates precoated with 0.05% Poly-L-Lysine for better attachment.

Inhibitors

GBA1 was irreversibly inhibited with 300 μM of conduritol B-epoxide (CBE) (Enzo Life sciences, Farmingdale, NY, USA) or 100 nM Inhibody Red (MDW941) (synthesized at the Department of Bio-Organic Synthesis at Leiden University as described in reference [16]). Carmofur (Sigma-Aldrich, St. Louis, MO, USA) was used for AC inhibition at the indicated concentrations. GBA2 was selectively inhibited by incubation with 10 nM AMP-DNM (synthesized at the Department of Bio-Organic Synthesis at Leiden University as described in reference [17]) and 250 nM of Eliglustat [18] (Genzyme, Cambridge, MA, USA) was used to inhibit GCS activity.

Generation of a stable AC overexpressing Farber cell line

The human acid ceramidase (ASAH1, NM_177924.3) coding sequence was amplified by PCR using the primers sense 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCATGCCGGGCCGGAGTTG-3′ and antisense 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCACCAACCTATACAAGGGTCAGGGC-3′, cloned into pDNOR-221 and subcloned in pLenti6.3/TO/V5-DEST using the Gateway® system (Invitrogen, Carlsbad, CA, USA). Correctness of all constructs was verified by sequencing. HEK293T cells were transfected with pLenti6.3-ASAH1 to produce lentiviral particles that were subsequently collect and used for infection of a Farber fibroblast cell line. Selection with blasticidin for several weeks rendered cells stably expressing the *wt* AC as determined by activity assays and western blot (data not shown).

Feeding of cells with $^{13}C_5$-GlcCer

$^{13}C_5$-GlcCer was synthesised as previously described [19]. The isotope-labelled lipid contains five ^{13}C atoms in the sphingosine moiety (see Fig. 3A). Confluent control fibroblasts were fed 1 nmol of $^{13}C_5$-labelled GlcCer per well (2 mL). Cell pellets and medium were collected at indicated time points. GBA1, GBA2 and GCS were inhibited by preincubation (2 h) with the respective inhibitors (see above) before feeding with $^{13}C_5$-GlcCer (1 nmol). Inhibitors were kept in the medium during the experiment.

Lipid measurement

Lipids were extracted as previously described by a modification of the Bligh and Dyer method [20]. Briefly, 25 μL of plasma were extracted and GSLs were determined in the lower phase by HPLC using C17-sphinganine as internal standard (Avanti Polar Lipids, Alabaster, AL, USA) [21]. Lyso-GSLs were analysed in the upper-phase by LC-ESI-MS/MS using $^{13}C_5$-LysoGb3 and $^{13}C_5$-GlcSph as internal standard [5,22]. $^{13}C_5$-sphingoid bases contain five ^{13}C atoms in the sphingosine moiety. Lipid extraction of cell homogenates was performed with 100 μL of homogenate prepared in water.

Protein concentration

The Pierce BCA Protein Assay kit (Thermo Scientific, Waltham, MA, USA) was used to determine protein. Absorbance was measured in the EL808 Ultra Microplate Reader (BioTEK instruments, Winooski, VT, USA) at 550 nm.

Statistical analysis

Values in figures are presented as mean ± SD. Data were analysed by unpaired Student's *t*-test or Mann–Whitney u-test. *P*-values < 0.05 were considered significant. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

Results

Elevated lyso-GSL in plasma and cultured fibroblasts of GD1 and FD patients

Prominent increases in GlcSph (300-fold) and lysoGb3 (200-fold) were detected in plasma of symptomatic GD1 patients and male classic FD patients (Fig. 1A, B respectively). Excessive GlcSph is also demonstrable in cultured fibroblasts of a collodion GD patient (homozygous for the recombination RecNci allele) with virtually no residual GBA1 activity (Fig. 1C). In the case of fibroblasts of GD1 and neuronopathic GD2/3 patients the elevations in GlcSph are much more modest. Likewise, fibroblasts of FD males with a classic phenotype show the most marked elevations of lysoGb3 (see Fig. 1D). In parallel, fibroblasts of the collodion GD patient clearly accumulate GlcCer (Fig. S1A). Similarly, Gb3 tends to accumulate in FD fibroblasts, again most prominently for cells obtained from patients with a classic disease presentation (Fig. S1B).

Induction of GlcSph formation by inhibition of lysosomal GBA1 and its prevention by concomitant inhibition of lysosomal AC

GlcSph formation can be induced in cultured control fibroblasts by their exposure to CBE, an irreversible GBA1 inhibitor. As shown in Fig. 2A, incubation of control fibroblasts for 5 days with CBE results in the

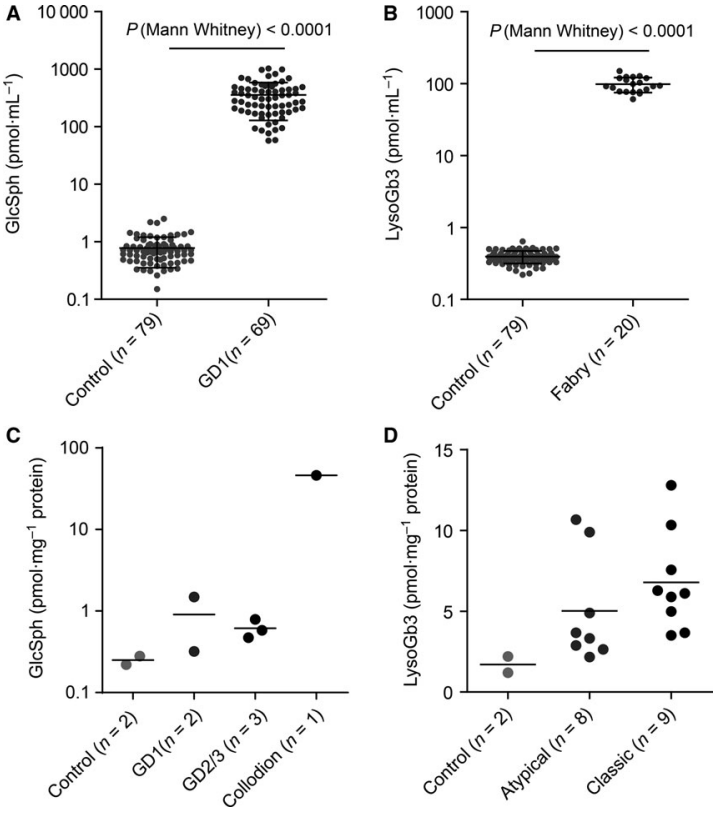


Fig. 1. GlcSph and lysoGb3 elevations in plasma and fibroblasts of Gaucher disease and Fabry disease patients respectively. (A) GlcSph (pmol mL^{-1}) in plasma of GD1 patients and healthy individuals. (B) LysoGb3 (pmol mL^{-1}) in plasma of classical Fabry patients and control individuals. (C) GlcSph (pmol mg^{-1} protein) in fibroblasts from GD1 and neuronopathic GD 2/3 patients, one collodion baby (no GBA1 activity) and healthy individuals. (D) LysoGb3 (pmol mg^{-1} protein) in fibroblasts from classic and atypical FD patients and control individuals. Data were analysed using the Mann–Whitney u-test.

prominent increase of cellular GlcSph levels. GlcCer levels are clearly elevated in these cells (Fig. S1C). The increase in GlcSph does not occur upon comparative treatment of fibroblasts obtained from a Farber disease patient, deficient in AC (Fig. 2A). Similar findings were earlier made by other investigators [23].

Carmofur is reported to irreversibly inhibit AC [24]. The inhibitory effect of Carmofur was confirmed by the demonstration of reduced AC enzymatic activity using C12-NBD ceramide as substrate (Fig. S2). Furthermore, a Carmofur-based activity-based probe has been found to result in specific labelling of AC in lysates of cultured fibroblasts [25]. We investigated the effect of the presence of Carmofur on GlcSph production in cells exposed to CBE. GlcSph was found to be reduced in cells cultured with increasing concentrations of Carmofur (Fig. 2B). Of note Mucolipidosis II (I-cell disease) fibroblasts, deficient in several lysosomal hydrolases including AC, failed to produce GlcSph upon inhibition of GBA1 (Fig. 2B).

Next, we comparatively studied the formation of lysoGb3 in α-galactosidase A-deficient fibroblasts obtained from two FD patients. LysoGb3 formation was found to be gradually reduced in Fabry fibroblasts when the cells were cultured in the presence of increasing concentrations of Carmofur (Fig. 2C). To further substantiate the role of AC in lyso-GSL formation, we transfected AC-deficient Farber fibroblasts with *wt* AC cDNA (Farber + ASAH1). A rescue of the phenotype was obtained as indicated by correction of ceramide levels (Fig. 2D). In parallel, GlcSph production was restored in the AC-transfected cells when incubated with the GBA1 inhibitor CBE (Fig. 2D).

Demonstration of deacylation of GlcCer in lysosomes

To directly demonstrate deacylation of GlcCer we made use of a newly synthesized $^{13}C_5$-GlcCer isotope labelled in its sphingosine moiety (Fig. 3A) [19]. Fibroblasts were incubated with $^{13}C_5$-GlcCer and its metabolism was followed with LC-MS/MS analysis of lipids (Fig. 3B). The $^{13}C_5$-GlcCer is rapidly metabolized to $^{13}C_5$-ceramide and next to $^{13}C_5$-sphingosine. This is

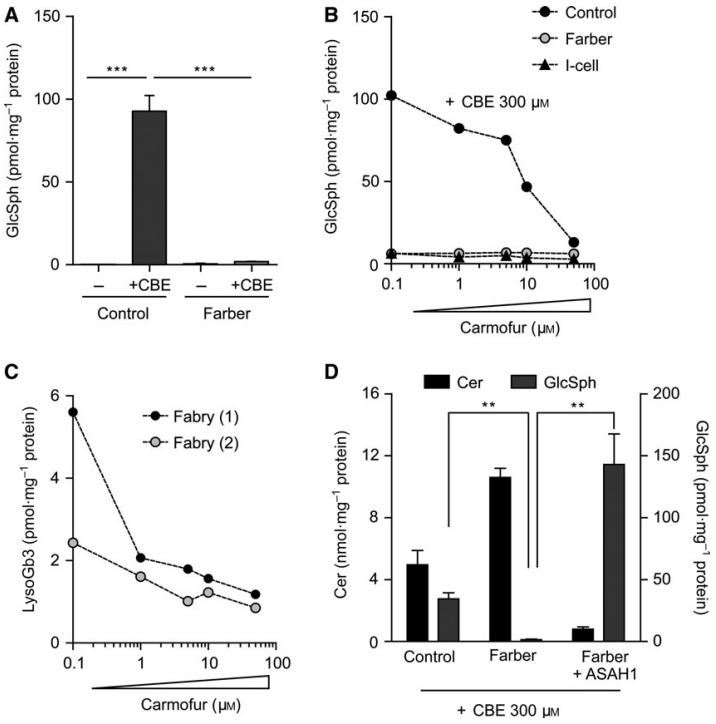


Fig. 2. Role for AC in formation of lyso-GSL in Gaucher and Fabry patient fibroblasts. (A) GlcSph (pmol mg^{-1} protein) in control and Farber disease fibroblasts cultured in the absence and presence of CBE (300 μM) inhibiting GBA1. (B) GlcSph (pmol mg^{-1} protein) in control, Farber and I cell (Mucolipidosis II) fibroblasts incubated with 300 μM CBE and in the presence of increasing concentrations of Carmofur. (C) LysoGb3 (pmol mg^{-1} protein) in two classically affected Fabry patients' fibroblasts incubated in the presence of increasing concentrations of Carmofur. (D) Ceramide (nmol mg^{-1} protein) and GlcSph (pmol mg^{-1} protein) in control, Farber and Farber fibroblasts overexpressing AC in the presence of 300 μM CBE. All incubations with inhibitors were performed for 5 days. Data were analysed using the unpaired *t*-test. $^{**}P < 0.01$ and $^{***}P < 0.001$.

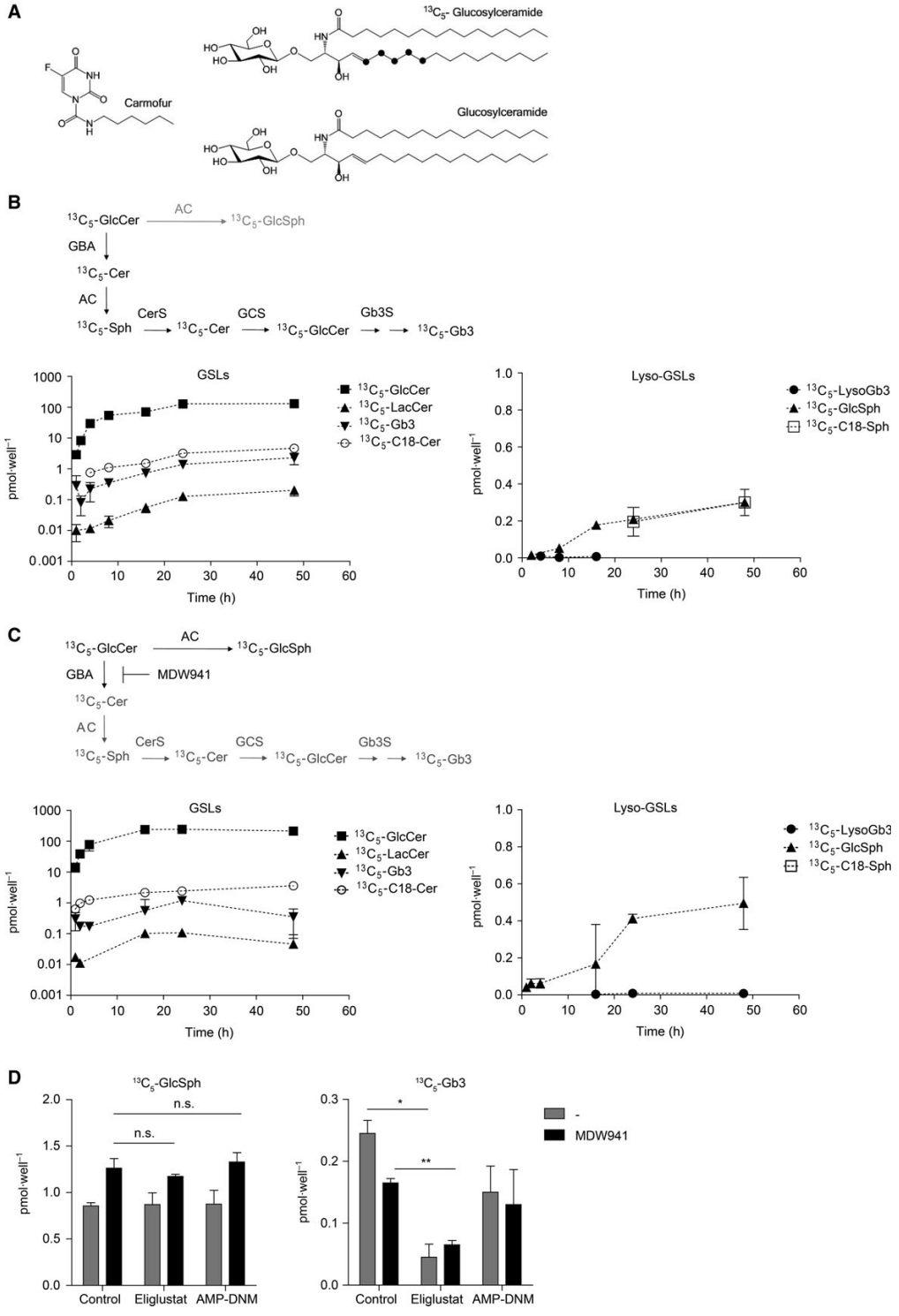
then reused in synthesis of $^{13}C_5$-ceramide and subsequently $^{13}C_5$-GSLs (Fig. 3B). When GBA1 is prior irreversibly inhibited in cells with Inhibody Red (100 nM), metabolism of $^{13}C_5$-GlcCer to $^{13}C_5$-ceramide is blocked and direct conversion into $^{13}C_5$-GlcSph is observed (Fig. 3C). This finding is consistent with intralysosomal conversion of accumulating GlcCer to GlcSph.

To exclude a role for GCS we fed, in an independent experiment, fibroblasts with $^{13}C_5$-GlcCer in the presence of the selective inhibitor Eliglustat [18]. Inhibition of GCS activity did not lead to a change in the formation of $^{13}C_5$-GlcSph (Fig. 3D, left panel). Effective inhibition of biosynthesis of GSLs by the inhibitor was demonstrated by reduced formation of $^{13}C_5$-Gb3 (Fig. 3D, right panel). Fibroblasts contain very little nonlysosomal β-glucosidase GBA2. We nevertheless checked the effect of selective inhibition of GBA2 with low nanomolar AMP-DNM [17]. No changes in the formation $^{13}C_5$-GlcSph were detected (Fig. 3D, left panel).

Occurrence of elevated lysoGb3 in plasma of GD patients

The noted broad specificity of AC in lipid-accumulating lysosomes illustrated by its ability to deacylate GlcCer as well as Gb3 prompted us to carefully examine plasma samples of GD1 patients with respect to lysoGb3 content. As shown in Fig. 4, significant increases in plasma lysoGb3 in GD1 plasma specimens were demonstrable. The concentrations of lysoGb3 in GD1 plasma coincided with those seen in female Fabry patients and even exceeded those in individuals with α-galactosidase A genetic variations of unknown

Fig. 3. Direct lysosomal formation of GlcSph from GlcCer. (A) Structure formula of Carmofur, GlcCer and $^{13}C_5$-labelled GlcCer. (B) Feeding of cells with $^{13}C_5$-GlcCer (1 nmol) and formation of $^{13}C_5$-labelled neutral GSLs and lyso-GSLs in time. (C) Formation of $^{13}C_5$-labelled neutral GSLs and lyso-GSLs in fibroblasts preincubated with 100 nM Inhibody Red (MDW941) before feeding with $^{13}C_5$-GlcCer (1 nmol). (D) $^{13}C_5$-GlcSph and GlcCer in fibroblasts preincubated with 100 nM Inhibody Red (MDW941), 250 nM Eliglustat and/or 10 nM AMP-DNM before feeding with $^{13}C_5$-GlcCer (1 nmol) for 16 h. CerS, ceramide synthase; GCS, glucosylceramide synthase; Gb3S, globotriaosylceramide synthase. Data were analysed using the unpaired *t*-test. $^{*}P < 0.05$ and $^{**}P < 0.01$.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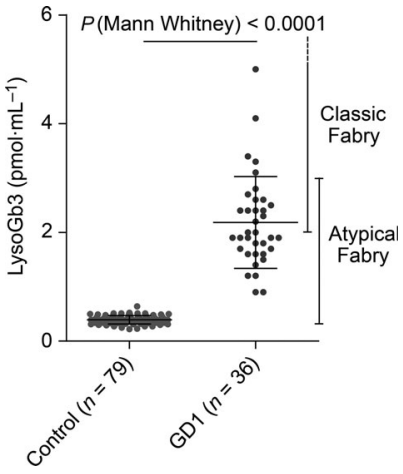

Fig. 4. LysoGb3 abnormalities in type 1 GD patients. Plasma LysoGb3 (pmol mL^{-1}) in GD1 patients and healthy individuals. Represented is the lysoGb3 interval range for atypical (0.3–3.0 pmol mL^{-1}) and classic (2–124 pmol mL^{-1}) FD patients [26]. Data were analysed using the Mann–Whitney U-test.

significance and presenting with an atypical course of FD [26].

Discussion

In the present investigation we demonstrate the crucial role of lysosomal AC in the formation of lyso-GSLs from GSLs accumulating in lysosomes as the result of glycosidase deficiency. Via AC, GlcSph is generated from accumulating GlcCer in lysosomes of cells from GD1 patients and lysoGb3 from accumulating Gb3 in FD patients. The experimental evidence for the role of AC in lyso-GSL formation is provided by two independent observations: the inhibitory effect of genetic deficiency of AC as well as that of selective inhibition by Carmofur. Direct demonstration of conversion of GlcCer to GlcSph during deficient activity of GBA1 is provided by the analysis of $^{13}C_5$-isotope-labelled GlcCer with the isotope in the sphingosine moiety. During GBA1 inhibition, $^{13}C_5$-GlcSph is formed from endocytosed $^{13}C_5$-GlcCer.

The physiological relevance of active formation of lyso-GSLs from GSLs by AC in glycosphingolipidoses deserves discussion. We noted that the amphiphilic and water-soluble sphingoid bases are released from cells (for $^{13}C_5$-GlcSph secretion see Fig. S3). This route may explain the marked elevations in plasma lysoGb3 and GlcSph in classic Fabry males and symptomatic GD1 patients respectively. Formation of lyso-GSLs might also have harmful side-effects. Sphingoid bases are known biologically active compounds that at excessive concentrations could exert negative effects (see references [27,28] for reviews). The actual risk for this is largely unclear because exact local concentrations of GlcSph and lysoGb3 in cells of GD1 patients and classic FD patients are unknown at the moment. The subcellular distribution of lyso-GSLs is also relevant in this connection. It should be kept in mind that lyso-GSLs may tend to accumulate in acidic compartments and consequently their concentration in the cytosol might be significantly less than that in the total cell. Toxic effects have been postulated for excessive galactosylsphingosine in Krabbe disease, GlcSph in GD1 and lysoGb3 in FD [27–29]. Of interest in this respect is the recent report on sensitization of peripheral nociceptive neurons by lysoGb3 at concentrations occurring in classic Fabry disease patients [30]. Earlier a correlation of plasma lysoGb3 levels and pain in FD patients has indeed been noted [31]. Theoretically, sphingoid bases might also interfere indirectly as structural mimics of sphingosine-1-phosphate, influencing processes governed by this sphingoid base and its receptors. The potential toxicity of lyso-GSLs and putative direct pathological role in glycosphingolipidoses warrants further research. On the other hand, formation of lyso-GSLs may be a blessing in disguise since it protects against generation of stressed and dysfunctional lipid-laden lysosomes. Moreover, the water solubility lyso-GSLs can offer a 'secret route' mediating the secretion from the body. Indeed, we observed more than hundred-fold increased amounts of lysoGb3 in bile of Fabry mice (*wt*: 0.83 ± 0.19; Fabry: 152.69 ± 26.13 pmol mL^{-1}).

Remarkable is the apparent switch in substrate specificity of lysosomal AC upon accumulation of GSLs. AC, like thiol proteases, uses a catalytic cysteine nucleophile (Cys-143) in its reaction mechanism [32]. The first step in catalysis is the deprotonation of the Cys-143 thiol in the active site by an adjacent histidine residue. The next step is the nucleophilic attack by the deprotonated cysteine's anionic sulphur on the sphingolipid substrate carbonyl carbon. In this step, sphingosine is released, the histidine residue in the enzyme is restored to its deprotonated form, and a thioester intermediate linking the new carboxy-terminus of the acyl to the cysteine thiol is formed. The thioester bond is subsequently hydrolysed to generate a free fatty acid, while regenerating the free enzyme. AC is synthesized as a 54-kDa protein that undergoes auto-proteolysis, after which the two generated subunits (14-kDa α-subunit and 40-kDa β-subunits starting with catalytic Cys-143) remain linked through one disulfide bridge [32]. We tested *in vitro* the activity of AC towards GlcCer at various conditions (pH range 4.0–7.0; presence of reducing

agent DTT or ascorbate). In all the examined conditions we were unable to demonstrate significant conversion of GlcCer to GlcSph. Thus, we could not recapitulate the apparent broad substrate specificity of the enzyme observed in cells with deficient GBA1. Of note, for the stratum corneum of the skin the presence of a deacylase converting sphingolipids to sphingoid bases has been reported [33,34]. It has been proposed, but not proven, that this broad enzyme activity is caused by the presence of free AC β-subunit. Limited homology of AC to conjugated bile acid hydrolase (CBAH) from *Clostridium perfringens*, with an available X-ray structure [35], has been used to generate homology structure models for AC [36,37]. These suggest that the α-subunit acts as a lid on the pocket. Speculatively, its release could indeed broaden access of GSL substrate to the catalytic β-subunit. Crystal structures of AC will be essential to further examine this possibility.

Our finding that plasma samples of GD1 patients not only show increased GlcSph but also elevated lysoGb3 warrants discussion. No data are available pointing to a prominent accumulation of Gb3 in tissues of GD1 patients. We, therefore, presume that the elevated lysoGb3 in GD patients stems from a changed substrate specificity of AC acquired during accumulation of GlcCer in lysosomes, allowing it to degrade efficiently GlcCer as well as Gb3 when entering lysosomes. Apart from the cause, the observed elevations in plasma lysoGb3 in GD1 patients are of practical importance for the FD clinic. In recent years increasing numbers of individuals with abnormalities of unknown significance in the *GLA* gene have been labelled as suffering from an atypical manifestation of FD [38–41]. Contrary to classic FD patients, these individuals do not express characteristic early signs of disease like acroparesthesia and corneal clouding, but only develop an isolated late onset symptom such as unexplained stroke, various forms of cardiomyopathy or kidney disease. These symptoms occur with relative high frequency in the general population, particularly among obese elderly individuals [42]. There is, therefore, a serious risk that based on a mere chance combination of a common *GLA* polymorphism with a common symptom like unexplained stroke, cardiomyopathy or urinary protein secretion, an incorrect conclusion is drawn and a faulty diagnosis of FD is made. In the worst scenario such *GLA* polymorphism is recorded in literature as disease-causing mutation and its carriers are subsequently erroneously labelled as future atypical Fabry patients. For an X-linked disorder as FD it implies that daughters of a male index case are viewed as obligate carriers that potentially develop disease in the future and may require preventive, extremely costly, therapeutic intervention by enzyme replacement therapy. In view of this it is important to point out that individuals with atypical FD generally show normal to only slightly increased plasma lysoGb3 levels. A threshold of 1.3 pmol mL^{-1} plasma lysoGb3 was recently proposed to distinguish atypical Fabry patients from normal individuals or those with α-galactosidase A abnormalities with unknown significance [43]. The threshold was based on a very small number of not age-matched controls ($n = 10$, each gender) and unfortunately lacked analysis of plasma from individuals with unexplained stroke, cardiomyopathy or renal disease in the presence of normal *GLA*. The strict use of the proposed threshold should in our view not be advocated since our study revealed plasma lysoGb3 levels above 1.3 pmol mL^{-1} in nearly every GD1 patient. It can presently not be excluded that multiple causes for lysosomal stress, including chronic exposure to lysomotropic drugs, may locally activate AC to generate sphingoid bases from GSLs present in the same compartment and cause modest elevations in the circulation. Modestly elevated lyso-GSLs in plasma should, therefore, be interpreted with caution in order to prevent misdiagnosis of disorders as FD.

In conclusion, the enzyme AC is involved in alternative lysosomal catabolism of GSLs during deficiencies in lysosomal glycosidase. The generated sphingoid bases in the process warrants further investigations on their potential toxic effects and role in pathophysiological processes. Furthermore, the beneficial effect of the alternative metabolic pathway catalysed by AC deserves detailed study.

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Author contributions

JMA and RGB conceived and supervised the study; PW and HSO provided reagents and new tools; MJF, ARAM, MDA, MV, AS and SS designed and performed experiments; MJF, ARAM, MM, CMO and DL analysed data; JMA, RGB, MJF and ARAM wrote and revised the manuscript. All authors have read and approved the final version of the manuscript.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:
Data S1. Supplemental method – Enzyme assay.
Fig. S1. Neutral GSLs in Gaucher, Fabry and CBE-treated fibroblasts.
Fig. S2. Inhibition of AC by Carmofur.
Fig. S3. Secretion of 13C5-GlcSph to the medium of fibroblasts.

Supporting Information

Supplemental Methods

Acid ceramidase activity assay

We followed the activity assay described by He *et al.* with slight modifications [1]. Firstly control fibroblasts were harvested in 0.25 M sucrose. The assay mixture (20 µL) consisted of 10 µL cell lysate (~50 µg of total protein) and 10 µL of 150 mM McIlvaine buffer (pH 4.5) with 300 mM sodium chloride, 0.1% bovine serum albumin (BSA), 0.2% Igepal Ca-630 and 100 µM N-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-D-*erythro*-sphingosine (C12-NBD-Cer) from Avanti Polar Lipids (Alabaster, AL, USA). The samples were incubated overnight at 37°C. Lipids were then extracted according to the Bligh and Dyer protocol [2]. Briefly, 500 µL methanol, 500 µL chloroform and 430 µL milliQ-H2O were added to the samples. The mixture was vortexed and centrifuged for 5 min at 16.100xg. The lower phase was taken to dryness at 40°C under nitrogen stream. The pellet was re-suspended in 15 µL 2:1 chloroform/methanol. 5 µL of the sample was applied on a TLC plate and developed with chloroform:methanol:ammonia (25%) 90:20:0.5 followed by detection of NBD-labelled lipid using a Typhoon Variable Mode Imager (GE Healthcare Bio-Science Corp., Piscataway, NJ, USA).

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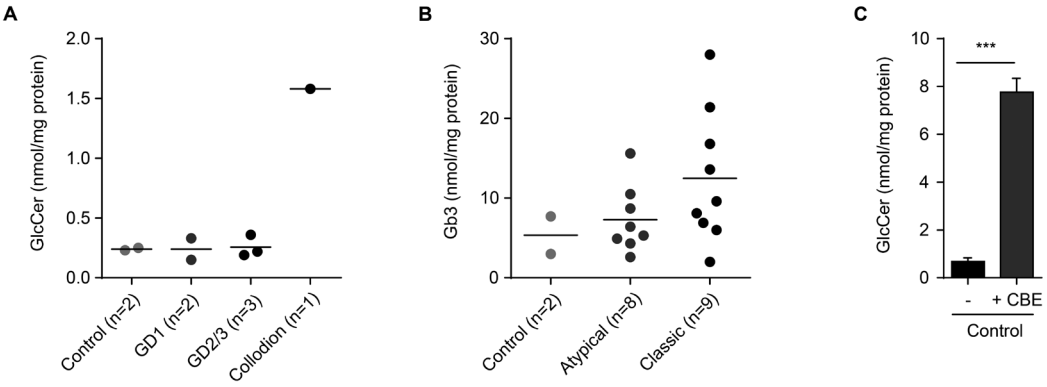
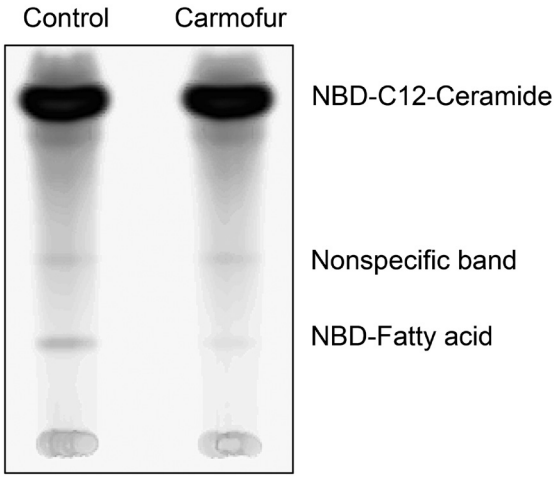
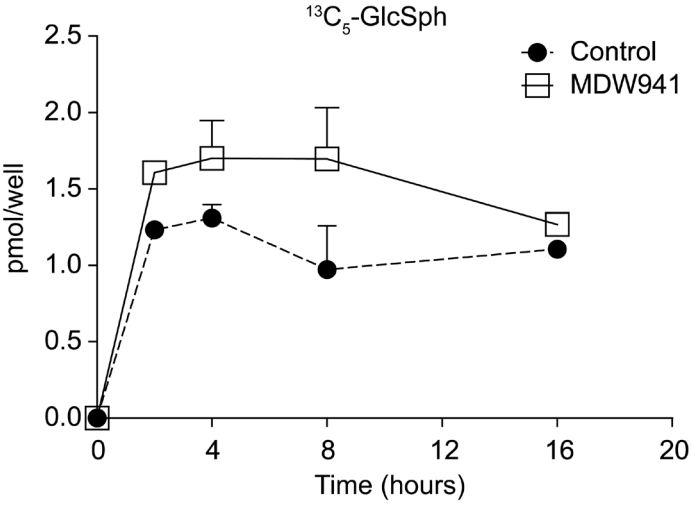


Supplemental Figure 1. Neutral GSLs in Gaucher, Fabry and CBE-treated fibroblasts.
*A. GlcCer (nmol/mg protein) in fibroblasts from GD1 and neuronopathic GD2/3 patients, one collodion baby (no GBA activity) and healthy individuals. B. Gb3 (nmol/mg protein) in fibroblasts from classic and atypical FD patients and control individuals. C. GlcCer (nmol/mg protein) in control fibroblasts in the presence of CBE (300 µM) inhibiting GBA. Data were analysed using the Mann-Whitney u-test. *** P < 0.001.*



Supplemental Figure 2. Inhibition of AC by Carmofur. *Lysates of control fibroblasts (containing AC) were incubated for 16 hours with NBD-C12-ceramide in the presence or absence of Carmofur (100 μM) to inhibit AC. Carmofur was added one hour prior to the addition of NBD-lipid.*



Supplemental Figure 3. Secretion of $^{13}C_5$-GlcSph to the medium of fibroblasts. *Cells were pre-incubated (for 2 h) with 100 nM Inhibody Red (MDW941) before feeding with $^{13}C_5$-GlcCer (1 nmol).*