

Erich Gulbins  
Irina Petrache *Editors*

# Sphingolipids: Basic Science and Drug Development

# Handbook of Experimental Pharmacology

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# Sphingolipids: Basic Science and Drug Development

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# Preface

Until the late 1980s, sphingolipids were believed to represent structural components of the plasma membrane, whose function was to provide a protective barrier to the cell. This picture dramatically changed within the last years. Sphingolipids are now recognized signals for fundamental cellular processes, such as proliferation, survival, cell death, adhesion, migration, angiogenesis, and embryogenesis. The explosion of knowledge regarding sphingolipids was facilitated by biochemical studies of their signaling properties, the cloning of enzymes of the sphingolipid metabolism, development of genetic models for determining their physiologic roles, and the establishment of biochemical, biophysical, and optical methods for their detection and quantitation. The next step in the evolution of sphingolipids will be the transfer of basic insights into the biochemistry and cell biology of human diseases. The recent success of the sphingolipid drug, fingolimod, a sphingosine-1-phosphate agonist, which rapidly became a therapy for multiple sclerosis, exemplifies the potential of targeting sphingolipids for the treatment of human disorders. The aim of our two volumes in this series—*Sphingolipids: Basic Science and Drug Development* and *Sphingolipids in Disease*—is to define the state of the art of sphingolipid biology and to present preclinical developments and early clinical applications of this fascinating class of lipids.

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**Part I**  
**Basic Mechanisms**

# The Genetics of Sphingolipid Hydrolases and Sphingolipid Storage Diseases

Edward H. Schuchman and Calogera M. Simonaro

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**Abstract** The relationship of sphingolipids with human disease first arose from the study of sphingolipid storage diseases over 50 years ago. Most of these disorders are due to inherited deficiencies of specific sphingolipid hydrolases, although a small number also result from defects in sphingolipid transport or activator proteins. Due to the primary protein deficiencies sphingolipids and other macromolecules accumulate in cells and tissues of affected patients, leading to a diverse presentation of clinical abnormalities. Over 25 sphingolipid storage diseases have been described to date. Most of the genes have been isolated, disease-causing mutations have been identified, the recombinant proteins have been produced and characterized, and animal models exist for most of the human diseases. Since most sphingolipid hydrolases are enriched within the endosomal/lysosomal system, macromolecules first accumulate within these compartments. However, these abnormalities rapidly spread to other compartments and cause a wide range of cellular dysfunction.

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This review focuses on the genetics of sphingolipid storage diseases and related hydrolytic enzymes with an emphasis on the relationship between genetic mutations and human disease.

**Keywords** Sphingolipids • Hydrolases • Lysosomes • Genes • Mutations

## 1 Introduction and Overview

Sphingolipids are a diverse group of over 100 bioactive lipids involved in many aspects of cellular function (see Chen et al. 2012; Furuya et al. 2011; Petrache et al. 2011; Podbielska et al. 2012 for recent reviews). They all share a common sphingosine (2-amino-4-octadecene-1,3-diol) backbone, which may be linked via an amide bond to a fatty acid (forming ceramide), or phosphorylated to form sphingosine-1-phosphate. Ceramide itself also may be modified to form glycosphingolipids, sphingomyelin, or ceramide-1-phosphate.

The metabolism of sphingolipids is complex and carefully regulated (e.g., Mullen et al. 2012; Wennekes et al. 2009; Worgall 2011). Abnormal metabolism can have profound effects on cellular function, leading to enhanced cell death, proliferation, and/or abnormal cellular differentiation. In general, the synthesis and breakdown of sphingolipids occur in distinct cellular compartments. For example, numerous enzymes contribute to the *de novo* synthesis of sphingolipids, mostly found in the endoplasmic reticulum and Golgi apparatus. In contrast, the breakdown of sphingolipids occurs primarily within endosomes and lysosomes by a series of hydrolytic enzymes that function optimally at acidic pH. Hydrolysis of sphingolipids also may occur in non-endosomal/lysosomal compartments, for example by enzymes located at the plasma membrane or within mitochondria. Overall, over 100 enzymes participate in the synthesis or breakdown of sphingolipids.

Historically, most information concerning the importance of sphingolipids in human disease stems from studies of the sphingolipid storage diseases (Schulze and Sandhoff 2011). These disorders are each due to deficient function of one or more of the hydrolytic enzymes found within the endosomal/lysosomal system, resulting in sphingolipid accumulation (Cox and Cachón-González 2012). Each of these diseases is inherited as a Mendelian trait (recessive or X-linked), the cDNAs/genes encoding the respective enzymes have been isolated, and many mutations causing the human diseases have been identified. In addition, genetic abnormalities in several of these enzymes/genes have now been linked to more common diseases (e.g., Parkinson's disease and beta-glucocerebrosidase) (Young et al. 2012). Table 1 lists the sphingolipid storage diseases and the respective enzymes involved.

This review focuses on the genetics of sphingolipid storage diseases and related hydrolytic enzymes. Each of the diseases/enzymes is briefly reviewed, with an emphasis on the importance of genetic mutations in disease.

**Table 1** Genetics of human sphingolipid hydrolases and diseases

Sphingolipid hydrolase/ activator	Disease	Primary accumulating lipid	Gene designation	Gene location
Acid ceramidase	Farber disease	Ceramide	ASAHI	8p22
Acid sphingomyelinase	Niemann-Pick (types A and B)	Sphingomyelin	SMPD1	11p15.4- p15.1
Beta glucocerebrosidase	Gaucher disease	Glucosylceramide	GBA1	1q21
Beta galactocerebrosidase	Krabbe disease	Galactosylceramide	GALC	14q31
Arylsulfatase A	Metachromatic leukodystrophy	Sulfatide	ARSA	22q13.31- qter
Alpha galactosidase A	Fabry disease	Globotriosylceramide	GLA	Xq22.1
Beta galactosidase	GM1 gangliosidosis	GM1 ganglioside	GLB1	3p22.31
Hexosaminidase	GM2 gangliosidosis			
Hexosaminidase A	Tay-Sachs disease	GM2 ganglioside	HEXA	15q24
Hexosaminidase B	Sandhoff disease	GM2 ganglioside	HEXB	5q13
GM2 activator	Tay-Sachs A/B variant	GM2 ganglioside	GMA2	5q31.1
Prosaposin	Variable	Variable	PSAP	10q21- q22

## 2 Acid Ceramidase Deficiency: Farber Disease

Patients with Farber disease (also known as Farber lipogranulomatosis) usually present within the first few months of life with a triad of clinical manifestations, including painful and progressively deformed joints, subcutaneous nodules, particularly near joints, and progressive hoarseness due to laryngeal involvement (Farber et al. 1957). About half of the patients have neurological deficits (Ahmad et al. 2009). As with all of the sphingolipid storage disorders, there is considerable clinical heterogeneity among Farber disease patients, and later onset, attenuated cases also have been described. Farber disease is the least common of the sphingolipid storage diseases, likely because severe mutations in the acid ceramidase (AC) gene (*ASAHI*) lead to embryonic lethality (see below).

Acid ceramidase (E.C. 3.5.1.23) was first identified and purified in the 1960s. It was recognized as the enzyme deficient in Farber disease in the 1970s (Sugita et al. 1972). The full-length cDNAs and genes encoding AC have been isolated from humans, mice, and other species. The human cDNA encodes a 395 amino acid precursor polypeptide, and the gene is located on chromosomal region 8p22. Of note, the newly synthesized AC precursor is inactive, and only assumes an active form after *N*-glycosylation and the internal cleavage of a peptide bond, resulting in alpha and beta subunits. Interestingly, this cleavage event is carried out by AC itself, and it is therefore considered a “self-regulating” enzyme. In addition, AC associates with other lipid hydrolases within lysosomes and other cell compartments, including acid sphingomyelinase (see below). The importance of

this multienzyme complex on AC activity, as well as on sphingolipid metabolism in general, is not clearly understood. Finally, full AC activity, at least in lysosomes, depends on the expression of a sphingolipid activator protein (SAP-D), which is expressed by a distinct gene (see Sect. 10, below).

Acid ceramidase is an “amidase” that hydrolyzes the amide bond between sphingosine and the fatty acid in ceramide. Ceramides are a heterogeneous group of lipids defined by the length of their fatty acid chains. While the substrate specificity of AC is not entirely clear, ceramides with fatty acid chains shorter than 12 carbons cannot be efficiently hydrolyzed by the enzyme *in vitro*. *In vivo*, it is likely that AC can recognize and cleave ceramides of varying chain lengths. The ceramides that accumulate in Farber disease patients are primarily found within lysosomes, leading to the original designation of AC as a lysosomal enzyme. AC also has maximal activity at lysosomal (acidic) pH, although it has been localized to sites other than the lysosomes (e.g., the spindle in oocytes) as well. It is therefore not entirely clear to what extent the biological activity of AC depends on the acidic environment, and in fact a “reverse” ceramidase reaction has been attributed to the enzyme at more neutral pH (Okino et al. 2003). Numerous *in vitro* assays have been developed for AC, and some can be used to diagnose Farber disease in cultured cells from suspected patients, or prenatally using amniocytes or cultured chorionic villi. However, due to the very hydrophobic nature of ceramides, it remains one of the more difficult diagnostic enzyme assays to carry out.

To date, only about 100 Farber disease patients have been reported in the literature (Park and Schuchman 2006). Several cases of hydrops fetalis also have been reported. Complete inactivation of the AC gene in mice leads to very early embryonic lethality (four-cell stage), suggesting that severe mutations in the human *ASAH1* gene will lead to embryonic lethality as well (Li et al. 2002). Of note, mouse embryos lacking AC can be rescued by the addition of recombinant AC (obtained from overexpressing Chinese hamster ovary [CHO] cells) to the culture media. Moreover, addition of recombinant AC to the culture media during *in vitro* fertilization (mouse, bovine) enhances embryo production and quality, resulting in more live births (Eliyahu et al. 2010).

Thus, AC is an essential enzyme required for very early mammalian development. One explanation for the lethal phenotype in mice is that in the absence of this enzymatic activity, early-stage embryos (four cells in mice) undergo apoptosis due to ceramide accumulation. It should be recognized, however, that an additional consequence of such AC “loss of function” during embryogenesis is reduced production of sphingosine, which in turn is converted to S1P, an important mitogenic/proliferative lipid. Thus, AC mutations also may reduce S1P production, contributing to the lethality. Indeed, embryos lacking AC can be partially rescued by S1P as well.

As noted above, the first relationship between AC and human disease arose from studies on Farber disease. To date over 20 mutations have been described in the *ASAH1* gene leading to Farber disease (Park and Schuchman 2006). Most are point mutations, although several splice variants leading to small deletions have been described as well. To date, no patients have been found homozygous for a complete

loss-of-function mutation, likely because such individuals do not survive embryonic development. Farber disease is inherited as an autosomal recessive disorder, and to develop the disorder mutations must be inherited on both *ASAH1* alleles. No reports of clinical findings in the parents of affected patients (obligate carriers) have been published; however such individuals are not usually followed by clinicians and follow-up analysis is lacking.

Recently, a “knock-in” mouse model of Farber disease has been reported where a specific human mutation was expressed (add ref). Preliminary analysis of these animals indicates that they develop a Farber disease-like phenotype. In addition, a conditional AC knockout mouse has been developed where AC can be inactivated at various stages of development or in specific tissues.

Other than Farber disease, which is due to the deficiency of AC, constitutive overexpression of the AC gene also occurs in many types of cancer (Park et al. 2005; Beckham et al. 2012). This may promote tumorigenesis and/or metastasis due to overproduction of SIP, or may lead to chemoresistance due to an enhanced ability to hydrolyze pro-apoptotic ceramide (Flowers et al. 2012; Furuya et al. 2011). The molecular basis of AC overexpression in cancer has not been studied extensively, but at least in some cases it appears to be due to elevated transcription. Gain-of-function mutations also could explain this finding, although to date these have not been described. In addition, a recent report showed that the *ASAH1* gene was down-regulated in a Chinese population of schizophrenic patients, and was associated with two single nucleotide polymorphisms in this gene (Zhang et al. 2012).

Finally, it must be noted that at least six other ceramidase activities have been described in man, and at least four other genes have been cloned (Mao and Obeid 2008). These ceramidases are defined by their unique pH, subcellular location, and other biological properties. These enzymes also play important roles in sphingolipid metabolism and/or signaling, and are likely to contribute to human disease pathogenesis. The biology of the other ceramidases has been reviewed extensively elsewhere (e.g., Mao and Obeid 2008).

### **3 Acid Sphingomyelinase Deficiency: Types A and B Niemann–Pick Disease**

Types A and B Niemann–Pick Disease (NPD) result from mutations in the gene (*SMPD1*) encoding acid sphingomyelinase (E.C. 3.1.4.12) (McGovern and Schuchman 2009; Schuchman 2009). Patients with Type A NPD present in early infancy with hepatosplenomegaly and failure to thrive, and develop a rapidly degenerative neurological phenotype that leads to death by 3 years of age. In contrast, patients with Type B NPD have a later onset disease, and usually lack neurological findings. Such individuals also frequently present with hepatosplenomegaly, and may have pulmonary involvement as well. Dyslipidemia (high LDL-C, low HDL-C, high triglycerides) also is common. Patients with Type B

NPD may survive into adulthood, although their life span is generally shortened by the disease. The cause of death is unknown, but may occur from complications related to liver disease, trauma-induced bleeding episodes due to thrombocytopenia, early-onset cardiovascular disease, or pulmonary disease.

Acid sphingomyelinase (ASM) was found to be deficient in Type A and B NPD patients in the late 1960s. Although the enzyme had been discovered several years earlier in rat tissues, it was not substantially purified until the late 1980s. The cDNAs and genes encoding human and mouse ASM were cloned in the early 1990s. The human gene is located on chromosome 11p15.1. Of interest, among genes encoding lysosomal enzymes, it is the only one for which genomic imprinting has been described. This form of genetic regulation is generally reserved for genes with important developmental roles.

Over 100 mutations have been found in the *SMPDI* gene leading to Types A or B NPD (<http://www.hgmd.org>). Of note, several of these mutations are common within specific regions or ethnic populations, allowing genetic screening programs to be established. Homozygosity for these mutations is required to develop NPD, and it is inherited as an autosomal recessive trait. However, heterozygous carriers of *SMPDI* mutations may develop dyslipidemia and late-onset features of the disorder. In part, the development of a phenotype in the carrier individuals may be related to their imprinting status (i.e., whether the mutations are inherited from the mother or the father) (Simonaro et al. 2006). Unlike acid ceramidase and several other sphingolipid hydrolases, ASM does not require an exogenous activator protein to achieve full function. Thus, mutations in the sphingolipid activator protein gene (see below) do not affect ASM activity or cause Types A and B NPD. Of interest, the lysosomal lipid, bis(monacyl) phosphate, may activate the enzyme in vitro and in vivo.

In addition to ASM, several other sphingomyelinases (nonacidic) exist in mammalian cells (Clarke et al. 2011). These are defined by their unique subcellular location or other biochemical properties. At least four human genes have been isolated encoding these sphingomyelinases. Definitive proof that mutations in the *SMPDI* gene were solely responsible for Types A and B NPD came from studies in the knockout mouse model (ASMKO), where neutral and other sphingomyelinase activities were normal, despite the fact that these animals completely lacked ASM and developed NPD-like pathology and clinical features (Horinouchi et al. 1995). In addition, these early studies in the ASMKO mice showed that the previously identified zinc-activated, serum (secreted) form of ASM was encoded by the same *SMPDI* gene as lysosomal ASM (Schissel et al. 1998). It is now known that ASM is, in fact, a zinc-requiring enzyme. However, the lysosomal form of the enzyme has high levels of zinc bound, and thus does not require exogenous zinc when measured in vitro. In contrast, the serum form of the enzyme requires exogenous zinc in the assay systems routinely used.

Another early finding in the ASMKO mouse was that ceramide, the product of sphingomyelin hydrolysis by ASM, also was elevated in tissues of these animals. While initially a surprising result, it is likely that the elevated ceramide derives from breakdown of the accumulating sphingomyelin in NPD tissues by other sphingomyelinases. Sphingomyelin levels may be up to 30-fold above normal in



the ASMKO mice, and ceramide two- to fivefold above normal. Most recently, sphingosine levels also were found to be high in tissues from ASMKO mice, probably due to breakdown of the accumulating ceramide by ceramidases (Schuchman et al., unpublished observations). Of interest, the degree of sphingosine accumulation in these animals (>20-fold above normal) cannot be explained by ceramide alone, perhaps suggesting some defect in converting the sphingosine to SIP.

The ASMKO mice develop a phenotype intermediate between Types A and B NPD. They die prematurely at ~6–8 months, secondary to neurological disease. They have been extensively used to study the pathobiology of this disease, and to develop/evaluate new therapies. In particular, enzyme replacement therapy (ERT) using recombinant ASM produced in CHO cells has been developed in the mouse model (Miranda et al. 2000), and a phase 1 clinical trial in adult patients with Type B NPD was recently completed (<http://clinicaltrials.gov>). A phase 2 trial is planned for 2012. Of interest, in the ASMKO mice dose-related toxicity was observed (which was not seen in healthy animals) using recombinant ASM, presumably due to rapid breakdown of the accumulating sphingomyelin to ceramide. This hypothesis was confirmed by pretreating animals with low dose of enzyme (“debulking” the accumulating sphingomyelin), which prevented any toxicity. In the patients treated by ERT, transient changes in several inflammatory biomarkers were observed, corresponding to dose and serum levels of ceramide.

Beginning in the late 1990s, the ASMKO mice also were extensively used to investigate the role of ASM in cell signaling. This is the subject of several reviews, and is not focused on in this chapter. However, it is now clear that ASM plays an important role in stress-induced ceramide generation and apoptosis, and that this occurs through hydrolysis of sphingomyelin at the cell surface within lipid rafts (Zeidan and Hannun 2010). How ASM, which has an acidic pH optimum *in vitro* and functions within lysosomes, participates in these processes has been the subject of much debate. It is now known that after the induction of stress or developmental signals, ASM may be rapidly translocated to lipid rafts at the cell surface. The mechanism underlying this rapid translocation is not entirely clear, but phosphorylation within the ER/Golgi may be responsible in part (Zeidan et al. 2008). It is also known that although ASM requires an acidic pH for optimal activity *in vitro*, *in vivo* it is capable of efficiently hydrolyzing sphingomyelin at physiological pH. This highlights the complex biology of this and other sphingolipid hydrolases, particularly in terms of pH, subcellular location, and other properties.

These observations on the ASMKO mice have also highlighted the important role of ASM in human disease, beyond NPD (e.g., Becker et al. 2010; Bikman and Summers 2011; Truman et al. 2011). Elevated ASM activity has now been detected in numerous common diseases, including diabetes, Alzheimer disease, cardiovascular diseases, pulmonary diseases, depression, and others. These will also be reviewed elsewhere and not discussed further here. At the present time it is not known if the elevated ASM found in these diseases is due to an activating genetic mutation, enhanced transcription, and/or posttranslational modification of the enzyme.

## 4 Beta-Glucocerebrosidase Deficiency: Gaucher Disease

Gaucher disease, due to the deficient activity of acid beta-glucocerebrosidase (also known as beta-glucosidase [E.C. 3.2.1.45]), is the most common sphingolipid storage disorder (Campbell and Choy 2012; Lee et al. 2012). There are three clinical subtypes of Gaucher disease, including an early-onset, neurological form (Type 2), an intermediate form (Type 3), and a late-onset, non-neurological form (Type 1) (Lo et al. 2012; Pastores 2010). All Gaucher patients present with hepatosplenomegaly, but the progression of the disease and survival are markedly different. For example, Type 3 children may only survive until 2–3 years of age, while Type 1 patients may survive into adulthood. If left untreated (see below), the hematological and bone lesions in Type 1 patients progress with age, seriously limiting quality of life. A massively enlarged spleen and frequent bone fractures may become common, requiring splenectomy, joint replacement, or other surgical interventions (Mikosch and Hughes 2010; Goker-Alpan 2011). Bleeding also is a common presenting feature of Gaucher disease due to thrombocytopenia. Pulmonary involvement may similarly occur. Of note, neoplastic disorders, particularly of the blood-forming organs, are more common in Gaucher disease patients than in the general population. These include chronic lymphocytic leukemia, multiple myeloma, and Hodgkin and non-Hodgkin lymphoma. The molecular basis of this increased cancer risk remains unknown (Choy and Campbell 2011).

All Gaucher patients are characterized by the presence of “Gaucher” cells in various organs, lipid-filled cells derived from the monocyte/macrophage system. Gaucher cells have a distinctive appearance by light microscopy, and can be readily distinguished from cells of other sphingolipid storage disorders. Gaucher cells are distributed throughout the body, wherever macrophages reside. The largest numbers are found in the spleen, sinusoids of the liver, bone marrow, and lymph nodes.

The major accumulating lipid in Gaucher disease is *N*-acyl-sphingosyl-1- $\beta$ -D-glucoside (glucosylceramide, also known as glucosylcerebroside, GC). GC is widely distributed in mammalian tissues in small quantities as a metabolic intermediate in the synthesis and degradation of complex glycosphingolipids, such as gangliosides or globoside. The deacylated form of GC, glucosylsphingosine, is not normally found in tissues, but also accumulates in Gaucher disease cells. GC levels in plasma may be 2- to 20-fold above normal.

The major site of GC turnover in cells is the lysosome. Beta-glucocerebrosidase is normally found in this organelle and functions optimally at an acidic pH. As with the other lipid hydrolases, *in vitro* detection of this enzymatic activity can be difficult, and there have been various factors (lipids, protein, sugars) shown to influence the assay systems. *In vivo*, sphingolipid activator-derived peptides, particularly saposin (SAP) C, appear to be required for full enzymatic activity (see below). Nonacidic glucocerebrosidases also have been described and are encoded by distinct genes. Whether these activities can be modifying factors for the Gaucher phenotype has been the subject of much discussion, but remains unclear.

The gene encoding beta-glucocerebrosidase (*GBAI*) has been mapped to chromosome 1q21. It is ~7 kb and contains 11 exons. Of note, a 5 kb “pseudogene” is located immediately downstream from the *GBAI* gene. This pseudogene has maintained a high degree of homology with the functional gene and is transcribed. However, the nucleotide changes within the pseudogene sequence prevent the occurrence of a long open reading frame, precluding the possibility of expressing an active beta-glucocerebrosidase (Brown et al. 2006). Full-length cDNAs from many species also have been obtained that can be used to express the active enzyme.

Numerous mutations have been found in the *GBAI* gene causing Gaucher disease (Hruska et al. 2008). As described for Niemann–Pick disease above, several common mutations are found in specific populations, and DNA-based screening is now routinely carried out in the Ashkenazi Jewish population (Scott et al. 2010). Estimates of the Gaucher disease carrier frequency in this population range from ~1:10 to 1:25. Some of the mutations also can be used to predict the occurrence of severe or mild Gaucher phenotypes, assisting in family planning and genetic counseling.

Recently, mutations in the *GBAI* gene also have been associated with the occurrence of Parkinson’s disease, although the precise relationship between the *GBAI* gene, glucocerebrosidase activity, and Parkinson’s disease remains uncertain (Kinghorn 2011). One of the first clues suggesting this linkage came from the small number of Gaucher patients who exhibited Parkinson-like features, while stronger associations later came from Gaucher relatives who were carriers for *GBAI* mutations. Currently, heterozygous *GBAI* mutations are considered to be one of the most common genetic risk factors for Parkinson’s disease (Anheim et al. 2012).

A complete knockout mouse model for Gaucher was constructed and had an early neonatal lethal phenotype, dying within 34 days after birth. These animals had massive GC storage, and while the cause of death was not entirely clear, skin abnormalities were prevalent. Several knock-in models also have been constructed, and some exhibited longer survival and later onset GC storage in macrophages, more closely resembling Gaucher disease patients (Farfel-Becker et al. 2011; Sardi et al. 2012).

Gaucher disease was the first sphingolipid storage disease for which ERT was developed in the early 1990s, first using beta glucocerebrosidase purified from placenta (Ceredase), and subsequently using recombinant enzyme produced in CHO cells (Cerezyme) (Hughes and Pastores 2010; Lachmann 2011). For non-neurological patients, ERT was life changing, resulting in reduction in spleen size and correction of the hematological abnormalities. Bone disease has been much less responsive to ERT, and remains a challenge in the treatment of Gaucher disease, as does treatment of the brain disease (Hughes and Pastores 2010). Another treatment strategy currently available for Gaucher patients uses a small molecule inhibitor of GC synthesis (Miglustat) to slow the accumulation of this lipid (Abian et al. 2011; Benito et al. 2011). The target of this inhibitor is the enzyme glucosylceramide synthase.

Of note, treatment of Gaucher patients either by ERT or with Miglustat should elevate ceramide, and could potentially result in ceramide-mediated toxicity. To date, however, such toxicity has not been observed. There are several factors that might explain this observation. First, compared to sphingomyelin, GC is generally found in small quantities in cells, and is not a major structural component of cell membranes. Thus, accumulation of GC in Gaucher cells is more exclusively found in the lysosomes than sphingomyelin in Niemann–Pick cells, where substantial amounts of the accumulating lipid also are found in the plasma membrane. Thus, ceramide-mediated raft reorganization and signaling are less likely to become activated in Gaucher disease compared to NPD. In addition, ceramide produced by ASM following ERT in NPD is likely to remain within the cell membrane, while the ceramide produced following ERT with Cerezyme or by inhibiting GC synthesis with Miglustat may be found in different cellular compartments, and therefore more readily accessible for biosynthetic pathways. Lastly, the ERT dose used to treat Gaucher patients (1.6 mg/kg, every 2 weeks) may be below the threshold needed to induce ceramide-mediated toxicity.

## 5 Galactocerebrosidase Deficiency: Krabbe Disease/Globoid Cell Leukodystrophy

Infantile globoid cell leukodystrophy (GLD), also known as Krabbe disease, is a rapidly progressive, fatal neurological disease of infants (Kohlschütter and Eichler 2011). The disease usually begins between 3 and 6 months of life with symptoms that may include irritability or hypersensitivity to stimuli. Patients rapidly exhibit severe mental and motor deterioration, and rarely survive beyond the second year (Duffner et al. 2011). Clinical manifestations in classical metachromatic leukodystrophy (MLD) patients are limited to the nervous system. Blindness and deafness are common, and peripheral neuropathy is almost always detectable. Patients with later onset forms of the disease, including adult-onset forms, may present with blindness, spastic paraparesis, and dementia (Malandrini et al. 2013; Perlman and Mars 2012).

The presence of numerous multinucleated globoid cells, almost total loss of myelin and oligodendrocytes, and astrocytic gliosis in the white matter are the morphologic basis of the disease. “Globoid cells” in Krabbe disease are enlarged macrophages that contain undigested galactosylceramide and other lipids. Demyelination, axonal degeneration, fibrosis, and histiocytic infiltration are also common in the peripheral nervous system. Consistent with the myelin loss, the white matter is severely depleted of all lipids, particularly glycolipids. Under normal circumstances, galactosylceramide is present almost exclusively in the myelin sheath. The ratio of galactosylceramide to sulfatide (3-O sulfogalactosylceramide) is abnormally high in MLD.

The genetic cause of Krabbe disease is mutations in the gene encoding galactocerebrosidase beta-galactosidase (*GALC*; E.C. 3.2.1.46) (see below). This lysosomal enzyme normally degrades galactosylceramide to ceramide and galactose. It is postulated that accumulation of a toxic metabolite, psychosine (galactosylsphingosine), which is also a substrate for the missing enzyme, leads to early destruction of the oligodendrocytes (see below). The total brain content of galactosylceramide is not increased in patients with Krabbe disease, supporting this hypothesis. It should be noted that mammalian tissues contain two genetically distinct lysosomal beta-galactosidases, *GALC* and GM1-ganglioside beta-galactosidase (see Sect. 8, below). These two enzymes have overlapping substrate specificities in vitro, which can present complications when attempting to make the enzymatic diagnosis of Krabbe disease (Sakai 2009).

The human *GALC* gene has been mapped to chromosome 14q24.3-32.1, and the genomic and/or full-length cDNA sequences have been cloned from multiple species. The human cDNA encodes a 669 amino acid polypeptide with six potential *N*-glycosylation sites. The fully glycosylated precursor polypeptide is ~85 kDa, and is processed into ~50 and 30 kDa subunits. To date, over 70 mutations in the *GALC* gene have been identified in infantile and late-onset *GALC*-deficient patients (<http://www.hgmd.org>). Most of these mutations are “private,” occurring in individual families, but some may be commonly found in specific regions or ethnicities. Among these, the most common mutation occurs in patients of European ancestry, and deletes a large segment of the *GALC* gene. Several polymorphisms also have been found in the *GALC* gene, and some of these may alter amino acids and thus potentially affect the function of the *GALC* polypeptide, although not enough to cause GLD.

In general, carriers of *GALC* mutations are considered clinically normal, although one interesting, recent report did show an association between the risk for primary open-angle glaucoma and a heterozygous deletion of the *GALC* gene (Liu et al. 2011). Another interesting recent paper also showed that *GALC* contributed to the maintenance of a functional hematopoietic stem cell niche, suggesting that genetic alterations in the *GALC* gene may be associated with hematological conditions as well. Indeed, hematopoietic stem cells retrieved from Twitcher mice (*GALC* deficient, see below) had defects in their frequency, proliferation, clonogenic potential, and engraftment.

As noted above, in 1972 Miyatake and Suzuki formulated a hypothesis to explain the unusually rapid and complete destruction of oligodendrocytes in GLD. It was already known at that time that psychosine (galactosyl sphingosine), due to its free amino group, was highly cytotoxic, and that this lipid served as a substrate for *GALC*, but not GM1 beta-galactosidase. In the central nervous system, psychosine is primarily formed within oligodendrocytes, the site of myelin synthesis, and this may explain the selective destruction of these cells in GLD brains. Indeed, in the white matter of brains from Krabbe patients, psychosine levels may be elevated up to 100-fold, while galactosylceramide levels are either normal or only elevated slightly.

GALC deficiency occurs in a number of naturally occurring animal models, including mice, sheep, several breeds of dogs, and the rhesus monkey. The mouse model in particular (also known as the “Twitcher” mouse) has been exploited for numerous therapeutic studies (Luzi et al. 2001). Affected mice develop clinical signs at ~20 days, with stunted growth, twitching, and hind leg weakness. By 40 days they reach a near-terminal stage. Twitcher mice have been treated by ERT, gene therapies, BMT, and other modalities (Neri et al. 2011; Reddy et al. 2011; Lin et al. 2011). BMT in particular may have positive therapeutic effects; however all of the treated mice eventually die of characteristic pathological findings. Thus, this and the other available therapies may slow the disease progress to some degree, but not prevent it.

Since 2006, New York State in the USA has screened all newborns for the occurrence of Krabbe disease. Pilot screening programs are now under way in other states and parts of the world. This screening is accomplished by GALC activity assays on dried blood spots, followed by DNA analyses. The rationale for this screening program is that the early identification of infants with Krabbe disease will permit early BMT and other therapeutic interventions, improving the quality of life (Duffner et al. 2012). However, since BMT has not been curative in the animal models, screening for severe neurological disorders such as this remains controversial.

## 6 Arylsulfatase A Deficiency: Metachromatic Leukodystrophy

MLD is an autosomal recessively inherited disorder in which the desulfation of 3-0-sulfogalactosylsphingosine or 3-0-sulfogalactosyl glycolipids is deficient. Among the accumulating glycolipids in MLD are sulfatide (galactosylceramide-3-sulfate), lysosulfatide (i.e., similar to sulfatide but lacking the fatty acid in the ceramide moiety), lactosylceramide-3-sulfate, and others. These sulfated glycolipids normally occur in the myelin sheaths in the central and peripheral nervous systems, and to a lesser extent in visceral organs.

The clinical onset and severity of MLD are highly variable (Renaud 2012). The late infantile form is usually recognized in the second year of life and fatal in early childhood. The juvenile form presents between age 4 and puberty; the adult form may occur at any age after puberty. In both of the later onset variants, gait disturbance and mental regression are the earliest signs. In the childhood variants, other common signs are blindness, loss of speech, quadriplegia, peripheral neuropathy, and seizures (Kehrer et al. 2011).

In 1963, Austin et al. reported the deficiency of arylsulfatase A (ASA, E.C. 3.1.6.8) activity in MLD, also known as sulfatide sulfatase, and mutations in this gene (*ARSA*) account for the vast majority of MLD patients. There also are two other minor, genetic causes of MLD. One is due to mutations in the gene (*PSAP*, see Sect. 10, below) encoding the sphingolipid activator protein, saposin B. Mutations in the saposin B encoding region of the *PSAP* gene lead to deficient ASA activity, and a clinical picture indistinguishable from MLD. In fact, these

observations in MLD patients provided the first *in vivo* proof that such sphingolipid activator proteins were required for *in vivo* activity of this and other sphingolipid hydrolases. In addition to ASA and SAP B mutations, patients with multiple sulfatase deficiency, due to mutations in the gene encoding sulfatase-modifying factor 1 (*SUMF-1*) (see below), also have a reduction in ASA and other sulfatase activities, and develop features of MLD.

As with the other sphingolipid hydrolases, full-length genomic and cDNA sequences encoding ASA have been isolated from several species. The human *ARSA* gene has been mapped to chromosomal region 22q13. It is a remarkably simple gene, and encompasses only 3.2 kb divided into eight exons. As noted above, almost all cases of MLD are due to mutations in this gene, except for the rare cases of *PSAP* or *SUMF-1* mutations. The human *ARSA* gene encodes a 507 amino acid polypeptide precursor that undergoes posttranslational modifications much like other lysosomal enzymes (e.g., *N*-glycosylation). Also, as with all known eukaryotic sulfatases, a formylglycine residue is substituted for cysteine 69 in human ASA. The formylglycine residue is generated by oxidation of the thiol group to an aldehyde. Mutations in the gene encoding the cysteine-modifying enzyme, *SUMF-1*, lead to the synthesis of inactive sulfatases, and are the cause for multiple sulfatase deficiency (Gieselmann et al. 1994).

Over 100 MLD-causing mutations have been described in the *ARSA* gene. The most common mutation in patients with late-infantile MLD is a G-to-A transition that eliminates the donor splice site at the start of intron 2 (Ługowska et al. 2011). This causes a loss of all ASA enzymatic activity, producing a severe, early-onset phenotype. In contrast, in adults with MLD the most frequent mutation is a C-to-T transition that results in substitution of a leucine for proline in amino acid residue 426. Patients with this mutation have a small amount of residual enzyme activity and therefore exhibit late-onset MLD. Common mutations in specific populations also have been described.

Notably, there also are two mutations that cause “pseudodeficiency” of ASA activity. The term “pseudodeficiency” reflects the fact that these mutations cause severe reduction of ASA activity *in vitro*, but do not result in MLD (Tinsa et al. 2010). One of these mutations alters an *N*-glycosylation site, but this has no effect on enzyme function. The other affects a polyadenylation site in the mRNA. The pseudodeficiency alleles may be found in ~3–8 % of the normal population, and therefore pose a diagnostic challenge since the artificial *in vitro* assay systems may reveal very low ASA activity, even though the individual is highly unlikely to develop MLD.

Alterations in the *ARSA* gene also have been linked with the occurrence of several common diseases. For example, as early as 1991, Kappler et al. found that among a small set of patients with multiple sclerosis (MS), there was a higher incidence of the ASA pseudodeficiency alleles than in the general population (Baronica et al. 2011). In addition, variants of the ASA protein and/or gene have been associated with depression and alcoholism. MLD itself also has been associated with several

psychiatric disorders, and there is at least one report where polymorphisms in the genes encoding the serotonin and dopamine pathways affected the MLD phenotype (Kumperscak et al. 2008).

Other than humans, naturally occurring mutations causing MLD do not occur in other species. ASA knockout mice have been constructed that exhibit a complete loss of ASA activity and sulfatide storage multiple organs (Geiselman et al. 1998). While these animals exhibit some neurological deficits, they do not develop the predicted severe phenotype of MLD and live a near-normal life span. They also do not develop demyelinating disease. Thus, these observations in the mouse suggest the existence of compensating pathways in this species that do not exist in humans, perhaps providing a link to novel therapies. Several therapies have been evaluated in the MLD knockout mouse model, including BMT, ERT, and gene therapies (Matthes et al. 2012; Stroobants et al. 2011). BMT also has been extensively used in MLD patients. While it is not effective in the infantile cases, BMT is often recommended for the later onset cases (Solders et al. 1998; Gassas et al. 2011). However, the data supporting an improved clinical outcome in the late-onset MLD cases are fragmented and incomplete, and it is unclear whether the benefit outweighs the potential risks.

## 7 Alpha Galactosidase A Deficiency: Fabry Disease

Fabry disease is inherited as an X-linked recessive trait, the only such X-linked disease among the sphingolipid storage disorders (Tarabuso 2011; Toyooka 2011). Therefore, all males (“hemizygotes”) carrying mutations in the Fabry gene (*GLA*, see below) develop symptoms of the disease, although the severity may vary depending on the nature of the individual mutation. Female heterozygotes who inherit one copy of the mutant gene on one of their X chromosomes also may exhibit features of the disease depending on the pattern of X-inactivation (“lyonization”) that occurs in their individual tissues.

The gene responsible for Fabry disease encodes an enzyme, alpha-galactosidase A (E.C. 3.2.1.22), that is required to hydrolyze glycosphingolipids with terminal alpha galactosyl moieties, predominately globotriosylceramide, and to a lesser extent, galabiosylceramide and blood group B substances. Affected males have extensive deposition of these lipids in body fluids and in the lysosomes of endothelial, perithelial, and smooth muscle cells of blood vessels. Deposition also occurs in ganglion cells, and in many cell types in the heart, kidney, eyes, and most other tissues (Aerts et al. 2011).

Clinical manifestations in classically affected hemizygotes with no detectable alpha galactosidase A activity include the onset during childhood or adolescence of pain and paresthesias in the extremities, angiokeratomas in the skin and mucous membranes, and hypohidrosis. Corneal and lenticular opacities also are early findings. With increasing age, proteinuria, hyposthenuria, and lymphedema appear. Severe renal impairment leads to hypertension and uremia. Death usually occurs



from renal failure or from cardiac or cerebrovascular disease. Atypical variant males with residual alpha galactosidase A activity may be asymptomatic or have late-onset disease. Heterozygous females may have an attenuated form of the disease depending on the pattern of X-inactivation. They usually are asymptomatic, although rarely present with clinical disease as severe as hemizygous males.

As noted above, in Fabry disease glycosphingolipids with terminal alpha-D-galactosyl residues are not properly broken down due to the alpha galactosidase A enzyme defect, and these lipids therefore accumulate in cells. The predominant accumulating lipid is globotriaosylceramide (Gal( $\alpha$ 1-4)Gal( $\alpha$ 1-4)Glc( $\beta$ 1-1')Cer). Since alpha galactosidase A is predominately found in lysosomes, the major site of lipid accumulation is this organelle. Elevated globotriaosylceramide is found in most tissues, but predominantly in kidney.

Other minor accumulating lipids include galabiosylceramide (Gal $\alpha$ 1-4)(Gal( $\beta$ 1-1')Cer) and the blood group B and P glycosphingolipids (Linthorst et al. 2004). In human erythrocytes there are two neutral glycosphingolipids with terminal alpha galactosyl residues that inhibit blood group B-specific hemagglutination. The structure of these blood group B glycosphingolipids has been determined, and they are found at high levels in Fabry patients. This raises an interesting and unique point about Fabry disease. Fabry hemizygotes and heterozygotes who have blood group B and AB accumulate four glycosphingolipids, while those who are blood group A or O only accumulate two (globotriaosylceramide and galabiosylceramide). A fifth neutral glycosphingolipid that can accumulate in Fabry is the P1 blood group antigen, which also has terminal alpha galactosyl residues.

In man, there are actually two alpha galactosidases (alpha galactosidases A and B). Fabry disease is caused by mutations in the gene (*GLA*) encoding the "A" type (see below) (Tomasic et al. 2010). However, when measuring alpha galactosidase activity in blood from Fabry patients in vitro, classic hemizygotes may exhibit up to 25 % of normal activity due to the presence of the "B" form. Alpha galactosidase B is encoded by a distinct gene (*NAGA*), and is now known to be an alpha-N-acetylgalactosaminidase that recognizes these moieties in glycoproteins, complex carbohydrates, and other molecules. Because the artificial substrates routinely used to measure alpha galactosidase activity do not readily distinguish these enzymes, substantial misdiagnosis can occur by simple enzyme testing.

The human gene encoding alpha galactosidase A resides on chromosomal region Xq22. It encompasses ~12 kb and contains seven exons. The full-length human cDNA contains a 1,290 bp open reading frame that encodes an unglycosylated precursor polypeptide of ~48 kDa. As with most lysosomal hydrolases, there are several N-glycosylation sites in the predicted polypeptide sequence and the oligosaccharide chains undergo mannose-6-phosphate modifications, which facilitates targeting of the enzyme to lysosomes. The mature, fully glycosylated alpha galactosidase A in lysosomes is ~51 kDa. Genomic and cDNA sequences encoding alpha galactosidases have now been isolated from many species. An unusual feature of most human alpha galactosidase A cDNAs is the lack of a 3' untranslated region. The polyadenylation signal sequence is often actually found in the coding region, 12 bp from the termination codon, followed by a poly(A) tract.

The full-length cDNA encoding human alpha galactosidase A had been used to express and purify the recombinant enzyme from CHO cells. This enzyme was extensively characterized and used in the Fabry disease mouse model to evaluate ERT (see below). Based on these results and after extensive clinical testing, a recombinant enzyme drug (Fabrazyme) was approved for use in human Fabry disease patients in 2003. This represents the second sphingolipid storage disorder for which ERT became available (Gaucher disease was the first). ERT in Fabry patients reduces pain, proteinuria, and endothelial cell storage of glycolipids, and improves the quality of life for Fabry patients (Rozenfeld and Neumann 2011). Since no naturally occurring animal model of Fabry disease has been found, the preclinical evaluation of ERT was performed in a knockout mouse model. These animals exhibit age-dependent glycosphingolipid storage, but there are only a few clinical findings of Fabry disease.

Numerous mutations have been found in the *GLA* gene causing Fabry disease, spread throughout the enzyme-coding region (Schaefer et al. 2005). Several regulatory (promoter) and splice site mutations also have been found. As noted above, the presence of one *GLA* mutation in hemizygous males will cause disease, but the severity will depend on the effect of this mutation on the residual alpha galactosidase A activity. In contrast, in female heterozygotes, because of X-inactivation, only some may develop disease (Bouwman et al. 2012). It should be noted that such “Fabry” females carrying single mutations will be essentially “mosaics” for alpha galactosidase A, with some cells expressing the mutant allele and others the normal allele (due to random inactivation of one X chromosome). Because the enzyme can be released from cells at low levels (as with all lysosomal hydrolases), and then taken up by neighboring cells, “cross-correction” of the lipid storage is possible. Aside from Fabry disease, there has been some evidence that mutations in the *GLA* gene also may be a previously unrecognized cause of stroke. Indeed, newborn screening programs have revealed that in some populations the incidence of *GLA* mutations is unexpectedly high, suggesting that this gene/disease association should be studied further as a potential risk factor for this and other common disorders of the vasculature (Spáčil et al. 2011).

## 8 Beta Galactosidase Deficiency: GM1 Gangliosidosis

An inherited deficiency of lysosomal acid beta galactosidase (E.C. 3.2.1.23) is expressed as two clinically distinct diseases, GM1 gangliosidosis and Morquio B disease (Brunetti-Pierri and Scaglia 2008; Morita et al. 2009; Caciotti et al. 2011). GM1 gangliosidosis affects both neurological and somatic tissues, occurring mainly in early infancy (type 1). Developmental arrest is usually observed a few months after birth, followed by progressive neurologic deterioration and generalized rigospasticity with sensorimotor and psycho/intellectual dysfunction. As with many of the other neurodegenerative lysosomal storage diseases, cherry-red maculae are common, as is facial dysmorphism, hepatosplenomegaly, and

generalized skeletal dysplasia. Later onset, juvenile and adult forms of beta galactosidase deficiency also have been described (types 2 and 3, respectively). They are observed as progressive neurologic diseases in childhood or in young adults. Dysmorphic skeletal changes are less prominent or absent in these clinical forms. A protracted course, mainly presenting as dystonia, is the major neurological manifestation in adults with GM1 gangliosidosis.

Morquio B disease is clinically expressed as a generalized skeletal dysplasia with corneal clouding, resulting in short stature, pectus carinatum, platyspondylia, odontoid hypoplasia, kyphoscoliosis, and genu valgum. There is no central nervous system involvement, although spinal cord compression may occur at late stages of the disease. Intelligence is normal and hepatosplenomegaly is not present.

In man, two lysosomal enzymes are known to be responsible for the hydrolysis of terminal beta-linked galactose residues at acidic pH in various glycoconjugates. One is the enzyme being discussed here, beta galactosidase (E.C. 3.2.1.23), and the other is galactocerebrosidase (E.C. 3.2.1.46), whose primary substrates include galactosylceramide, galactosylsphingosine, and other lipids. This is the enzyme deficient in globoid cell leukodystrophy (Krabbe disease), and was discussed in the section above. Beta galactosidase activity is severely deficient in cells and tissues from patients with both GM1 gangliosidosis and Morquio B disease, and both diseases are now known to be due to genetic mutations in the same gene (*GLB1*) (Hofer et al. 2009). The primary substrates for beta galactosidase include galactose-containing oligosaccharides (e.g., keratan sulfate), and GM1 ganglioside. The asialo derivative of GM1, GA1 ganglioside, also may be a substrate for this enzyme.

As expected, tissues from patients with beta galactosidase deficiency exhibit a broad spectrum of accumulating galactose-containing macromolecules, including GM1, GA1, and keratan sulfate. Gangliosides are normal components of plasma membranes, concentrated in neuronal membranes, especially in the regions of nerve endings and dendrites. GM1 is the major ganglioside in the brains of vertebrates. Gangliosides display a broad spectrum of interactions, and may act as binding molecules for toxins and hormones, and also are involved in cell differentiation, cell–cell interactions, and cell signaling (see below).

In the brains of patients with GM1 gangliosidosis, storage of GM1 is the most prominent observation (Nada et al. 2011). The accumulating GM1 has the same fatty acid composition, sugar composition and sequence, and glycosidic linkages as normal GM1. Visceral organs also show storage of GM1 ganglioside, but to a lesser degree. Also to a lesser degree, GA1 ganglioside may accumulate in the brain of GM1 gangliosidosis patients, as do glycosylceramide, lactosylceramide, and GM2 ganglioside. White matter shows chemical manifestations of myelin breakdown, including low proteolipid protein, low total lipid, and the presence of esterified cholesterol (Steenweg et al. 2010).

Other than gangliosides, the other major storage molecule in beta galactosidase-deficient patients is keratan sulfate (Ohto et al. 2012). This glycosaminoglycan normally exists in a proteoglycan linked with chondroitin sulfate. It is found primarily within connective tissues, particularly cartilage, explaining the skeletal