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Chapter 8

Gaucher disease and Fabry disease: new markers and insights in pathophysiology for two distinct glycosphingolipidosis

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Review

Gaucher disease and Fabry disease: New markers and insights in pathophysiology for two distinct glycosphingolipidoses☆

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ABSTRACT

Gaucher disease (GD) and Fabry disease (FD) are two relatively common inherited glycosphingolipidoses caused by deficiencies in the lysosomal glycosidases glucocerebrosidase and alpha-galactosidase A, respectively. For both diseases enzyme supplementation is presently used as therapy. Cells and tissues of GD and FD patients are uniformly deficient in enzyme activity, but the two diseases markedly differ in cell types showing lysosomal accumulation of the glycosphingolipid substrates glucosylceramide and globotriaosylceramide, respectively. The clinical manifestation of Gaucher disease and Fabry disease is consequently entirely different and the response to enzyme therapy is only impressive in the case of GD patients. This review compares both glycosphingolipid storage disorders with respect to similarities and differences. Presented is an update on insights regarding pathophysiological mechanisms as well as recently available biochemical markers and diagnostic tools for both disorders. Special attention is paid to sphingoid bases of the primary storage lipids in both diseases. The value of elevated glucosylsphingosine in Gaucher disease and globotriaosylsphingosine in Fabry disease for diagnosis and monitoring of disease is discussed as well as the possible contribution of the sphingoid bases to (patho)physiology. This article is part of a Special Issue entitled New Frontiers in Sphingolipid Biology.
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Chapter 8

1. Introduction

Glycosphingolipids (GSLs), ceramides with variable oligosaccharide moieties occur in lipoproteins and ubiquitously in cells where they are prominent at the surface [1]. Their continuous renewal involves degradation in lysosomes through sequential action of glycosidases and acid ceramidase, ultimately releasing free sugars, fatty acid and sphingosine [1]. A genetic deficiency of a lysosomal hydrolase involved in glycosphingolipid turnover may result in lysosomal accumulation of the corresponding substrate [2–4]. Albeit monogenetic diseases, inherited glycosphingolipidoses are complex disorders showing remarkable variation in nature, age of onset and progression of pathological processes and subsequent clinical presentations. The interest for glycosphingolipidoses has enormously increased after the success of enzyme supplementation for Gaucher disease, an approach copied for Fabry disease as well as other lysosomal storage disease like Pompe disease, glycogen storage due to acid alpha-glucosidase deficiency, and various Mucopolysaccharidoses.

This review focuses on two glycosphingolipidoses, Gaucher disease and Fabry disease, for which enzyme therapy is registered and widely applied. These two disorders show biochemical similarities, but also differ in many aspects. Their biochemical and clinical nature is below reviewed to make visible general consequences of deficiency in lysosomal glycosphingolipid degradation and the disease-specific pathological processes and adaptations. Discussed is a new chemical approach to visualize active glycosidase molecules in situ using specific activity-based probes. Described are (newly) identified biochemical markers for both disorders. Discovery of markers is boosted by the on-going capturing and careful investigation of patients by clinical experts, the increasing availability of animal and cell models with specific enzyme deficiency generated by knockdown of gene expression or inhibitors, and the emergence of high-throughput technologies such as next generation sequencing, transcriptomics, proteomics and metabolomics. Particular attention is given to the occurrence of sphingoid bases of the primary storage lipids in Gaucher disease as well as Fabry disease. Their value in diagnosis, (prognostic) differentiation of phenotypic variants of disease and monitoring of response to therapeutic intervention is described. In addition, the potential new insights in (patho)physiology of Gaucher and Fabry diseases stemming from lipid markers are discussed.

2. Gaucher disease, a lysosomal glycosphingolipid storage disorder

The prototypical glycosphingolipidosis is the Gaucher disease (GD) [5]. This recessively inherited disorder is named after the French

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clinician Philippe C.E. Gaucher who meticulously described patient's clinical manifestations and histological abnormalities a hundred and forty years ago. Soon after the clinical symptomatology was recognized as distinct disease entity and subsequently named Gaucher disease. A century of GD research led to elucidation of accumulating glycosphingolipid, underlying deficiency in lysosomal catabolism and genetic cause.

GD is a panethnic lysosomal storage disease with estimated overall frequencies of 1–2 in 100,000 live births [5]. A higher frequency was recently detected in a newborn screening in Austria (about 1 in 17,000 births) [6]. GD is relatively common among Ashkenazi Jews with a birth prevalence of 1 in 800 [4]. All GD patients suffer from mutations in the *GBA* gene encoding the lysosomal acid beta-glucosidase (EC. 3.2.1.45), known as glucocerebrosidase (GBA) [5,10,11]. This 497 amino acid glycoprotein removes the glucose group from the simplest glycosphingolipid glucosylceramide (GlcCer), the penultimate step in lysosomal breakdown of all glucosphingolipids. Characteristically, deficiency of GBA in GD patients results in lysosomal GlcCer accumulation in tissue macrophages [5] (Fig. 1). Despite the commonality in primary defect, GD presents with remarkable heterogeneous severity, ranging from lethal neonatal complications to an almost asymptomatic course. The most prevalent manifestation, type 1 GD, does not involve pathology of the central nervous system. Major symptoms are enlargement of spleen and liver, displacement of normal bone marrow cells by storage cells, thrombocytopenia and bleeding tendency, anemia and bone disease. More rare manifestations in advanced cases are lung involvement, pulmonary hypertension, and renal and cardiac involvement [5]. Next to type 1 GD, two phenotypic variants are distinguished based on occurrence of neurological symptoms: type 2 GD, the acute neuronopathic variant manifesting with fatal neurological symptomatology in the first year of life and type 3 GD, consisting of sub-acute neuronopathic forms with neurological manifestations at later age [5]. The most severe manifestation of GD is the so-called collodion baby, born with lethal skin permeability [12]. The boundaries between the GD types are not very strict [13]. Some type 1 GD patients show neurological signs and symptoms, but these are of a totally different kind from and usually much less severe than those associated with types 2 and 3 GD [14].

2.1. Diagnostic challenges

Deficiency of GBA can be shown by determining enzymatic activity towards the artificial fluorogenic substrate 4-methylumbelliferyl-beta-glucoside in leukocytes or cultured fibroblasts [15]. However, measured residual enzyme activity is a poor predictor of disease course in GD patients [16]. Determination of residual GBA activity in cultured cells, for instance catabolism of a fluorescein-diglucoside substrate or C12-NBD-GlcCer, a closer analogue of lipid substrate, in cultured fibroblasts or macrophages does not also allow accurate prediction of future course of an individual GD patient [16]. Bone marrow biopsies and quantification of lipid-laden storage macrophages (Gaucher cells) are not useful either in this respect. Actually, this invasive approach is no longer recommended for diagnosis since pseudo-Gaucher cells closely resembling the lipid-laden macrophages of GD patients in appearance are encountered in other disease conditions [5,17].

Numerous mutations in the *GBA* gene have by now been identified in GD patients [18]. Some mutations are associated with a benign disease course, e.g. the amino acid substitution N370S [18–22]. Homozygotes for N370S GBA, the most common GD genotype among Caucasians, may sometimes stay virtually asymptomatic, while others develop fulminant disease [5]. Heteroallelic presence of N370S GBA in patients is sufficient to prevent neurological manifestations of types 2 and 3 GD. Other GBA mutations, like the 84GG deletion and amino acid substitution L444P, are associated with more severe disease [5,18]. For example, homozygosity for L444P GBA results in type 3 GD, albeit with remarkable variability in severity. Compound heterozygotes for L444P and N370S GBA develop non-neuronopathic type 1 GD, but usually with more severe presentation than N370S GBA homozygotes.

The *GBA* genotype of a GD patient predicts only partially the actual clinical presentation. For several *GBA* genotypes considerable variability in disease severity is documented, even among monozygotic twins [23,24]. It has become clear that GD is not a simple monogenetic disorder, but rather a complex disease in which modifier genes, and possibly epigenetics and external factors, strongly modify expression of symptoms [25,26]. At present relatively little is known about the latter

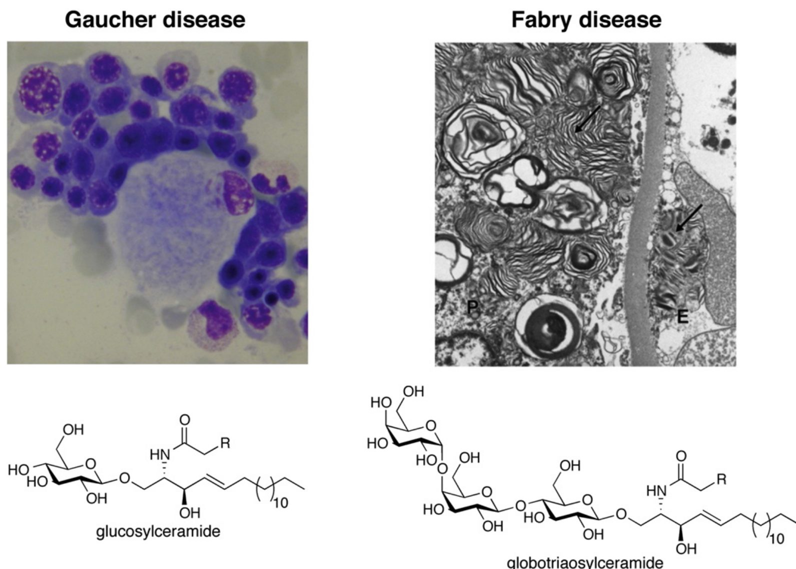

Fig. 1. Storage cells and primary storage lipids. Left: GD lipid-laden macrophage (Gaucher cell) in bone marrow aspirate, primary storage lipid glucosylceramide (GlcCer). Right: Electron micrograph of FD kidney with endothelial and podocyte storage cells (Ref. [157]), primary storage lipid globotriaosylceramide (Gb3).

aspects. Very recently a GWAS study has identified CLN8 as a possible modifier [27]. CLN8 is a transmembrane protein recycling between the ER and ER–Golgi intermediate compartment. Mutations in the CLN8 gene are associated with progressive epilepsy with mental retardation (EPMR), a subtype of neuronal ceroid lipofuscinosis. The role of CLN8 in glycosphingolipid metabolism is largely enigmatic [28]. Other proteins are known to directly influence the life cycle and activity of GBA. Newly formed GBA does not undergo phosphorylation of mannose molecules in its N-linked glycans as other lysosomal hydrolases do [29]. Consequently, the enzyme is transported independently of mannose-6-phosphate receptors to lysosomes [30]. Transport of newly formed GBA to lysosomes is mediated by the lysosomal membrane protein LIMP-2 encoded by the SCARB2 gene and even seems to involve specific vesicles [31,32]. Mutations in the SCARB2 gene underlie action myoclonus-renal failure syndrome (AMRF), a fatal disorder characterized by glomerulosclerosis, ataxia and progressive myoclonus epilepsy [33,34]. A mutation in the SCARB2 gene has been recently described as a modifier in GD [35]. In some cell types, such as fibroblasts, LIMP-2 deficiency results in almost complete absence of GBA from lysosomes. However, some tissues and blood cells of LIMP-2 deficient mice have significant residual enzyme, suggesting the existence of some cell type specific back-up transport mechanism [36]. Of note, unlike type 1 GD patients, AMRF patients develop no lipid-laden macrophages, suggesting that the residual lysosomal GBA in their macrophages is sufficient to cope with the influx of glycosphingolipids to degrade. In contrast, patients with a defect in saposin C, a lysosomal activator protein assisting GBA in degradation of GlcCer, develop disease reminiscent to GD patients [37]. Apparently, the lack of saposin C reduces lysosomal GBA activity in macrophages below the threshold required for adequate GlcCer catabolism in macrophages. Very recently, another potential modifier of Gaucher disease severity has been postulated, being polymorphisms in the gene encoding glucosylceramide synthase [37A]. It is indeed conceivable that the increased synthesis of glucosylceramide promotes lipid accumulation in individuals with an abnormal GBA genotype.

2.2. Gaucher cells and pathophysiology

In type 1 GD patients, storage of GlcCer is exclusively found in macrophages residing in the spleen, liver, bone marrow, lymph nodes and lung [5] (Fig. 1). These GlcCer-laden Gaucher cells have a characteristic morphology with an eccentric nucleus and a ‘wrinkled tissue paper’ appearance due to massive presence of tubular lipid deposits. At high concentration, the lipid GlcCer spontaneously forms bilayers, explaining the presence of the tubular storage structures in Gaucher cells as seen by electron microscopy [38]. The exclusive macrophage GlcCer storage is ascribed to their prominent role in degradation of senescent red and white blood cells rich in glycosphingolipids.

The presence of Gaucher cells in tissues underlies local pathological processes. For example, their abundance in bone marrow causes displacement of normal hematopoietic cells. In other tissues, lesions of Gaucher cells may lead to fibrosis, infarction, necrosis and scarring. Gaucher cells are not inert storage containers, but metabolically active cells, secreting various proteins [17]. Intriguingly, mature Gaucher cells resemble alternatively activated macrophages [39]. Histochemical investigation of GD spleens has revealed that storage lesions consist of a core of mature Gaucher cells surrounded by pro-inflammatory macrophages [39]. This mixture of different macrophages likely explains the observed complexity of cytokine, chemokine and protease abnormalities in the spleens and plasma of GD patients [40–44]. The increased plasma concentration of soluble CD14 and CD163 points to chronic activation of monocytes-macrophages in GD patients [45,46]. For type 1 GD, low-grade inflammation as well as low grade activation of both coagulation and the complement cascade has been reported [47,48]. Possible important roles for lipid mediators in the pathophysiology of type 1 GD are discussed below.

2.3. ERT of type 1 Gaucher disease

The realization of the crucial role of lipid-laden macrophages in pathology led Roscoe Brady to conceive a strategy for treatment of type 1 GD. He proposed targeted supplementation of macrophages with the lacking enzyme, an approach coined enzyme replacement therapy (ERT) [49]. To ensure targeting of enzyme to lysosomes of macrophages, GBA isolated from human placenta was modified in its N-linked glycans to expose terminal mannose groups. Intravenous administration of such modified enzyme results in selective uptake via the mannose receptor (or another mannose-accepting lectin) present at the surface of tissue macrophages and sinusoidal endothelial cells in the liver. Two-weekly ERT of type 1 GD patients with mannose terminated GBA has proven to be very effective in reversal of hepatosplenomegaly and correction of hematological abnormalities [50]. Moreover, it ameliorates bone disease and reduces storage cells in the bone marrow as can be visualized with non-invasive MRI techniques [51–53]. Seminal studies by Brady and colleagues in the 80s revealed that treatment interruption due to lack of enzyme, led to a relapse of anemia, indicating effective ERT of type 1 GD patients is a lifelong chronic treatment. The company Genzyme developed the modified human placental GBA to registered drug status for treatment of type 1 GD patients. Over decades, ERT has been found to be remarkably safe. The placental enzyme preparation was quickly replaced by purer recombinant human GBA produced in CHO cells [54]. From the start, ERT of type 1 GD has been very costly. Initially, enzyme from about 25,000 placentas was needed to treat a single adult patient for one year, explaining high costs. Nowadays several companies supply registered recombinant enzyme preparations for ERT of type 1 GD [55–58], but this has not led to a major price reductions yet. The availability of an effective, but very expensive chronic treatment raised considerable excitement as well as debate, culminating in 1995 in an NIH Technology Assessment Conference on Gaucher disease where an independent panel of fourteen renowned scientists from various fields formulated valid, and still appropriate, guidance [59]. They concluded: “Despite the success of enzyme therapy, treatment is limited by the cost of the agent. This makes it imperative to determine the lowest effective initial and maintenance doses to define the appropriate clinical indications for treatment and to establish uniform methods for optimal outcome assessment. The value of treatment for asymptomatic individuals has not been determined. General population screening for affected individuals and for carriers is not appropriate.” At the Academic Medical Center in Amsterdam, clinical and laboratory investigations were conducted in the 90s in the spirit of the conclusions of the NIH Technology Assessment Conference panel. As one of the first, studies were undertaken on individualized dosing regimens, an approach now being widely applied [60]. Furthermore, research focused on discovery of plasma markers of visceral Gaucher cells to be used as tools in diagnosis as well as to monitor the onset of disease, its progression and correction upon ERT at different dosing regimens [61,62]. Two plasma markers of Gaucher cells, now widely used, were discovered: the enzyme chitotriosidase and the chemokine CCL18, also known as PARC.

2.4. Plasma markers of Gaucher cells

Chitotriosidase is a chitinase named after the artificial substrate instrumental in its discovery, 4-methylumbelliferyl-chitotrioside [63,64]. The enzyme's activity is on average about 1000-fold elevated in the plasma of symptomatic type 1 GD patients [63]. More modestly elevated plasma chitotriosidase occurs in other lysosomal storage disorders and pathological conditions involving macrophages [61,65]. In type B Niemann Pick disease, where lipid-laden macrophages are prominent, plasma chitotriosidase is clearly increased [66]. Immunohistochemistry and in situ hybridization revealed that chitotriosidase is massively produced by Gaucher cells in GD patients [17]. Plasma chitotriosidase level does not reflect particular clinical symptoms, but rather total body burden of Gaucher cells. Of note, chitotriosidase was found to be rapidly

cleared from plasma by the liver in rats [67]. Due to the high first-pass liver clearance of chitotriosidase, splenic Gaucher cells might contribute relatively little to the total level of circulating enzyme. Indeed, following surgical removal of hugely (>20 times) enlarged spleens, plasma chitotriosidase was reduced by less than 40%, whereas a lung transplant in a very severely affected Gaucher patient led to a 70% reduction of plasma chitotriosidase (Aerts, unpublished observations). Plasma chitotriosidase levels generally correlate best with hepatomegaly. The striking elevation of chitotriosidase in the plasma of type 1 GD patients promotes its use to monitor body burden of Gaucher cells. There are however a number of pitfalls. Chitotriosidase shows apparent substrate inhibition, i.e. reduced generation of fluorescent 4-methylumbelliferone from the substrate 4-methylumbelliferyl-chitotrioside at increasing substrate concentrations [68]. For this reason, enzyme activity towards the 4-methylumbelliferyl-chitotrioside substrate is measured suboptimally at non-saturating substrate concentration. Cloning of the chitotriosidase (CHIT1) gene, production of recombinant enzyme and X-ray analysis of the enzyme's structure helped in elucidating its catalytic mechanism [69–71]. It was observed that chitotriosidase, like other endoglycosidases, acts as transglycosylase, explaining its exceptional kinetics [72]. To circumvent transglycosylase activity, we designed a new substrate, 4-methylumbeliferyl-4-deoxy-chitotrioside, that can't serve as an acceptor in transglycosylation [72]. With this substrate, chitotriosidase shows normal Michaelis–Menten kinetics, allowing the use of saturating substrate concentrations. Thus, a more accurate and robust assay is now available [73]. The novel substrate offers an additional advantage. A very common CHIT1 polymorphism encodes the amino acid substitution G102S in chitotriosidase [74]. Specific activities of chitotriosidase differ significantly between glycine-102 and serine-102 enzyme using 4-methylumbelliferyl-chitotrioside as substrate, but not using the improved 4-methylumbelliferyl-4-deoxy-chitotrioside [74]. The use of the novel substrate prevents underestimation of Gaucher cell burden in patients carrying the G102S CHIT1 polymorphism. Another common abnormality in the CHIT1 gene is a 24 base pair duplication that excludes synthesis of any active enzyme. In most ethnic groups, about one in three individuals is carrier for this duplication, and about one in sixteen individuals is homozygous and therefore completely lacking active chitotriosidase [75]. As the result of a gene-dosis effect, carriers of the 24 base pair duplication in the CHIT1 gene show halved plasma chitotriosidase levels.

An alternative circulating chemokine marker of Gaucher cells was next discovered [76,77]. Plasma specimens of untreated symptomatic type 1 GD patients show a 20–50 fold elevated level of CCL18/PARC [76]. Like chitotriosidase, the chemokine is secreted by Gaucher cells. The elevation of CCL18 in the plasma of GD patients is less spectacular than that of chitotriosidase and mildly affected patients may show plasma CCL18 levels close to the normal range. Regular determination of plasma chitotriosidase and/or CCL18 levels is presently performed worldwide to monitor GD patients and the data are used as additional guidance in clinical management [78,79].

Markedly elevated levels of the chemokines MIP-1α and MIP-1β have been detected in serum of symptomatic Gaucher patients [43]. Interestingly, these proteins were found to be produced by surrounding inflammatory spleen macrophages and not by mature Gaucher cells. A relation was observed between plasma MIP-1β and skeletal disease: stable high plasma MIP-1β levels during ERT correlated with on-going skeletal disease [43]. In another study, Pavlova and colleagues determined for 100 adult type 1 GD patients chemokines and cytokines by multiplex assays and related these to osteonecrosis. CCL18/PARC, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES and CXCL8/IL-8 were identified as potential biomarkers of osteonecrosis [44].

New potential markers of Gaucher cells were recently discovered by us. Using LC–MS^E proteomics [80], abundant proteins in laser dissected Gaucher cells from GD spleens were identified, including gp-NMB (to be published in detail elsewhere). A soluble fragment of this protein is found to be elevated about 500-fold elevated in the plasma of type 1 GD patients and can be employed as an additional plasma marker of Gaucher cells.

2.5. New research and diagnostic tools for Gaucher disease: activity-based labeling of GBA

In the last couple of years we have developed a new research toolbox for Gaucher disease consisting of fluorescent activity-based probes (ABPs) of which some allow specific labeling of catalytically active GBA molecules and others allow labeling of various beta-glucosidases (Fig. 2). The first category of ABPs exists of cyclophellitol beta-epoxide type suicide inhibitors of GBA, which covalently bind to the nucleophile E340 of the enzyme, with high affinity covalently [81,82]. Given their specificity for GBA molecules these ABPs have been coined Inhibodies. Several Inhibodies with differently colored fluorescent BODIPY-tags are meanwhile available. Their amphiphilic nature allows passage across membranes and in situ labeling of GBA in living cells from various species. As little as 10 nmol of intravenously administered cyclophellitol beta-epoxide ABP suffices to label all GBA in organs of a mouse within 2 h [81]. However, GBA in the brain is not labeled by i.v. administered Inhibody because its BODIPY-moiety leads to active removal from the brain by P-glycoproteins. Direct (i.c.v.) administration of the activity-based probe allows visualization of active GBA molecules in neurons, microglia and astrocytes (*to be published in detail*). Labeling of active GBA molecules in the brain offers an exciting new research tool for neurobiologists: deficiency in GBA, even carrier status, has been recognized as a significant risk factor for Parkinsonism [83,84]. The extreme specificity and sensitivity of Inhibody labeling of active GBA molecules can also be exploited for diagnostic purposes. Labeling of cultured fibroblasts derived from a type 1 GD patient, from a more severe type 3 GD patient, and from a collodion baby with the most severe GD form, reveals a downward gradient in the amount of active GBA molecules per cell [81] (Fig. 2b).

We more recently designed two other classes of ABPs. It was found that fluorescent 2-deoxy-2-fluoroglucosides with an N-phenyl trifluoroacetimidate leaving group at the anomeric center allow semi-irreversible labeling of active GBA molecules [85]. The other class of ABPs exists of cyclophellitol beta-aziridine structures, again tagged with a fluorescent BODIPY-group. These ABPs allow labeling of nucleophiles in several beta-glucosidases, including next to GBA, the enzymes GBA2 and GBA3 and the beta-glucosidase pocket III of LPH (lactase/phlorizin hydrolase) [86]. The broad specific cyclophellitol beta-aziridine ABPs are also named Anybodies. Among the human beta-glucosidases labeled by an Anybody is the enzyme GBA2, a non-lysosomal glucocerebrosidase discovered by us in the 90s [87]. A decade later Yildiz et al. and Boot et al. independently cloned the GBA2 gene [88,89].

2.6. Adaptations in GlcCer metabolism in Gaucher disease: degradation by GBA2

The enzyme GBA2 releases in the cytosol the pro-apoptotic lipid ceramide from GlcCer [90]. An excessively active GBA2, trying to compensate impaired GBA1 activity, might thus induce apoptosis. In view of this idea, we earlier speculated that GBA2 might play a role in neurological complications in patients with a severe GBA deficiency [91], either resulting from a direct defect in GBA as in GD patients or indirectly as in Niemann Pick type C (NPC) patients. For NPC, an attractive mouse model is available mimicking the course of disease in man. Daily oral treatment of NPC mice with as little as 25 μg of the nanomolar GBA2 inhibitor AMP-DNM ameliorates the neuropathic course of disease and prolongs lifespan significantly [92]. A comparable neuro-protective effect of the iminosugar AMP-DNM has also been reported for mice with Sandhoff disease, another neuropathic glycosphingolipidosis [93]. We, collaborating with Yildiz and colleagues, are currently studying the effect of introducing

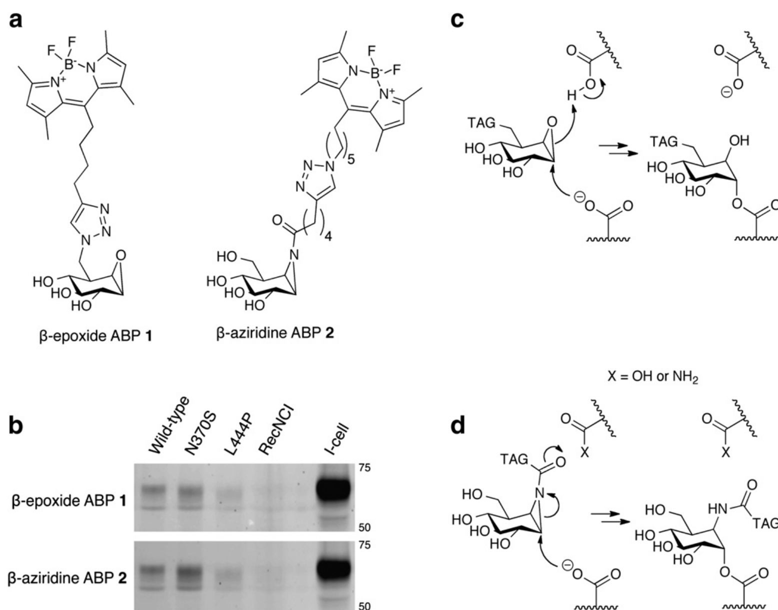

Fig. 2. Activity-based labeling of glucocerebrosidase. a. Chemical structures of cyclophellitol-epoxide and cyclophellitol-aziridine type activity-based probes. b. Labeling of GBA in comparable amount fibroblasts from normal subject (wild-type) and GD patients homozygous for N370S GBA, L444P GBA and lacking GBA protein (RecNCI). Fibroblast homogenates (equal amounts based on total protein), labeled with ABP, subjected to SDS-PAGE and labeled protein visualized by fluorescence scanning. c. Reaction mechanism of cyclophellitol-epoxide type ABP. d. Idem cyclophellitol-aziridine type ABP.

GBA2 deficiency into NPC mice to further validate GBA2 as therapeutic target. In addition, we are employing the Anybody ABP to visualize the location of active GBA2 in the brain.

2.7. Adaptations in GlcCer metabolism in Gaucher disease: increased gangliosides

In the plasma of GD patients the ganglioside GM3 is increased comparably to GlcCer [94]. GM3 is also elevated in the spleens of GD patients, suggesting increased anabolism of GlcCer to gangliosides in patient's cells. This phenomenon may be physiologically relevant, since it is known that elevated concentrations of GM3 contribute to insulin resistance [1]. Indeed, insulin insensitivity, without overt hyperglycemia, has been noted in type 1 GD patients [95-97]. It has been demonstrated that pharmacological reduction of gangliosides by inhibition of glucosylceramide synthase (GCS), the key enzyme in glycosphingolipid synthesis, improves glucose homeostasis and insulin signaling in insulin-resistant mouse and rats [98,99].

Substrate reduction therapy (SRT) of type 1 GD disease is based on inhibition of GCS activity. Several hydrophobic iminosugars inhibit GCS [100–102]. One of them, N-butyldeoxynojirimycin (Zavesca, Actelion), is already a registered drug for oral treatment of Gaucher disease and shown to reverse organomegaly and hematological symptoms in mildly affected type 1 GD patients [103–108]. More potent and specific iminosugar-type inhibitors of GCS, like AMP-DNM (N-(5-adamantane-1-yl-methoxy-pentyl)-deoxynojirimycin) [109], have been designed, as well as ceramide-analogues inhibiting the enzyme [110]. One of the latter compounds (Eliglustat, Sanofi-Genzyme) is already extensively studied in clinical trials in type 1 GD patients and reported to give beneficial responses, similar to ERT [111,112].

2.8. Adaptations in GlcCer metabolism in Gaucher disease: generation of glucosylsphingosine

Another noteworthy glycosphingolipid abnormality in type 1 GD patients concerns the deacylated form of GlcCer, known as glucosylsphingosine (GlcSph) (Fig. 3). Elevated levels of GlcSph in the brain and spleen of GD patients have been documented by several researchers [113–115]. After developing a sensitive LC–MS method, we were able to accurately quantify GlcSph in spleen and plasma samples of GD patients [116]. In the spleens from 4 type 1 Gaucher patients the concentrations of glucosylceramide (28.1, 38.1, 35.8 and 17.0 mmol/kg wet weight) and glucosylsphingosine (11.8, 7.8, 15.6 and 17.8 μmol/kg) were concomitantly increased compared with the concentrations of glucosylceramide (0.03 and 0.02 mmol/kg) and glucosylsphingosine (0.03 and 0.14 μmol/kg) in the spleens from 2 normal subjects. A spectacular, average 200-fold, increase in concentration of sphingoid base was observed in the plasma of symptomatic type 1 GD patients. While the concentration in normal individuals was found to be always less than 10 nM, most type 1 GD patients studied showed plasma GlcSph levels exceeding 100 nM. The magnitude of this abnormality in plasma GlcSph is far more spectacular than the one observed for plasma GlcCer, being on average only 3-fold higher in type 1 GD patients. Increased plasma GlcSph concentrations also occur in a mouse model of GD, generated by knockdown of GBA in blood cells [117]. To study the generation of GlcSph during GBA deficiency, we irreversibly inactivated the lysosomal GBA in cultured macrophages with Inhibody and/or conduritol B epoxide. Subsequently, GlcCer increased and after a short time GlcSph followed suit. The same phenomenon occurs in fibroblasts, but not in those derived from a Farber disease patient deficient in lysosomal acid ceramidase. Similar findings with Farber fibroblasts upon

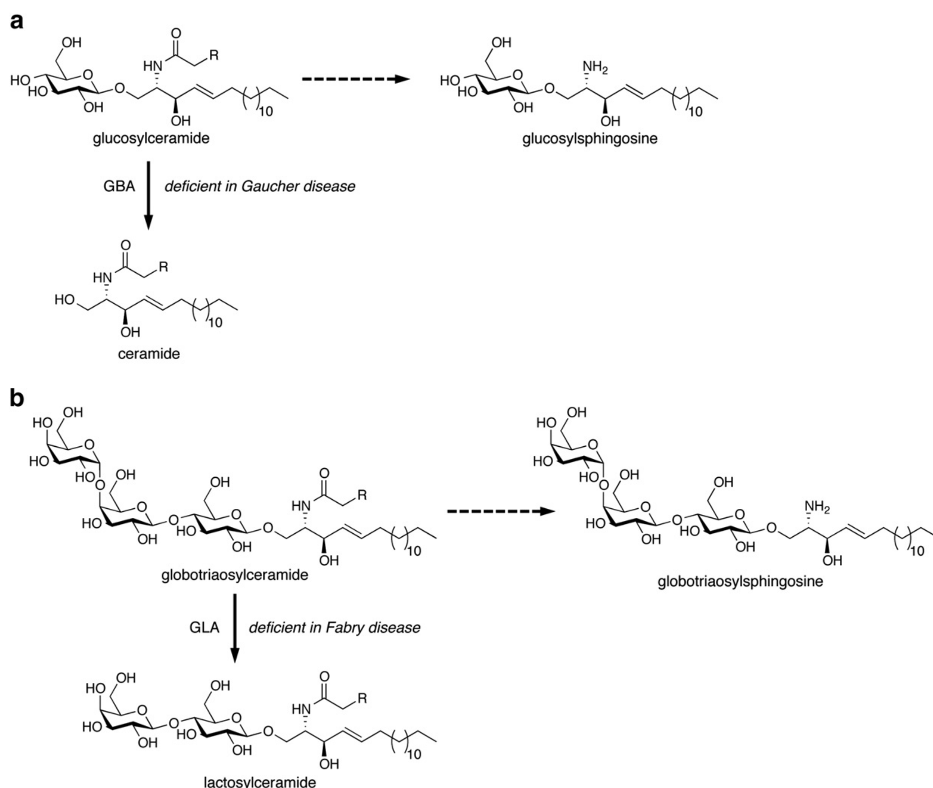

Fig. 3. Normal and alternative lysosomal catabolism of glycosphingolipid in Gaucher disease and Fabry disease. a. Gaucher disease: normal deglucosylation of glucosylceramide to ceramide by GBA (straight arrow down) and alternative deacylation in GBA-deficient cells to glucosylsphingosine (dotted horizontal arrow). b. Fabry disease: normal deglycosylation of globotriaosylceramide to lactosylceramide by αGal A (straight arrow down) and alternative deacylation in αGal A-deficient cells to globotriaosylsphingosine (dotted horizontal arrow).

conduritol B epoxide inhibition of GBA have been previously reported by other investigators [118]. These and other observations suggest that acid ceramidase is involved in GlcSph formation when GBA is impaired. It should be kept in mind that GlcSph might be also biosynthetically formed by glucosylation of sphingosine via the enzyme glucosylceramide synthase. Since GBA hydrolyzes GlcSph [119], the increased GlcSph in GD plasma and tissue may therefore partly stem from this biosynthetic pathway. We are presently conducting experiments with isotope-labeled (lyso)glycosphingolipids to differentiate better formation of GlcSph by biosynthesis and deacylation of GlcCer. Of note, GlcSph is an inhibitor of GBA activity with an IC_{50} of about 2.5 μM [119]. It is not clear whether such high concentrations are reached in the lysosomes of cells of GD patients.

In summary, the lipid GlcCer is normally degraded by the lysosomal GBA to ceramide which is next metabolized by lysosomal acid ceramidase to sphingosine and fatty acid. In GD, adaptations to GBA deficiency and excessive GlcCer occur. Synthesis of gangliosides such as GM3 is increased. Moreover, in some cell types increased degradation of GlcCer to ceramide by GBA2 might occur. On top of this, there appears to be a still poorly recognized alternative catabolic route during GBA deficiency. This 'secret route' probably involves lysosomal deacylation of GlcCer to GlcSph which subsequently leaves the lysosome and cell. We are presently investigating the metabolism of GlcSph in mice, using C13-isotope labeled lipid, especially focusing on potential elimination of the sphingoid base from the body via bile and/or urine.

2.9. Glucosylsphingosine in Gaucher disease, blessing or curse?

The tale of type 1 GD seems more complex than hitherto considered. The existence of the glucosylsphingosine pathway (see above) may help us understand how collodion babies completely lacking GBA manage to prevent massive GlcCer storage in most cell types. The obvious question now is whether conversion of GlcCer to GlcSph is only beneficial or also harmful to the GD patient. Literature suggests that GlcSph is potentially toxic since it can cause lysis of red blood cells, impair cell fission during cytokinesis, damage specific neurons, interfere with growth, impair osteoblasts, and promote inflammation via activation of phospholipase A2 (see Refs. [3,4] for reviews). These findings are strikingly reminiscent of signs and symptoms in GD patients such as occurrence of hemolysis, multinucleated macrophages, neuropathology, growth retardation, bone deterioration and chronic low grade inflammation.

Our present research focuses on the unequivocal identification of the amidase responsible for the formation of GlcSph during GBA deficiency, the subsequent metabolism of the water-soluble lyso-lipid and its potential elimination from the body. In hindsight, poor correlations between primary defect (*GBA* genotype), lipid storage deposition and

actual GD manifestation (phenotype) are already less surprising given various alternative metabolic pathways for GlcCer during GBA deficiency.

3. Fabry disease, another glycosphingolipidosis

Fabry disease (FD), is an X-linked glycosphingolipidosis caused by deficiency of the lysosomal enzyme alpha-galactosidase A (αGal A; E.C. 3.2.1.22) [120,121]. The *GLA* gene, located at Xq22, encodes a 429 amino acid precursor that is processed to a 398 amino acid glycoprotein functioning as a homodimer [122,123]. The N-linked glycans of αGal A acquire mannose-6-phosphate moieties and are sorted to lysosomes through interaction with mannose-6-phosphate receptors. The accumulating glycosphingolipids in FD contain terminal α-galactosyl moieties. The prominent storage lipid is globotriaosylceramide (Gb3; also named ceramidetrihexoside: CTH), with lesser amounts of galabiosylceramide (Gb2), blood group B, B1, and P_1 antigens accumulating [124,125]. FD was first described in 1898 independently by Johannes Fabry and William Anderson. Both dermatologists reported patients with characteristic skin lesions: angiokeratoma corporis diffusum. The disorder has subsequently become known as Anderson-Fabry disease or Fabry disease. The characteristic (classic) manifestation of FD in males involves (besides angiokeratomata), corneal opacity (cornea verticillata), neuropathic pain (acroparasthesias), intolerance to heat, inability to sweat and micro-albuminuria. Later in life, progressive kidney disease, cardiac symptoms and cerebrovascular disease (stroke) may develop [120]. The late-onset clinical abnormalities are non-specific, indistinguishable from similar complications of other origin commonly occurring in the general population. Fabry disease cannot be simply diagnosed in patients with routine electro-cardiographic, echographic or MRI techniques. The kidney disease in FD is usually associated with progressive proteinuria following a decline in glomerular filtration rate, leading finally to end-stage renal disease requiring dialysis and kidney transplantation. Cardiac complications are remarkably heterogeneous, including progressive hypertrophic cardiomyopathy with diastolic dysfunction, a variety of conduction defects and arrhythmia such as short P–R interval supraventricular and ventricular tachycardia, atrial fibrillation, valvular disease and coronary artery stenosis of large or small vessels. Ischemic stroke and transient ischemic attacks are relatively common, involving both small and large vessels, particularly in brain regions perfused by the posterior circulation. Asymptomatic lesions on brain MRI are often detected, usually in the white matter. Strokes in FD patients are similar to hypertensive or embolic strokes.

Intriguingly, many female heterozygotes also display attenuated forms of FD, although chronic renal insufficiency is rare [126,127]. The manifestation of disease in FD females is remarkable given the mozaic of αGal A-containing and -deficient cells in their tissues and the presence of considerable amounts of αGal A in circulation. The findings for FD females sharply contrast with the observed general lack of symptoms among heterozygous carriers of another X-linked lysosomal hydrolase deficiency, Hunter disease.

3.1. Diagnostic challenges

The diagnosis of classic FD males is straight-forward: mutations in the *GLA* gene encoding an absent or evidently dysfunctional αGal A protein can be identified, lacking to extremely low αGal A activity in leukocytes or fibroblasts can be demonstrated, elevated concentrations of plasma and urinary Gb3 can be detected and, crucially, a combination of characteristic disease manifestations is observed. More problematic is diagnosis of classic FD females and so-called atypical FD patients [120]. These latter individuals present with an uncharacteristic, relatively common, clinical symptom (e.g. albuminuria, left ventricular hypertrophy, or white matter lesions) in combination with an abnormality in the GLA gene with unknown consequences, a relatively high residual enzyme activity in cells, and no clear abnormality in plasma or urinary Gb3 concentration. The concomitant identification of decreased αGal A activity or a *GLA* mutation may not necessarily be related to the symptoms in these individuals. The incidence of atypical individuals is high. Recent newborn screening studies based on identification of abnormalities in the GLA gene or deficiency in αGal A activity suggest a birth prevalence of at least 1 in 4000 in European populations [9,128]. Higher frequencies have even been noted in Taiwan [129]. At present two atypical FD phenotypes are often discerned: a cardiac and a renal phenotype with symptoms restricted to a single organ [130]. The causal relationship between specific *GLA* mutations and atypical manifestation is far from established. For instance, the N215S *GLA* mutation is reported to be associated with the cardiac phenotype [131]. However, it is not known whether the presence of N215S GLA causes cardiac disease in each individual. For several mutations in the GLA gene, e.g. D313Y GLA [132], the functional consequences are the topic of debate: are they intrinsically disease-causing, do they only constitute a risk factor or even exemplify true pseudo-deficiencies? Demonstration of a functional deficiency may be a sound way to resolve these questions [133]. Some feel that biopsies and demonstration of deposits of storage lipid Gb3 are the most sensitive and robust way for this. Given the known risk on false positive findings with this approach [134–138], others feel that demonstration of abnormally elevated plasma or urinary Gb3 is best for confirmation of FD, at least that of the classic variant.

For the biochemical diagnosis of FD patients use is made of measurement of residual αGal A activity using artificial water-soluble substrates, like 4-methylumbelliferyl-alpha-galactoside. Likewise a similar artificial substrate, 4-methylumebbeliferyl-beta-glucoside, is used to assess GBA activity in relation to GD. It should be kept in mind that the activity of mutant enzymes towards these artificial substrates does not necessarily give a good impression of residual activity towards true lipid substrates, particularly since their degradation is known to be assisted by saposins [5,120]. Of note, activity-based labeling probes, as described above for GBA (Fig. 2), are presently also designed for αGal A. It is envisioned that with such ABP probes the concentration of active αGal A molecules in intact cells may be determined which would assist in diagnosis.

It should be kept in mind that, as in GD, other factors beyond the GLA gene (modifier genes, epigenetics and external factors) may very well influence FD disease manifestation. Indeed, there are already indications for modifier genes. Genotypes of polymorphisms G-174C of interleukin-6, G894T of endothelial nitric oxide synthase (eNOS), factor V G1691A mutation (factor V Leiden), and the A-13G and G79A of protein Z were found to be significantly associated with the presence of ischemic cerebral lesions on brain MRI [139]. Two polymorphisms in the NOS3 gene, encoding eNOS, seem to influence cardiomyopathy [140]. In this connection, the enzyme αGal B, the lysosomal N-acetylgalactosaminidase, warrants further investigation. The enzyme is able to cleave 4-methylumbelliferyl-alpha-galactosidase, although with poorer K_m and lower v_{max} than αGal A. It is still not completely clear whether αGal B might render some residual capacity to degraded Gb3 in completely αGal A-deficient classic FD males. This possibility is presently investigated by us.

3.2. Lipid storage and disease manifestation

Deficiency of α-Gal A results in accumulation of Gb3 in endothelial, perithelial, and smooth muscle cells of the vascular system, as well as renal epithelial cells and cells of the autonomic nervous system [120,125]. Lipid deposits have also been noted in podocytes and myocardial cells in some FD patients [141,142] (see Fig. 1). Mice lacking αGal A have been extensively studied on symptoms and lipid deposits [143,144]. Like classic FD males, male FD mice show extensive storage of Gb3 in the endothelium. The animals develop no prominent renal complications and no lipid deposition in their podocytes. Lipid deposition in cardiomyocytes of FD mice is relatively modest and the animals develop only mild myocardial alterations [145]. FD mice do not display a spontaneous vascular phenotype, but three models of induced

vasculopathy have been reported: oxidant-stimulated thrombosis in response to activation of rose Bengal, accelerated atherogenesis in mice bred on apolipoprotein E null background, and impaired vascular reactivity in aortic rings in response to β-adrenergic agonists and vasorelaxation in response to acetylcholine [146–148]. Decreased nitric oxide bioavailability has been noted in FD mice [149]. The FD mouse shows early, rather than later signs of renal disease [150]. There is no evidence of renal failure in 80-week old FD mice, but increases in urine volume and urinary Gb3 have been reported. Uromodulin, an abundant urinary protein, is abnormal in FD mice as in FD patients. FD patients show increased thresholds for both heat and cold stimuli and FD mice also develop a deficit in ability to respond to a heat stimulus. Abnormalities suggesting a peripheral nerve dysfunction have been observed in the Fabry mouse model [151,152]. FD mice develop a progressive thermal hypoalgesia (decreased sensitivity to painful stimuli) beginning at 5 months of age [150].

The lipid storage in podocytes of FD patients remains intriguing: the myelin-like deposits in podocytes are larger than those in endothelial cells and located more perinuclearly [145]. They are not strikingly reduced by ERT (see below) in contrast to the lipid material in various types of endothelial cells in the same patients [141]. The same difference in appearance and poor responsiveness to ERT is also observed for lipid deposits in cardiomyocytes [142]. The lack of prominent clearance of lipid deposits from podocytes during treatment is particularly surprising given the report that these cells can efficiently endocytose recombinant α-Gal A used in ERT [153]. At present the relationships between lipid deposition in podocytes, α-Gal A activity and symptoms is still obscure. Although lipid accumulation in podocytes also occurs in FD females, most heterozygotes show little alteration in renal function [154–156]. A strong correlation between podocyte lipid deposition and renal insufficiency is apparently lacking.

Even the relation of the characteristic Gb3 storage in endothelial cells with onset of classic FD manifestations is puzzling. Gb3 storage already occurs in utero in classic FD males completely lacking α-Gal A [157,158]. Nevertheless, symptoms in classic FD males develop much later in life. The same phenomenon is observed in α-Gal A-deficient FD mice. It is also well known that plasma or urinary Gb3 levels of classic FD males poorly correlate with actual disease [159–161]. Plasma protein markers of endothelial storage cells in FD patients, similar to chitotriosidase and CCL18 for Gaucher cells in GD patients, have to date not been identified.

3.3. ERT of Fabry disease

The success of ERT of type 1 GD prompted development of a similar approach for FD. In this case macrophages are not specifically targeted, but supplementation of several cell types in FD patients by the use of a therapeutic αGal A containing mannose-6-phosphate (M6P) moieties. The ubiquitous presence of M-6-P receptors on several cell types is supposed to ensure uptake of M6P-rich therapeutic αGal A and delivery to lysosomes. Two different recombinant αGal A preparations are used in Europe for treatment of Fabry disease [162,163]. Their European market authorization as orphan drug was obtained at the same day, 3 August 2001. One enzyme (agalsidase beta; Fabrazyme, Genzyme) is produced using CHO cells and classic recombinant technology and the other in cultured human skin fibroblasts with an activated promoter of the *GLA* gene (agalsidase alpha; Replagal, Shire). Both recombinant enzymes, differing slightly in glycan composition, were comparable in our hands when tested on in vitro specific activity and uptake by cultured fibroblasts [164]. Although both therapies (agalsidase beta at standard dose of 1 mg/kg bw/2 wks and agalsidase alpha at standard dose of 0.2 mg/kg bw/2 wks) result in clearance of Gb3 from endothelial cells, the clinical effects are not as robust as anticipated. In some FD patients stabilization of renal function and improvement in cardiac hypertrophy occur upon therapy, but a considerable number experiences progressive complications despite treatment [130,165]. Co-medication of FD patients with ACE inhibitors or angiotensin receptor blockers complicates interpretation of ERT response since such medication is known to exert beneficial effects on left ventricular hypertrophy and renal function.

Of note, in many countries ERT of FD is presently applied in almost all diagnosed patients: classic FD males with characteristic disease manifestation, FD females with an attenuated course, and atypical individuals with single symptoms. The heterogeneous nature of the ERT treated FD cohorts contributes to the difficulty to assess (cost) efficiency of this intervention. In contrast to this, ERT of Gaucher disease is now only applied in type 1 and type 3 GD patients. While ERT is largely successful in reversal and prevention of most symptoms in type 1 GD patients, in type 3 GD patients only visceral manifestations are improved. ERT of more severe type 2 GD patients has proven to be of little use and is not recommended [166]. The failure of ERT to prevent and ameliorate neurological symptoms in neuronopathic GD patients is ascribed to the inability of therapeutic GBA to pass effectively the blood–brain barrier (BBB) [167]. Attempts to distribute GBA into the brain through intraventricular delivery or BBB disruption gave disappointing results [168,169]. A better enzyme distribution in the brain was observed in rats and primates with direct convection-enhanced delivery of recombinant GBA [170].

The commonly used differentiation with respect to starting ERT of GD patients is appropriate. It helps to avoid ineffective invasive treatments and prevents false expectations. A similar conservative approach for ERT of FD is unfortunately not yet uniformly established.

3.4. Adaptations in Gb3 metabolism in Fabry disease: globotriaosylsphingosine (lysoGb3) as hallmark of classic disease

The striking discrepancies between lipid storage cells, plasma Gb3 levels and clinical symptoms, and the disappointing outcome of ERT, made us speculate that FD pathology involves a missing link, possibly some water-soluble toxic metabolite formed from the primary storage lipid [171]. In analogy to Krabbe disease and Gaucher disease [2,113], we hypothesized that in FD the lipid Gb3 is deacylated to water-soluble globotriaosylsphingosine (lysoGb3), at that time a still not described metabolite. We then demonstrated the existence of lysoGb3 in the plasma of FD patients [171] (see Fig. 3). Recently, we developed a superior method for quantification of lysoGb3 based on the use of isotope-labeled [5–9] (13)C5-lysoGb3 as internal standard and ultraperformance liquid chromatography–electrospray ionization-tandem mass spectrometry [172]. Plasma lysoGb3 concentration from 10 classically affected Fabry hemizygotes was 94.4 nM (range 52.7–136.8 nM), from 10 classically affected Fabry heterozygotes 9.6 nM (range 4.1–23.5 nM). Values for normal controls are below 1.5 nM [172].

Mirroring GD, in the plasma of classic FD males Gb3 is only 3-fold increased while lysoGb3 is concomitantly about 250-fold elevated, independent of age of patients. Classic FD in males is characterized by prominent endothelial Gb3 storage and concomitant high circulating lysoGb3. It is therefore likely that Gb3-laden endothelial cells contribute significantly to the elevated plasma lysoGb3 in FD males.

In classic FD females, often showing normal plasma Gb3 levels and hardly endothelial Gb3 deposits, an abnormal high plasma lysoGb3 is generally found, but the concentrations are far less than those in FD males [171,173]. Residual enzyme activity in plasma or blood cells from female heterozygotes varies considerably due to random X-inactivation, ranging from normal to nearly completely absent, and is a poor predictor of the clinical course [174]. Female FD patients with skewed X-inactivation may develop severe disease accompanied by high plasma lysoGb3 levels [175]. Very young pre-symptomatic FD girls may still show normal plasma lysoGb3 levels [171]. Similar findings regarding plasma lysoGb3 were made for hemizygous and heterozygous FD mice [171]. Already at young age lysoGb3 is high in the plasma of male FD mice (and homozygous female FD mice), and the

level remains almost constant with increasing age [161]. Of note, a very high plasma lysoGb3 level has also been detected in the cord blood of a classic FD male. In heterozygous female FD mice, plasma lysoGb3 gradually increases with age as in female FD patients [171]. Our observations on plasma lysoGb3 in classic FD patients have meanwhile been confirmed by several other investigators, including demonstration of abnormal lysoGb3 in urine [176–178]. Very recently, the existence of additional isoforms of lysoGb3 has been reported by Auray-Blais and colleagues. These isoforms contain additional double bands and/or hydroxyl moieties and can be quite prominent in urine of FD patients (see Refs. [179,180] for a recent review).

3.5. Plasma lysoGb3 and FD diagnosis

The diagnostic value of plasma lysoGb3 warrants discussion. While plasma lysoGb3 is strikingly increased in classic FD males, we observed that it is not clearly abnormal in examined males with reduced α-Gal A activity in combination with atypical manifestations [171,181]. The same atypical individuals also show normal or close to normal plasma and urinary Gb3 concentrations. These cases offer a major dilemma in biochemical confirmation of FD diagnosis and decision making on offering costly ERT as therapeutic intervention. An illustrative example deserves discussion. The Dutch Fabry cohort presently includes 11 individuals with the R112H GLA mutation, of which one male presented with severe renal insufficiency necessitating dialysis and one other male family member very recently developed proteinuria and decline of renal function. Kidney biopsy of the oldest case showed extensive lipid storage in podocytes, but not in endothelial cells as usual in classic FD [181]. Literature also indicates that the R112H GLA mutation is not strictly linked to classic FD manifestation. It has been reported to be associated with an atypical mild cardiac phenotype and not with the renal complications of the Dutch cases [183]. Up till now several mutations in the GLA gene have been identified for which the clinical outcome is comparably unclear and might be very well individually determined (see also Refs. [133,182]). Relatively common alterations in the GLA gene are the D313Y and A143T mutations, encoding enzymes with reduced activity as detected with in vitro α-Gal A assays. Whether these common mutations obligatory cause (atypical) disease is debated. Of note, we could not detect significant elevations in lysoGb3 in examined plasma specimens of males with these latter mutations.

In conclusion, demonstration of markedly abnormal plasma lysoGb3 allows biochemical confirmation of the diagnosis of classic FD for males, and even for most females. Plasma lysoGb3 offers a useful check to ascertain the diagnosis of classic FD. Lack of prominent elevation of plasma lysoGb3 should alert clinicians that the individual may not suffer from classic FD and that careful clinical examination and exclusion of other factors causing symptoms is warranted.

3.6. Plasma lysoGb3, vascular abnormalities and disease manifestations

FD is a systemic vascular disease manifesting non-specific symptoms. Chronically elevated plasma lysoGb3 might theoretically exert toxic effects on the vasculature. Indeed, we have observed that exposure of cultured smooth muscle cells (SMCs) to lysoGb3 at concentrations encountered in classic FD males promotes their proliferation. Analogous lipids like lactosylsphingosine or Gb3 do not exert this effect [171]. We therefore have speculated that chronic exposure to lysoGb3 may promote thickening of the vessel wall through induced proliferation of SMCs, thus contributing to vasculopathy in classic FD patients. A recent study confirmed that classic FD males present an increased (intima media) vessel wall thickness, as earlier also reported by others [184–187]. These patients also were found to show abnormal flow mediated dilatation [187]. Similar abnormalities were not seen in atypical individuals or female FD patients with (near) normal plasma lysoGb3 [187].

A hypothesis has been formulated for the etiology of vasculopathy in classic FD patients [188], aiming to unify literature reports on elevated ROS levels and reduced endothelial NO activity [130,149,189,190]. Smooth muscle cell proliferation is put forward as the initial step in a complex pathogenesis. Consequential to intima media abnormalities, a hyperdynamic circulation and a less compliant vascular wall, upregulation of local renin angiotensin systems would result on increased reactive oxygen species (ROS), dysregulation of the NO-pathway and release of pro-thrombotic factors [188].

Assessing relationships between plasma lysoGb3 and clinical manifestations of FD seems crucial. If exposure to lysoGb3 indeed forms a risk factor for developing vascular dysfunction and subsequent symptoms, a strict correlation between current plasma lysoGb3 level and disease is not to be expected. Lifetime exposure to plasma lysoGb3 (estimated as the product of plasma lysoGb3 concentration and age) should correlate better with disease, especially in male FD patients showing high plasma lysoGb3 from birth which remain fairly constant.

For Dutch FD patients, plasma lysoGb3 levels have been compared with several disease manifestations [181,187]. A significant correlation of lysoGb3 lifetime exposure with overall disease severity was noted for classic male and female FD patients [181]. Carotid intima media thickness (IMT) in female FD patients significantly correlated with plasma lysoGb3 levels measured. All classic FD males showed increased IMT (and flow mediated dilatation) with concomitant elevated plasma lysoGb3 [187]. Left ventricular mass in FD females also correlated with plasma lysoGb3. In the case of FD males there was a significant correlation of left ventricular mass with lifetime exposure to lysoGb3. No correlation was observed for plasma lysoGb3 and renal function in FD males or females. Others however, reported that urinary lysoGb3 correlates with serum creatinine as well as micro-albuminuria and proteinuria [191]. IMT is known to correlate with risk of stroke, possibly explaining the finding that white matter lesions in Dutch classic FD males correlate well with plasma lysoGb3 [181]. The observed associations for the small and clinically heterogeneous Dutch Fabry cohort between plasma lysoGb3, or life time exposure, and certain disease manifestations are exciting, but can only hint at causal relationships. Experimental investigations in animals have to reveal whether increased lysoGb3 itself is indeed causing symptoms, either directly or as risk factor.

Of particular interest is the relation between pain and lysoGb3 [192]. Neuropathic pain and pain attacks are often the first symptoms of FD presenting at an average age of 9 years in male patients and somewhat later in female patients. Small fiber neuropathy (SFN) is thought to play a role in pain in FD. SFN might arise from storage of lipids in ganglia leading to a so-called dying-back neuropathy, and/or from direct damage to small nerve fiber axons. SFN in Fabry disease is length-dependent and progresses with age. Pain sensation takes another time course, possibly due to peripheral sensitization in earlier stages of nerve fiber damage and disappearance of pain in later stages. Since small Aδ-fibers, carrying cold sensation and mechanical pain sensitivity, are preferentially affected in FD patients, the ganglia or axons of these small myelinated nerve fibers seem more vulnerable than those of small unmyelinated C-type fibers. Lifetime exposure to lysoGb3 was found to correlate very significantly with the cold detection threshold and thermal sensory limen at the upper limb [192]. Possibly, lysoGb3 itself exerts a direct pathological effect on the nervous system. Hereditary sensory neuropathy type 1 (HSN1) is caused by plasma accumulation of two sphingoid bases, 1-deoxy-sphinganine and 1-deoxymethyl-sphinganine, that structurally resemble lysoGb3 [193]. The neuropathy in HSN1 and FD shows clear similarities, supporting direct neurotoxicity of lysoGb3.

A pathogenic role for lysoGb3 in kidney disease has also been proposed by Sanchez-Nino and colleagues who reported that the sphingoid base dose and time dependently increased the expression of TGF-β1, extracellular matrix proteins (fibronectin and type IV collagen) and CD74 in podocytes [194]. They proposed that lysoGb3 plays a role in

glomerular injury in FD by promoting the release of secondary mediators of glomerular injury common to diabetic nephropathy [194].

3.7. ERT, antibodies and plasma lysoGb3 response

The impact of ERT on plasma lysoGb3 levels in FD patients has been investigated by us and others [171,173,181,191,195]. We reported that lysoGb3 levels in classic FD patients decrease rapidly after the start of ERT with several regimens (agalsidase alpha (0.2 mg/kg/2 wks), agalsidase beta (0.2 mg/kg/2 wks), and agalsidase beta (1.0 mg/kg/2 wks) [173]. Plasma lysoGb3 levels decrease dose-dependently, reaching almost stable levels within three months, however usually still above the normal range. The reduction in plasma lysoGb3 during ERT was found to correlate with correction of the left ventricular mass in female FD patients [181]. Furthermore, reduction in lysoGb3 level correlated with a lower hazard ratio for developing cerebral white matter lesions [196]. The potentially prognostic value of plasma lysoGb3 reduction during ERT for clinical outcome clearly warrants further investigation with a large cohort of FD patients.

Our studies so far revealed no difference in plasma lysoGb3 correction following ERT with one of the two enzymes, at least when administered at a same dose of 0.2 mg/kg bw/2 wks [173]. Due to unforeseen shortage of agalsidase beta, ERT of some Dutch FD patients had to be changed. Patients previously receiving agalsidase beta at 1.0 mg/kg bw/2 wks were reduced in dose to 0.2 mg/kg bw/2 wks and others switched to agalsidase alpha at similar lower dose [197]. Almost all FD patients undergoing a dose reduction showed a relapse in plasma lysoGb3 elevation within a few months. This finding illustrates that there is an ERT dose-effect on plasma lysoGb3 levels and that the biochemical response to ERT is suboptimal at a dose of 0.2 mg/kg bw/2 wks in the majority of classic FD patients [197].

We were the first to report the occurrence of antibodies in about 70% of classic FD males receiving ERT [198], a finding later confirmed [199]. Most classic FD males completely lack α-Gal A protein and an immunological response to the infused foreign protein (agalsidase alpha or beta) is not surprising. The antibodies formed in classic FD male patients receiving agalsidase alpha or beta comparably bind to both recombinant enzymes in vitro and comparably neutralize enzyme activity in vitro [198]. Biochemically, the presence of anti-(α-Gal A) antibodies in classic FD males has consequences. The reduction in plasma lysoGb3 during ERT is much less prominent in FD males with antibodies than those without [173,196]. In parallel, urinary Gb3 levels also hardly correct in FD males with antibodies [196,198].

The clinical impact of the presence of anti-(α-Gal A) antibodies in male FD patients is still unclear as well as its effect on responsiveness to ERT [196]. The vast majority of FD males does not develop tolerance to infused α-Gal A, and maintains high titers of anti-(α-Gal A) antibodies over many years [196]. Looking close into the consequences of anti-(α-Gal A) antibodies is particularly relevant given the current studies on preventive treatment of FD in pre-symptomatic boys with mutations predicting complete lack of α-Gal A protein and concomitant high chance on antibody formation.

4. Discussion

4.1. Gaucher and Fabry diseases: different or similar tales beyond lysosomal lipid storage?

Autosomal Gaucher disease and X-linked Fabry disease are both disorders characterized by lysosomal accumulation of a glycosphingolipid (GlcCer in GD and Gb3 in FD). The lysosomal lipid storage is thought to be the primary pathogenic event in both diseases. Nevertheless, the clinical presentation of both glycosphingolipidoses is entirely different. The difference in involved organ systems in GD and FD is due to the fact that the prominent lysosomal lipid deposits largely concern different cell types: GlcCer storage cells in viscera of type 1 GD patients are exclusively macrophages, whereas in FD patients several cell types store Gb3. The prominent Gb3 storage in endothelial cells of classic FD males could be due to chronic uptake of lipoproteins. Newly synthesized VLDL and LDL are known to contain significant amounts of Gb3 [200]. As in GD, lipid-laden macrophages are demonstrable in classic FD males [201]. The prominence of storage of lipids in lysosomes of macrophages in several glycosphingolipidoses results from the role of these cells in uptake and degradation of senescent and apoptotic cells. Blood cells, particularly leukocytes and platelets, are rich in glycosphingolipids ultimately rendering GlcCer for lysosomal catabolism. The globoside content of erythrocytes, a source of Gb3 destined for degradation in macrophages, is relatively high. In view of the latter, it is remarkable that lipid-laden macrophages are not more dominant in the pathophysiology of FD. Blood groups, particularly the αGal A substrates blood group B, B1, and P_1 antigens, have little influence on disease severity [202]. Somehow, the completely αGal A-deficient tissue macrophages of classic FD males are able to limit on-going lipid deposition with subsequent increase in lipid-laden macrophages. Probably, this is also the case in type 1 GD patients where the number of Gaucher cells can stay relatively constant, sometimes even spontaneously diminish, despite on-going turnover of blood cells [203].

The discovery of the formation of water-soluble sphingoid bases, lysoGb3 in FD and GlcSph in GD, might provide answers to some questions. Conversion of primary storage lipid to corresponding sphingoid base may allow exit from lysosomes and cells, and possibly even from the body via bile. In analogy to the bodily homeostasis of cholesterol, such route could offer FD and GD patients a way out for non-degradable glycosphingolipids. On the other hand, the concomitant chronic exposure to biologically active sphingoid bases may have (patho)physiological consequences for GD and FD patients. Toxic actions of lysoGb3 and GlcSph might underlie some of the symptoms of FD and GD patients, respectively. At present one can only speculate about the role of sphingoid bases in acute and late-onset clinical manifestations of GD and FD. In this respect, investigation of the outcome of inhibition of lysosomal sphingoid base formation in animal models of GD and FD will be of great interest.

Intriguingly, GD and FD differ in severity of disease manifestation in relation to relative enzyme deficiency. For example, absence of GBA results in severe neonatal disease, the collodion GD baby, and marked reduction of this enzyme leads to acute neuronopathic type 2 GD. However, completely αGal A-deficient FD males develop symptoms surprisingly late in life. Many female FD patients, despite their mosaic of αGal A-deficient and enzyme-competent cells and the presence of considerable circulating αGal A, develop late onset vascular disease manifestations. Contrary again, carriers of GD do not develop lipid-laden macrophages nor visceral symptoms such as shown by patients. Intuitively, marked deficiency of GBA should have more serious consequences than that of αGal A. GBA catalyzes the penultimate step in degradation of almost all GSLs: GlcCer, lactosylceramide, gangliosides and globosides. In contrast, lysosomal αGal A only catalyzes degradation of Gb3 stemming from globosides only. However, predictions on comparative outcome of deficiencies in the two lysosomal enzymes are hampered by the existence of alternative metabolic pathways for both GlcCer and Gb3 (see above). At present, the late-onset complications in FD patients, slowly developing in FD females and even in completely αGal A deficient FD males, are most difficult to understand. The slow kinetics of lipid deposition in podocytes and cardiomyocytes of FD patients is puzzling. More information on the composition and formation of lipid deposits in these cells, known to poorly respond to ERT, is probably needed to develop a more successful strategy for therapeutic intervention.

To better understand all manifestations of FD and GD it might be required to look also beyond the lysosome. It is possible that secondary lipid abnormalities outside the lysosome, as a consequence from primary lysosomal defects, contribute to particular symptoms. Excessive activity of GBA2 or formation of gangliosides in GD discussed

above are examples of this. In line with these excessive concentrations of GlcCer or GlcSph in the ER might influence calcium homeostasis and cell function [204]. Additional secondary lipid abnormalities in GD might very well occur and be physiologically relevant, for instance abnormalities in plasmalogens or bis(monoacylglycero)phosphate [205–207]. In the case of FD abnormal concentrations of Gb3 at plasma membrane microdomains might underlie the decreased nitric oxide bioavailability noted in FD mouse [149,208].

Concluding remarks

The present limitation in predicting specific symptoms in individual FD and GD patients by genotype or in vitro measured residual enzyme activity is sobering. For many inherited lysosomal enzymopathies present knowledge of consequences of deficiencies, and adaptations to these, is scarce. Moreover, the impact of modifier genes, epigenetics and external factors is largely obscure. Present learning is therefore still too incomplete to reliably predict disease course for individual patients. This missing knowledge will become even more apparent and challenging in the upcoming age of full exome sequencing. Inevitably, more individuals will be identified with enzyme encoding genes carrying mutations/polymorphisms of which the consequences are far from clear. Do particular mutations indeed predict disease and with which severity and with which penetrance among individuals? Capturing genetic, biochemical and clinical data in international patient registries is of utmost importance for improved learning in this respect [209]. Better diagnosis and sub-classification of patients based on objective criteria like functional deficiency (in different cells and organs) is also pivotal for better design and assessment of efficacy of therapeutic interventions in variably affected patients. Different phenotypic variants may very well differently profit from treatments. Appreciation of the heterogeneity of phenotypic variants of an enzymopathy seems essential for optimal clinical management and counseling. The increasing availability of therapeutic options for enzyme deficiencies such as ERT promotes early identification of individuals with abnormalities in corresponding genes and enzymatic activity. Given the present limitations in interpreting such abnormalities, an urgent discussion with all stakeholders is needed to handle the challenges posed by these developments. An inappropriate labeling of individuals with only presumed future disease should be avoided, likewise inappropriate burdening of individuals with invasive (pre-symptomatic) therapeutic intervention. In the new era, detection of functional enzyme deficiency at an early (st)age through demonstration of abnormal metabolites, using both biochemical methods, radiological techniques and microscopy, will have to increasingly contribute to optimal counseling and clinical management.

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