Zebrafish as research model to study Gaucher disease: Insights into molecular mechanisms

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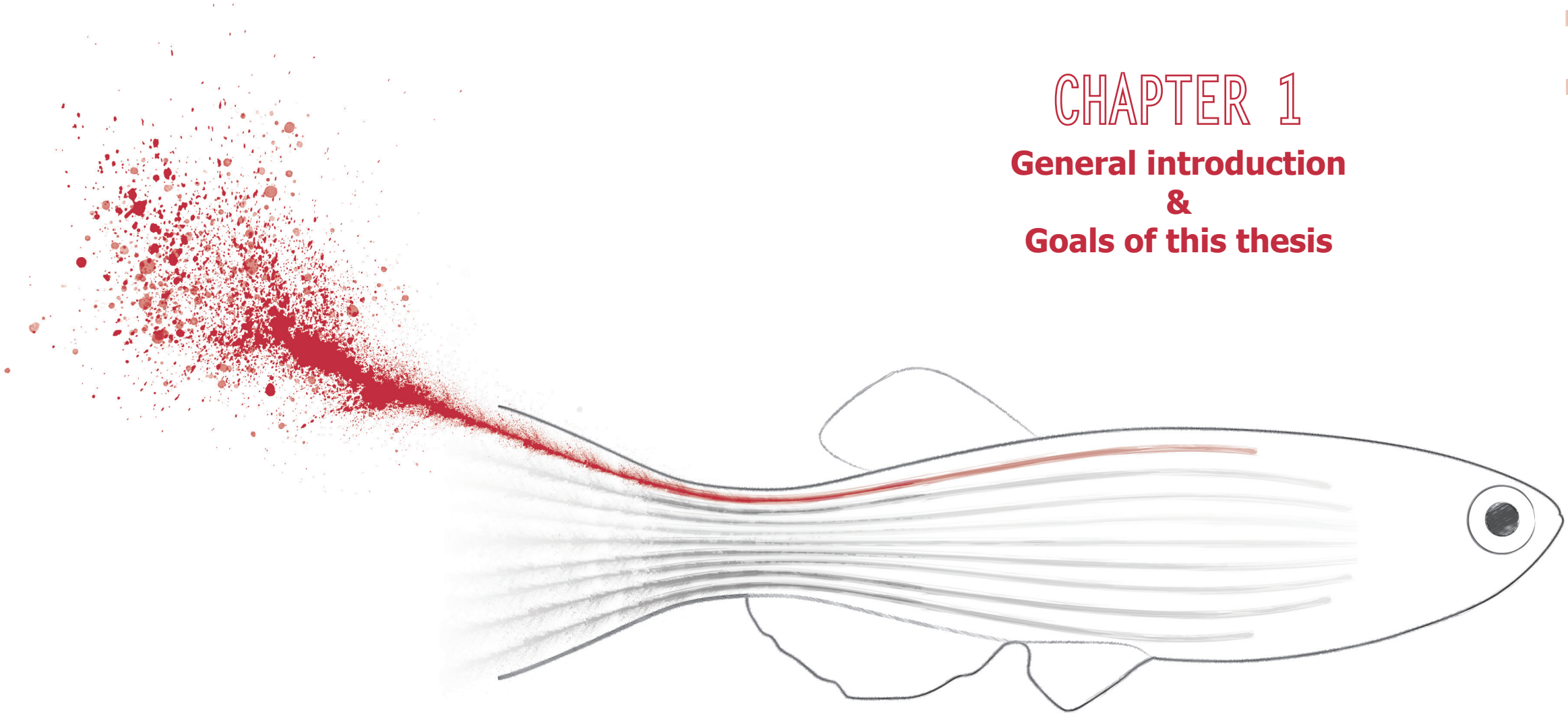
CHAPTER 1

General introduction & Goals of this thesis



Glycosphingolipids

In the nineteenth century, the chemist Johannes Ludwig Thudichum had a large impact on neurochemistry by extracting, purifying and elucidating many substances from the brain which he named cerebrosides, sphingosine and sphingomyelin[1]. These lipids are classified as sphingolipids and chemically defined by a long-chain aliphatic amino alcohol, also called sphingoid base, to which an amide-linked fatty acid is attached, thereby forming ceramide[2-4]. In mammals, the most abundant sphingoid base is sphingosine containing 18 carbons and one double bond (d18:1). In contrast, the length of the sphingoid base in glycosphingolipids of *Drosophila melanogaster* is shorter, with mainly C14 and C16 chains[5,6]. The attached fatty acid in glycosphingolipids shows considerable variation in structure[3].

GSL biosynthesis

Sphingolipids are generated via *de novo* synthesis or a salvage pathway (**Figure 1A**)[3]. *De novo* synthesis of sphingolipids starts in the endoplasmic reticulum (ER) with condensation of the amino acid L-serine and an acyl-CoA thioester into a 3-ketosphinganine. In mammals this reactions is mediated by serine palmitoyltransferase (SPT) which prefers a palmitoyl-CoA (C16:0 palmitate), thereby generating mainly sphingolipids with a d18:1 sphingoid base. 3-Ketosphinganine is reduced to sphinganine by 3-ketosphinganine reductase, followed by acylation mediated by a member of the ceramide synthase (CerS) family. The formed dihydroceramide is subsequently desaturated by a desaturase, forming ceramide[3]. DES1 generates the 4,5-trans double bond of ceramide while DES2 adds a hydroxyl group forming the t18:0 backbone of phytoceramide. Catabolism of ceramide by neutral and acid ceramidase enzymes results in sphingosine that, via the so-called 'salvage pathway', can be reacylated by one of the CerS enzymes.

Six CerS isoforms have been detected in mammals and the fatty acid composition of sphingolipids originates from the expression level, tissue distribution and acyl-CoA substrate selectively of the different CerS enzymes[3]. CerS1 is structurally and functionally different from the other CerS isoforms with a high expression in brain and a preference for stearoyl-CoA generating C18-dihydroceramide[7]. Both CerS2 and CerS3 prefer longer chain acyl-CoAs, between C20 and C26. CerS2 is highly expressed with a broad tissue distribution, but detected higher in kidney and liver[8]. Whereas CerS1 is highly expressed in neurons of the mouse brain, CerS2 expression is high in oligodendrocytes and Swann cells. Moreover, the transient increase in CerS2 mRNA during the period of active myelination suggests a role for longer fatty acids in myelin sphingolipids[9]. CerS3 expression seems restricted to epidermal keratinocytes and male germ cells and generates abundant epidermal ceramide species such as ceramides with very long fatty acids (C26-C36), 2-hydroxy- and ω-hydroxy fatty acids, the latter which are precursors of esterified glucosylceramide and ceramide lipids[10-12]. CerS4 is expressed ubiquitously at low levels and generates ceramides with C18-22 fatty acids[3]. CerS5 and CerS6 both make ceramides with C16 fatty acid, while CerS6 also utilizes myristoyl-CoA to make C14-dihydroceramide[7]. The majority of the membrane sphingolipids are composed of saturated fatty acids[3].

The produced ceramide species are subsequently transported to different parts of the ER or Golgi complex, where they are modified into more complex sphingolipids (**Figure 1A**).

Intracellular transport of ceramide is often mediated by CERT in a non-vesicular manner[13]. The major plasma membrane component, sphingomyelin, is generated by a transfer from the phosphocholine headgroup of phosphorylcholine to the 1-hydroxyl position of ceramide by either SMS1, located in Golgi membranes, or SMS2, present in plasma membranes[3].

Another group of more complex sphingolipids are the glycosphingolipids (GSLs), whereby the C1 hydroxyl of ceramide is modified by one or a chain of glycan moieties[2]. Two simple glycosphingolipids are the starting point of more complex GSLs: galactosylceramide (GalCer) and glucosylceramide (GlcCer). In general, the first sugar attached to ceramide is either a glucose- or galactose moiety and attached via a β-linkage, although α-linked GalCer has been found in bacteria and a marine sponge[14-16]. The ER localized ceramide galactosyltransferase (CGT) transfers a galactosyl moiety to the 1-hydroxyl position of ceramide, generating a β-linked GalCer, an important lipid in the brain as major constituent of myelin[17]. The formation of GlcCer is mediated by UDP-GlcCer glucosyltransferase (UGCG), also called glucosylceramide synthase (GCS) located on the cytosolic leaflet of the ER or early cis-Golgi membranes[3]. Newly formed GlcCer can be metabolized by the non-lysosomal membrane-associated glucosylceramidase GBA2[18-20], but most of it is relocated to the luminal leaflet of trans-Golgi membranes. Here, β4-galactosyltransferase (B4GALT5) mediates the transfer of a β-linked galactose to GlcCer generating lactosylceramide (LacCer), a Galβ(1,4)Glcβ(1)-Cer. LacCer is an important intermediate as starting point for more complex GSL lipids by addition of various sugars, including glucose, galactose, mannose, xylose, fucose, glucuronic acid, N-acetyl glucosamine and N-acetyl galactosamine. The complex GSLs are classified as members of globo-, isoglobo-, lacto-, neolacto- and ganglio-families and only a limited number of glycosyltransferases are required to obtain a large variety of root-structured GSLs by addition of different α- or β-linked glycan moieties to the 3-O- or 4-O position (**Figure 1A**)[3,21].

GSL function

The eukaryotic plasma membrane is formed of a bilayer of amphiphilic phospholipids, sphingolipids and sterols. In the plasma membrane, sphingolipids and sterols are thought to organize in transient semi-ordered lipid microdomains, called lipid rafts[22-25].

In particular, cholesterol and sphingomyelin are very abundant among the plasma membrane lipids (30-40 mol% and 10-20 mol% respectively), while GSLs are typically present at low levels however depending on the cell type[22,26]. Most of the sphingolipids are present in the outer leaflet of the plasma membrane bilayer, thereby having their hydrophilic head group facing the extracellular environment. The different head groups of sphingolipids and GSLs offer a variety of chemical surface characteristics. Sphingolipids and GSLs in lipid rafts are suggested to play a role in a variety of important biological functions, ranging from signal transduction, apoptosis, cell adhesion, synaptic transmission, organization of the cytoskeleton and protein sorting to the cellular entry of viruses, bacteria and toxins[22,27-30].

The synthesis of distinct GSLs is cell-type specific and different tissues can have different compositions of specific surface GSLs. For example, gangliosides are present in the plasma membrane of neuronal cells, while galactosylceramide, together with cholesterol, is a major lipid constituent in the myelin sheaths surrounding axons[31,32]. Myelin is formed by

specific glia cells, either oligodendrocytes in the central nervous system or Schwann cells in the peripheral nervous system. A change in lipid composition of myelin could contribute to myelin destabilization and breakdown[32]. Another distinct feature is the role of sphingolipids and GSLs in the skin of terrestrial animals. The specific ceramide species with very long fatty acids (C26-C36), 2-hydroxy-, ω-hydroxy fatty acids and esterified GlcCer and ceramide lipids are of importance for the organization of the corneocytes in the stratum corneum, in order to prevent trans-epidermal water loss and thereby lethal dehydration[12,33,34]. Ceramide species with very long fatty acids are also important for male fertility in sperm cell maturation[10].

Sphingolipids and GSLs might also have intracellular biological functions, as not all sphingolipids are associated with the plasma membrane[35,36]. Specific types of sphingolipids and GSLs are involved in different biological processes. Sphingolipids such as sphingosine and ceramide are thought to be involved in the eukaryotic stress response, mediating differentiation, cell cycle arrest, apoptosis and senescence, whereas sphingosine-1-phosphate, as ligand for G-protein coupled receptors, appears to promote proliferation and survival[30,37-41].

GSL catabolism

Catabolism of glycosphingolipids predominantly takes place in endosomes and lysosomes (**Figure 1B**). GSLs end up in endosomal-lysosomal compartments via various ways, including receptor-mediated endocytosis or internalization of cellular membranes through intraluminal vesicles, also called multivesicular bodies[2,26]. Macrophages are specialized in the phagocytosis of larger cellular debris, dying or dead cells and pathogens. The resulting phagosome is fused with lysosomes which contains the necessary enzymes to break down its content. Generally (glyco)sphingolipid breakdown is achieved by hydrolysis of the complex sphingolipid to separate components via lysosomal enzymes with acidic pH optima, however hydrolytic enzymes are also reported at other locations in the cell with other pH optima[2]. The stepwise removal of the terminal glycan moieties of the GSLs occurs sequentially by specific glycosidases[42,43]. For example, sialidase enzymes mediate removal of terminal sialic acid residues, β-hexosaminidase enzymes of N-acetyl-Glc or -Gal moieties, α-galactosidase removes terminal α-Gal moieties, β-galactosidase of terminal β-Gal moieties, glucocerebrosidase mediates hydrolysis of GlcCer and acid sphingomyelinase mediates removal of the phosphocholine head group of sphingomyelin. The biosynthetic enzymes in the ER and Golgi are generally membrane-bound, while the catabolic glycosidases in the lysosomes are not bound to membranes. In contrast, the sphingolipids and GSLs destined for hydrolysis are typically embedded in the lysosomal membrane. Several glycosidases therefore require the assistance of membrane-perturbing and lipid-binding proteins in order to efficiently interact with their target substrate lipid in the membrane.

Four sphingolipid activator proteins (saposins, Sap A-D) are known and originate from a single encoded prosaposin polypeptide sequence. The best studied Sap variant is Sap C which is essential for glucocerebrosidase (lysosomal acid β-glucosidase; GCase) to hydrolyse GlcCer located in the lysosomal membrane[26,44,45].

The final step in GSL catabolism is the cleavage of ceramide into sphingosine and a free fatty acid. This step is performed in the lysosome by acid ceramidase but could also be performed in other parts of the cell by one of the neutral ceramidases[2,46].

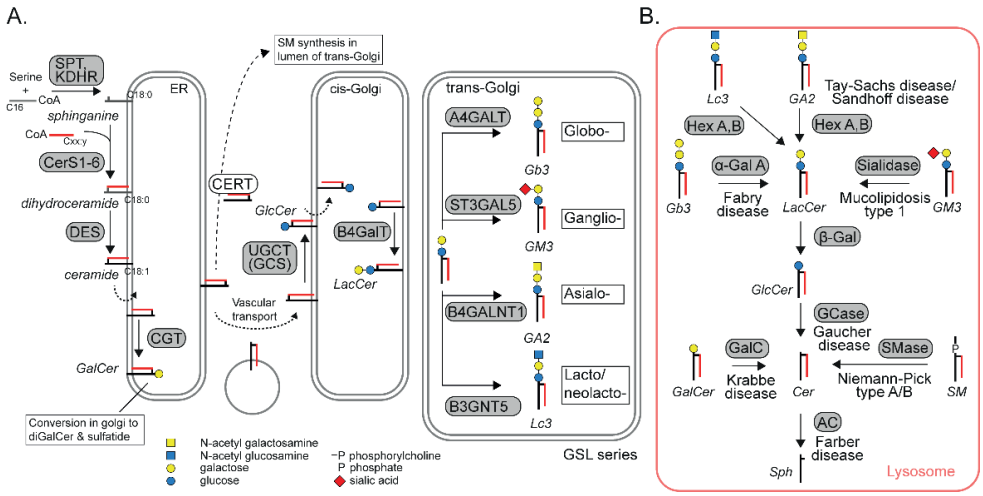


Figure 1 | Glycosphingolipid metabolism
Schematic representation of (glyco)sphingolipid biosynthesis and catabolism. Enzymes are depicted in grey. **(A)** (Glyco)sphingolipids (given in italic) are synthesized in ER and Golgi compartments. Root-structured GSLs are grouped in families (globo-, ganglio-, asialo and lacto/neolacto- series). The first GSL of the series is depicted and others are generated by sequential addition of different glycan moieties. **(B)** Catabolism of GSLs occurs in lysosomes through the sequential action of lysosomal glycosidases. A defect in one of the lysosomal glycosidases results in accumulation of GSLs and clinical symptoms specific for that lysosomal storage disorder.

Lysosomal storage disorders

Inherited lysosomal storage disorders (LSDs) are a group of orphan diseases characterized by impaired lysosomal catabolism or lysosomal dysfunction. More than 70 LSDs occur, summarized by Platt et al., and affect collectively about 1 in 5000 live births[43]. The majority of the LSDs is due to mutations in genes encoding specific lysosomal glycosidases, however LSDs can also occur when related lysosomal activator proteins, lysosomal transporters or integral membrane proteins are not functioning properly[21,43,47]. The so-called (glyco) sphingolipidoses are those LSDs that are characterized by accumulation of (glyco) sphingolipid. Despite the similarity in the chemical nature of the storage material, the (glyco)sphingolipidoses are clinically quite distinct and with each of these diseases there is variability among patients in severity, nature of symptoms and age of onset. The primary defect and prominent clinical features of a number of relevant LSDs are summarized in **Table 1**, including selected murine and zebrafish models.

Three LSDs and their compromised lysosomal hydrolyses receive particular attention in this thesis and therefore warrant more detailed introduction. These LSDs are Gaucher disease (GD) caused by deficiency of lysosomal acid β-glucosidase, Fabry disease caused by deficiency of lysosomal α-galactosidase A and Farber disease caused by deficiency of acid ceramidase.

Gaucher disease – A relatively common lysosomal storage disorder is Gaucher disease (GD), with a frequency of approximately 1 in 40.000 live births in the general population and 1 in 450 in the Ashkenazi Jewish population[43]. GD is caused by a defect in lysosomal acid β-glucosidase (glucocerebrosidase, GCase; EC 3.2.1.45) and leads to lysosomal accumulation of GlcCer, particularly in lysosomes of tissue macrophages that transform into Gaucher cells[48-50]. GCase is encoded by the *GBA* gene, located in man on locus 1q21, and several hundred mutations in this gene have meanwhile been associated with GD. GCase (Glycosyl Hydrolase (GH) family 30) is a 497 amino acid glycoprotein with four N-linked glycans and a retaining β-glucosidase with a characteristic $(\alpha/\beta)_8$ TIM barrel catalytic domain[51,52]. In this domain, Glu 340 acts as catalytic nucleophile and Glu 235 as acid/base residue[53]. Cyclophellitol and its synthetic derivative conduritol B-epoxide (CBE) are potent suicide inhibitors of GCase that bind covalently and irreversibly to the nucleophile Glu 340 of GCase. Recently, superior inhibitors for GCase have been designed with a bulky hydrophobic substituent at C8 of the cyclophellitol[54]. These cyclophellitol derivatives inactivate GCase with even higher affinity and specificity than CBE and cyclophellitol, the latter inhibitors have shown to react with GBA2 and/or acid α-glucosidase[55]. Selective activity-based probes (ABPs) have been designed by attaching a reporter group (biotin or BODIPY) to the C8 of cyclophellitol, enabling visualization and identification of active GCase[56]. Next, cyclophellitol aziridine ABPs with attached reporter groups have also been developed that target multiple retaining β-glucosidases including GCase, the cytosol faced membrane-associated protein GBA2 and the cytosolic GBA3[57].
GCase is transported to lysosomes independent of the mannose-6-phosphate receptor pathway unlike other lysosomal hydrolyses[58]. Instead, lysosomal integral membrane

protein 2 (LIMP2) binds newly formed GCase in the ER and transports it to lysosomes[59-61]. Mutations in the *SCARB2* gene, encoding LIMP2, cause action myoclonus-renal failure syndrome (AMRF)[62,63]. Interestingly, AMRF patients do not develop lipid-laden macrophages, indicating that the residual activity of GCase in these cell, acquired by re-uptake of faulty secreted enzyme, is sufficient[64]. The activator lipid-binding protein saposin C (Sap C) is required for optimal intralysosomal functioning of GCase as is illustrated by the finding that defective Sap C causes symptoms similar to GD[44,65].

GD is clinically remarkable heterogeneous[48]. Patients with considerable residual GCase activity develop only visceral symptoms, including hepatosplenomegaly, thrombocytopenia, abnormalities in coagulation, anaemia due to reduced erythrocyte populations and skeletal manifestations such as bone pain and bone fractures[66-68]. This non-neuronopathic type 1 GD variant differs from the acute and subacute neuronopathic GD variants type 2 and type 3. The very low residual GCase activity in types 2 and 3 GD patients causes a more severe visceral pathology with additional neurological manifestations, like epilepsy, apraxia and scoliosis[48,69]. Subcategories are distinguished for the heterogeneous neuronopathic GD variants and it has been proposed to consider the disorder as continuum of disease manifestations, each variable among individual patients[70]. An almost complete deficiency of GCase causes a unique phenotype, the collodion baby with a disturbed skin permeability barrier incompatible with terrestrial life[33,71]. The same lethal impairment is observed in mice with a complete genetic knockout of GCase[72]. GCase has an essential role in the stratum corneum where it converts extruded GlcCer lipids into ceramide which are required for formation of optimal lipid lamellae[33,73].

The molecular basis for the marked phenotypic heterogeneity among GD patients is still not fully known. It is apparent that some of the several hundred *GBA* mutations are associated with a milder course of disease. For example, patients with the common amino acid substitution N370S in GCase typically display no neuropathology[74]. The amino acid substitution D409H in GCase is associated with cardiac symptoms involving the aortic and mitral valve[75,76]. On the other hand, within one *GBA* genotype, variation in severity of disease is encountered. Most strikingly, monozygotic GD twins have been reported with discordance in phenotype[77,78]. Modifier genes, epigenetics and external factors have been proposed to contribute to variability of disease manifestations[79,80]. Polymorphisms in the genes encoding LIMP2, GCS and Sap C have been put forward as GD modifiers[81-83]. Another candidate modifier, based on a GWAS study in patients homozygous for N370S mutation, is the ER transmembrane protein CLN8[84]. CLN8 has recently been shown to be involved in the transport of newly formed lysosomal enzymes between ER and Golgi apparatus[85].

Mice have been used to study more closely the pathology associated with GCase deficiency. However, complete GCase deficiency in mice is lethal due to the altered permeability barrier function, as described for man[33,72]. Amino acid substitutions known to cause GD types 1 and 2 in man are introduced in the mouse genome, but these mice do not always show disease manifestations as observed in patients[86]. No phenotype or GlcCer accumulation has been observed in mice with the amino acid substitutions V394L, D409H and D409V, while mice with the common amino acid substitutions N370S, leading to type 1 GD, or L444P, causing a neuronopathic form of GD, die soon after birth[87,88].

In addition to the mice with point mutations, conditional knockouts have been generated with GCase deficiency restricted to specific cell lineages, such as the hematopoietic and mesenchymal cell lineage (*Mx1*-Cre-*loxP*[89]), the neuron stem cell lineages (Nestin-flox/flox[90]) or GCase deficiency in all tissues except skin (K14-lnl/lnl[90]). Defective GCase in the hematopoietic cell lineage is sufficient to induce formation of Gaucher cells, hepatosplenomegaly and haematological symptoms[91], however for skeletal deterioration a combined GCase deficiency in hematopoietic and mesenchymal cell lineages is needed[89]. Studies with mice developing neuropathology, either by genetically induced deficiency in neuronal cells or the overall inactivation of GCase with the suicide inhibitor CBE, have revealed that activated microglia and neuroinflammation are associated with damaged brain areas[90,92]. Activated microglia has been proposed by some researchers as key mediator of GD neuropathology, however whether the inflammation precedes neuron death or is concomitant with it remains unclear[93-95].

No strict correlation of GCase activity levels, as measured *in vitro*, with neuropathology has been observed and intriguingly neither with GlcCer levels or those of its metabolite glucosylsphingosine (see below)[95]. Thus, beyond the primary defective GCase other factors might influence GD neuropathology. A considered candidate in this respect is the enzyme GBA2 that is abundant in Purkinje neuronal cell bodies and dendrites. Prominent ABP labelling of GBA2, confirmed by antibody staining, is seen in the cerebellar cortex and thalamus which coincides closer with areas involved in neuropathology than the distribution of GCase[96,97]. Intriguingly, carriers of mutations in the *GBA* gene have an increased risk of developing Parkinson's disease or Lewy body disorders[98,99]. Active GCase molecules were detected in regions involved in motor functioning, which are areas susceptible to develop Lewy Bodies[96]. The 20-fold increased incidence of α-synucleinopathies in carriers of GD remains yet unexplained and is worldwide investigated.

It has become recognized that metabolic adaptation occur during GCase deficiency[100]. Firstly, in plasma and spleen of GD patients increases of the ganglioside GM3 have been noted, possibly due to increased shuttling of newly formed GlcCer to gangliosides and/ or impaired recycling of gangliosides[101]. The elevated concentrations of GM3 in GD patients are accompanied by insulin insensitivity, however without overt hyperglycemia[102]. Secondly, the β-glucosidases GCase and GBA2 can act as transglucosylase and form glucosylated cholesterol (GlcChol) from GlcCer and a cholesterol acceptor[103-105]. It has been suggested that GCase is typically involved in hydrolysis of lysosomal GlcChol, while the membrane-associated GBA2 generates GlcChol. Increased GlcChol levels were detected in plasma and spleen of GD patients and GD mouse models, while reduced GlcChol levels in plasma and liver of GBA2 deficient mice were observed[103]. It is conceivable that also other metabolites are glucosylated by GBA2 using GlcCer as sugar donor, which are accumulated during GCase deficiency. The possible role of excessive glucosylated metabolites in the complex GD pathology is yet unknown. Finally, accumulating GlcCer in lysosomes is converted to its sphingoid base glucosylsphingosine (GlcSph) by lysosomal acid ceramidase (ACase)[106]. Toxicity of excessive GlcSph in GD patients is considered in various GD disease manifestations. The lipid has been implicated in osteopenia, the common reduced bone mineral density in GD patients, through impairing osteoblasts[107].

In addition, GlcSph is found to promote α-synuclein aggregation, a hallmark of Parkinson disease[108]. It has been proposed that antigenicity of GlcCer, and possibly GlcSph, underlies the common gammopathies in GD patients that can evolve into multiple myeloma[109]. In addition, GlcSph has been proposed to activate the complement cascade activation and associated local tissue inflammation[110,111]. GlcSph is also hypothesized to diminish the cerebral microvascular density in GD mice[112]. In line with signs and symptoms of GD, it has been earlier hypothesized that high concentrations of GlcSph promote lysis of red blood cells, impair cell fission during cytokinesis leading to multi-nucleated cells, damage specific neurons, interfere with growth and activate pro-inflammatory phospholipase A2[49].

The lipid-laden Gaucher cells are viable, alternatively activated macrophages that release specific proteins, leading to elevated plasma levels in symptomatic GD patients[113]. Such proteins, used as biomarkers of Gaucher cells, include the chitinase, chitotriosidase[114,115], the chemokine CCL18/PARC (Chemokine (C-C motif) ligand 18; Pulmonary and activation-regulated chemokine)[116] and a soluble fragment of glycoprotein nonmetastatic melanoma protein B (gpNMB)[117,118]. Levels of these proteins as well as GlcSph levels in plasma are employed as biomarkers to assist diagnosis of GD patients and monitor treatment[116,119,120].

Since Gaucher cells play a prominent role in many of the visceral symptoms of type 1 GD patients, rational therapies have been designed aiming to prevent and/ or correct the lipid-laden macrophages. The first effective treatment of type 1 GD has been enzyme replacement therapy (ERT) which is aimed to supplement the GCase-lacking macrophages by repeated intravenous enzyme infusion[121]. The infused recombinant GCase is modified with mannose-terminal N-glycans to ensure targeting to macrophages. ERT results in prominent reduction of excessive liver and spleen volumes and correction of haematological symptoms[122]. However, at present, ERT does not prevent neurological symptoms due to the inability of the enzyme to pass the blood brain barrier[123]. An alternative treatment is substrate reduction therapy (SRT), which is aimed to balance synthesis of GlcCer with the reduced GCase activity of GD patients[124,125]. Miglustat and Eliglustat are two approved oral inhibitors of GCS which have been used in the clinic for years to treat type 1 GD patients without major adverse effects[126-129]. Brain-permeable inhibitors of GCS are presently tested[130]. Other types of brain-permeable small compounds are also actively studied, including chemical chaperones for improved folding of mutant GCase in the ER also called pharmacological chaperone therapy (PCT). Ongoing studies with Ambroxol, a weak inhibitor of GCase, have revealed impressive reductions in spleen and liver volumes in treated type 1 GD patients as well as neurological improvements in type 3 GD patients[131,132]. Another approach investigated is enzyme enhancement therapy with small compounds (EET). An example is arimoclomol, a heat shock protein amplifier found to improve refolding, maturation and lysosomal activity of GCase in GD fibroblasts and neuronal cells[133].

Fabry disease – In 1898, two dermatologists, Johannes Fabry and William Anderson, independently published case reports of patients with characteristic skin lesions called angiokeratoma corporis diffusum[134]. It was soon realized that such individuals represent a distinct disease entity, now named Anderson-Fabry disease (AFD) or Fabry disease (FD). Presently, FD is thought to be the most common glycosphingolipidosis among Caucasians with a birth prevalence of at least 1 in 4000 in European populations[135]. FD is an X-linked disorder in man caused by deficiency of the lysosomal enzyme α-galactosidase A (α-GAL A; EC 3.2.1.22) encoded by the *GLA* gene (human locus Xq22)[134]. The gene encodes a 429 amino acid precursor that is processed to a 398 amino acid glycoprotein functioning as a homodimer[134]. The N-linked glycans of α-Gal A acquire mannose-6-phosphate moieties and are sorted to lysosomes through interaction with mannose-6-phosphate receptors. The enzyme α-GAL A is a retaining α-galactosidase belonging to the GH 27 family, with a catalytic $(\alpha/\beta)_8$ TIM barrel domain and Asp 170 acting as nucleophile and Asp 231 as acid/base[136]. A highly homologous α-N-actelygalactosaminidase (α-NAGAL; (EC 3.2.1.49) is encoded in mammals by the *NAGA* gene (human locus 22q13) due to an ancient gene duplication. The α-NAGAL enzyme hydrolyses terminal α-N-acetylgalactosyl moieties but shows minor α-galactosidase activity *in vitro*, while α-GAL A can only accommodate and hydrolyse α-galactose configured lipids[137,138]. Substitution of two specific amino acids of the α-NAGAL protein into the respective residue of α-GAL A, Ser 188 into glutamic acid (Glu) and Ala 191 into leucine (Leu), renders an enzyme (α-NAGAL^{EL}) with improved activity towards the primary substrate of α-GAL A, the glycosphingolipid globotriaosylceramide (Gb3)[137,138]. ABPs consisting of an α-galactosyl configured cyclophellitol aziridine, have been designed allowing labelling of α-GAL A and α-NAGAL enzymes from human, mouse and plant material[139,140].

In FD, glycosphingolipids accumulate with terminal α-galactosyl moieties in endothelial, perithelial and smooth muscle cells of the vascular system, as well as renal epithelial cells and cells of the autonomic nervous system[134]. No plasma protein biomarkers of storage cells have yet been identified for FD, contrary to GD[49]. The most prominent storage lipid is Gb3, also named ceramidetrihexoside (CTH), with lesser amounts of galabiosylceramide (Gb2) and blood group B, B1 and P1 antigens[141-144]. Characteristic disease manifestations of FD in males range from skin lesions, corneal opacity, neuropathic pain (acroparasthesias), heat intolerance, inability to sweat and micro-albuminuria, while later in life progressive kidney disease, cardiac symptoms and cerebrovascular disease (stroke) may develop[134]. Intriguingly, many female heterozygotes display attenuated forms of FD, although with a later onset than displayed in affected hemizygous males[145]. The severity of manifestations has been suggested to depend on the degree of inactivation of the normal X-chromosome. A variation of 25-75% in enzyme levels has been observed with random skewing of X-inactivation[145]. Two atypical FD phenotypes are discerned: a cardiac and a renal variant with symptoms restricted to a single organ[146,147]. The N215S α-GAL A substitution appears associated with the cardiac phenotype[148]. However, among the more than 300 reported mutations in the *GLA* gene, a large number is of unknown significance, being either disease-causing or a polymorphism[149]. Again, modifiers might play a role in FD manifestation. For example two polymorphisms in the *NOS3* gene, encoding eNOS, seem to influence cardiomyopathy[150].

Interestingly, GD and FD differ in severity of disease manifestation in relation to residual enzyme capacity. For example, almost complete GCase deficiency results in the collodion baby and marked reduction of the activity leads to acute neuronopathic GD type 2, while α-GAL A deficient FD males manifest symptoms surprisingly late in life. The correlation between Gb3-laden cells and clinical symptoms in FD patients is poor[49]. Therefore, it is not surprising that the present ERT treatments for FD did not prevent disease progression, despite the observed clearance of vascular endothelial Gb3 deposits[151,152]. The poor response of male classic FD patients can be partly ascribed to generation of neutralizing antibodies against the therapeutic enzyme[153-155].

The striking discrepancies between lipid storage cells, plasma Gb3 levels and clinical symptoms as well as the disappointing outcome of ERT, prompted a search for missing pathogenic factors in FD pathology. It was discovered that in FD the lipid Gb3 is deacylated by acid ceramidase to water-soluble globtriaosylsphingosine (lysoGb3), as also observed for GlcCer in GD[106,156]. LysoGb3 levels are generally over hundred-fold elevated in plasma of classic male FD patients. Classic FD females often show normal plasma Gb3 levels and hardly any endothelial Gb3 deposits, while abnormal high plasma lysoGb3 is detected, thereby assisting diagnosis[156,157]. Chronically elevated plasma lysoGb3 is thought to be toxic. Exposure of cultured smooth muscle cells (SMCs) to lysoGb3 at concentrations encountered in classic FD males promotes their proliferation and might contribute to the increased vessel wall thickness in FD patients and associated vasculopathy[158]. More recently, inhibition of eNOS by lysoGb3 at concentrations as occur in plasma of GD patients has been observed, rendering an explanation for the abnormalities in FD patients in nitrogen oxide, vital for normal vasculature biology[159]. In addition, excessive lysoGb3 is proposed to promote fibrosis and toxic effects towards podocytes and nociceptive neurons have been documented[160-162]. These findings might offer an explanation for the peripheral pain and renal complications in FD patients.

Farber disease – Lipogranulomatosis, also known as ceramidosis or Farber disease, is due to deficiency of the lysosomal acid ceramidase (AC; N-acylsphingosine deacylase; EC 3.5.1.23)[46]. The human *ASAH1* gene is located on the short arm of chromosome 8 (8p21.3-p.22). AC is synthetized as 50 kDa that, via autoproteolysis in endosomes and lysosomes, matures into 13 and 40 kDa α- and β-subunits, respectively[163,164]. The β-subunit has 5 N-linked oligosaccharide chains but the α-subunit is not glycosylated[165]. In AC, the nucleophilic thiol of Cys143 is exposed at the N-terminus of the β-subunit after the autoproteolytic cleavage of the precursor protein[163]. Recently, crystal structures of the proenzyme and autocleaved forms of mammalian AC enzymes were described[164,166]. These findings suggested a conformational change of AC upon autocleavage uncovering a narrow hydrophobic channel leading to the active site. This is thought to constrain the orientation of the ceramide lipid due to the location of the catalytic cysteine and oxyanion hole. Interestingly, it was found that head groups of larger sphingolipids cannot be fitted in the catalytic pocket in a similar manner as ceramide[164]. Based on the non-specific AC inhibitor Carmofur as scaffold, ABPs labelling the enzyme were designed[167]. Recently superior and specific ABPs reacting with AC have been designed and characterized[168].

Farber published the first case report in 1952 of a lipid metabolic disorder with lipogranulomas[169]. Farber disease results in tissue accumulation of ceramide and is typically characterized by the manifestation of subcutaneous skin nodules which progresses to joint stiffness and immobilization[170]. Severe cases develop neurological symptoms such as paralysis of the arms and legs (quadriplegia), seizures, loss of speech and involuntary muscle jerks (myoclonus)[171,172]. The involvement of neuronal dysfunction depends on the residual lysosomal ceramide turnover[170]. Farber disease patients without neurological involvement can be treated with allogeneic hematopoietic stem cell transplantation[170]. Of note, spinal muscular atrophy with myoclonic epilepsy (SMA-PME; OMIM #159950) is also caused by mutations in the *ASAH1* gene[173-175].

Glycosphingoid bases in glycosphingolipidoses – In recent years it has become apparent that the lysosomal AC plays a key role in several glycosphingolipidoses by mediating the deacylation of the primary accumulating GSL in the lysosome, thereby generating the respective glycosphingoid base[21,106]. This phenomenon is documented for Gaucher disease with GlcSph, Fabry disease with lysoGb3, but also Krabbe disease with galactosylsphingosine (GalSph) and GM2 gangliosidosis (β-hexosaminidase deficiency)[157,176,177]. Likewise, acid ceramidase seems responsible for deacylation of sphingomyelin to lysoSM in Niemann-Pick disease type A/B (acid sphingomyelinase deficiency) and Niemann-Pick disease type C[178]. The availability of (isotope-encoded) standards of the various sphingoid bases allows multiplex assays for various glycosphingolipidoses[179]. The marked increase of glycosphingoid bases assists diagnostics, monitoring disease progression and corrections by therapy. The presumed toxicity of GlcSph and lysoGb3 in GD and FD has been addressed above. A recently published double genetic mouse model of Krabbe disease and AC deficiency showed a less progressive phenotype compared to the Krabbe mouse model alone, with an increased life span, improved motor activity and only minor neuroinflammation, and suggests an acute toxicity of GalSph[180]. AC is envisioned as novel target for glycosphingoid base reduction therapy. However, the window for such type of intervention might be very small, due to ceramide accumulation and the induction of Farber-like symptoms by AC inhibition. The popular AC inhibitor Carmofur is notoriously aspecific, however more specific inhibitors of AC have been designed in recent years[168]. In conclusion, it is still unknown to which extent clinical symptoms of glycosphingolipidoses can be attributed to 'toxic' glycosphingoid bases, a possibility that deserves extensive investigation.

Table 1 | List of sphingolipidoses

Overview of various human sphingolipidoses with the defective protein, human gene name and accumulating material as well as reported mouse models and zebrafish models.

Disorder	Defective protein	Gene	Accumulating material	Mouse model	Zebrafish model
Gaucher disease	Glucocerebrosidase; GCase	*GBA*	GlcCer, GlcSph, LacCer, GlcChol	Mice KO: not viable *Mx1*-Cre-*LoxP*: white blood cell lineage, GD type 1[89] Nestin-flox/flox: neuronal stem cell lineage, GD type 2[90] K14-lnl/lnl: *GBA* KO in all tissues except skin, GD type 2[90]	*gba*: Complete KO[181,182] Zf GCase studied in **Ch. 2 & 3** Mutants studied in **Ch. 5-7**
Fabry disease	α-galactosidase A; α-Gal A	*GLA*	Gb3, lyso-Gb3, diGalCer, blood group B antigens	Mouse KO; only minor cardiorenal phenotype[183-185] Rat KO; cardiorenal phenotype and pain[186-188]	Not reported Zf α-Gal A studied in **Ch. 8**
Krabbe disease	Galactocerebrosidase	*GALC*	GalCer, GalSph	Naturally occurring Twitcher KO mouse[189] GALC/ACase: improved phenotype[180]	*galca/galcb*: morphant[190]
Schindler disease	α-N-acetyl-galactosaminidase; α-NAGAL	*NAGA*	Sialylated or asialo glycopeptides and GSLs	-	Not reported
Farber disease	Acid ceramidase; AC	*ASAH*	Ceramide	Mouse KO[191]	*asah1b*: morphant[174] *asah1a/asah1b*: KO, **Ch. 6**
Tay-Sachs disease	Hexosaminidase A	*HEXA*	GM2 ganglioside, GSLs and oligosaccharides	Reviewed in [192] Mouse KO: limited clinical signs	
Sandhoff disease	Hexosaminidase B	*HEXB*	GM2 ganglioside, GA2 glycolipid and oligosaccharides	Reviewed in [192] Mouse KO: [193]	*Hexb* KO[194]
Niemann-Pick disease type A & B	Acid sphingomyelinase; aSMase	*SMPD1*	SM, lyso-SM, lyso-509	aSMase mouse KO: [195]	Not reported
Niemann-Pick disease type C	NPC intracellular cholesterol transporter 1 and -2	*NPC1* *NPC2*	Cholesterol, sphingolipids, lyso-SM, lyso-509, GlcChol	Naturally occurring mouse model:[97,196]	*Npc1*: KO[197] Mutant made in **Ch. 4**

Zebrafish as vertebrate model organism in research

Zebrafish (*Danio rerio*) are common domesticated freshwater fish, but have emerged over the past decades into a popular vertebrate model organism for research, including vertebrate genetics, development, toxicology and human diseases[198].

The zebrafish is a teleost fish that is part of the Cyprinidae family, which also includes carps. They naturally occur in still waters in South Asia, however different environments have been reported including temperature ranges from 24-38 °C with no reported heat stress response[199]. Laboratory zebrafish are maintained according to standardized protocols, such as a fixed temperature of 28 °C, a controlled circadian rhythm, optimized water quality and feeding, in order to improve the health of the zebrafish and the amount and quality of the offspring. Breeding occurs at the onset of light: courtship by the fish is followed by egg-laying and sperm release, resulting in external fertilization. Embryonic development of zebrafish off-spring is fast at 28 °C with most major organ systems developed around 36 hours post-fertilization (hpf)[200]. The first cell cycles are rapid and rely on the maternally provided mRNA. The tenth cell cycle initiates the mid-blastula transition, when the cells of the embryo start transcribing and translating their own genome. A chorion protects the developing embryo from external factors and the fish hatches typically between 2 to 3 days post-fertilization (dpf). The swim bladder inflates around 3 to 4 dpf, which initiates upright swimming and feeding behaviour[200]. Larvae of 4 to 5 dpf have a functional digestive system, including intestine, liver, gallbladder and pancreas as well as intestinal microbiota, required to digest external food. The latter process is essential since the nutrients from the maternally deposited yolk are exhausted around 5 dpf[200]. Zebrafish typically become sexually mature around 10-12 weeks post-fertilization (wpf), with growth and sexual maturation depending on temperature, density and individual differences[198]. Zebrafish can live up to 3-4 years, however they start showing signs of aging around 2 years including a redundancy in the amount and the quality of the off-spring[201].

The cost-effective maintenance, fertility, *ex vivo* fertilization and rapid development are attractive features of zebrafish for use as vertebrate animal model. Moreover, the embryonic and larval zebrafish off-spring offers additional advantages such as ex-uteral development, transparency, small size and ease of genetic manipulation with techniques such as CRISPR/Cas9. Additionally, the zebrafish genome has been sequenced and more than 26.000 protein-coding genes are annotated. There is a high conservation of genes between humans and zebrafish with 70% of the human genes having at least one orthologue in the zebrafish genome. This percentage is even higher for genes implicated in human disorders, with approximately 82% of the human genes having at least one orthologue in the zebrafish genome[202]. However the evolutionary divergence between man and fish (± 450 million years) is larger than between man and rodents (± 40 million years)[203]. Moreover, the teleost genome has undergone an additional genome duplication, which means that a variety of genes have two copies in the zebrafish[204,205]. It is not always known whether one gene copy is redundant, degenerated as pseudo-gene, or both gene copies are functional and have undergone neofunctionalization, acquiring novel functional properties, or subfunctionalization in which the ancestral function is divided and each

paralog independently performs its respective function.

In the vertebrate fish, organs and tissues are present with analogous functions to those of mammals, including brain, heart, liver, kidney, pancreas, intestinal tract and spleen. Lacking are the typical mammalian organs such as lung, skin with a stratum corneum and mammary gland. Anatomical and physiological similarities and differences have been reviewed by Lieschke and Currie[206]. For the study of lysosomal disorders certain organ systems are noteworthy. Firstly, the liver contains hepatocytes, endothelial cells and bile duct epithelial cells, but Kupffer cells, specialized macrophages in the mammalian liver, seem absent in fish[207]. Since zebrafish lack bone marrow, the kidney interstitium acts as main site for haematopoiesis, consisting of the same cell types such as erythrocytes (but nucleated), neutrophils, eosinophils, lymphocytes and macrophages[206,208]. Finally, the basic structure of the central nervous system in the zebrafish brain is similar to mammals, although distinct brain areas, such as the telencephalon and tectum, display more pronounced differences[206,209]. In contrast to mammals, zebrafish lack a distinct neocortex and neuronal populations in the midbrain are absent[210]. Dopamineric neurons in the posterior tuberculum have been suggested as functional homolog of the mammalian cluster in the substantia nigra, while the fish optic tectum is suggested to perform vision-related functions performed by the neocortex of mammals[211].

Zebrafish models of lysosomal storage disorders

Various inherited and acquired human disorders are studied in zebrafish larvae or adults, ranging from cancer, inflammation, haematological, neurological and lysosomal disorders. Genetic disorders can be generated using injection of antisense morpholino oligonucleotides, resulting in a transient knockdown, also called 'morphants'[212]. The antisense morpholino, injected in fertilized eggs, targets both maternal and zygotic transcripts, however off-target effects are problematic and the induced phenotype is transient and can only be studied for a limited period of time[212]. A stable knockout is rapidly becoming the standard, assisted by the development of convenient gene-editing techniques such as CRISPR/Cas9 technology, that also allow examination of mutant adult zebrafish. Obviously, generating a stable knockout line is more labour intensive and requires more time. Published morphant and stable knockout models in zebrafish of lysosomal sphingolipid storage disorders are discussed below and summarized in **Table 1**.

Gaucher disease: *gba* – Three zebrafish models of GD were generated by means of a complete knockout of *gba*[181,213,214]. The fish are viable in contrast to complete GCase deficient mice and humans, which die immediately after birth due to trans-epidermal water loss[33]. Marked accumulation of GlcSph and GlcCer develops soon in zebrafish larvae (5 dpf) as well as microglial activation[181,214]. Around 8 wpf the phenotype worsens with an apparent curved back and reduced motor activity at 12 wpf. Infiltration of Gaucher-like cells occurs in brain and liver, with extensive microglia invasion and autophagy in the brain. Dopaminergic neuronal cell count is reduced in the caudal hypothalamus and posterior tuberculum in the presence of ubiquitin-positive, intra-neuronal inclusions in the larger hindbrain neurons[181].

In another study it was noted that GCase deficiency in zebrafish is associated with defective canonical Wnt signalling, a reduction in bone mineralization and impaired osteoblast differentiation[213]. Chapters 5, 6 and 7 of this thesis describe *gba* knockout zebrafish as larvae and adults, recapitulating findings observed in the earlier generated models.

Non-lysosomal GBA2 – The non-lysosomal β-glucosidase, GBA2, has so far been limited studied in zebrafish. An antisense morpholino knockdown of *gba2* was found to develop abnormal motor behaviour and impaired axonal outgrowths[215]. In chapters 5 and 7, own findings with *gba2* knockout zebrafish are described for larvae and adult fish, as well as concomitant knockouts of *gba* and *gba2*.

Acid ceramidase deficiency: *asah1b* – Zebrafish possess two acid ceramidase genes: *asah1a* and *asah1b* (Chapter 6 of this thesis). Previous research using transient *asah1b* morphant showed reduction of axonal outgrowths accompanied by cellular death in the spinal cord[174].

Krabbe disease: *galc* – The zebrafish genome encodes two orthologues of human galactocerebrosidase (GALC). Both Galca and Galcb have high amino acid similarity to the human protein (both 61% identity) with a fully conserved active site with topologically conserved positions of residues required for substrate specificity[190]. A transient knockdown of both zebrafish *galc* co-orthologues, using morpholinos, was found to develop a neurological phenotype[190]. Of note, *galca*/*galcb* double morphants had partial Galc activity and no elevated GalSph levels were detected.

Tay-Sachs/Sandhoff disease: *Hexb* – The dimeric β-hexosaminidase enzyme mediates hydrolysis of terminal β-N-acetylgalactosamine or β-N-acetylglucosamine residues of glycolipids and oligosaccharides[216]. Mutations in the gene encoding subunit HEXB leads to the lysosomal disorder Sandhoff disease, while a defect in HEXA leads to Tay-Sachs which has clinically identical symptoms as Sandhoff disease[216,217]. Patients and mouse models accumulate the ganglioside GM2 or oligosaccharides with terminal β-N-acetylglucosamine moieties. This metabolite accumulation is accompanied by severe neuropathology with neuronal loss and infiltration of microglia[216-218]. A single *hexb* gene is present in the zebrafish genome and the encoding Hexb protein shares high amino acid identity with the human orthologue (approximately 64%)[194]. Hexb deficient 5 dpf larvae show abnormal lysosomes in microglia and radial glia, accompanied by reduced locomotor activity. Adult *hexb*$^{-/-}$ zebrafish accumulate oligosaccharides in the brain, but no neurological symptoms were observed[194].

Fabry disease: *gla* – Although the zebrafish genome contains an orthologue of α-GAL A, no transient knockdown or stable knockout of *gla* have been generated to study FD in the fish. A plausible explanation for this is the lack of globosides in fish due to the absence of the enzyme generating Gb3, Gb3 synthase also called A4GALT, which is the subject of investigations described in chapter 7.

Niemann-Pick type C – Low-density lipoprotein particles (LDL) end up in lysosomes following endocytosis where cholesterol esters are processed by the lysosomal acid lipase to cholesterol. Next, free cholesterol binds to NPC2, a small soluble glycoprotein that transfers it to the large, transmembrane protein NPC1, which mediates export from the lysosome[219]. Niemann-Pick disease type C (NPC) is caused by mutations in the genes encoding NPC1, encompassing approximately 95% of the clinical cases, or encoding NPC2. The impaired efflux of cholesterol from lysosome causes secondary deficiencies in activities of acid sphingomyelinase and GCase in lysosomes. As a result, cholesterol, sphingomyelin and GlcCer accumulate in liver, spleen and brain of NPC patients[97]. The clinical presentation of NPC is heterogeneous and includes visceral symptoms and progressive neurodegeneration with an onset in infancy or childhood, although lethal, prenatal onset has been described in severe NPC cases[43,220].

Zebrafish Npc1 shows 60% of identity with the polypeptide sequence of human NPC1 and shares the NPC-like region (NPCL) as well as the SSD motif crucial for correct functioning of NPC1[221]. *Npc1* knockouts have been generated. The mutant fish are significantly smaller than *npc1*$^{+/+}$ siblings and die sooner[197,222]. Mutants show an early-onset liver defect, with large vacuole-like structures and accumulation of unesterified cholesterol. Later in life, *npc1* mutant fish present hepatosplenomegaly, a severe liver defect and develop discoordinated swimming suggesting a CNS defect[197,222]. The abnormal phenotype starts with an impaired ability to maintain an upright position during swimming, which progresses rapidly into rapid spinning and tumbling movements[197]. Neurological abnormalities are observed, including axonal spheroids in the hindbrain and disorganized Purkinje neurons in the cerebellum, corresponding to findings in patients and mammalian models[196,223,224].

Tools to study zebrafish

Several standard histopathological, biochemical and analytical techniques can be used to study zebrafish (**Table 2** and **Figure 2**). Additional techniques exploit the beneficial features of zebrafish such as extra-uterine development and transparency. In the past years, numerous zebrafish lines have been generated expressing fluorescent reporters that either mark specific cell-types, sub-cellular compartments or under regulation of an inducible promoter[225,226]. These lines are also summarized on zfin.org[227]. Generation of a knockout in zebrafish using CRISPR/Cas9 or overexpression of a given gene is experimentally straightforward and can be achieved in a matter of months. The small size of larvae make them fit in 96-well plates, allowing easy administration of small molecules by addition to the swimming water, often in combination with phenotypic high-throughput screenings[228].

For the investigation of lysosomal glycosidases, several tools are available. Enzyme activities can be evaluated by studying the activity towards commercial fluorogenic substrates, such as 4-methylumbelliferone (4MU)-sugars, or nitrobenzoxadiazole (NBD)-labelled lipids. A novel tool-box comprise cyclophellitol activity-based probes (ABPs) that label specific retaining glycosidases[229]. As discussed above, the first ABP was developed for GCase[56]. This approach has been extended to other glycosidases with tuning of the configuration of the broad-spectrum aziridine-cyclophellitol scaffold to match the configuration of the target lysosomal retaining glycosidase (**Table 2**). The mechanism-based binding of the ABP allows profiling of glycosidases from man to plant and bacterial origin[57,140]. ABPs are particularly useful for zebrafish materials as limited zebrafish specific antibodies are available and antibodies reacting with protein from man or mouse origin generally do not cross-react. Of note, activity-based and affinity-based probes are also available for other enzyme classes including kinases[230], serine hydrolases[231,232] and cathepsins[233]. In principle, ABPs could be employed to visualize active enzymes in transparent zebrafish larvae. However, further optimization of probes and methodology is however still required to reach better window between enzyme labelling and non-specific labelling stemming from unreacted probe.

Cyclophellitol analogues can also be employed to specifically inactivate glycosidases of interest. It has been recently demonstrated that GCase in zebrafish can be inactivated on demand by exposure of the animals to ME656, a cyclophellitol with a bulky hydrophobic group at C8[54]. Pharmacological inactivation of GCase or other glycosidases nicely complements the study of genetic knockouts of these glycosidases[214].

Finally, ultrasensitive LC-MS/MS techniques are available to quantitatively measure glycosphingolipid abnormalities[103,179]. These protocols are exploited, optimized and broadened in this thesis in order to measure a broad range of sphingolipids and GSL in the limited zebrafish material. In particular, the impressive sensitivity of GSL detection enabled monitoring of individual zebrafish larvae[214].

Table 2 | Toolbox

Overview of genetic-, biochemical-, pathological- and *in vivo* tools used to study zebrafish larvae or adults.

Type of tool			Chapter
Genetic-			
	Genome-editing	CRISPR/Cas9 and Tol2 transposase	**Chapter 4**
	Transgenic zebrafish	Expression of reporter tag using (sub)cellular promoter	
Biochemical-			
	Fluorogenic substrates		**Chapter 2, 3, 8**
	Activity-based probes	Cyclophellitol-configured molecule modified with a reporter tag	**Chapter 3, 5**
		GCase[56]	**Chapter 3, 5**
		β-glucosidases[57]	**Chapter 3**
		α-glucosidases[234]	-
		β-galactosidases[235]	-
		α-galactosidases[139]	**Chapter 8**
		α-fucosidases[236]	-
		β-Glucuronidases[237]	-
		α-Iduronidases[238]	**Chapter 3**
		β-mannosidases	-
		α-Mannosidases	-
	Mass spectrometry	Quantitative detection of sphingolipids and GSLs (LC-MS/MS)	**Chapter 2-8**
	Protein expression	Immunoblotting using antibodies	**Chapter 6 & 7**
	RNA expression	RT-qPCR using specific primers, RNA sequencing	**Chapter 6 &7**
Pathological-			
	Histology	Standard stains for microscopic anatomy For example: haematoxylin & eosin	**Chapter 6 & 7**
	Immunohistochemistry	Visualize (sub)cellular localization of target protein	-
	***In situ* hybridization**	Visualize (sub)cellular localization of target mRNA	-
In vivo-			
	Transgenic lines	See above	-
	Life stains	Visualize specific cells or compartments *in vivo* and in real-time. For example: lysotracker to visualize lysosomes	-
	Phenotype	Morphology & behavioural studies	**Chapter 6, 7**
	Drug administration	Emerge ZF in swimming water with small molecule	**Chapter 3, 5**

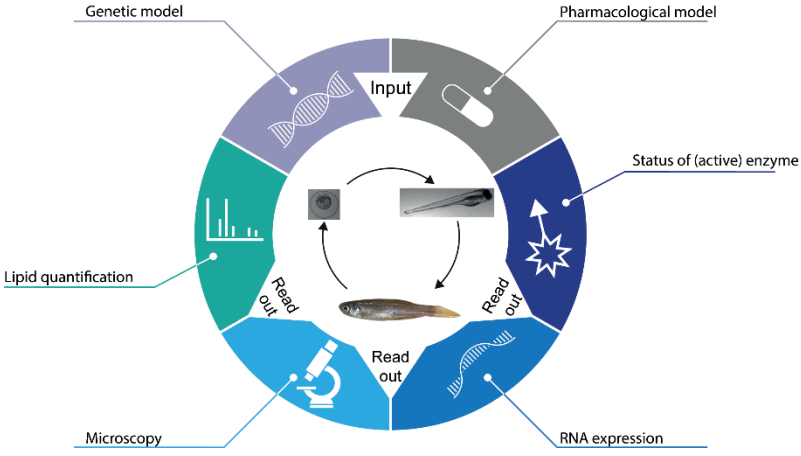


Figure 2 | Zebrafish as research model for glycosphingolipidoses

Goals of the thesis investigations

The primary goal of this thesis has been to use zebrafish as vertebrate animal model for the investigation of lysosomal storage disorders, in particular Gaucher disease (GD). Several biochemical and genetic techniques have been used and optimized in order to study the catalytic features of zebrafish glucocerebrosidase (GCase) and to investigate the consequences of its defect in zebrafish larvae and adults. In addition, the impact of two other enzymes, non-lysosomal GBA2 and lysosomal acid ceramidase, on GCase-deficient zebrafish received attention.

In **chapter 2**, GCase enzymes from different species (man, zebrafish, frog and turtle) are compared in enzymatic features by means of biochemical assays and *in silico* structure analysis using homology modelling of human GCase. Subtle differences were noted among the different GCase enzymes. Zebrafish and frog GCase required no additives for *in vitro* hydrolysis contrary to the human enzyme, while the fish enzyme was not able to perform transglucosylation in contrast to frog and human enzyme. Over-expression of the GCase enzymes from the different species corrected increased GlcCer and GlcSph levels in human GCase deficient cells.

Chapter 3 reports on the pharmacological inactivation of GCase in zebrafish and compares conduritol B epoxide, cyclophellitol and newly synthesized cyclophellitol derivatives in selectivity towards GCase and other retaining glycosidases. Adult zebrafish allowed the assessment of brain permeability of the new superior GCase inhibitors.

In **chapter 4** a detailed protocol for the generation of knockouts in zebrafish by CRISPR/Cas9 technology is described, including notes and considerations. The chapter also includes information on the genomic location and obtained mutations in the genes of *gba1*, *gba2*, *gpnmb*, *asah1a*, *asah1b*, *npc1* and *cln8*. Finally, proof-of-concept is provided for the introduction of exogenous target DNA sequences in the zebrafish genome using the Tol2 transposase technique.

Chapter 5 evaluates the metabolism of glucosylceramide (GlcCer) in zebrafish larvae (up to 5 days post-fertilization, 5dpf) that are single or double knockouts of *gba1* and *gba2*. Adaptations in lipid metabolism as the result of enzyme deficiencies were monitored by LC-MS/MS methods. Recapitulating GD, g*ba1* knockout larvae massively generate glucosylsphingosine (GlcSph). In addition, the feasibility of pharmacological modulation of GlcCer metabolism in individual 5 dpf larvae is reported.

In **chapter 6** the detrimental role of excessive GlcSph in GD pathology is evaluated. A specific acid ceramidase (Asah1b) is reported which is responsible for conversion of GlcCer to GlcSph. The other acid ceramidase (Asah1a) is not able to generate GlcSph, however its presence prohibits ceramide accumulation. Comparing *gba1* knockout zebrafish with excessive GlcSph to *gba1:asah1b* knockout zebrafish without GlcSph, rendered new insight in the toxicity of the sphingoid base and the role of storage cells, neuroinflammation and neurodegeneration in the pathophysiology of GD.

Chapter 7 reports an overview and comparison of biochemical and pathological findings of adult zebrafish with a knockout of *gba1*, *gba2* and *asah1b* and combinations thereof. In addition mutant zebrafish at different developmental stages are examined with emphases on morphology and accompanying lipid-, protein and RNA abnormalities. The investigation sheds further light on the effects of non-functional Gba2 or acid ceramidase during GCase deficiency in zebrafish.

Chapter 8 concerns the potential use of zebrafish to study a different lysosomal storage disorder, Fabry disease. The presence of α-Gal A and α-Nagal enzymes in zebrafish cells, larvae and organs is described.

Chapter 9 discusses the obtained results of the undertaken investigations and describes future prospects of research. Opportunities are described including established and novel techniques to address other common clinical manifestations of GD patients and study their underlying molecular mechanisms. In addition, anatomical and physiological similarities and differences of the zebrafish compared to mammals are discussed in order to put the use of the zebrafish GD model in perspective.

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