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Review

Inherited monogenic defects of ceramide metabolism: Molecular bases and diagnoses

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A B S T R A C T

Ceramides are membrane lipids implicated in the regulation of numerous biological functions. Recent evidence suggests that specific subsets of molecular species of ceramide may play distinct physiological roles. The importance of this family of molecules in vertebrates is witnessed by the deleterious consequences of genetic alterations in ceramide metabolism. This brief review summarizes the clinical presentation of human disorders due to the deficiency of enzymes involved either in the biosynthesis or the degradation of ceramides. Information on the possible underlying pathophysiological mechanisms is also provided, based on knowledge gathered from animal models of these inherited rare conditions. When appropriate, tools for chemical and molecular diagnosis of these disorders and therapeutic options are also presented.

1. Introduction/foreword

Studies on ceramides and sphingolipid metabolism have attracted a lot of attention recently. This is largely related to the multiplicity of actions of this group of lipids in eukaryotic biology and human health. Since the initial description of Farber disease, then recognized as an inherited disorder of ceramide breakdown, much progress has been made not only on the characterization of the disorder and understanding of its pathogenic mechanisms, but also on the identification of novel genetic conditions that alter ceramide metabolism.

We will first introduce ceramide metabolism in humans and briefly summarize current knowledge of the biological effects of this class of molecules. Then, human monogenic disorders that primarily affect ceramide biosynthesis or breakdown will be presented with emphasis on the available tools to diagnose such inherited human conditions (see Table 1). With the aim to approach the pathophysiological bases of these diseases and test possible therapies, the phenotype of animal (vertebrate) models for these disorders will also be described. Indeed, animal models are powerful tools in the study of many genetic disorders, especially for relatively rare disorders such as lysosomal storage disorders. Sometimes genetically modified mice recapitulate human disorders closely; sometimes they do not. When they do, such mouse models offer the tangible possibility to study genotype-phenotype relationships that are relevant for unraveling the biological role of these genes and gene products in humans.

2. Outline of ceramide metabolism in man

Ceramides are sphingolipids, a large and complex family of molecules including glycolipids and phospholipids. Ceramides all contain a sphingoid base, the most abundant being sphingosine. As N-acylated molecules, ceramides may be viewed at the center of sphingolipid metabolism, as all sphingolipids are generated from ceramide and because the breakdown of all complex sphingolipids converges to ceramide [1] (see Fig. 1). There are numerous molecular species of ceramide, depending on the chain length, unsaturation and hydroxylation of both the sphingoid base and fatty acyl moieties of ceramide [2]. This molecular heterogeneity may underlie the multiplicity of ceramide biological roles and the absence of redundancy, that is, a given molecular species of ceramide could not substitute others for a specific function (as illustrated by the consequences of some genetic defects of ceramide metabolism; see below).

Ceramides can be produced by three different pathways: the *de novo* synthesis, the salvage pathway, and by sphingolipid catabolism. The *de novo* synthesis and the salvage pathways both occur in (or at the cytosolic surface of) the endoplasmic reticulum [3,4], and involve

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Table 1
Inherited defects of ceramide biosynthesis and catabolism in man.
When appropriate, vertebrate models are mentioned.

Gene/ID	Enzyme/protein	Expression pattern	Phenotype MIM number and Mode of inheritance	Main biochemical features	Main symptoms	Animal (vertebrate) models
CERS1 10715	Ceramide synthase 1	CNS, skeletal muscle	616230 AR	Reduced C18-ceramide[a]	PME, cognitive decline	Spontaneous mouse strains (flincher & toppler); [47] Transgenic mouse strain [48]
CERS2 29956	Ceramide synthase 2	Ubiquitous	AD?	Reduced C24-C26-ceramides	PME	Transgenic mouse strain [56–58]
CERS3 204219	Ceramide synthase 3	Testis, skin	615023 AR	Reduced C26-ceramide	Congenital ichthyosis	Transgenic mouse strain [63]
DEGS1 8560	Dihydroceramide desaturase 1	Ubiquitous, high expression in CNS	AR	Increased DH-ceramide (and increased DH-ceramide/ceramide ratio)	Psychomotor arrest, nystagmus, dystonia, spasticity, seizures, failure to thrive	Transgenic mouse strain [66]; Zebrafish [64]
ASAH1 427	Acid lysosomal ceramidase	Ubiquitous	228000 and 159950 AR	Accumulation of ceramides	Farber lipogranulomatosis SMA-PME	Transgenic mouse strains [78,80]; Zebrafish [86]
PSAP 5660	Saposin precursor	Ubiquitous	611721 AR	Accumulation of glycosphingolipids, lyso-sphingolipids and ceramides	Early-onset seizures, hypotonia, myoclonus, poor feeding, and hepatosplenomegaly	Transgenic mouse strain [91]
ACER3 55331	Alkaline phytoceramidase	Ubiquitous	617762 AR	Accumulation of C18:1 and C20:1-ceramides and DH-ceramides	Childhood-onset progressive leukodystrophy	Transgenic mouse strain [96]

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; CNS, central nervous system; DH-ceramide, dihydroceramide; PME, progressive myoclonic epilepsy, Gene ID as provided by NCBI.
[a] In some instances, the biochemical changes reported here were observed on cultured cells (such as HeLa cells), not in the patient's organs or plasma.

ceramide synthases. Six ceramide synthases have been described so far, CERS1 to 6, which condense an acyl-CoA to the amino group of the sphingoid base. Whereas *de novo* biosynthesis first leads to a saturated sphingoid backbone (sphinganine) and then to dihydroceramide, the salvage pathway utilizes the recycled unsaturated sphingoid base (sphingosine), directly producing ceramide. The relative contribution of these two pathways to the overall production of ceramide appears to depend on the cell type and its regulation remains poorly understood. It is now generally accepted that CERS1 to 6 differ in their specificity for defined acyl-chain lengths [5,6]. Despite the paucity of our understanding of the substrate specificity and regulation of ceramide synthases [7], these enzymes are known to be required for distinct biological responses. In addition, ceramide synthase expression varies according to the tissues considered and developmental stage [5].

Interestingly, the possibility that the enzymes dedicated to ceramide hydrolysis, *i.e.* ceramidases, may catalyze the reverse reaction in some contexts was reported years ago [8,10]. Whether this enigmatic reverse reaction (as it would use a free fatty acid instead of an acyl-CoA) operates *in vivo* and under which circumstances still requires further investigation.

Ceramides can also be generated by catabolism of more complex sphingolipids [11]. This catabolism occurs mainly in the lysosomes, but enzymatic reactions producing ceramide also take place in other subcellular compartments such as the endoplasmic reticulum/Golgi, plasma membrane and mitochondria. Sphingomyelin is the most abundant sphingolipid in the cell and is hydrolysed to ceramide by a sphingomyelinase. On the other hand, the breakdown of all glycosphingolipids, which include neutral glycolipids and acidic molecules such as gangliosides and sulfatides, and are important components of the plasma membrane of numerous cell types, leads to the release of a monohexosylceramide. Then, this simple glycolipid is cleaved into ceramide owing to the action of a glucosyl or galactosyl-ceramidase. Because of a large pool of membrane sphingolipids, hydrolases are able to rapidly provide an important quantity of ceramide as compared to the *de novo* synthesis and salvage pathways. This may explain the important role these degradation pathways play in the generation of ceramides as bioactive molecules (see paragraph 2). Lastly, dephosphorylation of ceramide-1-phosphate represents another, yet poorly characterized source of ceramide production.

Ceramide degradation is catalyzed by ceramidases, which cleave ceramide into sphingosine and fatty acid. Sphingosine can then be reused in the salvage pathway or phosphorylated into sphingosine 1-phosphate (S1P) before being irreversibly cleaved by a lyase [12] to terminate sphingolipid metabolism. Three isoforms of ceramidases are classically distinguished according to the pH required for their optimal activity [13]. Acid ceramidase (ACDase) is the best documented one as it likely ensures the hydrolysis of the major part of the cell ceramides in the lysosomes. This lysosomal hydrolase requires the presence of an activator called saposin D to properly exert its catalytic activity. Saposin D is a small lysosomal protein that could disrupt lysosomal membrane in order to improve substrate presentation to the enzyme [14,15]. Neutral ceramidase localizes at the plasma membrane [16], but also in the endoplasmic reticulum/Golgi complex and the mitochondria [17,18]. Neutral ceramidase seems to play an active role in intestinal sphingolipid digestion [19] and the development of colon cancer [20]. Three alkaline ceramidases have been described, all harboring several transmembrane domains. ACER1 is present in the endoplasmic reticulum, especially in epidermal keratinocytes, where it plays a major role in regulating skin homeostasis [21]. ACER2 has been shown to be located in the Golgi complex, while ACER3 is found in the endoplasmic reticulum and the Golgi apparatus. Finally, and quite intriguingly, adiponectin receptors have recently been reported to behave as adiponectin-activated ceramidases [22].

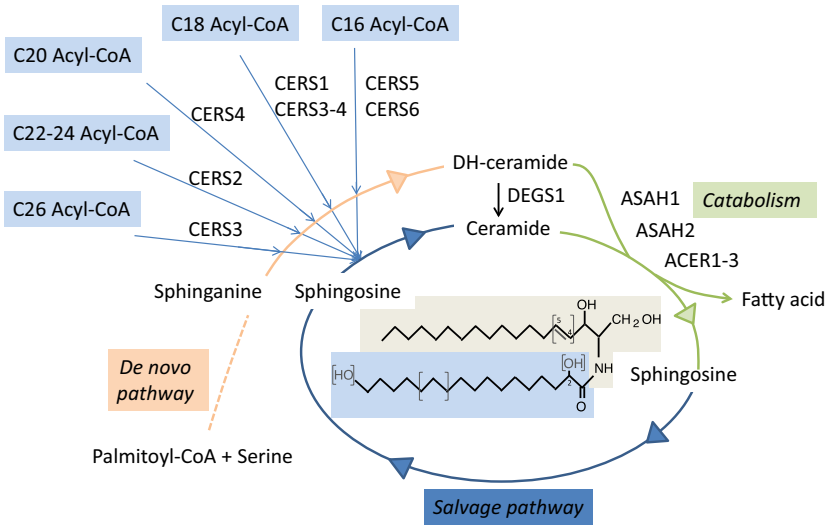

Fig. 1. Simplified view of ceramide metabolism. The name of the genes/enzymes involved in ceramide biosynthesis and degradation are indicated.

3. Biological functions of ceramide

While in most tissues the relative abundance of ceramides is very low as compared to other lipids, their concentration is high in the most superficial layer of the epidermis, *i.e.*, the stratum corneum, where ceramides represent up to 50% by weight of the lipids [23]. Of particular interest is the fact that peculiar ceramide species are present in the stratum corneum, with very-long and ultra-long chain (containing 24–32 carbons) and ω-hydroxylated fatty acids [24]. These ω-hydroxylated ceramides can be esterified by a linoleic acid or covalently bound to proteins at the corneocyte surface to form a lipid-bound envelope that prevents water loss. Ceramides are thus crucial players in the permeability barrier function of the epidermis [25]. In addition, ceramides may serve as a reservoir to generate sphingosine and sphinganine – thereby playing an antibacterial role at the skin surface [26].

One can anticipate that changes in the fine lipid composition of the stratum corneum may lead to disease. As a matter of fact, structural modifications of ceramide fatty acids were shown to occur with aging [27], and in common, *e.g.*, atopic dermatitis, or rare skin disorders, *e.g.*, Netherton syndrome, with subsequent permeability barrier dysfunction [28]. To further illustrate the key role of ceramides in skin homeostasis, mutations in the genes encoding several enzymes of ceramide biosynthesis, including *ELOVL1* and *ELOVL4* (fatty acid elongase), *CYP4F22* (fatty acid ω-hydroxylation), *KDSR* (3-ketodihydrosphingosine reductase), *CERS3*, and *PNPLA1* (ω-O-acylceramide synthesis), all lead to a human disease that presents with severe skin lesions [29–37].

In addition to the aforementioned role in the epidermis, ceramide and its degradation product, sphingosine, play critical functions as bioactive lipids. Increased cellular concentration of both lipids triggers cell death. For ceramide, this may occur due to its enhanced synthesis or decreased hydrolysis in response to various insults, and result in different modes of death, including apoptosis, necrosis, necroptosis, autophagy, mitophagy, cell cycle arrest, and senescence (for a recent review, see [1]). How ceramides (as well as sphingosine) molecularly promote such biological effects is still elusive as specific targets interacting with ceramides remain to be identified. Nevertheless, some actions of ceramides are known to be mediated through the activation of protein phosphatases such as PP1 and PP2A, which in turn inactivate several proteins [38,39]. Besides interactions with specific signal transducers, ceramides may affect, as a membrane component, the ability of the cell to respond to extracellular signals that act through binding to membrane receptors [40]. Sphingolipids are key constituents of membrane microdomains (and rafts), which are believed to serve as platforms to drive receptor-mediated signaling. One can anticipate that changes in the composition of these membrane microdomains and local concentration of ceramides may affect membrane fluidity, impacting ligand-receptor interaction and internalization, and subsequent signal outputs [41]. Ceramides also appear to modulate the formation and secretion of exosomal vesicles, which are implicated in intercellular communications [42]. Finally, one should keep in mind that sphingolipid metabolism is a very dynamic process, and that primary changes in the ceramide content may well be accompanied by secondary variations in the concentrations of other sphingolipid mediators, such as sphingosine, sphingosine 1-phosphate, and ceramide 1-phosphate, which could either exacerbate or attenuate the ceramide-induced actions.

As illustrated below in various genetic conditions, ceramides and complex sphingolipids play key functions not only in the skin but also in the nervous system (for reviews, see [43,44]).

4. Defects of ceramide biosynthesis

These conditions seem to be extremely rare and, have so far, been diagnosed through genetic studies.

4.1. CERS1 defect

4.1.1. Clinical presentation

Mutations in *CERS1*, the gene encoding for ceramide synthase 1, have been discovered in one single family of Algerian origin [45]. Four out of six siblings presented an unusual form of progressive myoclonus epilepsy (PME), characterized by a severe myoclonic syndrome, the persistence of frequent generalized tonic-clonic seizures and unresponsiveness to common antimyoclonic agents. In addition, moderate-to-severe intellectual disability/deterioration was observed [45]. The diagnosis there was established by homozygosity mapping, revealing the following homozygous mutation c.549C > G, p.H183Q (numbered according to NM_021267; rs200024180). CERS1 typically catalyzes the biosynthesis of C18-ceramide and is mainly expressed in the brain, especially in neurons [7]. The study of this variant enzyme expressed in transfected (HeLa) cells showed a decrease of C18:0 and C18:1-dihydroceramides and ceramides as compared to cells transfected with the wild-type construct [46]. The specific role of these ceramide species is still unknown; as components of sphingolipids (ganglioside and sphingomyelin) present in the neuronal membrane, they may well modulate cellular functions. Of note, other sphingolipids

than the above-mentioned species, *e.g.*, other ceramide species (produced by the non-affected CERS isoforms) or other metabolic products may also participate in the pathophysiology of the disease [this may apply for the other genetic disorders described here].

4.1.2. Mouse models

Two mouse strains with spontaneous recessive mutations in the *Cers1* gene, known as *flincher* and *toppler*, demonstrated a complete loss of CerS1 which catalyzes the synthesis of C18-ceramide [47]. The enzyme variations resulted in a reduction of sphingolipid biosynthesis in the brain along with dramatic changes in sphingolipid composition. Purkinje cell loss and lipofuscin accumulation was also observed, mainly in brain regions [47]. Aging brain is often accompanied by an increased accumulation of lipofuscin, so these results suggest a link between lipid biosynthesis deficiencies and lipofuscin accumulation in neurodegenerative disorders. Further, Ginkel *et al* [48] reported up to a 60% decrease in the level of gangliosides and myelin-associated glycoproteins in the cerebellum and forebrain of mice carrying a mutation in the coding region of the *Cers1* gene. That mutation resulted in deletion of the catalytic domain of the CerS1 protein. Those deficient mice experienced a foliation defect, reduced cerebellum size, and neuronal apoptosis with impaired locomotion and motor coordination. Compared to glial cells, post-mitotic neurons express especially high levels of complex gangliosides whose composition is altered throughout brain development [49,50]. CerS1 thus plays a pivotal role in brain development and physiology through formation of C18-ceramide - which constitutes an intermediate step in the synthesis of complex gangliosides [48].

4.2. CERS2 defect

4.2.1. Clinical presentation

As for *CERS2* mutations, one publication reported the case of a 30-year-old man also suffering from PME since infancy, in whom a heterozygous deletion was found containing the entire *CERS2* gene. Biochemical analysis of the patient's fibroblasts showed a 50% reduction in CERS2 mRNA, protein, and activity compared to parental controls [51]. Ceramide synthase 2 catalyzes the biosynthesis of very-long chain (C24-C26) ceramides. Lipidomic analysis of the patient's cells revealed a decrease in these very-long chain ceramides compared to controls and an increase in long chain (C16-C18) ceramides [51]. Similar results had already been observed in neuroblastoma cells in which CERS2 was down-regulated [52]. Those cells exhibited cell cycle arrest, activation of autophagy, and endoplasmic reticulum stress. Whether the decrease of very-long chain ceramides and/or the accompanying increase of long chain ceramides are responsible for the symptoms observed in the heterozygous patient is still uncertain. In mouse, *CerS2* is mainly expressed in kidney and liver and at moderate levels in the brain [53,54] especially in oligodendrocytes and Schwann cells, which are involved in myelination. Also, the very-long chain ceramides are components of glycosphingolipids in brain [53]. Thus, changes in the fine ceramide subspecies distribution in brain may contribute to the neurological defects seen. Clearly, further cases with *CERS2* mutations need to be documented.

4.2.2. Mouse models

Similar to CerS1 deficiency discussed above, CerS2 deficiency was believed to be manifested mainly in neurons [55]. However, Imgrund *et al* [56] reported significantly reduced levels of ceramide species with very-long chain fatty acid residues (> or = C22) in the liver, kidney, and brain of a mouse model harboring an insertion in intron 1 of the *Cers2* gene. Reduced or absent enzyme activity toward particular fatty acyl-CoA species (such as C18:0, C22:0 and C24:1 in the brain or liver) was also detected. As a consequence, the mice developed myelin degeneration accompanied by an 80% loss of myelin basic proteins, loss of cerebellar granule cells, and hepatocarcinomas [56]. These results are in accordance with those observed by Futerman and coworkers [57,58]. Furthermore, Pewzner-Jung *et al* reported a significantly elevated amount of both neutral sphingomyelinase 2 activity and sphingoid long-chain bases, both most likely being involved in dramatic pathological changes in the liver [58]. They also highlighted how the change in membrane lipid composition can affect fluidity and curvature, which may modulate different membrane processes [58]. CerS2-deficient mice also developed severe hepatopathy at about 30 days of age accompanied by increased hepatocyte apoptosis and proliferation [57]. Interestingly, changes in renal function and pathological changes in the kidney were not observed, suggesting that CerS2 may function primarily in maintenance of normal liver homeostasis [57].

4.3. CERS3 defect

4.3.1. Clinical presentation

Contrary to the neurological involvement seen in ceramide synthase 1 or ceramide synthase 2 deficiency, a primary skin disorder is the hallmark of *CERS3* mutations. Two independent teams reported dermatological features in two consanguineous families with mutations in *CERS3* [59,60]. These patients presented an autosomal recessive congenital ichthyosis (ARCI), a heterogeneous group of disorders of keratinization. Each patient presented collodion membranes at birth, generalized erythema, scaling, and localized hyperkeratosis. More recently, Youssefian and co-workers, using next generation sequencing, studied 140 consanguineous families suffering from ARCI and revealed six new *CERS3* mutations [61]. The CERS3 isoform typically catalyzes the synthesis of very- (or ultra-) long chain ceramides, which are essential components of the skin. Histological studies performed by Radner and co-workers showed the location of the enzyme at the interface between the stratum granulosum and the stratum corneum in the epidermidis in control biopsies but absent in the patient specimens. Also, Eckl and co-workers showed that there was a strong reduction of very-long chain ceramides in patients' keratinocytes as well as in HEK293T cells transfected with mutant CERS3 as compared to control cells. Finally, a reconstructed skin model containing patients' fibroblasts and keratinocytes showed an immature cornification process and reduced permeability barrier activity [59]. The lack of very-long chain ceramides in the skin is likely to be responsible for the dermatological involvement.

4.3.2. Mouse models

Rabionet and coworkers [62,63] described the correlation between expression of ultra-long polyunsaturated sphingolipids (with C26-C32 fatty acids) in differentiating mouse spermatogenic cells and the distribution pattern of CerS3 during spermatogenesis. This points to CerS3 as a key player in this pathway. Moreover, CerS3 synthesizes unique ceramide membrane anchors that are subject to further modification by common enzymes of the sphingolipid pathway to produce glycosphingolipids or sphingomyelin. Loss of these anchors causes increased apoptosis during meiosis, formation of multinuclear giant cells, and spermatogenic arrest [63].

4.4. Dihydroceramide desaturase defect

4.4.1. Clinical presentation

Nineteen patients from thirteen families with a hypomyelinating leukodystrophy have been shown recently to be homozygous or compound heterozygous for mutations in the *DEGS1* gene [64]. A further adult patient has been described independently [65]. The described variants include missense, non-sense and frameshift mutations. *DEGS1* encodes the Δ4-dihydroceramide desaturase that introduces a double bond in ceramide in the last step of the *de novo* sphingolipid synthesis pathway. With a quite early onset (mean 5.6 months), the majority of the patients suffered from a severe phenotype including delayed psychomotor development, dystonia, severe spasticity, seizures, and abnormal eye movements. Failure to thrive was present in all but three

patients. Four patients presented with a less severe evolution of the disorder beginning with progressive spasticity leading to motor regression occurring between 2 and 16 years. Hypomyelination was evident in all the patients by magnetic resonance imaging; along with basal ganglia abnormalities and thinning of the corpus callosum in almost all the severe cases. Thalamus and cerebellar atrophy was sometimes noted. Biochemically, a marked elevation of the dihydroceramide/ceramide ratio was observed in the patient's fibroblasts or muscle [64], and an increased plasma level of several dihydrosphingolipids (dihydro-ceramides, sphingomyelins, and monohexosylceramides) [65], indicating a reduced activity of the DEGS1 desaturase. Based on experiments performed on patient's cells, dihydroceramide accumulation was suggested to trigger abnormal ROS production. Interestingly, Karsai and collaborators detected, in the patient plasma and in DEGS-deleted cells, a novel sphingoid base characterized by the presence of a double bond in the C14 position instead of position C4 [65]. The relevance of this novel metabolite as a disease biomarker and in terms of pathophysiology remains to be explored.

4.4.2. Animal models

In zebrafish, knockdown of DEGS1 roughly recapitulates biochemical and clinical anomalies observed in humans, and, is accompanied by a decreased number of myelinated oligodendrocytes [64]. This model was used to show the efficacy of FTY720, a sphingosine homolog acting as an inhibitor of ceramide synthases, in improving zebrafish locomotor performance, increasing the number of myelinated oligodendrocytes, as well as improving the dihydroceramide/ceramide ratio. FTY720 also prevented ROS production upon exposure of patient fibroblasts to dihydroceramide.

The homozygous loss of dihydroceramide desaturase in mice, due to the insertion of a gene trapping vector in the first intron of the *Degs1* gene [66], is responsible for a more complex phenotype, including scaly skin and sparse hair, tremor, along with numerous blood chemistry and hematological abnormalities. The deficient mice were smaller in size and exhibited other signs of growth retardation such as decreases in mean total tissue mass, lean body mass, bone mineral content, and bone density. No detectable ceramide levels were observed in the heart, liver, pancreas, white adipose tissue, and soleus muscle in 7-week-old mutant mice. Moreover, abnormal liver function tests including increases in serum alkaline phosphatase, alanine aminotransferase, and total bilirubin levels were also detected. The mutant mice ultimately failed to thrive and died within 8 to 10 weeks of age. Heterozygous mice, otherwise showing a normal phenotype, demonstrated enhanced insulin sensitivity and were refractory to dexamethasone-induced insulin resistance compared to controls, supporting the conclusion that ceramide synthesis is essential for glucocorticoid-induced insulin resistance.

5. Defects of ceramide degradation

5.1. ASAH1 deficiency

5.1.1. Clinical presentation

Acid ceramidase (ACDase) is a ubiquitously expressed enzyme that releases fatty acid and sphingosine from ceramide, and its deficiency causes a progressive lysosomal storage disorder classically known as Farber disease (FD). FD is the best described defect of ceramide metabolism. In a recent review article [67], information on about 200 cases of ACDase deficiency was collected, emphasizing the broad clinical spectrum of this condition. Two main disorders can be distinguished (see also Table 2). The first is FD, or Farber's lipogranulomatosis, in which patients classically present with subcutaneous nodules, joint contractures, a hoarse voice, hepatosplenomegaly, and neurological and/or respiratory complications. More or less severe forms of FD have been described according to the age of onset (ranging from the perinatal period to first years of age) or the nature of organ involvement (visceral

Table 2
Clinical classification of acid ceramidase deficiencies.

Current classification			Gene involved	Proposed classification	
Subtype	Main clinical features	Mean age of death (years)[a]		Subtype	Remarks
Type 1 - Classical	Subcutaneous nodules, joint contractures, voice hoarseness, ± hepatosplenomegaly and neurological symptoms	1.5–2.6	*ASAH1*	Infantile	
Type 2 - Intermediate	Similar to Type 1 but longer lifespan	6.2		Juvenile	Mild neurological involvement
Type 3 - Mild	Similar to Type 2 but longer lifespan	15.8			
Type 4 - Neonatal	Massive organomegaly	0.2		Neonatal	
Type 5 - Neurologic progressive	Progressive neurological deterioration, seizures, nodules, joint involvement	3.6		Predominantly neuronopathic	
SMA-PME and SMA-PME-like	Progressive proximal weakness with or without myoclonic epilepsy	14.4–21.9		SMA phenotype	Absence of the clinical triad typical of the infantile form
Type 6 - Combined Farber & Sandhoff	Clinical triad of Type 1, cherry-red macular spot, severe neurogenerative disease	1.75	*ASAH1* + *HEXB*	Combined Farber & Sandhoff	Exceptional case of two coincident diseases
Type 7 - Combined saposin deficiency	Hypotonia, myoclonus, hepatosplenomegaly	0.25	*PSAP*	Prosaposin deficiency	Clinical presentation rather suggestive of type II Gaucher disease

[a] See Refs. [67, 97].

or neurological). The second disorder has been recognized quite recently; it is Spinal Muscular Atrophy with Progressive Myoclonic Epilepsy (SMA-PME), whose onset typically occurs later during infancy. This latter disorder is characterized by a myoclonic epilepsy and a lower motor neuron disease with gait abnormality and muscle weakness progressing toward respiratory complications. In a review describing 12 SMA-PME cases, electromyography showed a chronic denervation process and muscle biopsies revealed a neurogenic atrophy [68].

Whether FD and SMA-PME are distinct phenotypes (possibly linked to different pathogenic mechanisms) or different forms belonging to a wide spectrum of clinical presentations of the same disorder is still unclear. Detailed characterization of additional case reports may help address this issue. Meanwhile, we are proposing a modification to the standard classification of *ASAH1*-related disorders (see Table 2).

For both disorders, diagnosis is based on a substantially (> 70%) decreased enzymatic activity of ACDase in leukocytes or cultured skin fibroblasts, either measured in cell lysates or in intact living cells after sphingolipid loading, and/or accumulation of ceramides as demonstrated by lipid analysis of cultured cells or biopsy specimens. Recently, Cozma and co-workers have proposed that the increase of C26-ceramide by LC/MS from dried blood spots could be a sensitive and specific biomarker for FD and SMA-PME [69]. Alternatively, detection of pathogenic mutations in the *ASAH1* gene can lead to the diagnosis of ACDase deficiency; in any case, identification of *ASAH1* variants must be accompanied by functional tests to demonstrate their pathogenic role. Quite surprisingly, some mutations can lead to both of the two disorders [67].

ACDase catalyzes the last step of sphingolipid catabolism in lysosomes, by hydrolyzing ceramides into sphingosine and free fatty acid. Under pathological conditions of lysosomal burden, ACDase may also deacylate glycosphingolipids to generate psychosine or lyso-Gb3 [70]. The functional deficit of ACDase leads to the accumulation of ceramides in lysosomes in many organs, including brain, visceral organs, skin, and lymph nodes. No deficient production of ceramide metabolites has been documented, likely because of the action of other (alkaline and neutral) ceramidases or regulation of ceramide metabolic pathways. As previously discussed, ceramides are bioactive lipids involved in many cellular processes. Is the accumulation of ceramides in general (with many molecular species) or ceramide metabolites, or even only one specific ceramide molecule responsible for these disorders? This issue has not yet been resolved (see also the discussion below for animal models). A study of the complete plasma ceramide profile in Farber patients has shown a positive correlation between disease and dihydro-C12-ceramide, dihydro-C24:1-ceramide, and α-hydroxy-C18-ceramide levels [71].

In all likelihood, inflammation plays an important pathogenic role in FD, supported by the presence of subcutaneous nodules, joint contractures and a hoarse voice, and the histiocytic infiltration found in many tissues [72]. In Farber patients, the plasmatic cytokine profile showed an inflammatory phenotype with an increase of monocyte chemotactic protein (MCP-1), keratinocyte chemoattractant (KC), interferon gamma-induced protein-10 (IP10), interleukin-6 and chitotriosidase, and the activation of lipid-filled macrophages in subcutaneous nodules [71]. How the ceramides stored in lysosomes get into the extracellular space (*e.g.*, plasma) and how they trigger inflammation still remains to be thoroughly investigated. Regarding SMA-PME, the pathophysiological mechanisms still remain mysterious: is this condition indeed a different entity than FD? Why in certain patients does the ACDase deficiency only seem to affect a very restricted subset of cells, resulting in either FD or SMA-PME?

Therapy for ACDase deficiency should aim to not only correct the metabolic defect by supplying native enzyme but also reduce inflammation. To this end, hematopoietic stem cell transplantation (HSCT) is currently used, which appears to be an effective means in the absence of neurological involvement [73]. Enzyme replacement therapy (ERT) using recombinant ACDase represents another potential strategy that is under ongoing development. Indeed, initial studies in mice showed the efficiency of this treatment [74]. Finally, based on the progress made in animal models, gene therapy could also be considered [75–78].

5.1.2. Animal models

Three mouse models of FD have been generated so far by differing mutations in the *Asah1* gene. Li *et al* [79] produced a large insertion with the pmACgKO vector into intron 12 of the endogenous mouse *Asah1* gene leading to an embryonic lethal phenotype in homozygous mice. A subsequent study from this group found that the transgenic embryos in this case died at the 4-cell stage [79]. Their results suggest that ACDase activity is essential during normal mouse development. In contrast to the outcomes seen in the homozygous mice, heterozygous animals exhibited a normal phenotype until 6 months of age when significant pathological signs such as numerous lipid-laden inclusions in the liver, lung, skin, and bone began to appear. We [78] generated the first viable mouse model of systematic ACDase deficiency by introducing a single-nucleotide mutation into exon 13 leading to the replacement of a highly conserved proline residue with an arginine at position 361. The third model of FD was generated by Beckmann *et al* [80], who created a deletion in the region of exon 1 of *Asah1* that encodes the signal sequence. This deletion results in the formation of a variant ACDase protein from an alternative start codon, that lacks lysosomal targeting.

The latter two models mentioned above represent progressive and lethal phenotypes of FD; they display reduced ACDase activity in lysates from various organs along with ceramide and sphingomyelin accumulation. Both mouse models demonstrate marked damage of the lungs and nerve tissues, corresponding to a severe phenotype in FD patients (type 1 and 4) [81]. Significant behavioral changes such as reduced voluntary locomotion and exploration, increasing thigmotaxis, impaired muscle grip strength, and defects in motor coordination were also observed in the first model [82]. A wide range of CNS cell types were affected, dominated by granuloma-like subcellular substrate accumulation in CD68+ microglia and/or macrophages in white matter and perivascular cuffs of the cerebrum [82]. Manifestations in the lung included low compliance, increased airway resistance, decreased blood oxygenation - probably due to significant inflammation, increased vascular permeability, and the presence of vacuolated foamy histiocytes full of storage material [83].

Involvement of hematopoietic organs is a very common feature in FD patients. This includes enlargement of the spleen, thymus, and liver. Similar manifestations have been observed in our mouse model as well. Interestingly, this organ enlargement has been accompanied by a reduced number (40–85%) of the total cells. Histiocytic infiltration and accumulation of Mac-2+ foamy macrophages (increase in both size and numbers) are attributed to disruption of the parenchyma and enlargement of the organs [84]. We also observed a change in composition of particular blood cells during progression of the disorder. Some of these occurrences could be attributed to the effect of inflammatory cytokines such as MCP-1 [83]. Yet, whereas the *Asah1* point mutation model demonstrated a significant increase in leukocytes, red blood cells, and hemoglobin level [78,84], deletion of the signal peptide of ACDase led to leukopenia and a normal hemoglobin level [80]. These findings may reflect the differing background strains between the two models and simultaneously illustrate genotype-phenotype correlations in FD. Another paper of Yu *et al* [85] further describes severe ocular pathology and visual impairment in the first mouse model of FD that was characterized by progressive inflammation, retinal dysplasia, and increased prevalence of storage material in various cell types of the retina and optic nerves. Detailed pathological studies of these mouse models of FD complement knowledge gained in the patients. Moreover, these small animal models can contribute not only to the development of the proper treatments but also to more precise methods of diagnosis of FD [84].

Lastly, a zebrafish model of SMA-PME has been created by injections of antisense morpholino oligonucleotides into 1- to 4-cell-stage embryos leading to knockdown of *ASAH1* gene [86]. Two days after fertilization, deficient embryos exhibited morphology changes such as curved body shape or decreased motor-axon collateral formation. Decrease in motor-neuron axonal branching, associated with increased apoptosis in the spinal cord, support the finding that *ASAH1* mutations are responsible for SMA-PME [86].

5.2. PSAP deficiency

The degradation of glycosphingolipids with relatively short carbohydrate chains is accomplished with the aid of small lysosomal glycoproteins. GM2-activator protein and 4 sphingolipid activator proteins (saposins A-D) act as cofactors of particular lysosomal hydrolases. Saposins A-D are homologous to each other and are derived from a common precursor protein (prosaposin) encoded by a single gene, *PSAP*.

5.2.1. Clinical presentation

ACDase requires an activator protein called saposin D (SapD) for its optimal activity. Mutations in the *PSAP* gene can lead to a defect of the entire prosaposin or of a single saposin. In humans, no saposin D defect has been described so far. As for the prosaposin defect (or combined saposin deficiency (OMIM 611721)), the review from Motta and coworkers [87], reporting a total of 9 published cases, showed that it is a very early onset disease with a poor prognosis (life expectancy of a few months). Each patient presented with an acute neurovisceral dystrophy with hepatosplenomegaly and in most cases a hypotonia, myoclonus, and seizures that were poorly responsive to antiepileptics. Microcephaly, respiratory, and ophthalmic involvement have also been described. When brain imaging was performed, abnormalities were always found, whether it be in white matter or a cortical atrophy. Biochemical studies showed a variable decrease of enzymatic activities of ACDase, glucosylceramidase, and galactosylceramidase, along with increased ceramide, glucosylceramide, lactosylceramide, and globotriaosylceramide in patient-derived non-neuronal cells compared to control cells [87–89]. In two patients, the quantitative analysis of plasma lysosphingolipids showed a slight increase of lysoGb3 and glucosylsphingosine compared to controls [87]. Diagnosis of prosaposin deficiency can be delayed because symptoms overlap with other sphingolipidoses. The pathophysiology of this disease still remains unknown. Nevertheless, two hypotheses can be considered: the first proposes that lipid storage results in an autophagy impairment in non-neuronal cells; the second involves the absence of the prosaposin protein, which has neurotrophic functions on its own [90]. Diagnosis can be established by enzyme assays of sphingolipid hydrolases and molecular genetics but these cases underscore the interest in implementation of urinary lipid screening, which is an easy, accessible and non-invasive test.

5.2.2. Mouse models

Mutant mice homozygous for an inactivated prosaposin gene [91] have been generated. These animals exhibited two distinct clinical phenotypes: neonatally fatal and later-onset. Up to half of deficient mice died *in utero* or within the first 1–2 days after birth. Affected mice with the neonatal form showed progressive neurological involvement including severe hypomyelination and periodic acid-Schiff-positive material throughout the nervous system at 30 days of age. Abnormal cells were also observed in the liver and spleen. Activities of several lysosomal enzymes (such as glucosylceramidase or galactosylceramidase) relevant to the sphingolipid activator proteins were decreased in liver extracts. Accordingly, the concentrations of these enzymes' substrates were increased in the brain, liver, and kidney. Clinical, pathological, and biochemical abnormalities described were similar to those observed in human patients with a combined saposin deficiency [91].

5.3. ACER3 deficiency

Among ceramidases other than ACDase, only alkaline ceramidase 3, encoded by the *ACER3* gene, has been described to be defective in humans. Two siblings from a consanguineous family [92] presented with developmental stagnation then regression in their first year of life with a motor development impairment (truncal hypotonia, limb spasticity, areflexia, and dystonia) that progressively worsened. The magnetic resonance imaging of brain indicated a leukodystrophy. The diagnosis was established through exome analysis, which found a catalytic site mutation (p.E33G; rs1554988032) [93]. The alkaline ceramidase 3 hydrolyzes unsaturated long chain ceramides, especially C18:1 and C20:1-ceramides and dihydroceramides [94]. Study of the patients' cells showed an undetectable enzymatic activity. An increased blood content of unsaturated long chain ceramides and -dihydroceramides, as well as glycosphingolipids, sphingoid bases, and their phosphates was also observed. Alkaline ceramidase is expressed in several cell types [94] and has a function in cell proliferation *via* its enzymatic activity. ACER3 knockdown in HeLa cells led to an increase of unsaturated long chain ceramides and -dihydroceramides and a decrease of long chain and very-long chain ceramides along with an increase of sphingosine and sphingosine 1-phosphate, respectively. Interestingly, an upregulation of alkaline ceramidase 2 (ACER2), which hydrolyzes long chain and very-long chain ceramides, was noted in these cells, which could explain the above unexpected results. The pathophysiology of ACER3 deficiency is presently unknown, but Hu and co-workers suggested that the accumulation of unsaturated long chain ceramides in HeLa cells leads to the inhibition of cell proliferation by upregulating p21 [94]. Could the accumulation of these ceramide species underlie the leukodystrophy? In the murine model (see below), where C18:1-ceramide also accumulates in CNS, there is no leukodystrophy but a loss of Purkinje cells [95]. Finally, regarding therapeutic options for this disorder, at least for the reported cases, chaperone therapy could be envisioned since the *ACER3* mutation destabilizes the enzyme [93].

5.3.1. Mouse models

Wang and coworkers [96] found that the mRNA levels of ACER3 as well as its activity (but not the activity of other alkaline ceramidases) were increased in both the cerebrum and cerebellum of 8-month-old wild-type mice compared to 6-week-old control mice, suggesting that the enzyme is upregulated with age in the mouse brain. To elucidate the physiological function of ACER3 during aging, they created a mouse model of ACER3 deficiency by replacement of exon 8 of the *Acer3* gene by the neomycin resistant gene cassette [96] leading to reduction of the enzyme activity in brain, liver, and lung tissue. ACER3 deficiency decreased alkaline ceramidase activity on C18:1-ceramide, but not C16:0, C18:0, C24:1, or C24:0-ceramide in the tissues examined. As mentioned above, ACER3 catalyzes the hydrolysis of ceramide into sphingosine, which in turn can be phosphorylated to form sphingosine 1-phosphate (S1P). An age-dependent increase in the levels of both sphingosine and S1P was also observed in the mouse brain. The knockout of ACER3 then significantly decreased the levels of sphingosine but not S1P in the cerebrum of 6-week-old mice whereas both compounds were found in an increased amount in both the cerebrum and cerebellum of 8-month-old animals. The ACER3 mutant mice developed impaired motor coordination, balance capabilities, and reduced grip strength, yet did not show major defects in development. The ACER3 mutant mice also exhibited a significant decrease in the number of Purkinje cells at 8 months of age compared to 6-week-old deficient mice where no significant differences were observed compared to wild-type mice. These results indicate that ACER3 plays a critical role in controlling the homeostasis of various sphingolipids, mainly ceramides, sphingosine, and S1P, and protects Purkinje cells from premature degeneration and

thereby cerebellar ataxia.

6. Concluding remarks

Ceramides may have different biological functions depending on the acyl chain, playing essential roles as both signaling molecules and as intermediates in the biosynthesis of more complex glycosphingolipids. For maintenance of their functional levels at an organismal and cellular level, it is essential to understand not only how sphingolipids are synthesized but also how they are degraded, and to know the consequences of inadequate degradation pathways.

With the exception of CERS3 deficiency, a number of genetic conditions described here highlight the critical importance of ceramides and dihydroceramide/ceramide ratio in myelin development and maintenance. Whether this lipid imbalance directly impacts myelin structure and composition or could stimulate signaling pathways leading, for example, to oligodendrocyte death remains to be elucidated. Moreover, thorough investigation of additional cases with defects in ceramide metabolism may help characterize genotype-phenotype relationships and understand the molecular pathophysiology of these rare conditions.

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