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

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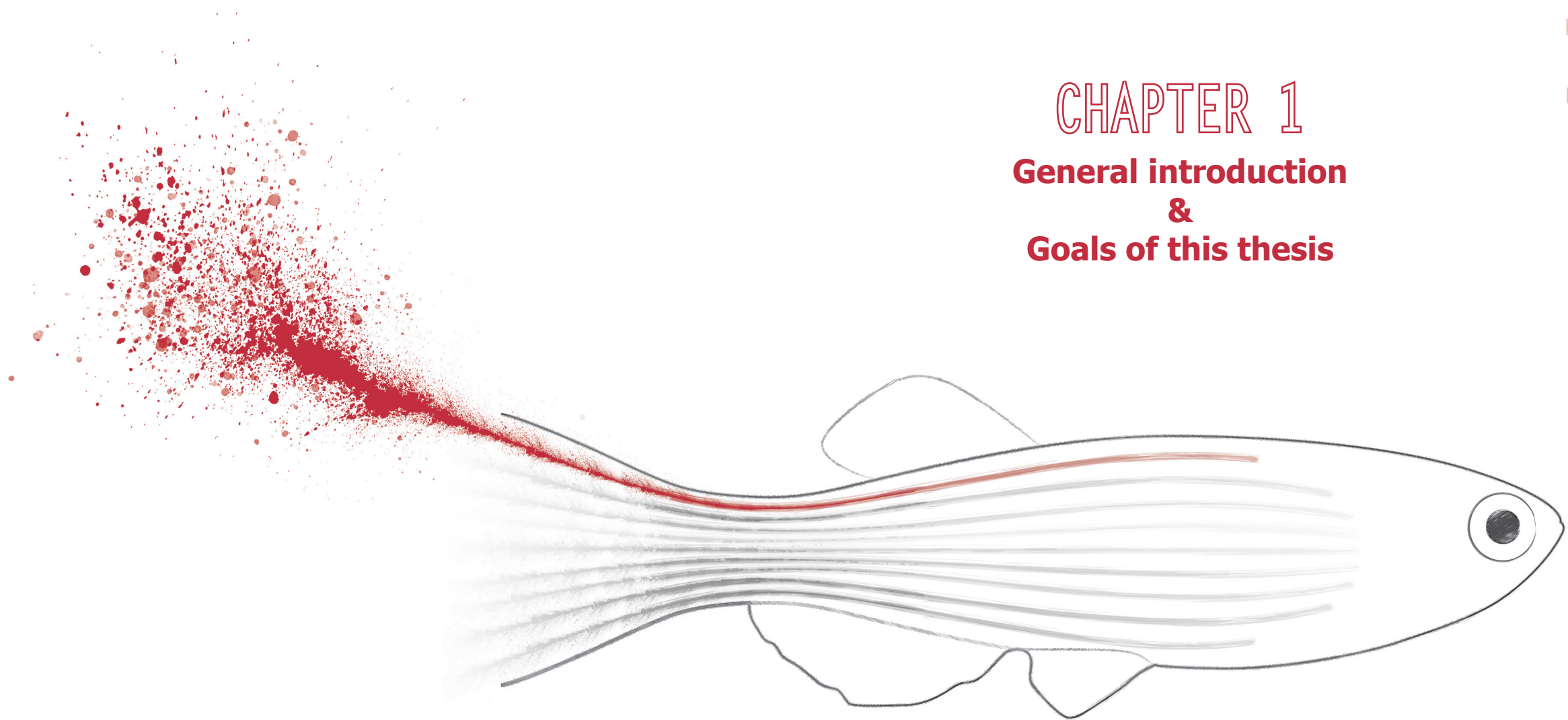
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CHAPTER 1

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 sphingomyelin¹. 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 ceramide²⁻⁴. 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 structure³.

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 reaction 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 ceramide³. 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 selectivity of the different CerS enzymes³. 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-dihydroceramide⁷. 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 liver⁸. Whereas CerS1 is highly expressed in neurons of the mouse brain, CerS2 expression is high in oligodendrocytes and Schwann cells. Moreover, the transient increase in CerS2 mRNA during the period of active myelination suggests a role for longer fatty acids in myelin sphingolipids⁹. 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 lipids¹⁰⁻¹². CerS4 is expressed ubiquitously at low levels and generates ceramides with C18-22 fatty acids³. CerS5 and CerS6 both make ceramides with C16 fatty acid, while CerS6 also utilizes myristoyl-CoA to make C14-dihydroceramide⁷. The majority of the membrane sphingolipids are composed of saturated fatty acids³.

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 manner¹³. 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 membranes³.

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 moieties². 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 sponge¹⁴⁻¹⁶. 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 myelin¹⁷. 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 membranes³. Newly formed GlcCer can be metabolized by the non-lysosomal membrane-associated glucosylceramidase GBA2¹⁸⁻²⁰, but most of it is relocated to the luminal leaflet of trans-Golgi membranes. Here, β 4-galactosyltransferase (B4GALT5) mediates the transfer of a β -linked galactose to GlcCer generating lactosylceramide (LacCer), a Gal β (1,4)Glc β (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 rafts²²⁻²⁵.

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 breakdown³². 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 maturation¹⁰.

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 optima². 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}.

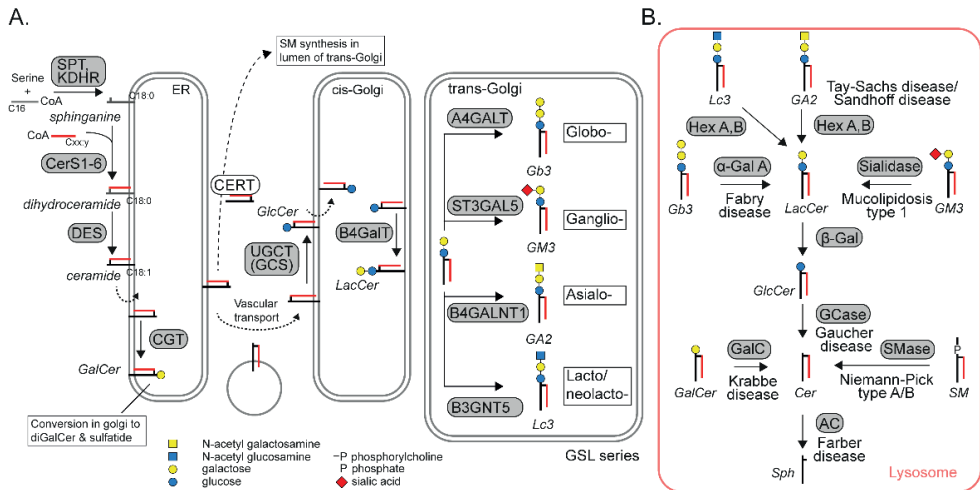


Figure 1 | Glycosphingolipid metabolism

Schematic representation of (glyco)sphingolipid biosynthesis and catabolism. Enzymes are depicted in grey. **(A)** (Glyco)sphingolipids (given in *italics*) 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 births⁴³. 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 population⁴³. 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 cells⁴⁸⁻⁵⁰. 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 residue⁵³. 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 cyclophellitol⁵⁴. 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 -glucosidase⁵⁵. 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 GCase⁵⁶. 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 GBA3⁵⁷.

GCase is transported to lysosomes independent of the mannose-6-phosphate receptor pathway unlike other lysosomal hydrolyses⁵⁸. Instead, lysosomal integral membrane

protein 2 (LIMP2) binds newly formed GCase in the ER and transports it to lysosomes⁵⁹⁻⁶¹. 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 sufficient⁶⁴. 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 heterogeneous⁴⁸. 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 fractures⁶⁶⁻⁶⁸. 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 patients⁷⁰. 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 GCase⁷². 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 neuropathology⁷⁴. 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 modifiers⁸¹⁻⁸³. Another candidate modifier, based on a GWAS study in patients homozygous for N370S mutation, is the ER transmembrane protein CLN8⁸⁴. CLN8 has recently been shown to be involved in the transport of newly formed lysosomal enzymes between ER and Golgi apparatus⁸⁵.

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 patients⁸⁶. 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-Inl/Inl*⁹⁰). Defective GCase in the hematopoietic cell lineage is sufficient to induce formation of Gaucher cells, hepatosplenomegaly and haematological symptoms⁹¹, however for skeletal deterioration a combined GCase deficiency in hematopoietic and mesenchymal cell lineages is needed⁸⁹. 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 unclear⁹³⁻⁹⁵.

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 Bodies⁹⁶. 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 deficiency¹⁰⁰. 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 gangliosides¹⁰¹. The elevated concentrations of GM3 in GD patients are accompanied by insulin insensitivity, however without overt hyperglycemia¹⁰². Secondly, the β -glucosidases GCase and GBA2 can act as transglucosylase and form glucosylated cholesterol (GlcChol) from GlcCer and a cholesterol acceptor¹⁰³⁻¹⁰⁵. 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 observed¹⁰³. 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 osteoblasts¹⁰⁷.

In addition, GlcSph is found to promote α -synuclein aggregation, a hallmark of Parkinson disease¹⁰⁸. It has been proposed that antigenicity of GlcCer, and possibly GlcSph, underlies the common gammopathies in GD patients that can evolve into multiple myeloma¹⁰⁹. 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 mice¹¹². 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 A2⁴⁹.

The lipid-laden Gaucher cells are viable, alternatively activated macrophages that release specific proteins, leading to elevated plasma levels in symptomatic GD patients¹¹³. 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 infusion¹²¹. 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 symptoms¹²². However, at present, ERT does not prevent neurological symptoms due to the inability of the enzyme to pass the blood brain barrier¹²³. 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 effects¹²⁶⁻¹²⁹. Brain-permeable inhibitors of GCS are presently tested¹³⁰. 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 cells¹³³.

Fabry disease – In 1898, two dermatologists, Johannes Fabry and William Anderson, independently published case reports of patients with characteristic skin lesions called angiokeratoma corporis diffusum¹³⁴. 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 populations¹³⁵. 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 homodimer¹³⁴. 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/base¹³⁶. A highly homologous α -N-acetylgalactosaminidase (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 system¹³⁴. No plasma protein biomarkers of storage cells have yet been identified for FD, contrary to GD⁴⁹. The most prominent storage lipid is Gb3, also named ceramidetrihexoside (CTH), with lesser amounts of galabiosylceramide (Gb2) and blood group B, B1 and P1 antigens¹⁴¹⁻¹⁴⁴. 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 develop¹³⁴. Intriguingly, many female heterozygotes display attenuated forms of FD, although with a later onset than displayed in affected hemizygous males¹⁴⁵. 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-inactivation¹⁴⁵. 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 phenotype¹⁴⁸. 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 polymorphism¹⁴⁹. Again, modifiers might play a role in FD manifestation. For example two polymorphisms in the *NOS3* gene, encoding eNOS, seem to influence cardiomyopathy¹⁵⁰.

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 poor⁴⁹. 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 enzyme¹⁵³⁻¹⁵⁵.

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 globotriaosylsphingosine (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 vasculopathy¹⁵⁸. 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 biology¹⁵⁹. In addition, excessive lysoGb3 is proposed to promote fibrosis and toxic effects towards podocytes and nociceptive neurons have been documented¹⁶⁰⁻¹⁶². 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 synthesized 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 glycosylated¹⁶⁵. In AC, the nucleophilic thiol of Cys143 is exposed at the N-terminus of the β -subunit after the autoproteolytic cleavage of the precursor protein¹⁶³. 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 ceramide¹⁶⁴. Based on the non-specific AC inhibitor Carmofur as scaffold, ABPs labelling the enzyme were designed¹⁶⁷. Recently superior and specific ABPs reacting with AC have been designed and characterized¹⁶⁸.

Farber published the first case report in 1952 of a lipid metabolic disorder with lipogranulomas¹⁶⁹. 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 immobilization¹⁷⁰. 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 turnover¹⁷⁰. Farber disease patients without neurological involvement can be treated with allogeneic hematopoietic stem cell transplantation¹⁷⁰. Of note, spinal muscular atrophy with myoclonic epilepsy (SMA-PME; OMIM #159950) is also caused by mutations in the *ASAH1* gene¹⁷³⁻¹⁷⁵.

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 C¹⁷⁸. The availability of (isotope-encoded) standards of the various sphingoid bases allows multiplex assays for various glycosphingolipidoses¹⁷⁹. 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 GalSph¹⁸⁰. 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 years¹⁶⁸. 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	<i>GBA</i>	GlcCer, GlcSph, LacCer, GlcChol	Mice KO: not viable <i>Mx1-Cre-LoxP</i> : white blood cell lineage, GD type 1 ⁸⁹ Nestin-flox/flox: neuronal stem cell lineage, GD type 2 ⁹⁰ K14-Inl/Inl: <i>GBA</i> KO in all tissues except skin, GD type 2 ⁹⁰	<i>gba</i> : 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	<i>GLA</i>	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	<i>GALC</i>	GalCer, GalSph	Naturally occurring Twitcher KO mouse ¹⁸⁹ GALC/ACase: improved phenotype ¹⁸⁰	<i>galca/galc</i> : morphant ¹⁹⁰
Schindler disease	α -N-acetyl-galactosaminidase; α -NAGAL	<i>NAGA</i>	Sialylated or asialo glycopeptides and GSLs	-	Not reported
Farber disease	Acid ceramidase; AC	<i>ASAH</i>	Ceramide	Mouse KO ¹⁹¹	<i>asah1b</i> : morphant ¹⁷⁴ <i>asah1a/asah1b</i> : KO, Ch. 6
Tay-Sachs disease	Hexosaminidase A	<i>HEXA</i>	GM2 ganglioside, GSLs and oligosaccharides	Reviewed in ¹⁹² Mouse KO: limited clinical signs	
Sandhoff disease	Hexosaminidase B	<i>HEXB</i>	GM2 ganglioside, GA2 glycolipid and oligosaccharides	Reviewed in ¹⁹² Mouse KO: ¹⁹³	<i>Hexb</i> KO ¹⁹⁴
Niemann-Pick disease type A & B	Acid sphingomyelinase; aSMase	<i>SMPD1</i>	SM, lyso-SM, lyso-509	aSMase mouse KO: ¹⁹⁵	Not reported
Niemann-Pick disease type C	NPC intracellular cholesterol transporter 1 and -2	<i>NPC1</i> <i>NPC2</i>	Cholesterol, sphingolipids, lyso-SM, lyso-509, GlcChol	Naturally occurring mouse model. ^{97,196}	<i>Npc1</i> : 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 diseases¹⁹⁸.

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 response¹⁹⁹. 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 behaviour²⁰⁰. 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 dpf²⁰⁰. Zebrafish typically become sexually mature around 10-12 weeks post-fertilization (wpf), with growth and sexual maturation depending on temperature, density and individual differences¹⁹⁸. 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-spring²⁰¹.

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-uterine 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 genome²⁰². However the evolutionary divergence between man and fish (\pm 450 million years) is larger than between man and rodents (\pm 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 Currie²⁰⁶. 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 fish²⁰⁷. 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 absent²¹⁰. Dopaminergic 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 mammals²¹¹.

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 time²¹². 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 loss³³. 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 neurons¹⁸¹.

Chapter 1

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 differentiation²¹³. 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 outgrowths²¹⁵. 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 cord¹⁷⁴.

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 specificity¹⁹⁰. A transient knockdown of both zebrafish *galc* co-orthologues, using morpholinos, was found to develop a neurological phenotype¹⁹⁰. 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 oligosaccharides²¹⁶. 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 microglia²¹⁶⁻²¹⁸. 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 observed¹⁹⁴.

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 lysosome²¹⁹. 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 patients⁹⁷. 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 NPC1²²¹. *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 disorganized 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 movements¹⁹⁷. 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.org²²⁷. 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 screenings²²⁸.

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 glycosidases²²⁹. As discussed above, the first ABP was developed for GCase⁵⁶. 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 kinases²³⁰, serine hydrolases^{231,232} and cathepsins²³³. 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 C8⁵⁴. Pharmacological inactivation of GCase or other glycosidases nicely complements the study of genetic knockouts of these glycosidases²¹⁴.

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 larvae²¹⁴.

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
			Chapter 3, 5
			Chapter 3
			Chapter 3
			Chapter 8
			Chapter 3
			Chapter 3
	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 <i>in vivo</i> 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

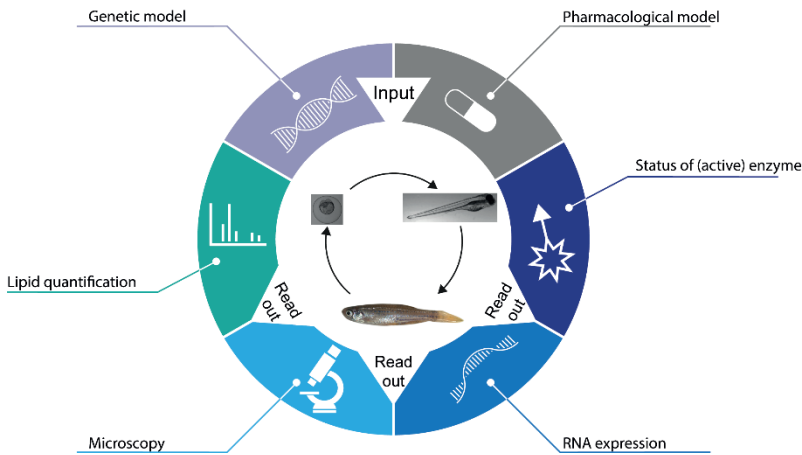


Figure 2 | Zebrafish as research model for glycosphingolipidoses

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, *gba1* 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.

References

1. Thudichum J.L.W. (1884) A treatise on the chemical constitution of the brain, (London: Bailliere Tindall and Cox).
2. Wennekens T., van den Berg R.J., Boot R.G., van der Marel G.A., Overkleeft H.S. and Aerts J.M. (2009) Glycosphingolipids--nature, function, and pharmacological modulation. *Angewandte Chemie* **48**, 8848-8869.
3. Merrill A.H., Jr. (2011) Sphingolipid and glycosphingolipid metabolic pathways in the era of sphingolipidomics. *Chem Rev* **111**, 6387-6422.
4. Carter H.E., Glick F.J., Norris W.P. and Phillips G.E. (1947) Biochemistry of the Sphingolipides .3. Structure of Sphingosine. *J Biol Chem* **170**, 285-294.
5. Kraut R. (2011) Roles of sphingolipids in Drosophila development and disease. *J Neurochem* **116**, 764-778.
6. Cabasso O., Paul S., Dorot O., Maor G., Krivoruk O., Pasmanik-Chor M.,... and Horowitz M. (2019) Drosophila melanogaster Mutated in its GBA1b Ortholog Recapitulates Neuronopathic Gaucher Disease. *J Clin Med* **8**.
7. Levy M. and Futerman A.H. (2010) Mammalian ceramide synthases. *IUBMB Life* **62**, 347-356.
8. Laviad E.L., Albee L., Pankova-Kholmyansky I., Epstein S., Park H., Merrill A.H., Jr. and Futerman A.H. (2008) Characterization of ceramide synthase 2: tissue distribution, substrate specificity, and inhibition by sphingosine 1-phosphate. *J Biol Chem* **283**, 5677-5684.
9. Becker I., Wang-Eckhardt L., Yaghootfam A., Gieselmann V. and Eckhardt M. (2008) Differential expression of (dihydro)ceramide synthases in mouse brain: oligodendrocyte-specific expression of CerS2/Lass2. *Histochem Cell Biol* **129**, 233-241.
10. Sandhoff R. (2010) Very long chain sphingolipids: tissue expression, function and synthesis. *FEBS Lett* **584**, 1907-1913.
11. Mizutani Y., Kihara A., Chiba H., Tojo H. and Igarashi Y. (2008) 2-Hydroxy-ceramide synthesis by ceramide synthase family: enzymatic basis for the preference of FA chain length. *J Lipid Res* **49**, 2356-2364.
12. Rabionet M., Gorgas K. and Sandhoff R. (2014) Ceramide synthesis in the epidermis. *Biochimica et biophysica acta* **1841**, 422-434.
13. Hanada K., Kumagai K., Yasuda S., Miura Y., Kawano M., Fukasawa M. and Nishijima M. (2003) Molecular machinery for non-vesicular trafficking of ceramide. *Nature* **426**, 803-809.
14. Wieland Brown L.C., Penaranda C., Kashyap P.C., Williams B.B., Clardy J., Kronenberg M.,... and Fischbach M.A. (2013) Production of alpha-galactosylceramide by a prominent member of the human gut microbiota. *Plos Biol* **11**, e1001610.
15. Natori T., Koezuka Y. and Higa T. (1993) Agelasphins, Novel Alpha-Galactosylceramides from the Marine Sponge Agelas-Mauritianus. *Tetrahedron Lett* **34**, 5591-5592.
16. von Gerichten J., Schlosser K., Lamprecht D., Morace I., Eckhardt M., Wachten D.,... and Sandhoff R. (2017) Diastereomer-specific quantification of bioactive hexosylceramides from bacteria and mammals. *J Lipid Res* **58**, 1247-1258.
17. Sprong H., Kruitthof B., Leijendekker R., Slot J.W., van Meer G. and van der Sluijs P. (1998) UDP-galactose:ceramide galactosyltransferase is a class I integral membrane protein of the endoplasmic reticulum. *J Biol Chem* **273**, 25880-25888.
18. van Weely S., Brandsma M., Strijland A., Tager J.M. and Aerts J.M. (1993) Demonstration of the existence of a second, non-lysosomal glucocerebrosidase that is not deficient in Gaucher disease. *Biochimica et biophysica acta* **1181**, 55-62.
19. Yildiz Y., Matern H., Thompson B., Allegood J.C., Warren R.L., Ramirez D.M.,... and Russell D.W. (2006) Mutation of beta-glucosidase 2 causes glycolipid storage disease and impaired male fertility. *The Journal of clinical investigation* **116**, 2985-2994.
20. Boot R.G., Verhoek M., Donker-Koopman W., Strijland A., van Marle J., Overkleeft H.S.,... and Aerts J.M. (2007) Identification of the non-lysosomal glucosylceramidase as beta-glucosidase 2. *J Biol Chem* **282**, 1305-1312.
21. Aerts J., Kuo C.L., Lelieveld L.T., Boer D.E.C., van der Lienden M.J.C., Overkleeft H.S. and Artola M. (2019) Glycosphingolipids and lysosomal storage disorders as illustrated by gaucher disease. *Current opinion in chemical biology* **53**, 204-215.
22. Munro S. (2003) Lipid rafts: elusive or illusive? *Cell* **115**, 377-388.
23. Mukherjee S. and Maxfield F.R. (2004) Membrane domains. *Annu Rev Cell Dev Biol* **20**, 839-866.
24. Sonnino S. and Prinetti A. (2013) Membrane domains and the "lipid raft" concept. *Curr Med Chem* **20**, 4-21.
25. Lingwood D. and Simons K. (2010) Lipid rafts as a membrane-organizing principle. *Science* **327**, 46-50.
26. Kolter T. and Sandhoff K. (2010) Lysosomal degradation of membrane lipids. *FEBS Lett* **584**, 1700-1712.
27. Aerts J., Artola M., van Eijk M., Ferraz M.J. and Boot R.G. (2019) Glycosphingolipids and Infection. Potential New Therapeutic Avenues. *Front Cell Dev Biol* **7**, 324.
28. Langeveld M. and Aerts J.M. (2009) Glycosphingolipids and insulin resistance. *Prog Lipid Res* **48**, 196-205.

29. Nakayama H., Nagafuku M., Suzuki A., Iwabuchi K. and Inokuchi J.I. (2018) The regulatory roles of glycosphingolipid-enriched lipid rafts in immune systems. *FEBS Lett* **592**, 3921-3942.
30. Garcia-Ruiz C., Morales A. and Fernandez-Checa J.C. (2015) Glycosphingolipids and cell death: one aim, many ways. *Apoptosis* **20**, 607-620.
31. Yu R.K., Tsai Y.T., Ariga T. and Yanagisawa M. (2011) Structures, biosynthesis, and functions of gangliosides--an overview. *J Oleo Sci* **60**, 537-544.
32. Schmitt S., Castelvetti L.C. and Simons M. (2015) Metabolism and functions of lipids in myelin. *Biochimica et biophysica acta* **1851**, 999-1005.
33. Holleran W.M., Ginns E.I., Menon G.K., Grundmann J.U., Fartasch M., McKinney C.E.,... and Sidransky E. (1994) Consequences of beta-glucocerebrosidase deficiency in epidermis. Ultrastructure and permeability barrier alterations in Gaucher disease. *The Journal of clinical investigation* **93**, 1756-1764.
34. Doering T., Holleran W.M., Potratz A., Vielhaber G., Elias P.M., Suzuki K. and Sandhoff K. (1999) Sphingolipid activator proteins are required for epidermal permeability barrier formation. *J Biol Chem* **274**, 11038-11045.
35. Gillard B.K., Thurmon L.T. and Marcus D.M. (1993) Variable subcellular localization of glycosphingolipids. *Glycobiology* **3**, 57-67.
36. Contreras F.X., Ernst A.M., Haberkant P., Bjorkholm P., Lindahl E., Gonen B.,... and Brugger B. (2012) Molecular recognition of a single sphingolipid species by a protein's transmembrane domain. *Nature* **481**, 525-529.
37. Taha T.A., Mullen T.D. and Obeid L.M. (2006) A house divided: ceramide, sphingosine, and sphingosine-1-phosphate in programmed cell death. *Biochimica et biophysica acta* **1758**, 2027-2036.
38. Spiegel S. and Milstien S. (2003) Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol* **4**, 397-407.
39. Rohrbach T., Maceyka M. and Spiegel S. (2017) Sphingosine kinase and sphingosine-1-phosphate in liver pathobiology. *Crit Rev Biochem Mol Biol* **52**, 543-553.
40. Maceyka M., Harikumar K.B., Milstien S. and Spiegel S. (2012) Sphingosine-1-phosphate signaling and its role in disease. *Trends Cell Biol* **22**, 50-60.
41. Coant N., Sakamoto W., Mao C. and Hannun Y.A. (2017) Ceramidases, roles in sphingolipid metabolism and in health and disease. *Adv Biol Regul* **63**, 122-131.
42. Breiden B. and Sandhoff K. (2018) Ganglioside Metabolism and Its Inherited Diseases. *Methods Mol Biol* **1804**, 97-141.
43. Platt F.M., d'Azzo A., Davidson B.L., Neufeld E.F. and Tift C.J. (2018) Lysosomal storage diseases. *Nat Rev Dis Primers* **4**, 27.
44. Tytki-Szymanska A., Czartoryska B., Vanier M.T., Poorthuis B.J., Groener J.A., Lugowska A.,... and Jurkiewicz E. (2007) Non-neuronopathic Gaucher disease due to saposin C deficiency. *Clin Genet* **72**, 538-542.
45. Tytki-Szymanska A., Groener J.E., Kaminski M.L., Lugowska A., Jurkiewicz E. and Czartoryska B. (2011) Gaucher disease due to saposin C deficiency, previously described as non-neuronopathic form--no positive effects after 2-years of miglustat therapy. *Mol Genet Metab* **104**, 627-630.
46. Park J.H. and Schuchman E.H. (2006) Acid ceramidase and human disease. *Biochimica et biophysica acta* **1758**, 2133-2138.
47. Ballabio A. and Gieselmann V. (2009) Lysosomal disorders: from storage to cellular damage. *Biochimica et biophysica acta* **1793**, 684-696.
48. Beutler E. and Grabowski G.A. (2001) Gaucher disease. In *The Metabolic and Molecular Bases of Inherited disease*, Volume III, 8th Edition, C.R. Scriver, A.L. Beaudet, W.S. Sly and D. Valle, eds. (New York: McGraw-Hill), pp. 3635-3668.
49. Ferraz M.J., Kallemeijn W.W., Mirzaian M., Herrera Moro D., Marques A., Wisse P.,... and Aerts J.M. (2014) Gaucher disease and Fabry disease: new markers and insights in pathophysiology for two distinct glycosphingolipidoses. *Biochimica et biophysica acta* **1841**, 811-825.
50. Brady R.O., Kanfer J.N., Bradley R.M. and Shapiro D. (1966) Demonstration of a deficiency of glucocerebrosidase-cleaving enzyme in Gaucher's disease. *The Journal of clinical investigation* **45**, 1112-1115.
51. Dvir H., Harel M., McCarthy A.A., Toker L., Silman I., Futerman A.H. and Sussman J.L. (2003) X-ray structure of human acid-beta-glucosidase, the defective enzyme in Gaucher disease. *EMBO Rep* **4**, 704-709.
52. Brumshtein B., Greenblatt H.M., Butters T.D., Shaaltiel Y., Aviezer D., Silman I.,... and Sussman J.L. (2007) Crystal structures of complexes of N-butyl- and N-nonyl-deoxyojirimycin bound to acid beta-glucosidase: insights into the mechanism of chemical chaperone action in Gaucher disease. *J Biol Chem* **282**, 29052-29058.
53. Kallemeijn W.W., Witte M.D., Voorn-Brouwer T.M., Walvoort M.T., Li K.Y., Codee J.D.,... and Aerts J.M. (2014) A sensitive gel-based method combining distinct cyclophellitol-based probes for the identification of acid/base residues in human retaining beta-glucosidases. *J Biol Chem* **289**, 35351-35362.
54. Artola M., Kuo C.L., Lelieveld L.T., Rowland R.J., van der Marel G.A., Codee J.D.C.,... and Overkleeft H.S. (2019) Functionalized Cyclophellitols Are Selective Glucocerebrosidase Inhibitors and Induce a Bona Fide

- Neuropathic Gaucher Model in Zebrafish. *Journal of the American Chemical Society* **141**, 4214-4218.
55. Kuo C.L., Kallemeijn W.W., Lelieveld L.T., Mirzaian M., Zoutendijk I., Vardi A.,... and Artola M. (2019) In vivo inactivation of glycosidases by conduritol B epoxide and cyclophellitol as revealed by activity-based protein profiling. *FEBS J* **286**, 584-600.
 56. Witte M.D., Kallemeijn W.W., Aten J., Li K.Y., Strijland A., Donker-Koopman W.E.,... and Aerts J.M. (2010) Ultrasensitive in situ visualization of active glucocerebrosidase molecules. *Nature chemical biology* **6**, 907-913.
 57. Kallemeijn W.W., Li K.Y., Witte M.D., Marques A.R., Aten J., Scheij S.,... and Overkleeft H.S. (2012) Novel activity-based probes for broad-spectrum profiling of retaining beta-exoglucosidases in situ and in vivo. *Angewandte Chemie* **51**, 12529-12533.
 58. Aerts J.M., Schram A.W., Strijland A., van Weely S., Jonsson L.M., Tager J.M.,... and Murray G.J. (1988) Glucocerebrosidase, a lysosomal enzyme that does not undergo oligosaccharide phosphorylation. *Biochimica et biophysica acta* **964**, 303-308.
 59. Rijnboutt S., Aerts H.M., Geuze H.J., Tager J.M. and Strous G.J. (1991) Mannose 6-phosphate-independent membrane association of cathepsin D, glucocerebrosidase, and sphingolipid-activating protein in HepG2 cells. *J Biol Chem* **266**, 4862-4868.
 60. Reczek D., Schwake M., Schroder J., Hughes H., Blanz J., Jin X.,... and Saftig P. (2007) LIMP-2 is a receptor for lysosomal mannose-6-phosphate-independent targeting of beta-glucocerebrosidase. *Cell* **131**, 770-783.
 61. Saftig P. and Klumperman J. (2009) Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function. *Nat Rev Mol Cell Biol* **10**, 623-635.
 62. Berkovic S.F., Dibbens L.M., Oshlack A., Silver J.D., Katerelos M., Vears D.F.,... and Bahlo M. (2008) Array-based gene discovery with three unrelated subjects shows SCARB2/LIMP-2 deficiency causes myoclonus epilepsy and glomerulosclerosis. *Am J Hum Genet* **82**, 673-684.
 63. Dibbens L.M., Michelucci R., Gambardella A., Andermann F., Rubboli G., Bayly M.A.,... and Berkovic S.F. (2009) SCARB2 mutations in progressive myoclonus epilepsy (PME) without renal failure. *Ann Neurol* **66**, 532-536.
 64. Gaspar P., Kallemeijn W.W., Strijland A., Scheij S., Van Eijk M., Aten J.,... and Aerts J.M. (2014) Action myoclonus-renal failure syndrome: diagnostic applications of activity-based probes and lipid analysis. *J Lipid Res* **55**, 138-145.
 65. Vaccaro A.M., Motta M., Tatti M., Scarpa S., Masuelli L., Bhat M.,... and Salvio R. (2010) Saposin C mutations in Gaucher disease patients resulting in lysosomal lipid accumulation, saposin C deficiency, but normal prosaposin processing and sorting. *Human molecular genetics* **19**, 2987-2997.
 66. Hollak C.E., Levi M., Berends F., Aerts J.M. and van Oers M.H. (1997) Coagulation abnormalities in type 1 Gaucher disease are due to low-grade activation and can be partly restored by enzyme supplementation therapy. *Br J Haematol* **96**, 470-476.
 67. Wenstrup R.J., Roca-Espiau M., Weinreb N.J. and Bembi B. (2002) Skeletal aspects of Gaucher disease: a review. *Br J Radiol* **75 Suppl 1**, A2-12.
 68. Stirnemann J., Belmatoug N., Camou F., Serratrice C., Froissart R., Caillaud C.,... and Berger M.G. (2017) A Review of Gaucher Disease Pathophysiology, Clinical Presentation and Treatments. *Int J Mol Sci* **18**.
 69. Wong K., Sidransky E., Verma A., Mixon T., Sandberg G.D., Wakefield L.K.,... and Schiffmann R. (2004) Neuropathology provides clues to the pathophysiology of Gaucher disease. *Mol Genet Metab* **82**, 192-207.
 70. Sidransky E. (2004) Gaucher disease: complexity in a "simple" disorder. *Mol Genet Metab* **83**, 6-15.
 71. Stone D.L., Carey W.F., Christodoulou J., Sillence D., Nelson P., Callahan M.,... and Sidransky E. (2000) Type 2 Gaucher disease: the collodion baby phenotype revisited. *Arch Dis Child Fetal Neonatal Ed* **82**, F163-166.
 72. Sidransky E., Sherer D.M. and Ginns E.I. (1992) Gaucher disease in the neonate: a distinct Gaucher phenotype is analogous to a mouse model created by targeted disruption of the glucocerebrosidase gene. *Pediatr Res* **32**, 494-498.
 73. van Smeden J., Dijkhoff I.M., Helder R.W.J., Al-Khakany H., Boer D.E.C., Schreuder A.,... and Bouwstra J.A. (2017) In situ visualization of glucocerebrosidase in human skin tissue: zymography versus activity-based probe labeling. *J Lipid Res* **58**, 2299-2309.
 74. Boot R.G., Hollak C.E., Verhoek M., Sloof P., Poorthuis B.J., Kleijer W.J.,... and van Weely S. (1997) Glucocerebrosidase genotype of Gaucher patients in The Netherlands: limitations in prognostic value. *Hum Mutat* **10**, 348-358.
 75. Abrahamov A., Elstein D., Gross-Tsur V., Farber B., Glaser Y., Hadas-Halpern I.,... and Zimran A. (1995) Gaucher's disease variant characterised by progressive calcification of heart valves and unique genotype. *Lancet* **346**, 1000-1003.
 76. Chabas A., Cormand B., Grinberg D., Burguera J.M., Balcells S., Merino J.L.,... and Vilageliu L. (1995) Unusual expression of Gaucher's disease: cardiovascular calcifications in three sibs homozygous for the D409H mutation. *J Med Genet* **32**, 740-742.

77. Biegstraaten M., van Schaik I.N., Aerts J.M., Langeveld M., Mannens M.M., Bour L.J.,... and Hollak C.E. (2011) A monozygotic twin pair with highly discordant Gaucher phenotypes. *Blood Cells Mol Dis* **46**, 39-41.
78. Lachmann R.H., Grant I.R., Halsall D. and Cox T.M. (2004) Twin pairs showing discordance of phenotype in adult Gaucher's disease. *QJM* **97**, 199-204.
79. Goker-Alpan O., Hruska K.S., Orvisky E., Kishnani P.S., Stubblefield B.K., Schiffmann R. and Sidransky E. (2005) Divergent phenotypes in Gaucher disease implicate the role of modifiers. *J Med Genet* **42**, e37.
80. Lo S.M., Choi M., Liu J., Jain D., Boot R.G., Kallemijn W.W.,... and Mistry P.K. (2012) Phenotype diversity in type 1 Gaucher disease: discovering the genetic basis of Gaucher disease/hematologic malignancy phenotype by individual genome analysis. *Blood* **119**, 4731-4740.
81. Velayati A., DePaolo J., Gupta N., Choi J.H., Moaven N., Westbroek W.,... and Sidransky E. (2011) A mutation in SCARB2 is a modifier in Gaucher disease. *Hum Mutat* **32**, 1232-1238.
82. Alfonso P., Navascues J., Navarro S., Medina P., Bolado-Carrancio A., Andreu V.,... and Giraldo P. (2013) Characterization of variants in the glucosylceramide synthase gene and their association with type 1 Gaucher disease severity. *Hum Mutat* **34**, 1396-1403.
83. Siebert M., Westbroek W., Chen Y.C., Moaven N., Li Y., Velayati A.,... and Sidransky E. (2014) Identification of miRNAs that modulate glucocerebrosidase activity in Gaucher disease cells. *RNA Biol* **11**, 1291-1300.
84. Zhang C.K., Stein P.B., Liu J., Wang Z., Yang R., Cho J.H.,... and Mistry P.K. (2012) Genome-wide association study of N370S homozygous Gaucher disease reveals the candidacy of CLN8 gene as a genetic modifier contributing to extreme phenotypic variation. *Am J Hematol* **87**, 377-383.
85. di Ronza A., Bajaj L., Sharma J., Sanagasetti D., Lotfi P., Adamski C.J.,... and Sardiello M. (2018) CLN8 is an endoplasmic reticulum cargo receptor that regulates lysosome biogenesis. *Nat Cell Biol* **20**, 1370-1377.
86. Farfel-Becker T., Vitner E.B. and Futerman A.H. (2011) Animal models for Gaucher disease research. *Dis Model Mech* **4**, 746-752.
87. Xu Y.H., Quinn B., Witte D. and Grabowski G.A. (2003) Viable mouse models of acid beta-glucosidase deficiency: the defect in Gaucher disease. *Am J Pathol* **163**, 2093-2101.
88. Liu Y., Suzuki K., Reed J.D., Grinberg A., Westphal H., Hoffmann A.,... and Proia R.L. (1998) Mice with type 2 and 3 Gaucher disease point mutations generated by a single insertion mutagenesis procedure. *Proc Natl Acad Sci U S A* **95**, 2503-2508.
89. Mistry P.K., Liu J., Yang M., Nottoli T., McGrath J., Jain D.,... and Zaidi M. (2010) Glucocerebrosidase gene-deficient mouse recapitulates Gaucher disease displaying cellular and molecular dysregulation beyond the macrophage. *Proc Natl Acad Sci U S A* **107**, 19473-19478.
90. Enquist I.B., Lo Bianco C., Ooka A., Nilsson E., Mansson J.E., Ehinger M.,... and Karlsson S. (2007) Murine models of acute neuronopathic Gaucher disease. *Proc Natl Acad Sci U S A* **104**, 17483-17488.
91. Dahl M., Doyle A., Olsson K., Mansson J.E., Marques A.R.A., Mirzaian M.,... and Karlsson S. (2015) Lentiviral gene therapy using cellular promoters cures type 1 Gaucher disease in mice. *Mol Ther* **23**, 835-844.
92. Vardi A., Zigdon H., Meshcheriakova A., Klein A.D., Yaacobi C., Eilam R.,... and Futerman A.H. (2016) Delineating pathological pathways in a chemically induced mouse model of Gaucher disease. *J Pathol* **239**, 496-509.
93. Farfel-Becker T., Vitner E.B., Pressey S.N., Eilam R., Cooper J.D. and Futerman A.H. (2011) Spatial and temporal correlation between neuron loss and neuroinflammation in a mouse model of neuronopathic Gaucher disease. *Human molecular genetics* **20**, 1375-1386.
94. Vitner E.B., Farfel-Becker T., Eilam R., Biton I. and Futerman A.H. (2012) Contribution of brain inflammation to neuronal cell death in neuronopathic forms of Gaucher's disease. *Brain* **135**, 1724-1735.
95. Farfel-Becker T., Vitner E.B., Kelly S.L., Bame J.R., Duan J., Shinder V.,... and Futerman A.H. (2014) Neuronal accumulation of glucosylceramide in a mouse model of neuronopathic Gaucher disease leads to neurodegeneration. *Human molecular genetics* **23**, 843-854.
96. Herrera Moro Chao D., Kallemijn W.W., Marques A.R., Orre M., Ottenhoff R., van Roomen C.,... and Aerts J.M. (2015) Visualization of Active Glucocerebrosidase in Rodent Brain with High Spatial Resolution following In Situ Labeling with Fluorescent Activity Based Probes. *PLoS one* **10**, e0138107.
97. Marques A.R., Aten J., Ottenhoff R., van Roomen C.P., Herrera Moro D., Claessen N.,... and Aerts J.M. (2015) Reducing GBA2 Activity Ameliorates Neuropathology in Niemann-Pick Type C Mice. *PLoS one* **10**, e0135889.
98. Tsuang D., Leverenz J.B., Lopez O.L., Hamilton R.L., Bennett D.A., Schneider J.A.,... and Zabetian C.P. (2012) GBA mutations increase risk for Lewy body disease with and without Alzheimer disease pathology. *Neurology* **79**, 1944-1950.
99. Sidransky E., Nalls M.A., Aasly J.O., Aharon-Peretz J., Annesi G., Barbosa E.R.,... and Ziegler S.G. (2009) Multicenter analysis of glucocerebrosidase mutations in Parkinson's disease. *N Engl J Med* **361**, 1651-1661.
100. Aerts J.M., Ferraz M.J., Mirzaian M., Gaspar P., Oussoren S.V., Wisse P.,... and Marques A.R.A. (2017) Lysosomal Storage Diseases. For Better or Worse: Adapting to Defective Lysosomal Glycosphingolipid Breakdown. *In eLS*. (John Wiley & Sons, Ltd), pp. 1-13.

Chapter 1

101. Ghauharali-van der Vlugt K., Langeveld M., Poppema A., Kuiper S., Hollak C.E., Aerts J.M. and Groener J.E. (2008) Prominent increase in plasma ganglioside GM3 is associated with clinical manifestations of type I Gaucher disease. *Clin Chim Acta* **389**, 109-113.
102. Langeveld M., Ghauharali K.J., Sauerwein H.P., Ackermans M.T., Groener J.E., Hollak C.E.,... and Serlie M.J. (2008) Type I Gaucher disease, a glycosphingolipid storage disorder, is associated with insulin resistance. *J Clin Endocrinol Metab* **93**, 845-851.
103. Marques A.R., Mirzaian M., Akiyama H., Wisse P., Ferraz M.J., Gaspar P.,... and Aerts J.M. (2016) Glucosylated cholesterol in mammalian cells and tissues: formation and degradation by multiple cellular beta-glucosidases. *J Lipid Res* **57**, 451-463.
104. Akiyama H. and Hirabayashi Y. (2017) A novel function for glucocerebrosidase as a regulator of sterylglucoside metabolism. *Biochim Biophys Acta Gen Subj* **1861**, 2507-2514.
105. Akiyama H., Kobayashi S., Hirabayashi Y. and Murakami-Murofushi K. (2013) Cholesterol glucosylation is catalyzed by transglucosylation reaction of beta-glucosidase 1. *Biochem Biophys Res Commun* **441**, 838-843.
106. Ferraz M.J., Marques A.R., Appelman M.D., Verhoek M., Strijland A., Mirzaian M.,... and Aerts J.M. (2016) Lysosomal glycosphingolipid catabolism by acid ceramidase: formation of glycosphingoid bases during deficiency of glycosidases. *FEBS Lett* **590**, 716-725.
107. Reed M.C., Schiffer C., Heales S., Mehta A.B. and Hughes D.A. (2018) Impact of sphingolipids on osteoblast and osteoclast activity in Gaucher disease. *Mol Genet Metab* **124**, 278-286.
108. Taguchi Y.V., Liu J., Ruan J., Pacheco J., Zhang X., Abbasi J.,... and Chandra S.S. (2017) Glucosylsphingosine Promotes alpha-Synuclein Pathology in Mutant GBA-Associated Parkinson's Disease. *J Neurosci* **37**, 9617-9631.
109. Nair S., Branagan A.R., Liu J., Boddupalli C.S., Mistry P.K. and Dhodapkar M.V. (2016) Clonal Immunoglobulin against Lysolipids in the Origin of Myeloma. *N Engl J Med* **374**, 555-561.
110. Pandey M.K., Burrow T.A., Rani R., Martin L.J., Witte D., Setchell K.D.,... and Grabowski G.A. (2017) Complement drives glucosylceramide accumulation and tissue inflammation in Gaucher disease. *Nature* **543**, 108-112.
111. Pandey M.K., Grabowski G.A. and Kohl J. (2018) An unexpected player in Gaucher disease: The multiple roles of complement in disease development. *Semin Immunol* **37**, 30-42.
112. Smith N.J., Fuller M., Saville J.T. and Cox T.M. (2018) Reduced cerebral vascularization in experimental neuronopathic Gaucher disease. *J Pathol* **244**, 120-128.
113. Boven L.A., van Meurs M., Boot R.G., Mehta A., Boon L., Aerts J.M. and Laman J.D. (2004) Gaucher cells demonstrate a distinct macrophage phenotype and resemble alternatively activated macrophages. *Am J Clin Pathol* **122**, 359-369.
114. Hollak C.E., van Weely S., van Oers M.H. and Aerts J.M. (1994) Marked elevation of plasma chitotriosidase activity. A novel hallmark of Gaucher disease. *The Journal of clinical investigation* **93**, 1288-1292.
115. Boot R.G., Renkema G.H., Strijland A., van Zonneveld A.J. and Aerts J.M. (1995) Cloning of a cDNA encoding chitotriosidase, a human chitinase produced by macrophages. *J Biol Chem* **270**, 26252-26256.
116. Deegan P.B., Moran M.T., McFarlane I., Schofield J.P., Boot R.G., Aerts J.M. and Cox T.M. (2005) Clinical evaluation of chemokine and enzymatic biomarkers of Gaucher disease. *Blood Cells Mol Dis* **35**, 259-267.
117. Kramer G., Wegdam W., Donker-Koopman W., Ottenhoff R., Gaspar P., Verhoek M.,... and van Eijk M. (2016) Elevation of glycoprotein nonmetastatic melanoma protein B in type 1 Gaucher disease patients and mouse models. *FEBS Open Bio* **6**, 902-913.
118. Murugesan V., Liu J., Yang R., Lin H., Lischuk A., Pastores G.,... and Mistry P.K. (2018) Validating glycoprotein non-metastatic melanoma B (gpNMB, osteoactivin), a new biomarker of Gaucher disease. *Blood Cells Mol Dis* **68**, 47-53.
119. Dekker N., van Dussen L., Hollak C.E., Overkleeft H., Scheij S., Ghauharali K.,... and Aerts J.M. (2011) Elevated plasma glucosylsphingosine in Gaucher disease: relation to phenotype, storage cell markers, and therapeutic response. *Blood* **118**, e118-127.
120. van Dussen L., Hendriks E.J., Groener J.E., Boot R.G., Hollak C.E. and Aerts J.M. (2014) Value of plasma chitotriosidase to assess non-neuronopathic Gaucher disease severity and progression in the era of enzyme replacement therapy. *J Inher Metab Dis* **37**, 991-1001.
121. Brady R.O. (2003) Enzyme replacement therapy: conception, chaos and culmination. *Philos Trans R Soc Lond B Biol Sci* **358**, 915-919.
122. Barton N.W., Furbish F.S., Murray G.J., Garfield M. and Brady R.O. (1990) Therapeutic response to intravenous infusions of glucocerebrosidase in a patient with Gaucher disease. *Proc Natl Acad Sci U S A* **87**, 1913-1916.
123. Sechi A., Deroma L., Dardis A., Ciana G., Bertin N., Concolino D.,... and Bembi B. (2014) Long term effects of enzyme replacement therapy in an Italian cohort of type 3 Gaucher patients. *Mol Genet Metab* **113**, 213-218.

124. Aerts J.M., Hollak C.E., Boot R.G., Groener J.E. and Maas M. (2006) Substrate reduction therapy of glycosphingolipid storage disorders. *J Inher Metab Dis* **29**, 449-456.
125. Platt F.M., Jeyakumar M., Andersson U., Priestman D.A., Dwek R.A., Butters T.D.,... and Zimran A. (2001) Inhibition of substrate synthesis as a strategy for glycolipid lysosomal storage disease therapy. *J Inher Metab Dis* **24**, 275-290.
126. Giraldo P., Andrade-Campos M., Alfonso P., Irun P., Atutxa K., Acedo A.,... and Pocovi M. (2018) Twelve years of experience with miglustat in the treatment of type 1 Gaucher disease: The Spanish ZAGAL project. *Blood Cells Mol Dis* **68**, 173-179.
127. Cox T.M., Drelichman G., Cravo R., Balwani M., Burrow T.A., Martins A.M.,... and Peterschmitt M.J. (2017) Eliglustat maintains long-term clinical stability in patients with Gaucher disease type 1 stabilized on enzyme therapy. *Blood* **129**, 2375-2383.
128. Peterschmitt M.J., Freisens S., Underhill L.H., Foster M.C., Lewis G. and Gaemers S.J.M. (2019) Long-term adverse event profile from four completed trials of oral eliglustat in adults with Gaucher disease type 1. *Orphanet J Rare Dis* **14**, 128.
129. Lukina E., Watman N., Dragosky M., Lau H., Avila Arreguin E., Rosenbaum H.,... and Peterschmitt M.J. (2019) Outcomes after 8 years of eliglustat therapy for Gaucher disease type 1: Final results from the Phase 2 trial. *Am J Hematol* **94**, 29-38.
130. Shayman J.A. and Larsen S.D. (2014) The development and use of small molecule inhibitors of glycosphingolipid metabolism for lysosomal storage diseases. *J Lipid Res* **55**, 1215-1225.
131. Maegawa G.H., Tropak M.B., Buttner J.D., Rigat B.A., Fuller M., Pandit D.,... and Mahuran D.J. (2009) Identification and characterization of ambroxol as an enzyme enhancement agent for Gaucher disease. *J Biol Chem* **284**, 23502-23516.
132. Narita A., Shirai K., Itamura S., Matsuda A., Ishihara A., Matsushita K.,... and Suzuki Y. (2016) Ambroxol chaperone therapy for neuronopathic Gaucher disease: A pilot study. *Ann Clin Transl Neurol* **3**, 200-215.
133. Fog C.K., Zago P., Malini E., Solanko L.M., Peruzzo P., Bornaes C.,... and Kirkegaard T. (2018) The heat shock protein amplifier arimoclomol improves refolding, maturation and lysosomal activity of glucocerebrosidase. *EBioMedicine* **38**, 142-153.
134. Desnick R.J., Ioannou Y. and Eng C.M. (2001) α -Galactosidase A deficiency: Fabry disease. In *The metabolic and Molecular Bases of Inherited Disease*, C.R. Scriver, A.L. Beaudet, W.S. Sly and D. Valle, eds. (New York: McGraw-Hill), pp. 3733-3744.
135. Spada M., Pagliardini S., Yasuda M., Tükel T., Thiagarajan G., Sakuraba H.,... and Desnick R.J. (2006) High incidence of later-onset fabry disease revealed by newborn screening. *Am J Hum Genet* **79**, 31-40.
136. Garman S.C. and Garboczi D.N. (2004) The molecular defect leading to Fabry disease: structure of human alpha-galactosidase. *J Mol Biol* **337**, 319-335.
137. Tajima Y., Kawashima I., Tsukimura T., Sugawara K., Kuroda M., Suzuki T.,... and Sakuraba H. (2009) Use of a modified alpha-N-acetylgalactosaminidase in the development of enzyme replacement therapy for Fabry disease. *Am J Hum Genet* **85**, 569-580.
138. Kytidou K., Beenakker T.J.M., Westerhof L.B., Hokke C.H., Moolenaar G.F., Goosen N.,... and Aerts J. (2017) Human Alpha Galactosidases Transiently Produced in Nicotiana benthamiana Leaves: New Insights in Substrate Specificities with Relevance for Fabry Disease. *Front Plant Sci* **8**, 1026.
139. Willems L.I., Beenakker T.J., Murray B., Scheij S., Kallemeijn W.W., Boot R.G.,... and Overkleeft H.S. (2014) Potent and selective activity-based probes for GH27 human retaining alpha-galactosidases. *Journal of the American Chemical Society* **136**, 11622-11625.
140. Kytidou K., Beekwilder J., Artola M., van Meel E., Wilbers R.H.P., Moolenaar G.F.,... and Aerts J. (2018) Nicotiana benthamiana alpha-galactosidase A1.1 can functionally complement human alpha-galactosidase A deficiency associated with Fabry disease. *J Biol Chem* **293**, 10042-10058.
141. Sweeley C.C. and Klionsky B. (1963) Fabry's Disease: Classification as a Sphingolipidosis and Partial Characterization of a Novel Glycolipid. *J Biol Chem* **238**, 3148-3150.
142. Rybova J., Kuchar L., Hulkova H., Asfaw B., Dobrovolny R., Sikora J.,... and Ledvinova J. (2018) Specific storage of glycoconjugates with terminal alpha-galactosyl moieties in the exocrine pancreas of Fabry disease patients with blood group B. *Glycobiology* **28**, 382-391.
143. Kuchar L., Faltyskova H., Krasny L., Dobrovolny R., Hulkova H., Ledvinova J.,... and Havlicek V. (2015) Fabry disease: renal sphingolipid distribution in the alpha-Gal A knockout mouse model by mass spectrometric and immunohistochemical imaging. *Anal Bioanal Chem* **407**, 2283-2291.
144. Asfaw B., Ledvinova J., Dobrovolny R., Bakker H.D., Desnick R.J., van Diggelen O.P.,... and Schindler D. (2002) Defects in degradation of blood group A and B glycosphingolipids in Schindler and Fabry diseases. *J Lipid Res* **43**, 1096-1104.

145. Deegan P.B., Bahner F., Barba M., Hughes D.A. and Beck M. (2006) Fabry disease in females: clinical characteristics and effects of enzyme replacement therapy. *In Fabry Disease: Perspectives from 5 Years of FOS*, A. Mehta, M. Beck and G. Sunder-Plassmann, eds. (Oxford).
146. Lin H.Y., Chong K.W., Hsu J.H., Yu H.C., Shih C.C., Huang C.H.,... and Niu D.M. (2009) High incidence of the cardiac variant of Fabry disease revealed by newborn screening in the Taiwan Chinese population. *Circ Cardiovasc Genet* **2**, 450-456.
147. Schiffmann R. (2009) Fabry disease. *Pharmacol Ther* **122**, 65-77.
148. Eng C.M., Resnick-Silverman L.A., Niehaus D.J., Astrin K.H. and Desnick R.J. (1993) Nature and frequency of mutations in the alpha-galactosidase A gene that cause Fabry disease. *Am J Hum Genet* **53**, 1186-1197.
149. Smid B.E., Hollak C.E., Poorthuis B.J., van den Bergh Weerman M.A., Florquin S., Kok W.E.,... and Linthorst G.E. (2015) Diagnostic dilemmas in Fabry disease: a case series study on GLA mutations of unknown clinical significance. *Clin Genet* **88**, 161-166.
150. Rohard I., Schaefer E., Kampmann C., Beck M. and Gal A. (2008) Association between polymorphisms of endothelial nitric oxide synthase gene (NOS3) and left posterior wall thickness (LPWT) of the heart in Fabry disease. *J Inherit Metab Dis* **31 Suppl 2**, S349-356.
151. Rombach S.M., Hollak C.E., Linthorst G.E. and Dijkgraaf M.G. (2013) Cost-effectiveness of enzyme replacement therapy for Fabry disease. *Orphanet J Rare Dis* **8**, 29.
152. Rombach S.M., Smid B.E., Bouwman M.G., Linthorst G.E., Dijkgraaf M.G. and Hollak C.E. (2013) Long term enzyme replacement therapy for Fabry disease: effectiveness on kidney, heart and brain. *Orphanet J Rare Dis* **8**, 47.
153. Linthorst G.E., Hollak C.E., Donker-Koopman W.E., Strijland A. and Aerts J.M. (2004) Enzyme therapy for Fabry disease: neutralizing antibodies toward agalsidase alpha and beta. *Kidney Int* **66**, 1589-1595.
154. Lenders M., Neusser L.P., Rudnicki M., Nordbeck P., Canaan-Kuhl S., Nowak A.,... and Brand E. (2018) Dose-Dependent Effect of Enzyme Replacement Therapy on Neutralizing Antidrug Antibody Titers and Clinical Outcome in Patients with Fabry Disease. *J Am Soc Nephrol* **29**, 2879-2889.
155. Arends M., Biegstraaten M., Hughes D.A., Mehta A., Elliott P.M., Oder D.,... and Hollak C.E.M. (2017) Retrospective study of long-term outcomes of enzyme replacement therapy in Fabry disease: Analysis of prognostic factors. *PLoS one* **12**, e0182379.
156. Aerts J.M., Groener J.E., Kuiper S., Donker-Koopman W.E., Strijland A., Ottenhoff R.,... and Poorthuis B.J. (2008) Elevated globotriaosylsphingosine is a hallmark of Fabry disease. *Proc Natl Acad Sci U S A* **105**, 2812-2817.
157. Gold H., Mirzaian M., Dekker N., Joao Ferraz M., Lugtenburg J., Codee J.D.,... and Poorthuis B.J. (2013) Quantification of globotriaosylsphingosine in plasma and urine of fabry patients by stable isotope ultraperformance liquid chromatography-tandem mass spectrometry. *Clin Chem* **59**, 547-556.
158. Rombach S.M., van den Bogaard B., de Groot E., Groener J.E., Poorthuis B.J., Linthorst G.E.,... and Aerts J.M. (2012) Vascular aspects of Fabry disease in relation to clinical manifestations and elevations in plasma globotriaosylsphingosine. *Hypertension* **60**, 998-1005.
159. Kaissarian N., Kang J., Shu L., Ferraz M.J., Aerts J.M. and Shayman J.A. (2018) Dissociation of globotriaosylceramide and impaired endothelial function in alpha-galactosidase-A deficient EA.hy926 cells. *Mol Genet Metab* **125**, 338-344.
160. Weidemann F., Sanchez-Nino M.D., Politei J., Oliveira J.P., Wanner C., Warnock D.G. and Ortiz A. (2013) Fibrosis: a key feature of Fabry disease with potential therapeutic implications. *Orphanet J Rare Dis* **8**, 116.
161. Choi L., Vernon J., Kopach O., Minett M.S., Mills K., Clayton P.T.,... and Wood J.N. (2015) The Fabry disease-associated lipid Lyso-Gb3 enhances voltage-gated calcium currents in sensory neurons and causes pain. *Neurosci Lett* **594**, 163-168.
162. Sanchez-Nino M.D., Carpio D., Sanz A.B., Ruiz-Ortega M., Mezzano S. and Ortiz A. (2015) Lyso-Gb3 activates Notch1 in human podocytes. *Human molecular genetics* **24**, 5720-5732.
163. Shtraizent N., Eliyahu E., Park J.H., He X., Shalgi R. and Schuchman E.H. (2008) Autoproteolytic cleavage and activation of human acid ceramidase. *J Biol Chem* **283**, 11253-11259.
164. Gebai A., Gorelik A., Li Z., Illes K. and Nagar B. (2018) Structural basis for the activation of acid ceramidase. *Nature communications* **9**, 1621.
165. Ferlinz K., Kopal G., Bernardo K., Linke T., Bar J., Breiden B.,... and Sandhoff K. (2001) Human acid ceramidase: processing, glycosylation, and lysosomal targeting. *J Biol Chem* **276**, 35352-35360.
166. Dementiev A., Joachimiak A., Nguyen H., Gorelik A., Illes K., Shabani S.,... and Doan N. (2019) Molecular Mechanism of Inhibition of Acid Ceramidase by Carmofur. *Journal of medicinal chemistry* **62**, 987-992.
167. Ouairy C.M., Ferraz M.J., Boot R.G., Baggelaar M.P., van der Stelt M., Appelman M.,... and Overkleeft H.S. (2015) Development of an acid ceramidase activity-based probe. *Chemical communications* **51**, 6161-6163.
168. Ordóñez Y.F., Abad J.L., Aseeri M., Casas J., García V., Casasampere M.,... and Fabrias G. (2019) Activity-Based Imaging of Acid Ceramidase in Living Cells. *Journal of the American Chemical Society* **141**, 7736-7742.

169. Farber S. (1952) A lipid metabolic disorder: disseminated lipogranulomatosis; a syndrome with similarity to, and important difference from, Niemann-Pick and Hand-Schuller-Christian disease. *AMA Am J Dis Child* **84**, 499-500.
170. Ehler K., Frosch M., Fehse N., Zander A., Roth J. and Vormoor J. (2007) Farber disease: clinical presentation, pathogenesis and a new approach to treatment. *Pediatr Rheumatol Online J* **5**, 15.
171. Tanaka T., Takahashi K., Hakozaaki H., Kimoto H. and Suzuki Y. (1979) Farber's disease (disseminated lipogranulomatosis)--a pathological, histochemical and ultrastructural study. *Acta Pathol Jpn* **29**, 135-155.
172. Bonafe L., Kariminejad A., Li J., Royer-Bertrand B., Garcia V., Mahdavi S.,... and Superti-Furga A. (2016) Brief Report: Peripheral Osteolysis in Adults Linked to ASAH1 (Acid Ceramidase) Mutations: A New Presentation of Farber's Disease. *Arthritis Rheumatol* **68**, 2323-2327.
173. Gan J.J., Garcia V., Tian J., Tagliati M., Parisi J.E., Chung J.M.,... and Pierson T.M. (2015) Acid ceramidase deficiency associated with spinal muscular atrophy with progressive myoclonic epilepsy. *Neuromuscul Disord* **25**, 959-963.
174. Zhou J., Tawk M., Tiziano F.D., Veillet J., Bayes M., Nolent F.,... and Melki J. (2012) Spinal muscular atrophy associated with progressive myoclonic epilepsy is caused by mutations in ASAH1. *Am J Hum Genet* **91**, 5-14.
175. Schuchman E.H. (2016) Acid ceramidase and the treatment of ceramide diseases: The expanding role of enzyme replacement therapy. *Biochimica et biophysica acta* **1862**, 1459-1471.
176. Ferraz M.J., Marques A.R., Gaspar P., Mirzaian M., van Roomen C., Ottenhoff R.,... and Aerts J.M. (2016) Lyso-glycosphingolipid abnormalities in different murine models of lysosomal storage disorders. *Mol Genet Metab* **117**, 186-193.
177. Marshall J., Nietupski J.B., Park H., Cao J., Bangari D.S., Silvescu C.,... and Cheng S.H. (2019) Substrate Reduction Therapy for Sandhoff Disease through Inhibition of Glucosylceramide Synthase Activity. *Mol Ther* **27**, 1495-1506.
178. Kuchar L., Sikora J., Gulinello M.E., Poupetova H., Lugowska A., Malinova V.,... and Ledvinova J. (2017) Quantitation of plasmatic lysosphingomyelin and lysosphingomyelin-509 for differential screening of Niemann-Pick A/B and C diseases. *Anal Biochem* **525**, 73-77.
179. Mirzaian M., Wisse P., Ferraz M.J., Marques A.R.A., Gaspar P., Oussoren S.V.,... and Aerts J.M. (2017) Simultaneous quantitation of sphingoid bases by UPLC-ESI-MS/MS with identical (13)C-encoded internal standards. *Clin Chim Acta* **466**, 178-184.
180. Li Y., Xu Y., Benitez B.A., Nagree M.S., Dearborn J.T., Jiang X.,... and Sands M.S. (2019) Genetic ablation of acid ceramidase in Krabbe disease confirms the psychosine hypothesis and identifies a new therapeutic target. *Proc Natl Acad Sci U S A* **116**, 20097-20103.
181. Keatinge M., Bui H., Menke A., Chen Y.C., Sokol A.M., Bai Q.,... and Bandmann O. (2015) Glucocerebrosidase 1 deficient Danio rerio mirror key pathological aspects of human Gaucher disease and provide evidence of early microglial activation preceding alpha-synuclein-independent neuronal cell death. *Human molecular genetics* **24**, 6640-6652.
182. Watson L., Keatinge M., Gegg M., Bai Q., Sandulescu M.C., Vardi A.,... and Bandmann O. (2019) Ablation of the pro-inflammatory master regulator miR-155 does not mitigate neuroinflammation or neurodegeneration in a vertebrate model of Gaucher's disease. *Neurobiol Dis* **127**, 563-569.
183. Ohshima T., Murray G.J., Swaim W.D., Longenecker G., Quirk J.M., Cardarelli C.O.,... and Kulkarni A.B. (1997) alpha-Galactosidase A deficient mice: a model of Fabry disease. *Proc Natl Acad Sci U S A* **94**, 2540-2544.
184. Nguyen Dinh Cat A., Escoubet B., Agrapart V., Griol-Charhbil V., Schoeb T., Feng W.,... and Jaisser F. (2012) Cardiomyopathy and response to enzyme replacement therapy in a male mouse model for Fabry disease. *PLoS one* **7**, e33743.
185. Rodrigues L.G., Ferraz M.J., Rodrigues D., Pais-Vieira M., Lima D., Brady R.O.,... and Sa-Miranda M.C. (2009) Neurophysiological, behavioral and morphological abnormalities in the Fabry knockout mice. *Neurobiol Dis* **33**, 48-56.
186. Miller J.J., Aoki K., Mascari C.A., Beltrame A.K., Sokumbi O., North P.E.,... and Dahms N.M. (2019) alpha-Galactosidase A-deficient rats accumulate glycosphingolipids and develop cardiorenal phenotypes of Fabry disease. *FASEB J* **33**, 418-429.
187. Miller J.J., Aoki K., Moehring F., Murphy C.A., O'Hara C.L., Tiemeyer M.,... and Dahms N.M. (2018) Neuropathic pain in a Fabry disease rat model. *JCI Insight* **3**.
188. Miller J.J., Aoki K., Reid C.A., Tiemeyer M., Dahms N.M. and Kassem I.S. (2019) Rats deficient in alpha-galactosidase A develop ocular manifestations of Fabry disease. *Sci Rep* **9**, 9392.
189. Kobayashi T., Yamanaka T., Jacobs J.M., Teixeira F. and Suzuki K. (1980) The Twitcher mouse: an enzymatically authentic model of human globoid cell leukodystrophy (Krabbe disease). *Brain Res* **202**, 479-483.
190. Zizioli D., Guarienti M., Tobia C., Gariano G., Borsani G., Bresciani R.,... and Presta M. (2014) Molecular cloning and knockdown of galactocerebrosidase in zebrafish: new insights into the pathogenesis of Krabbe's

- disease. *Biochimica et biophysica acta* **1842**, 665-675.
191. Alayoubi A.M., Wang J.C., Au B.C., Carpentier S., Garcia V., Dworski S.,... and Medin J.A. (2013) Systemic ceramide accumulation leads to severe and varied pathological consequences. *EMBO Mol Med* **5**, 827-842.
 192. Lawson C.A. and Martin D.R. (2016) Animal models of GM2 gangliosidosis: utility and limitations. *Appl Clin Genet* **9**, 111-120.
 193. Sango K., Yamanaka S., Hoffmann A., Okuda Y., Grinberg A., Westphal H.,... and Proia R.L. (1995) Mouse models of Tay-Sachs and Sandhoff diseases differ in neurologic phenotype and ganglioside metabolism. *Nat Genet* **11**, 170-176.
 194. Kuil L.E., Lopez Marti A., Carreras Mascaro A., van den Bosch J.C., van den Berg P., van der Linde H.C.,... and van Ham T.J. (2019) Hexb enzyme deficiency leads to lysosomal abnormalities in radial glia and microglia in zebrafish brain development. *Glia* **67**, 1705-1718.
 195. Horinouchi K., Erlich S., Perl D.P., Ferlinz K., Bisgaier C.L., Sandhoff K.,... and Schuchman E.H. (1995) Acid sphingomyelinase deficient mice: a model of types A and B Niemann-Pick disease. *Nat Genet* **10**, 288-293.
 196. Loftus S.K., Morris J.A., Carstea E.D., Gu J.Z., Cummings C., Brown A.,... and Pavan W.J. (1997) Murine model of Niemann-Pick C disease: mutation in a cholesterol homeostasis gene. *Science* **277**, 232-235.
 197. Tseng W.C., Loeb H.E., Pei W., Tsai-Morris C.H., Xu L., Cluzeau C.V.,... and Porter F.D. (2018) Modeling Niemann-Pick disease type C1 in zebrafish: a robust platform for in vivo screening of candidate therapeutic compounds. *Dis Model Mech* **11**.
 198. Meyers J.R. (2018) Zebrafish: Development of a Vertebrate Model Organism. *Current Protocols Essential Laboratory Techniques* **16**, e19.
 199. Engeszer R.E., Patterson L.B., Rao A.A. and Parichy D.M. (2007) Zebrafish in the wild: a review of natural history and new notes from the field. *Zebrafish* **4**, 21-40.
 200. Kimmel C.B., Ballard W.W., Kimmel S.R., Ullmann B. and Schilling T.F. (1995) Stages of embryonic development of the zebrafish. *Developmental dynamics : an official publication of the American Association of Anatomists* **203**, 253-310.
 201. Gerhard G.S., Kauffman E.J., Wang X., Stewart R., Moore J.L., Kasales C.J.,... and Cheng K.C. (2002) Life spans and senescent phenotypes in two strains of Zebrafish (*Danio rerio*). *Exp Gerontol* **37**, 1055-1068.
 202. Howe K., Clark M.D., Torroja C.F., Torrance J., Berthelot C., Muffato M.,... and Stemple D.L. (2013) The zebrafish reference genome sequence and its relationship to the human genome. *Nature* **496**, 498-503.
 203. Kumar S. and Hedges S.B. (1998) A molecular timescale for vertebrate evolution. *Nature* **392**, 917-920.
 204. Meyer A. and Schartl M. (1999) Gene and genome duplications in vertebrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene functions. *Curr Opin Cell Biol* **11**, 699-704.
 205. Meyer A. and Van de Peer Y. (2005) From 2R to 3R: evidence for a fish-specific genome duplication (FSGD). *Bioessays* **27**, 937-945.
 206. Lieschke G.J. and Currie P.D. (2007) Animal models of human disease: zebrafish swim into view. *Nat Rev Genet* **8**, 353-367.
 207. Menke A.L., Spitsbergen J.M., Wolterbeek A.P. and Woutersen R.A. (2011) Normal anatomy and histology of the adult zebrafish. *Toxicol Pathol* **39**, 759-775.
 208. Jagannathan-Bogdan M. and Zon L.I. (2013) Hematopoiesis. *Development* **140**, 2463-2467.
 209. Panula P., Chen Y.C., Priyadarshini M., Kudo H., Semenova S., Sundvik M. and Sallinen V. (2010) The comparative neuroanatomy and neurochemistry of zebrafish CNS systems of relevance to human neuropsychiatric diseases. *Neurobiol Dis* **40**, 46-57.
 210. Rink E. and Wullmann M.F. (2001) The teleostean (zebrafish) dopaminergic system ascending to the subpallium (striatum) is located in the basal diencephalon (posterior tuberculum). *Brain Res* **889**, 316-330.
 211. Friedrich R.W., Jacobson G.A. and Zhu P. (2010) Circuit neuroscience in zebrafish. *Curr Biol* **20**, R371-381.
 212. Eisen J.S. and Smith J.C. (2008) Controlling morpholino experiments: don't stop making antisense. *Development* **135**, 1735-1743.
 213. Zancan I., Bellesso S., Costa R., Salvalaio M., Stroppiano M., Hammond C.,... and Moro E. (2015) Glucocerebrosidase deficiency in zebrafish affects primary bone ossification through increased oxidative stress and reduced Wnt/beta-catenin signaling. *Human molecular genetics* **24**, 1280-1294.
 214. Lelieveld L.T., Mirzaian M., Kuo C.L., Artola M., Ferraz M.J., Peter R.E.A.,... and Aerts J. (2019) Role of beta-glucosidase 2 in aberrant glycosphingolipid metabolism: model of glucocerebrosidase deficiency in zebrafish. *J Lipid Res* **60**, 1851-1867.
 215. Martin E., Schule R., Smets K., Rastetter A., Boukhris A., Loureiro J.L.,... and Stevanin G. (2013) Loss of function of glucocerebrosidase GBA2 is responsible for motor neuron defects in hereditary spastic paraplegia. *Am J Hum Genet* **92**, 238-244.
 216. Gravel R.A., Kaback M.M., Proia R.L., Sandhoff K., Suzuki K. and Suzuki K. (2001) The G_{M2} Gangliosidoses. In *The Metabolic & Molecular Bases of Inherited Disease*, Volume 3, C.R. Scriver, A.L. Beaudet, W.S. Sly and D. Valle, eds. (McGraw-Hill), pp. 3827-3876.

217. Suzuki K. and Chen G.C. (1967) Brain ceramide hexosides in Tay-Sachs disease and generalized gangliosidosis (GM1-gangliosidosis). *J Lipid Res* **8**, 105-113.
218. Jeyakumar M., Thomas R., Elliot-Smith E., Smith D.A., van der Spoel A.C., d'Azzo A.,... and Platt F.M. (2003) Central nervous system inflammation is a hallmark of pathogenesis in mouse models of GM1 and GM2 gangliosidosis. *Brain* **126**, 974-987.
219. Pfeffer S.R. (2019) NPC intracellular cholesterol transporter 1 (NPC1)-mediated cholesterol export from lysosomes. *J Biol Chem* **294**, 1706-1709.
220. Gumus E., Haliloglu G., Karhan A.N., Demir H., Gurakan F., Topcu M. and Yuce A. (2017) Niemann-Pick disease type C in the newborn period: a single-center experience. *Eur J Pediatr* **176**, 1669-1676.
221. Schwend T., Loucks E.J., Snyder D. and Ahlgren S.C. (2011) Requirement of Npc1 and availability of cholesterol for early embryonic cell movements in zebrafish. *J Lipid Res* **52**, 1328-1344.
222. Lin Y., Cai X., Wang G., Ouyang G. and Cao H. (2018) Model construction of Niemann-Pick type C disease in zebrafish. *Biol Chem* **399**, 903-910.
223. Sarna J.R., Larouche M., Marzban H., Sillitoe R.V., Rancourt D.E. and Hawkes R. (2003) Patterned Purkinje cell degeneration in mouse models of Niemann-Pick type C disease. *J Comp Neurol* **456**, 279-291.
224. Paul C.A., Boegle A.K. and Maue R.A. (2004) Before the loss: neuronal dysfunction in Niemann-Pick Type C disease. *Biochimica et biophysica acta* **1685**, 63-76.
225. Felker A. and Mosimann C. (2016) Contemporary zebrafish transgenesis with Tol2 and application for Cre/lox recombination experiments. *Methods Cell Biol* **135**, 219-244.
226. Udvadia A.J. and Linney E. (2003) Windows into development: historic, current, and future perspectives on transgenic zebrafish. *Dev Biol* **256**, 1-17.
227. Howe D.G., Bradford Y.M., Conlin T., Eagle A.E., Fashena D., Frazer K.,... and Westerfield M. (2013) ZFIN, the Zebrafish Model Organism Database: increased support for mutants and transgenics. *Nucleic acids research* **41**, D854-860.
228. Zon L.I. and Peterson R.T. (2005) In vivo drug discovery in the zebrafish. *Nature reviews. Drug discovery* **4**, 35-44.
229. Kuo C.L., van Meel E., Kytidou K., Kallemeijn W.W., Witte M., Overkleeft H.S.,... and Aerts J.M. (2018) Activity-Based Probes for Glycosidases: Profiling and Other Applications. *Methods Enzymol* **598**, 217-235.
230. Zhao Q., Ouyang X., Wan X., Gajiwala K.S., Kath J.C., Jones L.H.,... and Taunton J. (2017) Broad-Spectrum Kinase Profiling in Live Cells with Lysine-Targeted Sulfonyl Fluoride Probes. *Journal of the American Chemical Society* **139**, 680-685.
231. Baggelaar M.P., Janssen F.J., van Esbroeck A.C., den Dulk H., Allara M., Hoogendoorn S.,... and van der Stelt M. (2013) Development of an activity-based probe and in silico design reveal highly selective inhibitors for diacylglycerol lipase- α in brain. *Angewandte Chemie* **52**, 12081-12085.
232. Simon G.M. and Cravatt B.F. (2010) Activity-based proteomics of enzyme superfamilies: serine hydrolases as a case study. *J Biol Chem* **285**, 11051-11055.
233. Paulick M.G. and Bogoy M. (2011) Development of activity-based probes for cathepsin X. *ACS Chem Biol* **6**, 563-572.
234. Jiang J., Kuo C.L., Wu L., Franke C., Kallemeijn W.W., Florea B.I.,... and Aerts J.M. (2016) Detection of Active Mammalian GH31 α -Glucosidases in Health and Disease Using In-Class, Broad-Spectrum Activity-Based Probes. *ACS Cent Sci* **2**, 351-358.
235. Marques A.R., Willems L.I., Herrera Moro D., Florea B.I., Scheij S., Ottenhoff R.,... and Aerts J.M. (2017) A Specific Activity-Based Probe to Monitor Family GH59 Galactosylceramidase, the Enzyme Deficient in Krabbe Disease. *Chembiochem* **18**, 402-412.
236. Jiang J., Kallemeijn W.W., Wright D.W., van den Nieuwendijk A., Rohde V.C., Folch E.C.,... and Overkleeft H.S. (2015) In vitro and in vivo comparative and competitive activity-based protein profiling of GH29 α -l-fucosidases. *Chem Sci* **6**, 2782-false.
237. Wu L., Jiang J., Jin Y., Kallemeijn W.W., Kuo C.L., Artola M.,... and Davies G.J. (2017) Activity-based probes for functional interrogation of retaining beta-glucuronidases. *Nature chemical biology* **13**, 867-873.
238. Artola M., Kuo C.L., McMahon S.A., Oehler V., Hansen T., van der Lienden M.,... and Aerts J. (2018) New Irreversible α -l-Iduronidase Inhibitors and Activity-Based Probes. *Chemistry* **24**, 19081-19088.