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

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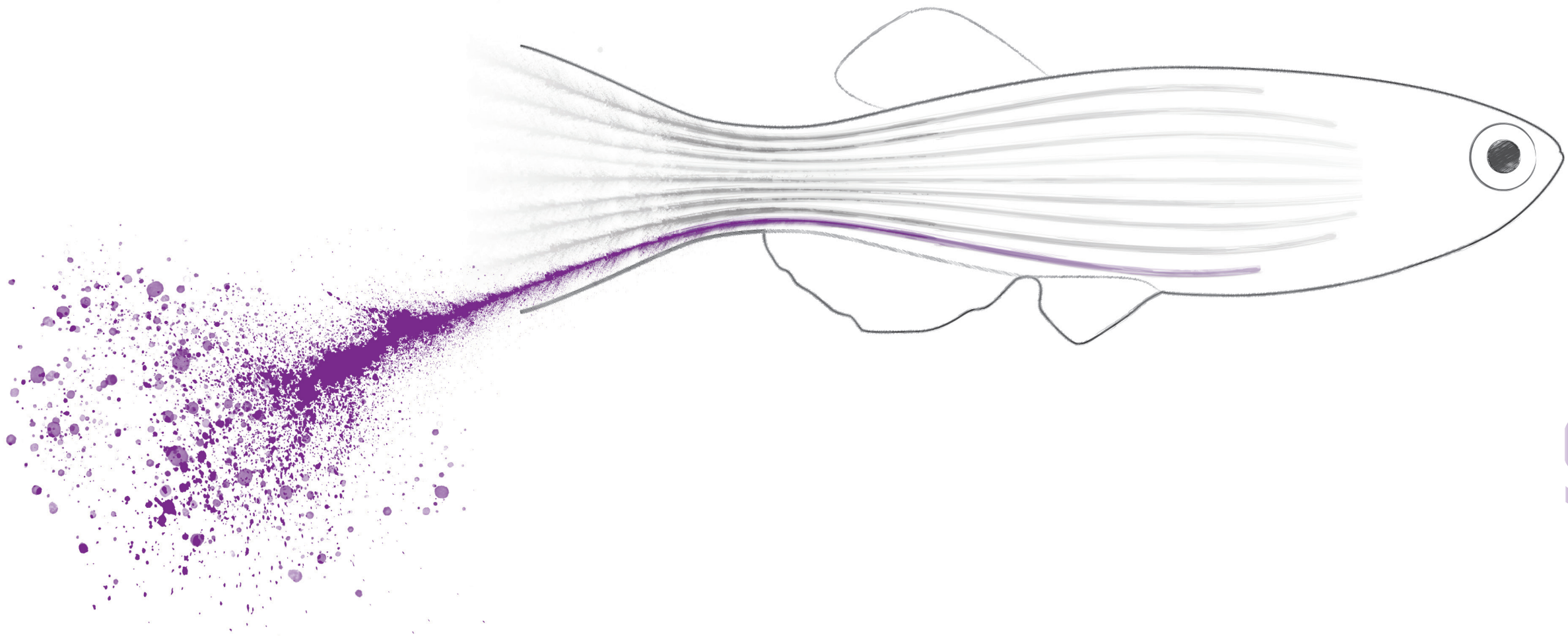
CHAPTER 9

General discussion & Future prospects



In this thesis, zebrafish are used as vertebrate model to study the lysosomal storage disorder Gaucher disease (GD). GD is characterized by mutations in the gene encoding glucocerebrosidase (GCase). Lysosomal GCase hydrolyses the glucosylceramide (GlcCer) as penultimate step in the catabolism of glycosphingolipids. Zebrafish are at first glance an appealing model organism to study GD disease since they produce the lipid GlcCer, their genome contains an orthologue of the human *GBA* gene and an active acid β-glucosidase is expressed. Zebrafish also express candidate modifier proteins, such as non-lysosomal Gba2 and lysosomal acid ceramidase (ACase).

Zebrafish GCase –To establish whether the highly homologous zebrafish GCase enzyme has similar enzymatic properties to its human counterpart, a detailed comparison of GCase enzymes was performed (chapter 2). For this purpose, GCase of zebrafish and humans was comparatively expressed in GCase-deficient HEK293T cells and examined regarding catalytic features. Both enzymes showed an acid pH optimum and a similar sensitivity for inhibitors. In addition, over-expression of both GCase enzymes corrected the increased levels of GlcCer and its deacylated sphingoid base, glucosylsphingosine (GlcSph), in the GCase-deficient HEK293T cells. Differences between the human and zebrafish GCase were also noted. Firstly, the human GCase enzyme required additives for optimal *in vitro* hydrolysis while no additives were necessary for zebrafish GCase activity. Secondly, the fish enzyme was equally active at 10 and 37 °C, whereas the human enzyme showed about three-fold higher activity at 37 °C. Lastly, the zebrafish enzyme was unable to transglucosylate, i.e. form glucosylated cholesterol (GlcChol) from GlcCer as sugar donor and with cholesterol as acceptor. A modest transglucosylation activity of zebrafish GCase was noted with ceramide as acceptor that is more flexible than the rigid cholesterol. Of note, zebrafish GCase, like human enzyme, is able to hydrolyse GlcChol, both *in vitro* (chapter 2) and *in vivo* (chapters 5 and 7).

Evolutionary aspects and protein dynamics – In the same investigation, GCase of turtle and frog were studied as well to reveal an evolutionary trend. Subtle differences in characteristics were noted for each of the enzymes when compared to human GCase. The ability to transglucosylate cholesterol was demonstrable for frog and turtle enzyme. Thus, the zebrafish is unique in its incapacity to position cholesterol as acceptor for transglucosylation. Taken together, it may be speculated that the fish GCase, over-expressed in mammalian cells, is more permanently present in an active status as compared to the other enzymes studied. Such more 'rigid' conformation of zebrafish GCase may as downside limit the enzyme's ability to transfer glucose to structurally rigid acceptors like cholesterol. For human GCase, it has been proposed that specific side-chain conformations favour a more ordered region near the catalytic pocket and reduce unfolding at neutral pH[1]. In an acidic environment, GCase shows an increased half-life, higher melting temperature and lower sensitivity to tryptic digestion[2]. Binding of (semi-)covalent inhibitors increases the structural stability of recombinant GCase further[2]. *In silico* comparison of modelled structures of zebrafish and human GCases revealed divergent residues in the three loops surrounding the catalytic pocket. Site-directed mutagenesis of specific residues in loops of the zebrafish GCase did not change hydrolytic activity or ability to transglucosylate.

Swapping of loops among the GCases of different species might be more helpful in future experiments. Of interest will be future meticulous comparative investigations of GCases of various species regarding protein dynamics by means of measuring melting curves and performing deuterium exchange mass spectrometry[2].

Generation of double knockout zebrafish – An enormous practical advantage of the zebrafish, compared to mice, is the speed and convenience with which new gene knockouts (KOs) can be generated and these KOs crossed with fish with other genetic traits. Chapter 4 provides a detailed protocol for the generation of gene KOs in zebrafish by means of CRISPR/Cas9 technology. Knockouts of *gba1*, *gba2*, *asah1a*, and *asah1b* have already been successfully established and are topics of the investigations described in chapters 5-7 of which the results are discussed below. Chapter 4 also describes generated mutations in the zebrafish *gpnmb*, *cln8* and *npc1* genes. Follow-up investigations with fish of these gene KOs are of interest as well as the combined KO with GCase deficiency.

gpNMB – The protein gpNMB is remarkably induced in plasma and Gaucher cells of GD patients as well as in GlcCer-laden macrophages of GD and NPC mice[3,4]. Exposing cultured RAW cells to stressors of lysosomes, e.g. HEPES, chloroquine or sucrose, leads to prominent increases in gpNMB[5]. Zebrafish with a GCase deficiency show a remarkable increase in the expression of *gpnmb* mRNA (chapter 6). The question remains whether the marked overexpression of this protein during lysosomal stress of macrophages serves a particular function. Crossing *gpnmb* KO fish, with or without a concurrent GCase deficiency, with fish expressing a fluorescent reporter in macrophages, under regulation of the *mpeg* promoter[6], will allow an assessment of the impact of Gpnmb on macrophages. Moreover, careful examination of *gpnmb* KO fish regarding their sensitivity for infections should be considered.

CLN8 – Appealing is a follow-up investigation on the role of CLN8 during GCase deficiency in zebrafish. CLN8 is an ER protein that is considered as modifier of clinical severity of GD. Its possible impact on disease severity was first indicated by a GWAS study involving more than a hundred type 1 GD patients homozygous for N370S mutation[7]. Several SNPs in the locus of the *CLN8* gene were associated with a more severe outcome of type 1 GD. Moreover, a higher expression of *CLN8* mRNA was observed in fibroblasts of mild GD patients compared to severe GD patients. Independently, the *Cln8* gene locus was linked to the variation in plasma GlcCer levels in inbred mouse sub-strains (Argmann, Aerts unpublished observations). More recently, it was reported that the CLN8 protein is involved in the ER-to-Golgi transfer of many newly formed soluble lysosomal enzymes, such as cathepsins[8,9]. Upon CLN8 deficiency, maturation of lysosomal enzymes was delayed and a reduction of several enzymes in the lysosome observed[8]. In chapter 4, c*ln8* KO zebrafish larvae with concurrent GCase deficiency show a significant reduction in the accumulating GlcSph, warranting further investigation. Possibly, acid ceramidase is reduced but other explanations for the observation can't be excluded yet. It will of great interest to examine whether CLN8 modulates the clinical course of gba1$^{-/-}$ fish.

NPC1 – Finally, the viability of *npc1* mutant fish opens possibilities for exciting novel research. There are indications for an intricate interplay between cholesterol and GSL metabolism. In Niemann Pick disease (NPC), the efflux of cholesterol from lysosomes is impaired, due to defects in either NPC1 or NPC2. In cholesterol-laden lysosomes of cells of NPC patients and NPC mouse models, secondary partial deficiencies in activities of sphingomyelinase (ASMase) and GCase cause accumulation of SM and GlcCer[4,10]. In NPC lysosomes, GlcChol is actively formed by GCase. Normally, GlcChol is synthesized in cells by the cytosol-faced enzyme GBA2 and degraded by GCase[11]. It has been reported that *npc1* KO zebrafish develop prominent accumulation of lysosomal cholesterol similar to NPC patients[12,13]. In the presented study, no glycosphingolipid abnormalities were detected in the generated *npc1* KO larvae up to 5 days post-fertilization (dpf) (chapter 4). In young larvae, compensatory maternal Npc1 might be available or the burden of lysosomal cholesterol might yet not be high enough to induce secondary deficiencies of ASMase and GCase. Raising the *npc1* KO fish to adulthood will enable analysis of cholesterol and glycosphingolipid levels at adult age as well as close monitoring of the expected symptoms, such as hepatosplenomegaly, neuropathology and uncoordinated swimming[12,13]. In addition, analysis of Npc1-deficient, with and without concurrent GCase deficiency, will indicate whether they also start to accumulate GlcChol as occurs in NPC mice and patients[11]. If so, it could be attempted to prohibit formation of excessive GlcChol with potent iminosugar inhibitors of GBA2 and GlcCer synthase (GCS)[14]. Inhibition of GBA2 in NPC mice, either by pharmacological or genetic ablation, was earlier found to ameliorate disease manifestations and increase life span of the animals[15,16]. Likewise, the available Gba2 KO fish could be used to test whether NPC disease can also be ameliorated by Gba2 modulation in zebrafish. For this, the clinical course of *npc1*$^{-/-}$*:gba2*$^{+/+}$, *npc1*$^{-/-}$*:gba2*$^{-/+}$ and *npc1*$^{-/-}$*:gba2*$^{-/-}$ fish could be compared.

ACase-deficient zebrafish – The generated GCase deficient zebrafish have already been combined with deficiencies of two other enzymes (Asah1b and Gba2) and the outcome of these double KOs has been investigated. Given the role of acid ceramidase (ACase) in formation of the presumed toxic GlcSph lyso-lipid in mouse and man[17], it was decided to generate zebrafish lacking such enzyme and consequently being unable to generate GlcSph during GCase deficiency. In zebrafish the genes *asah1a* and *asah1b* encode two orthologues of human ACase. Only the enzyme encoded by the *asah1b* gene was found to be able to generate GlcSph during GCase deficiency, while Asah1a and Asah1b seem complementary regarding degradation of ceramide (chapter 6). Combined deficiency of the two enzymes is not viable, likely as the result of massive ceramide accumulation mimicking Farber disease, a devastating disorder in humans. It will be of interest to study the cause for the differences in substrate specificity of the two fish ACases, Asah1a and Asah1b, that is presently not understood. Substrate specificity might be caused by subtle difference in amino acid composition of Asah1a and Asah1b. On the other hand, expression of the two enzymes in cells accumulating GlcCer during GCase deficiency could differ, with Asah1b being present far more prominently in lysosomes of macrophages. Recently, highly specific ABPs for ACase were developed by Fabrias and colleagues[18]. These probes could assist studies on the cellular and subcellular distribution of Asah1a and Asah1b. The available *asha1a* and *asah1b* KO zebrafish will be helpful in such investigations.

GCase-deficient fish unable to produce excessive GlcSph – *Gba1*$^{-/-}$*:asah1b*$^{-/-}$ fish were successfully generated as a model of GCase deficiency without concomitant GlcSph excess. The comparison of adult *gba1* KO fish, with excessive GlcSph, and adult *asah1b:gba1* KO zebrafish, without GlcSph, excites several topics for further discussion. Importantly, the *gba1:asah1b* KO fish developed a milder course of disease than *gba1* KO animals. This finding suggests that excessive GlcSph during GCase deficiency is indeed toxic. Secondly, *gba1*- and *asah1b:gba1* KO zebrafish accumulate comparably GlcCer in tissues and develop similarly Gaucher-like storage cells. In their brains, the two mutant fish showed similar abnormal autophagy, indicated by increased protein levels of p62, similar inflammation, reflected by comparably increased mRNA levels of *il1-β*, *tnf*β and *apoeb*, and indications for similar activation of the complement cascade. The infiltration of Gaucher-like storage cells, likely microglia, in the periventricular grey zone of the optic tectum also appeared comparable. Keatinge and colleagues earlier reported a reduction in dopaminergic neurons of *gba1* KO fish in the presence of ubiquitin-positive, intra-neuronal inclusions[19]. Own findings with *gba1*- and *gba1:asah1b* KO fish suggest that the presence of excessive GlcSph might accelerate dopaminergic neuron loss, by a presently unknown mechanism. It is noteworthy that the observed amelioration of disease in *asah1b:gba1* KO fish, lacking GlcSph, appears to be independent of storage cell burden, neuroinflammation and complement activation, commonly assumed major drivers of GD pathology[20-24]. In GD/PD mouse models and human neuronal cells, GlcSph has been found to trigger α-synuclein aggregation[24,25]. The zebrafish has no orthologue of human α-synuclein and only express a β-synuclein and two γ-synuclein orthologues[26,27]. In this respect zebrafish offer no simple copy of PD. Nevertheless, the available zebrafish that are able or unable to form GlcSph during GCase deficiency can be exploited to investigate GlcSph driven neuropathology independent of α-synuclein. It should be noted that the *asah1b:gba1* KO fish lacking excessive GlcSph do develop symptoms at 4 months. Thus, it seems that GlcSph indeed contributes to the onset of neuropathology but is not the only toxic factor during GCase deficiency and other pathological mechanisms are involved. In this respect, the impact of accumulating GlcCer and other glucosylated lipids deserves further attention.

It is tempting to consider inhibition of ACase as therapeutic avenue for neuronopathic GD with present unmet need. The more specific ACase inhibitors recently designed by Fabrias and colleagues could be tested in the now available zebrafish models of GCase-deficiency[18]. The window for such intervention is likely narrow given the fact that a partial ACase deficiency impairs spinal-cord motor neurons[28].

Regarding toxicity of GlcSph, it has to be taken into consideration that present investigations only quantified GlcSph with a regular C18-sphingosine moiety. Several species of GlcSph with distinct sphingosine moieties do however occur[29]. For example, the regular C18-sphingosine containing GlcSph is the most prominent species in plasma of GD patients, while in their urine it is only a very minor species and hydroxylated sphingoid bases are common[29]. It will be of interest to study more closely specific GlcSph isoforms in brain of GCase deficient zebrafish and to establish whether these contribute to severity of symptoms.

Other glycosphingolipidoses – The ACase-mediated deacylation of accumulating glycosphingolipids in lysosomes to sphingolipid bases is not unique to GD[30]. ACase also converts accumulating globotriaosylceramide (Gb3) in lysosomes of Fabry disease (FD) patients, having a defective α-galactosidase A (α-GAL A), into globotriaosylsphingosine (lysoGb3)[17]. At present many symptoms of FD patients are attributed to excessive lysoGb3, but caution is warranted since solid proof for the physiological relevance in patients is largely lacking[31-34]. In analogy to chapter 6, *asah1b* KO fish could be exploited to increase insight in the toxicity of lysoGb3. Chapter 8 reports the presence of an acid α-galactosidase (α-Gal A) and acid α-N-acetylgalactosaminidase (α-Nagal) in zebrafish cells, larvae and adult tissues. Zebrafish α-Gal A, despite prominent homology with the human enzyme, seems unable to degrade fluorescent NBD-Gb3 *in vitro*, but shows correction of Gb3 accumulation *in situ*. Noteworthy, fish lack the enzyme responsible for the synthesis of Gb3 from lactosylceramide, Gb3 synthase (a.k.a. A4GALT). Consequently, the globo-series glycosphingolipids could not be detected in the investigated zebrafish materials. Introducing *A4GALT* should allow generation of the non-endogenous Gb3 in fish. Next, accumulation of lysoGb3 should be induced by α-Gal A inactivation in fish expressing A4GALT and it should be prohibited in *asah1* KO fish expressing A4GALT. In this way, organismal models could be obtained allowing investigation of the toxicity of endogenous lysoGb3 *in vivo*.

The same approach as described above could be considered for other diseases in which ACase generates sphingoid bases from accumulating sphingolipids in lysosomes[30]. Recently, Li and colleagues describe the amelioration of disease manifestations in a Krabbe mouse model by introducing ACase deficiency, thereby lacking the ability to from galactosylsphingosine (GalSph) from accumulating galactosylceramide[35]. In contrast to the described AC deficient mice, *asah1b* KO zebrafish show no features of Farber disease until the relatively old age of 2 years. Other interesting LSDs in this respect are Sandhoff disease where lysoGM2 is formed from the ganglioside GM2[36] and acid sphingomyelinase deficiency, a.k.a. Niemann-Pick disease type A/B type (NP A/B), where lyso-sphingomyelin is formed from sphingomyelin[37]. Orthologues of the defective lysosomal hydrolases involved in these LSD are present in the zebrafish genome (**Table 1**), as well as most of the enzymes of glycosphingolipid synthesis (**Supplementary Table 1**).

Table 1 | Zebrafish orthologues of human lysosomal hydrolases defective in LSDs. The zebrafish gene is given, as well as the genomic location and Uniprot code of the encoded proteins.

	gene		Protein		gene		Protein
Gaucher disease	*gba*	Chr 16	E7EZM1	**Tay-sachs disease/ Sandhoff disease**	*hexa*	Chr 25	F1RDQ5
Krabbe disease	*galca*	Chr 20	Q5SNX7	**Sandhoff disease**	*hexb*	Chr 5	A2BHD8/ F1QUU0
	galcb	Chr 17	F1QYH2	**NP type A/B**	*smpd1*	Chr 10	F8W546
Fabry disease	*gla*	Chr 14	F1Q5G5	**MLD**	*arsa*	Chr 18	A5WV48
Schindler disease	*naga*	Chr 4	A0A0R4IJL2	**GM1-gangliosidosis**	*glb1*	chr 1	A2BG54
Farber disease	*asah1a*	Chr 14	Q5XJR7	**Sialidosis**	*neu1*	Chr 19	Q1LWP9
	asah1b	Chr 1	Q6PH71	**Pompe disease**	*gaa*	Chr 3	E7FGC0/ F1R7G7

Combined GCase and Gba2 deficiency in zebrafish – GBA2 is a β-glucosidase tightly associated with membranes that catalyzes metabolism of GlcCer in the cytosolic membrane-leaflet. It has been demonstrated that GBA2 is able to act as transglucosylase and generates GlcChol using GlcCer as sugar donor and cholesterol as acceptor, both in zebrafish (chapter 5) and in mammals[11]. Lack of Gba2 in the zebrafish is therefore predicted to reduce GlcChol and to increase GlcCer in cytosolic membrane leaflets. Double *gba1:gba2* KO zebrafish were generated to examine the impact of the proposed reduction of GlcChol on disease manifestations. Investigations in chapter 7 reveal that *gba2* KO fish lack a clear phenotype, while *gba1:gba2* KO individuals do not show apparent phenotypic improvements when compared to *gba1* KO fish.

A prominent increase of GlcCer lipids with specific fatty acyls is observed in brains of *gba2* KO fish, as also reported for cerebellum of GBA2-deficient mice[38]. Moreover, levels of these particular GlcCer lipid species in brains of *gba1:gba2* KO fish exceed those observed in *gba1* KO brains. It is likely that these additional 18:0 and 20:0 fatty acyl GlcCer lipids accumulate at the cytosolic membrane leaflets in *gba1:gba2* KO fish, instead of intra-lysosomal. The findings regarding GlcChol warrant discussion. GlcChol is increased in GCase-deficient fish and it is reduced in young *gba2* KO fish, consistent with the role of Gba2 in synthesis of GlcChol and the role of GCase in hydrolysis of GlcChol (chapter 5 and 7[11]). Unexpectedly, GlcChol levels in older Gba2 deficient fish were found to be quite comparable to those in WT fish. The presence of GlcChol in the diet of the zebrafish, receiving both plant- and animal-based food, possibly explains this discrepancy. Supporting this hypothesis is the finding by Akiyama and co-workers that brain of chicken contains GlcChol, but also plant sterols and glucosylated plant sterols[39]. This suggests that food-derived glucosylated sterols indeed are taken up and even reach the brain. Therefore, it is of interest to raise the *gba1:gba2* KO zebrafish without the supplementary animal food, likely high in GlcChol, to examine disease manifestations in fish receiving different diets.

Macrophage storage cells – GCase-deficient zebrafish develop lipid-laden cells, reminiscent to Gaucher cells in GD patients. Besides *gpnmb*, GCase-deficient fish show increased expression of the chitinase, *chia.6*. The most extreme biomarker of human Gaucher cells is also a chitinase called chitotriosidase[40,41]. Similarly, GD fruit flies deficient in GCase over-express a chitinase[42]. While the zebrafish genome encodes six homologous *chia* genes, *chia.1-chia.6,* with different tissue distributions[43] only *chia.5* and *chia.6* mRNA levels are increased in brains of GCase-deficient fish. Of interest for future experiments is examining the spatial location of *gpnmb* and *chia.6* expression in the GCase-deficient zebrafish by for example *in situ* hybridization. It will also be of interest to study more closely what drives lipid-laden macrophages to massively overexpress proteins with unknown functions in GD, such as gpNMB and a chitinase with apparent antifungal and possible anti-microbial activity. For this purpose, the promoters of *chia.6* and *gpnmb* could be combined with a reporter to visualize the induction of lipid-laden macrophages in transparent larvae. Such visualization could be of particular use to study different disease conditions resulting in lipid-laden macrophages and microglia in developing larvae.

Genome-editing approaches – In this thesis, CRISPR/Cas9 technology has been used in zebrafish to generate gene knockouts, while Tol2 transposase technique introduced exogenous DNA in the zebrafish genome (chapter 4). Random integration of the target sequence and its expressional regulation by ubiquitous and reported cell-specific promoters are two limitations of the Tol2 transposase technique. Of particular interest for future research is optimization of so-called knockin approaches after CRISPR/Cas9 mediated DNA cleavage. Several applications can be envisioned including a study on the impact of human GD-causing mutations in zebrafish, generation of the described *gpnmb* or *chia.6* reporter fish or combining an endogenous gene sequence with a reporter tag. For example, the subcellular localization of the membrane-associated Gba2 is still poorly understood and a fluorescently tagged Gba2 may assist detailed analysis of subcellular localization of this enzyme in the transparent larvae.

Small compound interventions – Zebrafish offer a convenient organismal model to study small compound inhibitors. The investigation described in chapter 3 reveals comparable selectivity of GCase inhibitors, as cultured human cells. GCase was specifically inactivated when intact zebrafish larvae were exposed to ME656, a cyclophellitol modified at C8 with a bulky adamantyl moiety, in the swimming water. Moreover, it could be demonstrated with adult fish that ME656 cyclophellitol passes the blood brain barrier and inactivates GCase in the brain. Given the specificity of ME656, not targeting other retaining β-glucosidases[44,45] it appears feasible to generate pharmacologically a *bona fide* organismal model of neuronopathic Gaucher disease. Likewise, addition of small compound iminosugars inhibitors of GCS and GBA2 to the medium of GCase-deficient larvae resulted in expected corrections in lipid abnormalities as detected with sensitive LC-MS/MS allowing analysis of individual fish (chapter 5).
The studies in this thesis illustrate that in essence similar fluorogenic substrates, ABPs and LC-MS/MS techniques can be used to evaluate enzyme characteristics and relevant lipids, as those used for human and mouse materials.

Opportunities and limitations of zebrafish as disease model

GCase deficiency in zebrafish results in a number of similar biochemical abnormalities that are similar to those in GD patients and GD mice models. GlcSph and GlcCer massively increase and storage cells accumulate in tissues, likely being the source of increased *gpnmb* and *chia.6* expression. The focus of investigations in this thesis has been primarily the brain. The zebrafish models might equally be exploited to study other common GD manifestations, such as splenomegaly, hepatomegaly, focal lesions, blood abnormalities and bone involvements, such as bone crises and osteoporosis. In view of the common symptoms of GD patients, key organ systems are discussed regarding anatomical and physiological similarities and differences between fish and mammals. The impact of GCase deficiency in these organ systems in fish and humans is discussed.

Spleen and haematopoiesis – In mammals, haematopoiesis takes largely place in bone marrow and the spleen is involved in the removal of senescent blood cells from the circulation. The infiltration of viable Gaucher cells in bone marrow, spleen and liver of GD patients are thought to underlie cytopenia, hepatosplenomegaly and bone defects[46]. The zebrafish spleen, as in nearly all vertebrates, is a non-vital organ and similar in structure to a large lymph node located inside the serosal lining of the intestine[47]. It acts primarily as a blood filter and plays important roles regarding red blood cells and the immune system. As in GD patients, GCase-deficient fish accumulate Gaucher-like cells in their spleen, which are more apparent in the *gba1:gba2* and *gba1:asah1b* KO fish.

Zebrafish fundamentally differ from mammals in the site of haematopoiesis. In adult fish, the kidney marrow acts as the main site for haematopoiesis, analogous to specialized bone marrow in mammals[48]. Development of the haematopoiesis system is regulated by similar molecular mechanisms as the mammalian system[48,49]. Fish have similar blood cells as mammals: erythrocytes, however nucleated, neutrophils, eosinophils, lymphocytes and macrophages[48,49].

The kidneys of the *gba1-, gba1:gba2- and gba1:asah1b* KO fish appeared normal with standard haematoxylin and eosin staining. It will be of interest to study the haematological abnormalities and/or disturbed haematopoiesis in the three *gba1* mutants. Blood sampling is feasible from an adult zebrafish, although limited to only a few microliter[50]. Zancan and colleagues reported that in 3-10 dpf larvae hepatomegaly, thrombocytopenia, anaemia were already demonstrable[51]. In this respect, transgenic zebrafish lines can be used to investigate hepatocytes (*fabp10a* gene element), thrombocytes (*oitga2b*), erythrocytes (*gata1*) and blood vessel architecture (*krdl*)[51]. It will be particularly interesting to study blood abnormalities in *gba1:gba2* zebrafish as it has been described that ablation of GBA2 in mice with GCase deficiency in the haematopoietic lineage improves the observed cytopenia, amongst other symptoms[52].

Bone – Common among GD patients is the occurrence of bone defects, characterized by a reduction in bone mineral density, reduction of bone mass and a decrease in the trabecular volume of long bones. This results in brittle bones that are more prone to fracture. Osteoporosis is caused by a dysregulation of bone homeostasis with decreased bone metabolism, by reduced activity of osteoblasts and osteocytes, and increased catabolism, by enhanced osteoclast activity. Several studies have indicated that osteoblastic bone formation is particularly impaired in GD patients[52,53].
The skeletal physiology of the zebrafish shows strong similarities to mammals[54,55]. In zebrafish, as in mammals, bone is formed *de novo* by osteoblasts and remodelled by the coupled action of osteoclasts. Terrestrial vertebrates have thicker bones compared to zebrafish, however the relevant skeletal cell types and modes of regulation, including osteoblast and osteoclast coupling and bone remodelling, are conserved between zebrafish and higher vertebrates[54,56]. The first cartilaginous structures, chondrocytes and osteoblasts are formed in the few days of development, however osteoclasts are not formed until 10-12 dpf[54]. Therefore, true remodelling through combined activity of osteoblasts and osteoclasts starts in the second week of development. Previously discussed research, using transgenic zebrafish, also reveals reduced bone mineralization, impaired osteoblast differentiation and a defect in canonical Wnt signalling in the GCase deficient larvae[51]. Increased reactive oxygen species production was found to precede the Wnt signalling impairment. Reduced Wnt signalling activity was also observed in GD fibroblasts resulting from increased β-catenin degradation[51]. The three GCase-deficient zebrafish models have not been examined yet for skeletal abnormalities. Of note, the mutants do show a curvature of the back but it is presently unknown whether this abnormality has not a neurological nature. In view of the hypothesized interference of excessive GlcSph in normal osteoblast bone formatio [57], it will be particularly interesting to study the bone in *gba1:asah1b* fish lacking excessive GlcSph. Examining bone defects in the *gba1:gba2* KO zebrafish is also of interest, given the reported improvement of bone in GD mice with concomitant GBA2 deficiency[52].

In order to study bone defects in developing zebrafish larvae, reported transgenic zebrafish lines can be used, studying for example osteoblasts (*Ola.Sp7* gene element), osteoclasts (*ctsk*), chondrocytes (*col2a1*) or neural crest derived-skeleton (*sox10*)[51,56]. The skeletal phenotype of larvae and adult zebrafish can also be studied using histological stains, that bind calcium and calcium-containing materials, or X-ray based imaging of live animals[58]. Micro-computed tomography analysis has, for example, been used to evaluate developmental skeletal defects in a zebrafish model for mucopolysaccharidosis type II (MPSII)[59]. Underlying molecular mechanisms can be studied by means of protein or RNA analysis of different skeletal structures. For example, the tail fin of adult zebrafish is easily accessible, contains several separated vertebrae and has been extensively used as model for regenerating bone[54,60].

Liver – Mildly affected GD patients develop a moderate hepatosplenomegaly due to infiltration of Gaucher cells[61]. In severely affected GD patients, chronic liver injury and inflammation lead to collagen deposition and fibrosis which may lead to loss of metabolic and synthetic functions, also called hepatic cirrhosis[61,62]. The zebrafish liver differs in important aspects from the mammalian one. It encompasses three lobes, containing hepatocytes, endothelial cells and bile duct epithelial cells, but specialized macrophages, called Kupffer cells, seem absent[47]. Hepatocytes form plates and are lined with sinusoids and biliary ducts, but are not clearly organized in cords or lobules with portal triads as in the mammalian liver[47,63]. Hepatocytes of females are basophilic due to production of vitellogenin, which results in a slightly different staining of the female and male livers using standard haematoxylin and eosin staining[47].

Gaucher-like cells are apparent in liver of GCase-deficient fish, while hepatocytes in these livers appear relatively normal. No enlarged spleen or liver was observed during dissections of GCase-deficient zebrafish and their histopathological examination. Further characterization of the observed Gaucher-like cells by immunostaining for macrophage markers, and *in situ* hybridization of *gpnmb* or *chia.6* should be undertaken.

Brain – GD patients with very low residual GCase activity display neurological impairments, which are either severe and manifest at very young age (type 2 GD) or more mild and variable of nature (type 3 GD)[46]. Mutations in the *GBA* gene encoding GCase are a prominent risk factor for Parkinson's disease[64].

The zebrafish brain resembles the mammalian brain in many aspects, however there are differences to point out. The zebrafish brain is also anatomically divided into fore-, mid-and hindbrain with defined regions such as the diencephalon, telencephalon, cerebellum and spinal cord[55,65]. The telencephalon, or forebrain, is responsible for aspects of memory, colour vision as well as reproductive- and feeding behaviours[66]. The midbrain, or mesencephalon, is relatively large and anatomically subdivided in the tegmentum and optic tectum[47]. The optic tectum in fish appears to have a high degree of complexity and is suggested to perform vision-related functions performed by the neocortex of mammals[67]. The blood-brain barrier (BBB) develops fast in zebrafish and has a similar structure and function compared to that of higher vertebrates[68,69].

The zebrafish brain uses the same neurotransmitters as mammals such as dopamine, noradrenaline, serotonin, histamine and acetylcholine[68,70]. The adjustment of movement is regulated predominantly by the monoaminergic system that is highly conserved in vertebrates[68,70]. Tyrosine hydroxylase (TH) mediates the first step of catecholamine biosynthesis and is therefore an important marker of catecholaminergic neurons. Two orthologues of the mammalian *TH* gene exist in the zebrafish genome and the encoding Th1 and Th2 proteins are highly similar to mammalian TH. Th1 expressing dopaminergic neurons are found in the olfactory bulb, telencephalon, diencephalon and locus coeruleus, while Th2 expressing neurons are found in the diencephalon, ventral proptic region and hypothalamus. Th1 and Th2 neuron populations are intermingled in the diencephalon, however neurons mostly express either one of the enzymes. Importantly, only Th1 reacts with the available antibodies, therefore catecholiminergic populations visualized by immunohistochemistry are Th1-positive populations[70,71].

Several reported hallmarks of neuronopathic GD in mouse models are also observed in GCase-deficient zebrafish, such as infiltration of Gaucher-like cells, likely expressing *gpnmb* and *chia.6*, increased neuroinflammation, abnormal autophagy, complement system activation and neurodegeneration[72-74]. The various available mutant fish with GCase deficiency discussed above will allow follow-up investigations on the role of specific lipid abnormalities in neuropathology.

Given the interest in the relationship between GCase and Parkinson's disease (PD), it is of interest to point out that zebrafish lack the dopaminergic neuronal populations in the midbrain that are involved in PD[75]. In PD, selective degeneration of dopaminergic neurons occurs in the pars compacta of the substantia nigra, a basal ganglia structure located in the midbrain, which is thought to be responsible for its motor symptoms. In the zebrafish, the telencephalon contains a significant population of dopaminergic neurons that is not present in mammalian brain. Diencephalic dopaminergic cell populations in the posterior tuberculum of the zebrafish brain are considered as functional homolog of the mammalian dopaminergic neurons in the substantia nigra[75]. In PD, cytoplasmic inclusion bodies termed Lewy bodies, mainly composed of α-synculein, are a histopathological hallmark. As mentioned, above an orthologue of the human α-synuclein is not to be present in the zebrafish genome. Zebrafish express three synuclein isoform: β-, γ1- and γ2-synuclein, the first two showing involvement in normal dopaminergic neuron development[26,27]. Over-expression of γ1-synuclein in zebrafish results in formation of neuronal aggregates and neurotoxicity, similar to that observed for human α-synuclein in PD[76].

Levels of β- and γ1-synucleins were found to be reduced in brains of GCase-deficient zebrafish, both at mRNA (chapter 7) and protein level[19]. This reduction is likely attributed to loss of neurons[19]. This investigation by Keatinge and coworkers with *gba1* KO fish also revealed intra-neuronal inclusions, resembling Lewy bodies in PD patients[19]. It is difficult to judge at present whether *gba1* KO zebrafish provide a useful model for studying PD or only offer a model for neuronopathic GD[68,77].

Endogenous α-synuclein is expressed by another type of teleost fish, *Oryzias latipes* (medaka). *Gba1* KO medaka fish show infiltration of Gaucher-like cells in the brain, progressive neuronal loss and microgliosis[78]. Accumulation of α-synuclein in axonal swellings is observed, however disruption of the gene encoding α-synuclein does not improve lifespan, formation of axonal swellings, neuronal loss or neuroinflammation[78]. Medaka fish might intrinsically offer more attractive PD models than zebrafish.

Skin – Skin of collodion GD patients and null-allele GD mice show severe barrier abnormalities, incompatible with terrestrial life, with increased GlcCer levels and decreased ceramide levels in the stratum corneum[79,80]. The extracellular outer skin layer of mammals prevents against water loss and protects the epidermis and dermis[81]. The stratum corneum consists of terminally differentiated keratinocytes, called corneocytes, embedded in lipid lamellae that consist largely of ceramide, cholesterol and free fatty acids[81]. Keratinocytes extrude GlcCer and sphingomyelin, via lamellar bodies, into the interface with the stratum corneum as well as GCase and ASMase. These enzymes generate locally the ceramides from the lipid precursors, an essential process for formation of desired barrier properties of the skin[82].

Fish are, in sharp contrast to mammals, ectoterm (rely on environmental heat sources), poikilotherm (body temperature varies with the ambient temperature) and their skin is adapted to the aqueous environment[83,84]. The teleost skin consists of an epidermis and dermis and contains no keratin or water barrier[84]. The protective outer cuticle contains a mix of glycoproteins and immune component containing mucus, which is the mechanical and biochemical barrier against pathogens[83,84]. Fish lack a similar structure to the stratum corneum of mammals. The presence of sphingolipids in teleost skin is poorly studied. Only limited data is available for total skin, indicating the presence of quite similar GlcCer, SM and ceramide levels to those in other tissues[85,86]. The absence of a specialized, ceramide-enriched stratum corneum in fish might explain the observed viability of GCase deficient zebrafish (chapters 5, 6 and 7).

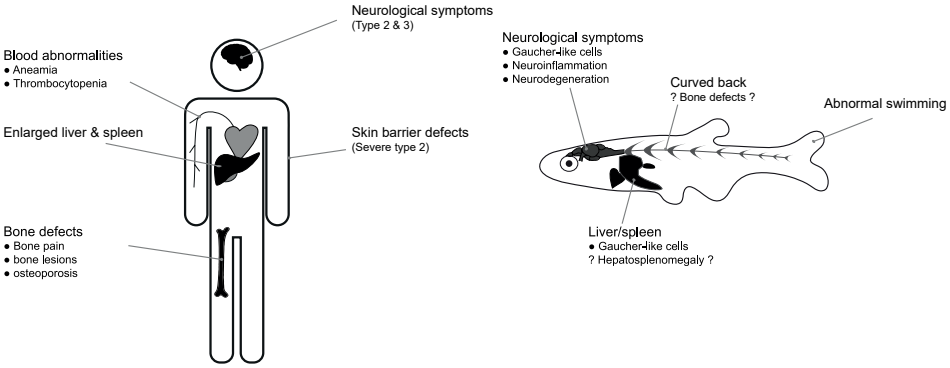


Figure 1 | GD disease manifestations in human patients and zebrafish model
Schematic representation of common disease manifestations of human GD patients and the observed symptoms of the established GCase-deficient zebrafish.

Concluding remarks

The studies in this thesis illustrate the great potential of the zebrafish as vertebrate model to study Gaucher disease. The practical advantages of zebrafish have enabled generation of various gene knockouts and screening of small molecule inhibitors. Similar techniques can be applied in zebrafish larvae and adult tissues as earlier established for human and mouse materials. Glycosphingolipid metabolism can be evaluated using fluorogenic substrates, ABPs and LC-MS/MS techniques, while standard pathology, protein and RNA analysis offer opportunities to study underlying molecular mechanisms.

Obviously, fish are no phenocopy of humans due to differences in anatomy and physiology. However, zebrafish prove to be an attractive model to study molecular mechanism of disease manifestation at an organismal level. Moreover, GD zebrafish allow convenient screening for drugs and the evaluation of their efficacy in correcting aberrant metabolism at organismal level. Insights that are obtained in this manner complement information obtained by studying cultured human cells and mouse models. In particular, the unique *asah1b* KO fish, not able to generate the presumed toxic lyso-lipid, could give valuable insights into the role of GlcSph, GalSph, lyso-Gb3 or other lyso-lipids in accelerating disease manifestations.

Supplementary information

Supplementary table 1 | Zebrafish (ZF) orthologues of human proteins responsible for GSL metabolism. Limited information is available on the cell-specific expression, localization and substrate specificity of these zebrafish enzymes as compared to their human orthologue.

	ZF gene		Uniprot		ZF gene		Uniprot
Serine palmitoyl transferase	*sptlc1*	Chr 10	Q502G6/F1QEL4	**GSL**	*ugt8*	Chr 1	E7FDZ0
	sptlc2a	Chr 17	F1QCX4		*gal3st1a*	Chr 5	A2BGH3
	sptlc2b	Chr 20	F1Q992		*gal3st1b*	Chr 10	E9QCM2
	sptlc3	Chr 13	F1QQP7		*ugcg*	Chr 10	F1QNT4
Desaturase	*segs1*	Chr 13	B8A4A2	**LacCer**	*b4galt5*	Chr 23	Q3YL68
	degs2	Chr17	F1QW74	**Globo- series**	*a4galt/ a3galt2*	Not found	
Ceramide synthase	*cers1*	Chr 22	F1Q5B1	**Lacto- series**	*b3gnt5a*	Chr 11	Q7T3S5
	cers2a	Chr 19	Q90YY7		*b3gnt5b*	Chr 2	A5WVT9
	cers2b	Chr 16	E7F9X7	**Ganglio- series**	*b4galnt1a*	Chr 23	A1L229
	cers3a	Chr 7	E7F1Q9		*b4galnt1b*	Chr 6	X1WFC5
	cers3b	Chr 18	F1QVG1		*st3gal5*	Chr 14	F1RCA6
	cers4a	Chr 22	Q1L8N1		*st8sia1*	Chr 4	Q6KC13/A1A5W4
	cers4b	Chr 2	E7FCM3		*st3gal2*	Chr 18	Q6EV31
	cers5	Chr 22	A7E7D4	**LacCer**	*b4galt5*	Chr 23	Q3YL68
	cers6	Chr 9	F1QPF3	**Globo- series**	*a4galt/ a3galt2*	Not found	
Cer1P	*cerk*	Chr 4	AWVZ0	**Lacto- series**	*b3gnt5a*	Chr 11	Q7T3S5
SM	*sgms1*	Chr 12	A0JMN0	**Lysosomal protein**	*Npc1*	Chr 2	F1QNG7
	sgms2a	Chr 1	B8A5Q0		*Npc2*	Chr 17	Q9DGJ3
	sgms2b	Chr 23	Q6DEI3	**Activator protein**	*Psap*	Chr 13	B8JI17

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