Molecular and Cellular Neuroscience 116 (2021) 103670

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Sulfatide in health and disease. The evaluation of sulfatide in cerebrospinal fluid as a possible biomarker for neurodegeneration

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ARTICLE INFO

Keywords:
Sulfatide
Biomarker
Myelin
Cerebrospinal fluid
White matter disorders
Neurodegeneration

ABSTRACT

Sulfatide (3-*O*-sulfogalactosylceramide, SM4) is a glycosphingolipid, highly multifunctional and particularly enriched in the myelin sheath of neurons. The role of sulfatide has been implicated in various biological fields such as the nervous system, immune system, host-pathogen recognition and infection, beta cell function and haemostasis/thrombosis. Thus, alterations in sulfatide metabolism and production are associated with several human diseases such as neurological and immunological disorders and cancers.

The unique lipid-rich composition of myelin reflects the importance of lipids in this specific membrane structure. Sulfatide has been shown to be involved in the regulation of oligodendrocyte differentiation and in the maintenance of the myelin sheath by influencing membrane dynamics involving sorting and lateral assembly of myelin proteins as well as ion channels. Sulfatide is furthermore essential for proper formation of the axo-glial junctions at the paranode together with axonal glycosphingolipids. Alterations in sulfatide metabolism are suggested to contribute to myelin deterioration as well as synaptic dysfunction, neurological decline and inflammation observed in different conditions associated with myelin pathology (mouse models and human disorders).

Body fluid biomarkers are of importance for clinical diagnostics as well as for patient stratification in clinical trials and treatment monitoring. Cerebrospinal fluid (CSF) is commonly used as an indirect measure of brain metabolism and analysis of CSF sulfatide might provide information regarding whether the lipid disruption observed in neurodegenerative disorders is reflected in this body fluid. In this review, we evaluate the diagnostic utility of CSF sulfatide as a biomarker for neurodegenerative disorders associated with dysmyelination/demyelination by summarising the current literature on this topic.

We can conclude that neither CSF sulfatide levels nor individual sulfatide species consistently reflect the lipid disruption observed in many of the demyelinating disorders. One exception is the lysosomal storage disorder metachromatic leukodystrophy, possibly due to the genetically determined accumulation of non-metabolised sulfatide. We also discuss possible explanations as to why myelin pathology in brain tissue is poorly reflected by the CSF sulfatide concentration. The previous suggestion that CSF sulfatide is a marker of myelin damage has

Abbreviations: AD, Alzheimer's disease; ApoE, apolipoprotein E; ASA, arylsulfatase A; BBB, blood-brain barrier; CerS, ceramide synthases; CGT, UDP-galactose: ceramide galactosyltransferase; CNS, central nervous system; CSF, cerebrospinal fluid; CST, cerebroside sulfotransferase; EAE, experimental autoimmune encephalomyelitis; EDSS, Expanded Disability Status Scale; EVs, extracellular membrane vesicles; FA2H, fatty acid 2-hydroxylate; Galcer, galactosylceramide; GBS, Guillain-Barré syndrome; GFAP, glial fibrillary acidic protein; GSL, glycosphingolipid; LC-MS/MS, liquid-chromatography tandem mass spectrometry; LRP1, low-density lipoprotein receptor-related protein 1; MAG, myelin-associated protein; MAL, myelin and lymphocyte protein; MBP, myelin basic protein; MLD, metachromatic leukodystrophy; MRI, magnetic resonance imaging; MS, multiple sclerosis; NF-155, neurofascin; NF-L, neurofilament light; NKT cells, natural killer T-cells; NPH, normal pressure hydrocephalus; PLP, myelin proteolipid protein; PNS, peripheral nervous system; SAE, subcortical arteriosclerotic encephalopathy; SapB, saposin B; SSVD, subcortical small vessel type of dementia; TLC-ELISA, thin-layer chromatography enzyme-linked immunosorbent assay; VaD, Vascular dementia; VLCFA, very-long-chain fatty acids.

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https://doi.org/10.1016/j.mcn.2021.103670
Received 25 March 2021; Received in revised form 14 September 2021; Accepted 17 September 2021
Available online 23 September 2021

thereby been challenged by more recent studies using more sophisticated laboratory techniques for sulfatide analysis as well as improved sample selection criteria due to increased knowledge on disease pathology.

1. Sulfatide

Glycosphingolipids (GSLs, see Fact box for sphingolipids) had been described already during the second half of the 19th century by a German physician named J. T. L. Thudichum (Thudichum, 1884). He isolated the first mammalian sulfoglycolipid from the brain, 3-O-sulfogalactosylceramide (sulfatide, SM4s, Fig. 1). However, it was not until 1933 that sulfatide was chemically characterised by Gunnar Blix, and it was eventually shown in 1963 to be a sulfate ester of its precursor galactosylceramide (galcer). It has since been established that sulfatide comprises many molecular species (isoforms), with structures differing in acyl chain length and hydroxylation and sphingoid base (Fig. 1). The nervous system is especially rich in GSLs, where over 70% of the myelin sheath is composed of lipids (O'Brien and Sampson, 1965; Norton and Poduslo, 1973; Månsson and Fredman, 2000; Rasband and Macklin, 2012). Sulfatide is particularly enriched in this membrane structure (4%–6% of the total myelin lipids) and, together with galcer, accounts for almost one third of the myelin lipids (Norton and Cammer, 1984). Both oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS) express sulfatide (Ishizuka, 1997). Sulfatide has also been shown to occur in neurons as well as astrocytes in the grey matter of the brain (Zalc et al., 1981; Berntson et al., 1998; Pernber et al., 2002). However, it is not known whether sulfatide is synthesised by these cells or is imported through lipoprotein endocytosis. Other organs rich in sulfatide are the kidney, trachea, gastrointestinal tract, spleen and pancreas, more specifically the islets of Langerhans (Ishizuka, 1997). Compared with the brain, these organs contain minor amounts of sulfatide. Sulfatide localises mainly to the plasma membrane, Golgi apparatus and lysosomes, as well as granules of pancreatic beta cells.

1.1. Sulfatide metabolism

The *de novo* synthesis of sulfatide begins with the production of ceramide, the precursor of all GSLs, in the endoplasmic reticulum, followed by transferring a UDP-galactose moiety to the ceramide backbone (Figs. 2 and 3). This reaction is catalysed by the enzyme UDP-galactose: ceramide galactosyltransferase (CGT, EC 2.4.1.45) (Morell and Radin, 1969; van der Bijl et al., 1996). Subsequently, galcer is transported to the Golgi apparatus and sulfatide is synthesised by sulfation of the 3′-OH group of the galactose moiety through the action of cerebroside sulfotransferase (CST, EC 2.8.2.11) (Farooqui et al., 1977; Honke et al., 1996; Hirahara et al., 2000). The *CGT* and *CST* genes show a tissue-specific expression; however, *CST* expression is not as strictly regulated as *CGT* (Stahl et al., 1994; Hirahara et al., 2000). Thus, the tissue distribution of sulfatide is mainly regulated by the *CGT* expression. The second synthesis pathway, termed the 'salvage pathway', involves degradation in the lysosome to generate ceramide to be used for resynthesis of GSLs (Fig. 3). The lysosomal enzyme arylsulfatase A (ASA, EC 3.1.6.8) specifically degrades sulfatide by hydrolysis of the sulfate group. This reaction requires an activator protein, saposin B (sap B), that enables the exposure of sulfatide to ASA (Kolter and Sandhoff, 2005). The 'salvage pathway' has been shown to be the main synthesis pathway of sulfatide in the beta cells of the islets of Langerhans (Fredman et al., 2000).

1.2. Structural diversity of sulfatide and implication in biological function

A main source of complexity in the GSL structure is the heterogeneity of the ceramide backbone. The fatty acid of the lipid moiety may vary in alkyl chain length, degree of unsaturation and hydroxylation (see Fig. 1 for sulfatide) (Karlsson, 1970; Merrill, 2011). The sphingosine base can also vary in the type and chain length. The specific GSL isoform distribution is regulated by differential expression of ceramide synthases as well as fatty acid hydroxylases (Alderson et al., 2004; Eckhardt et al., 2005; Park et al., 2014); however, the precise mechanisms of these metabolic pathways are still unknown. The structural variability of GSL shows a tissue- and cell-specific pattern as well as age- dependent distribution in humans (Rosenberg and Stern, 1966; Vanier et al., 1971; Mansson et al., 1978; Svennerholm et al., 1989), most likely reflecting different biological functions of various GSLs and their specific isoforms.

Sulfatide exhibits numerous structures in mammals, including

Fact box - Sphingolipid classification

Sphingoid bases

sphingosine, phytosphingosine, sphinganine (including derivates)

Ceramides

Phosphosphingolipids

ceramide phosphocholines (sphingomyelin), ceramide phosphoethanolamines
ceramide phosphoinositols

Glycosphingolipids (GSLs)

neutral GSLs (1-20 neutral monosaccharides)

acidic GSLs

gangliosides
glucuronosphingolipids
phospho-GSLs
sulfo-GSLs (sulfatides)

sulfatide (SM4s)
sulfated lactosylceramide (SM3)
sulfated glucosylceramide (SM4s-Glc)
SM4s-6 (6-sulfation)
for more examples see Ishizuka 1997

https://www.lipidmaps.org/data/classification/LM_classification_exp.php

Fact box. Sphingolipid classification

different lengths of the fatty acid chain (C16–C26 is most common), which can be hydroxylated and unsaturated introducing further complexity (Fig. 1). Concerning the sphingosine moiety of sulfatide, 4-sphingenine (d18:1) clearly dominates in mammals except for the intestine and kidney, where t18:0 is rather common (Karlsson, 1970; Kyogashima et al., 2006). This structural diversity results in different chemical properties of various sulfatide isoforms and thereby diverse biological functions. The fatty acid compositions of sulfatide are dependent on specific tissues. When comparing cell types of the nervous system, sulfatide isoform profiles show quite large differences, were sulfatide found in the adult myelin sheath of mammals mainly comprises very-long-chain fatty acids (VLCFA, C22–C26) and hydroxylated fatty acid chains (Svennerholm et al., 1992; Månsson and Fredman, 2000; Hirahara et al., 2017; Pintado-Sierra et al., 2017) and sulfatide found in astrocytes and neurons is dominated by shorter alkyl chains (C16–C18, based on rodent studies) (Berntson et al., 1998; Pernber et al., 2002; Isaac et al., 2006). This is in line with the high expression level of neuronal steaoryl-CoA specific ceramidase synthase 1 (CerS1) (Becker et al., 2008) creating a large pool of C18:0 fatty acids in neurons. This isoform distribution is very different compared with the islets of Langerhans of mammals, where sulfatide with saturated fatty acids of 16 or 24 carbon atoms are present (Fredman et al., 2000). These differences most likely reflect specific biological functions of distinct sulfatide isoforms in different cell types.

Sulfatide also show an age-dependent isoform expression during myelin formation, studied in rodents and cell cultures, which are described in more detail in the following sections (Marbois et al., 2000; Hirahara et al., 2017). In addition, the proportion of hydroxylated to non-hydroxylated fatty acids of CNS-associated sulfatide changes with age in mammals (Svennerholm and Stallberg-Stenhagen, 1968; De Haas and Lopes-Cardozo, 1995; Marbois et al., 2000; Pintado-Sierra et al., 2017). Comparing grey and white matter, sulfatide with hydroxylated fatty acids is distributed in the grey matter of the human brain, whereas non-hydroxylated fatty acid sulfatide dominate in the white matter. Because hydroxylated fatty acids originate from myelin-producing cells in the grey matter, researchers suggested that sulfatide with hydroxylated fatty acids influences myelin stability (Yuki et al., 2011).

1.3. Biological functions of sulfatide

The biological functions of sulfatide have been widely studied particularly in the nervous system and are described in detail in Section 2 (summarised in Table 2). The biological functions of sulfatides outside the nervous system are described below and summarised in Table 1.

1.3.1. Studies in animal models and cell culture systems

Biological functions of sulfatide involving host-pathogen recognition and infection, lymphocyte homing and monocyte adhesion by lectin/selectin and chemokine binding have been reported (Takahashi and Suzuki, 2012; Takahashi and Suzuki, 2015; Kim et al., 2020; Williams et al., 2020). Moreover, natural killer T (NKT) cells, innate-like T cells recognising lipid antigens presented by CD1, recognises naturally occurring sulfatide (Shamshiev et al., 2002; Zajonc et al., 2005; Blomqvist et al., 2009; Patel et al., 2012; Stax et al., 2017). CD1 presentation of sulfatide to type II NKT cells, as well as conventional T cells, results in priming of T helper (Th) 1-, Th 2- or Th17-like responses, as reviewed previously (Rhost et al., 2012; Marrero et al., 2015; Dasgupta and Kumar, 2016). Thus, through activation of type II NKT cells, sulfatide might control antigen-induced autoimmune disorders. For example, during experimental autoimmune encephalomyelitis (EAE) and diabetes mellitus type 1 progression, sulfatide-reactive type II NKT cells accumulate in target tissues/areas of these animal models, and experimental administration of sulfatide inhibits these disease processes (Jahng et al., 2004; Subramanian et al., 2012; Maricic et al., 2014). Importantly, long-chain sulfatides (C24:0 and C24:1) are specifically efficient in activating the type II NKT cells, further highlighting the importance of specific fatty acid isoforms for biological function. In addition, sulfatide-reactive type II NKT cells regulate type I NKT cells by modulating the function of antigen-presenting cells, which in turn results in protection from autoimmune disorders (Halder et al., 2007; Arrenberg et al., 2009). Sulfatide also ameliorates experimental autoimmune neuritis by regulating the balance of T helper cells (Th1/Th17) and regulatory T cells (Wang et al., 2019). Anti-inflammatory properties of sulfatide have also been demonstrated in an experimental *Staphylococcus aureus*-induced sepsis model through a CD1d-dependent pathway (Kwiecinski et al., 2013).

Interestingly, myelin-derived sulfatide activates human type I NKT cells through CD1 and is thereby a self-lipid for these cells, possibly participating in the pathogenesis of neuroinflammatory disorders (Stax et al., 2017). A pro-inflammatory role of sulfatide has also been described in autoimmune hepatitis and EAE, as well as in glia, possibly through L-selectin-dependent mechanisms (Kanter et al., 2006; Jeon et al., 2008; Sebode et al., 2019). Thus, sulfatide might act as a double-edged sword depending on the organ involvement and pathological context. (Stax et al., 2017) also addressed the importance of taking different sulfatide isoforms into account when studying the role of sulfatide in neuroimmune conditions, as well as in future therapeutic interventions.

Sulfatide has been described to have both coagulant and anticoagulant activities (Kyogashima et al., 2001; Ida et al., 2004). The anticoagulant activity is probably due to the ability of sulfatide to bind fibrinogen as well as its inhibitory effect on thrombin activity. On the other hand, accelerated coagulation has been suggested to involve annexin V and/or interaction with P-selectin on platelets. Sulfatide might act differently in the coagulation process depending on surrounding factors and the environment. The divergent results might also be explained by differences in experimental setups of separate cell culture experiments. Sulfatide binds to extracellular matrix components such as laminins, von Willebrand factor and thrombospondin; this binding is important for its function in cell adhesion processes (Roberts and Ginsburg, 1988). Membrane-bound sulfatide is also implicated in the passive sodium chloride transport from the lumen of kidney tubules into the interstitial space (Zalc et al., 1978). Furthermore, in the islets of Langerhans, sulfatide interacts with insulin crystals of beta cells and potassium channels regulating insulin secretion (Osterbye et al., 2001; Buschard et al., 2006).

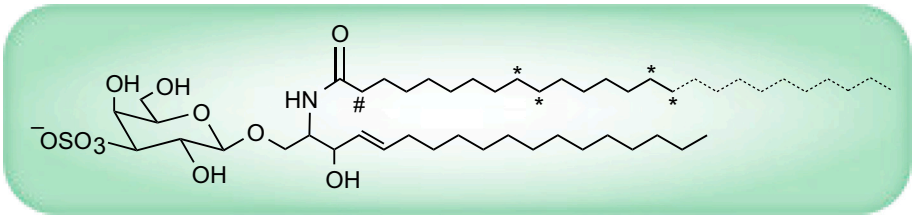

Fig. 1. The structure of sulfatide. Sulfatide (3-*O*-sulfogalactosylceramide, SM4s) is composed of a glycan part, 3-*O*-sulfated galactose and a hydrophobic part, ceramide, which in turn is composed of a sphingosine base and a fatty acid. The fatty acid can vary in alkyl chain length (illustrated by the dashed line, C16–C26 are most common), degree of unsaturation (double bounds introduced between the *) and hydroxylation (#). The sphingosine moiety of sulfatide is dominated by 4-sphingenine in mammals (d18:1, exemplified in the figure), apart from the intestine and kidney, where t18:0 is rather common.

1.3.2. Studies of human tissues/biofluids and human derived cell lines

There is elevated expression of sulfatide in mammalian malignancies and carcinoma cell lines, as reviewed by (Takahashi and Suzuki, 2012). Thus, specific isoforms of sulfatide might be used as a biomarker for some cancers. Sulfatide might play an important role in cancer progression exemplified by the involvement in adhesion of cancer cells through P-selectin expressed on endothelial cells (Aruffo et al., 1991) and by the contribution to apoptosis in breast cancer cells (Suchanski et al., 2018). Sulfatide has also been suggested as a serological biomarker for tuberculosis (Dos Santos et al., 2020).

2. The role of sulfatide in myelin biogenesis

2.1. Myelin synthesis and composition

Myelin is formed by oligodendrocytes in the CNS and Schwann cells in the PNS and shows a multilamellar structure rich in lipids that encloses segments of axons. Myelinated fibres organise into domains including nodes of Ranvier, paranodes, juxtaparanodes and internodes, each of which has specific functions depending on its molecular composition (Nave, 2010; Rasband and Macklin, 2012). At the paranodes, myelin loops attach to the axons to form paranodal axo-glial junctions. Myelin has important functions as an insulator and metabolic support for the neuronal axon, thereby increasing the nerve conduction velocity and reducing energy consumption (Nave, 2010, Rasband and Macklin, 2012). In other words, myelin is essential for motor, sensory and cognitive functions. Myelination starts during embryonic development (the first oligodendrocytes are detected by 45 days post-conception (Hajihosseini et al., 1996)), but the active phase of myelination starts after birth, around 3–4 months of age, at which time oligodendrocytes and Schwann cells produce enormous amounts of lipids in a relatively short period of time (first 2 years of postnatal life; Fig. 4A) (Morell and Quarles, 1999). Myelination occurs for another 2–3 decades in the human cerebral white matter (Yakovlev and Lecours, 1967; Benes et al., 1994; Sowell et al., 2003), albeit at a much lower rate compared with the active phase. Thus, severe developmental defects of myelin are not embryonically lethal, but instead cause disease later in life. When myelination is completed, the oligodendrocytes have synthesised approximately 40% of the total lipid amount in the brain (Norton, 1981).

The dry weight of CNS and PNS myelin contains a high percentage of lipid (70%–85%) and, consequently, a low percentage of protein (15%–30%) (Rasband and Macklin, 2012). This is very different compared with most biological membranes, which show a higher ratio of proteins to lipids. Actually, the overall molecular ratio of proteins to lipids in myelin has been estimated as almost 1 to 200 (O'Brien and Sampson, 1965). There is variation between the CNS and PNS in the protein composition (Patzig et al., 2011; Rasband and Macklin, 2012). The membrane-bound proteolipid protein (PLP, in the CNS), P0 (PNS) and periaxin (PNS) and cytosolic myelin basic protein (MBP) are the most abundant proteins (Boggs, 2006; Baron and Hoekstra, 2010), but as many as 500 different myelin proteins have been identified (Patzig et al., 2011). The lipid species of the CNS and PNS are remarkably similar, where cholesterol, galactosylceramide and ethanolamine plasmalogens dominate (Månsson and Fredman, 2000; Chrast et al., 2011; Rasband and Macklin, 2012). In addition, the fatty acid composition of many individual lipid classes of myelin is distinctive compared with other neuronal structures, further supporting different biological functions of lipid isoforms. The lipids are important for the close packing and tight organisation of molecules in this highly specialised membrane structure and thereby also crucial for the long-term maintenance of myelin, described in more detail below.

2.2. Lipids and myelin biogenesis

For myelination biosynthesis to occur, progenitor oligodendrocytes

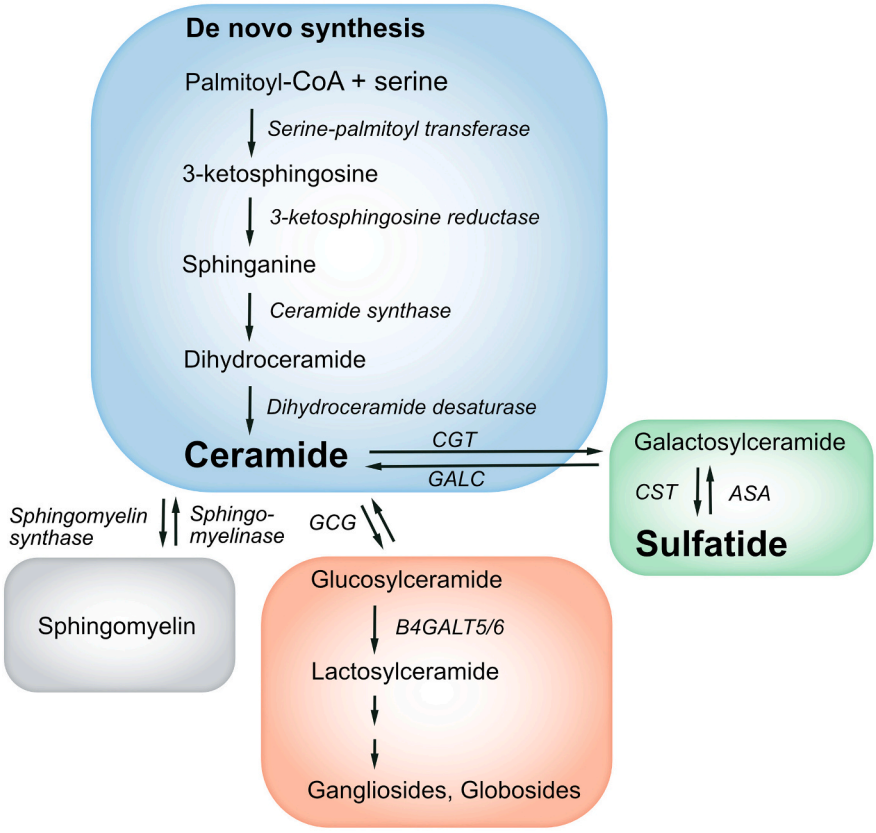

Fig. 2. Key reactions involved in glycosphingolipid (GSL) synthesis. Ceramide, the precursor of all GSLs, is synthesised by three pathways: *de novo* synthesis in the ER, the sphingomyelin pathway by degradation of sphingomyelin (plasma membrane and lysosomes) and the 'salvage pathway' by recycling of GSLs (lysosomal degradation). The *de novo* synthesis pathway begins by the condensation of serine with palmitoyl-CoA by serine-palmitoyl transferase to produce ceramide (*blue square*). Complex GSLs such as gangliosides and globosides are further synthesised in the endoplasmic reticulum (ER) and Golgi compartments through the production of glucosylceramide (enzyme *GCG: UDP-glucose-ceramide glucosyltransferase*) and lactosylceramide (enzyme *B4GALT5/6: beta-1,4-galactosyltransferase 5/6*) (*orange square*). The synthesis of sulfatide (green square) occurs through the action of *UDP-galactose:ceramide galactosyltransferase (CGT)*. The metabolism of sulfatide is described more in detail in Fig. 3. Enzymes are italicised. *GALC*, *galactosylceramidase*; *ASA*, *arylsulfatase A*.

go through a well-defined differentiation process in which the expression of lipids and proteins is tightly regulated. Cholesterol is an essential lipid component of the plasma membrane and has been described as one of the drivers of myelin synthesis (Schmitt et al., 2015). The entire pool of cholesterol is produced *de novo* in the CNS and, once incorporated into the myelin sheath, the turnover is slowed down, resulting in a half-life of cholesterol of several months/years (Petrov et al., 2016). Oligodendrocytes synthesise most of the cholesterol during brain development, however, microglia also produce sterols and thus contribute to myelination as well as repair of demyelinated lesions (Berghoff et al., 2021). Cholesterol is also important for the stability of myelin by regulating membrane fluidity and permeability. The galactolipids galactosylceramide and sulfatide are described as lipids typical of myelin but they are not myelin specific. The accumulation rate of these lipids in the mammalian brain can be used as a measure of myelin production (Norton and Poduslo, 1973; Månsson and Fredman, 2000). These lipids have been described as the stabilisers of myelin (Schmitt et al., 2015). Proper 'compartmentalisation' of lipids and proteins in the myelin sheath most likely has functional importance. Immunohistochemical studies show that the major myelin proteins PLP and MBP reside with galcer in compacted myelin, whereas sulfatide localises mainly to the paranodal region of non-compacted myelin (Baron and Hoekstra, 2010), described in more detail below. However, the inability to detect sulfatide in compacted myelin might be due to issues of antibody penetration and accessibility as well as the relatively low concentration of sulfatide compared with galcer. While we focus mainly on sulfatide, the importance of galactosylceramide for the maintenance and function of the myelin sheath should not be neglected.

2.2.1. Sulfatide and myelin biogenesis – studies in animal models and cell culture systems

As previously mentioned, sulfatide is highly expressed by myelin-producing cells, oligodendrocytes in the CNS and Schwann cells in the PNS. During oligodendrocyte differentiation in primary cultures, sulfatide is detected early in oligodendrocyte progenitor cells and has been shown to be upregulated just before cells wrap their myelin fibres around the neuronal axons (Hirahara et al., 2017). In addition, prior to the onset of myelin formation, the short chain fatty acid sulfatide isoforms (C16–C18) seem to dominate, whereas longer acyl chains (C22–C24) are upregulated from when the active phase of myelination begins (Marbois et al., 2000; Hirahara et al., 2017). The change in fatty acid composition observed during oligodendrocyte development may reflect different roles of specific sulfatide isoforms in oligodendrocyte maturation and myelin function. Developmental expression of fatty acid sulfatide isoforms in mammalian brain is further illustrated in Fig. 4B.

In earlier studies in primary cell cultures, researchers also suggested sulfatide as a key negative regulator of oligodendrocyte differentiation (Bansal et al., 1999; Hirahara et al., 2004). However, anti-sulfatide antibodies were found to block oligodendrocyte differentiation, which was not the case for anti-galcer antibodies (Bansal et al., 1999). The authors explained these results by the ability of anti-sulfatide antibodies to mimic an endogenous ligand activating the negative regulatory pathway and thereby blocking terminal differentiation (Bansal et al., 1999). Sulfatide also seems to be involved in oligodendrocyte survival and in the inhibition of myelin-associated axon outgrowth (Winzeler et al., 2011).

Although cell culture experiments suggest that sulfatide is important for the myelination process, sulfatide-independent pathways most likely

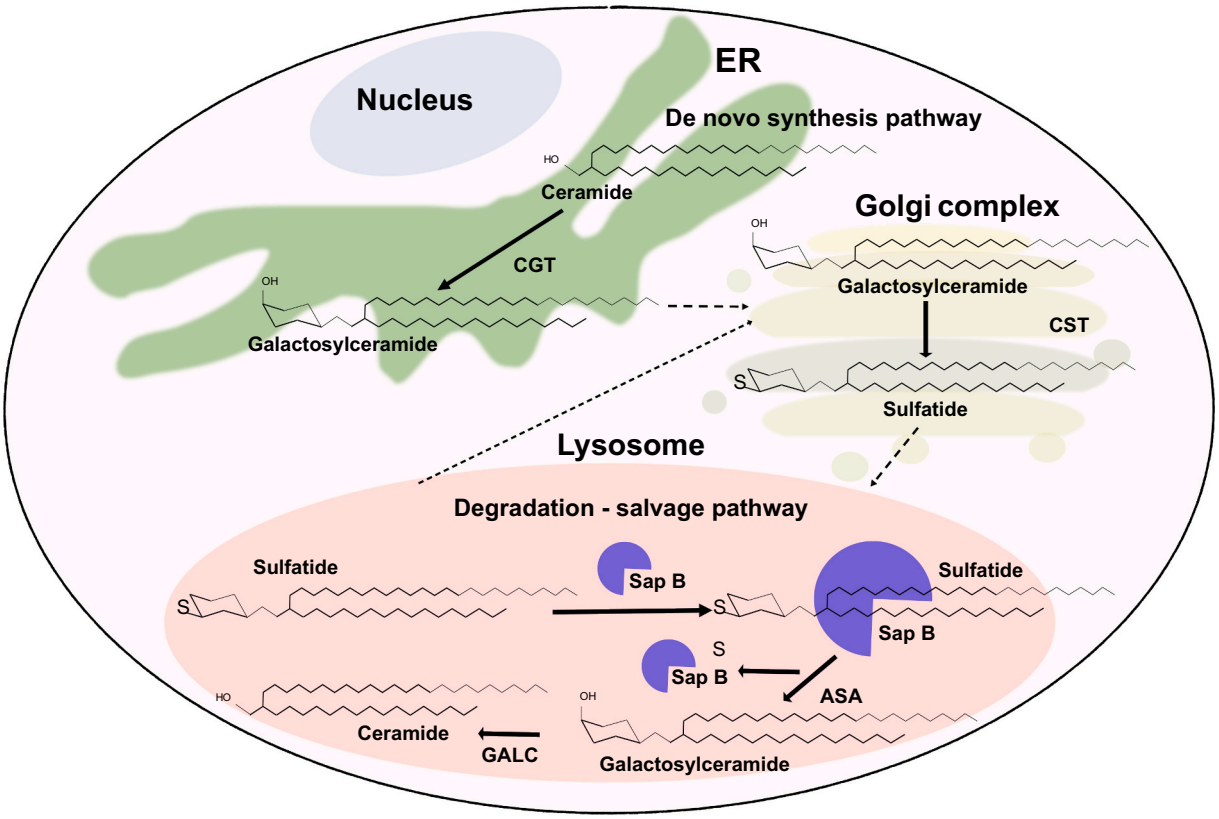

Fig. 3. The metabolism of sulfatide. The *de novo* synthesis of sulfatide begins with the production of ceramide in the endoplasmic reticulum (ER) followed by transferring a UDP-galactose moiety to the ceramide backbone by the action of *UDP-galactose:ceramide galactosyltransferase (CGT)*. Galactosylceramide is transported to the Golgi apparatus and sulfated at the 3′-OH group of the galactose moiety to produce sulfatide, by the action of *cerebroside sulfotransferase (CST)*. The second synthesis pathway of sulfatide, termed the 'salvage pathway' involves degradation through hydrolysis in the lysosomal compartment by the action of *arylsulfatase A (ASA)*. This reaction requires an activator protein, saposin B (sap B), that enables the exposure of sulfatide to *ASA* to generate galactosylceramide and subsequently ceramide (catalysed by *galactosyl ceramidase [GALC]*) to be used for re-synthesis of GSL in the Golgi compartment.

Table 1
Biological functions of sulfatide outside the nervous system.

Biological function	Experimental system	Reference
Host-pathogen recognition and infection		Takahashi and Suzuki, 2012, review
	Influenza A strains	Takahashi and Suzuki, 2015, review
	M. Pneumoniae strain M129	Williams et al., 2020
Lymphocyte homing and monocyte adhesion by lectin/selectin and chemokine binding		Takahashi and Suzuki, 2012, review
Regulation of antigen induced autoimmune disorders through CD1 presentation of sulfatide to type II NKT cells/ conventional T cells	Experimental autoimmune encephalomyelitis (EAE)	Shamshiev et al., 2002
	NOD/Del, NOD.Scid and NODCD1d$^{-/-}$ mice	Jahng et al., 2004
	SJL/J mice	Zajonc et al., 2005
	C57BL/6, C57BL6-CD1d$^{-/-}$, C57BL6-IL-12p40$^{-/-}$ and C57BL6-Jα18$^{-/-}$ mice	Halder et al., 2007
	CST$^{-\backslash-}$ mice	Arrenberg et al., 2009, review
	iNKT cell hybridoma/clones and hiNKT cells, dNKT cells	Subramanian et al., 2012
	XV19, K562 and RMAS cells,	Maricic et al., 2014
	NKT cell line M0,	Blomqvist et al., 2009
	hCSF, bovine brain myelin	Patel et al., 2012
	Peripheral blood mononuclear cells to obtain dendritic cells	Stax et al., 2017
	CR1 lymphoblastoma cells	
Anti-inflammatory properties in *Staphylococcus aureus* infection	C57BL/6, C57BL6-CD1d$^{-/-}$ and C57BL6-Jα18$^{-/-}$ mice	Kwiecinski et al., 2013
Anti-inflammatory properties by suppressing secretion of HMGB1 during LPS stimulation	C57BL/6 mice Raw 264.7 cells	Kim et al., 2020
Coagulant and anticoagulant activities	Rats and mice	Kyogashima et al., 2001
	HT29 cell line, human plasma	Ida et al., 2004
Contribute to cell adhesion processes by binding to extracellular matrix components		Roberts and Ginsburg, 1988, review
Interact with insulin crystals of beta cells and potassium channels regulating insulin secretion	Islet/beta cell isolation from Lewis rats	Osterbye et al., 2001
	αTC1–9 glucagonoma cells	Buschard et al., 2006
Serological biomarker for tuberculosis	Human material	Dos Santos et al., 2020
Passive sodium chloride transport in the kidney	Rabbit	Zalc et al., 1978
Biomarker of specific cancers and contributing to cancer progression as well as apoptosis	HL60, THP1 and COS cells, human buffy coats	Takahashi and Suzuki, 2012, review
	Breast cancer cell lines and tissue biopsies from patients with breast cancer	Aruffo et al., 1991
		Suchanski et al., 2018

Table 2
Biological functions of sulfatide in the myelin biogenesis.

Biological function	Experimental system	Reference
Oligodendrocyte differentiation – aspects of sulfatide isoforms	CGT$^{-\backslash-}$ mice	Bansal et al., 1999
	OLG cell cultures from Wistar rats, C57BL6 mice	Hirahara et al., 2004
		Hirahara et al., 2017
Oligodendrocyte survival and inhibition of myelin-associated axon outgrowth	Retinal ganglion cells (RGCs) CGT$^{-\backslash-}$ mice	Winzeler et al., 2011
Myelin maintenance	CST$^{-\backslash-}$ mice	Honke et al., 2002
		Ishibashi et al., 2002
		Marcus et al., 2006
Clustering of ion channels at the nodes of Ranvier	CST$^{-\backslash-}$ mice	Ishibashi et al., 2002
Influence lateral diffusion of MBP and PLP into lipid rafts	Rat derived OLG cell line OLN-93	Ozgen et al., 2014
	OLG cell cultures from Wistar rats, HepG2 cells	Baron et al., 2015
Promote the interaction between adjacent PLP extracellular domains	CST$^{-\backslash-}$ mice	Palavicini et al., 2016
Compartmentalisation and stabilisation of NF-155 and MAG at the paranodes, maintaining proper axo-glial interaction	CST$^{-\backslash-}$ mice GalNAc-T$^{-/-}$ mice	Ishibashi et al., 2002
		Palavicini et al., 2016
		McGonigal et al., 2019
Association with MAL, GSL clustering and transport	Lewis rat	Frank et al., 1998
Negative feedback on MAL synthesis	ASA$^{-/-}$ mice	Saravanan et al., 2004
		Frank, 2000, review

exist because the *Cst*-deficient (*Cst*$^{-/-}$) mouse, despite lacking sulfatide, produces normal compacted myelin (Honke et al., 2002; Ishibashi et al., 2002). This mouse model has an attenuated phenotype in terms of age of onset, life span and severity of symptoms compared with the *Cgt*$^{-/-}$ mouse, in which sulfatide as well as galactosylceramide are missing (Coetzee et al., 1996; Dupree et al., 1998). In the *Cgt*$^{-/-}$ mouse, myelin is initially synthetised using glucosylceramide instead of galcer, but after 3–4 weeks the myelin falls apart (Coetzee et al., 1996). This clearly demonstrates that a glucose derivative cannot replace galactose. With age, *Cst*$^{-/-}$ mice show conduction deficits and tremor combined with progressive ataxia. Further studies using the *Cst*$^{-/-}$ mouse model showed that adult mice have significantly thinner myelin sheaths compared with wild-type mice. There is redundant myelin, vacuolar degeneration and non-compacted myelin, suggesting an important role for sulfatide in the maintenance of the myelin sheath (Marcus et al., 2006). Furthermore, in line with the primary cell culture experiments (Bansal et al., 1999, Hirahara et al., 2004), the *Cst*$^{-/-}$ mouse shows more terminally differentiated oligodendrocytes due to increased proliferation and decreased apoptosis, which persist into adulthood (Shroff et al., 2009).

The clustering of ion channels at nodes of Ranvier, the gap between adjacent myelin sheaths, promote rapid saltatory conduction along myelinated axons. The *Cst*$^{-/-}$ mouse model furthermore revealed that sulfatide plays an important role for the clustering of ion channels at nodes of Ranvier (Ishibashi et al., 2002). At these nodes, myelin forms lateral loops that terminate in the paranodal region. Disruption of paranodal junctions in the model might be explained by impaired location of adhesion proteins to lipid rafts. These structures are believed to represent highly dynamic functional domains of the plasma membrane where selected lipids and membrane proteins merge to exert their biological functions such as receptor signalling and trafficking (Sonnino and Prinetti, 2013; Murate et al., 2015; Kim et al., 2020). The myelin galactolipids containing long saturated fatty acid chains in combination with their ability to form hydrogen bonding network are suitable to form ordered membrane domains such as lipid rafts. Thus, at these lipid raft domains, specific sulfatide isoforms might affect sorting and lateral assembly of myelin proteins and may also influence membrane dynamics.

The extracellular surfaces of myelin face each other in the multilayer myelin structure, which enables carbohydrate interactions of the galactolipids. The signalling areas enriched in GSLs between oligodendrocytes or myelin membranes are called the glycosynapse (Hakomori,

Table 3
Sulfatide alterations in conditions with myelin pathology.

Animal models	
CerS2 deficient mouse model (lacking VLCFA C22–C24). Decrease in sulfatide and galactosyl-ceramide in myelin. Myelin stainability is progressively lost in early adulthood.	Imgrund et al., 2009 Ben-David et al., 2011
FA2H deficient mouse model (lacking 2-hydroxylated GSL). Myelin stainability is progressively lost in early adulthood. The turnover of the oligodendrocytic myelin paranodal and inner loop protein (Opalin) is affected.	Zoller et al., 2008 Hardt et al., 2020
Elovl1 deficient mouse model (lacking VLCFA C22–C24). Reduced chain lengths of GSL, including sulfatide. Hypomyelination, subtle impairment of motor and auditory functions and decreased postnatal survival.	Isokawa et al., 2019
Experimental autoimmune encephalomyelitis (EAE, a murine model for MS). Anti-sulfatide T cell responses improve the course of the disease.	Jahng et al., 2004, Maricic et al., 2014
Cuprizone mouse model (MS model), Involvement of vitamin K in myelination and remyelination is coupled to increased production of individual brain sulfatide isoforms (C18–C24).	Popescu et al., 2018
Shiverer (Shi) mouse model (mutant MBP resulting in myelin destabilisation). Lipid distribution changes, sulfatide correlate with local morphological myelin changes.	Maganti et al., 2019
ASA deficient mouse model ($ASA^{-/-}$, MLD model). Sulfatide and lysosulfatide storage with MLD-like symptoms without demyelination.	Hess et al., 1996 Molander-Melin et al., 2004 Blomqvist et al., 2011 Moyano et al., 2014
Overexpression of sulfatide on the $ASA^{-/-}$ background gives typical hallmarks of infantile MLD with progressive demyelination and sulfatide storage.	Ramakrishnan et al., 2007
tgArcSwe transgenic mouse model (AD model). Sulfatide depletion (specific isoforms), disruption of the myelin sheet and ApoE deposition localize to amyloid plaques.	Kaya et al., 2018 Kaya et al., 2020
LPR1 deficient mouse (protein of lipoprotein metabolism). Global amelioration of lipid metabolism in the brain including decreased levels of sulfatide.	Liu et al., 2010
Experimental autoimmune neuritis (mirroring GB). Sulfatide ameliorates disease by regulating the balance of T helper cells (Th1/Th17) and regulatory T cells.	Wang et al., 2019
Lepr (db/db) mouse (obesity/diabetes model). Myelin lipids (including sulfatide) associates with neuropathy, progressing to CNS at advanced diabetic stages.	Palavicini et al., 2020
Chronic alcohol consumption causing neurodegeneration and white matter changes. Contribution of sulfatide to the toxic effect of smoking and alcohol abuse in rat.	Yalcin et al., 2015 Yalcin et al., 2017
Human disorders	
CerS1 gene. Autosomal recessive progressive myoclonus epilepsy.	Vanni et al., 2014
CerS2 gen. Progressive myoclonic epilepsy.	Mosbech et al., 2014
FA2H gene. Leukodystrophy involving spastic paraparesis and dystonia.	Edvardson et al., 2008
ELOVL1 gene. Neurocutaneous disorder involving skin ichthyosis, hypomyelination, spastic paraplegia and high frequency deafness.	Kutkowska-Kazmierczak et al., 2018 Mueller et al., 2019
Multiple sclerosis (MS). Immune-mediated demyelination disorder. Deficient remyelination over time results in persistent loss of myelin. Divided in relapsing-remitting MS and progressive MS.	Baronica et al., 2011, Marbois et al., 2000, Ilyas et al., 2003, Kanter et al., 2006, Brennan et al., 2011, Haghighi et al., 2012, Moyano et al., 2013, Novakova et al., 2018
Metachromatic leukodystrophy (MLD, deficiency of the enzyme arylsulfatase A). Progressive demyelination due to sulfatide storage.	van Rappard et al., 2015 Dali et al., 2015 Cao et al., 2020
Alzheimer's disease (AD). Progressive neurodegenerative disorder where extracellular amyloid-beta (Aβ), neuritic plaques and neurofibrillary tangles are characteristic for the disease. White matter changes are present.	Svennerholm and Gottfries, 1994, Han et al., 2002, Han et al., 2003a, 2003b, Cheng et al., 2010, Han, 2010, Cheng et al., 2013, Couttas et al., 2016 Gonzalez de San Roman et al., 2017
Vascular dementia (VaD). Disorder with white matter lesions, including subcortical changes (leukoaraiosis) and demyelination.	Wallin et al., 1989 Fredman et al., 1992 Tarkowski et al., 2003
HIV-associated dementia.	Gisslen et al., 1999
Peripheral neuropathy conditions including ***Guillian-Barre syndrome (GBS).***	Fredman et al., 1991, Fredman, 1998, Souayah et al., 2007, Schirmer et al., 2016, Peter et al., 2020
Children with white matter abnormalities of different origin show increased CSF-sulfatide.	Kristjansdottir et al., 2001

2002). (Boggs et al., 2010) suggest that lipid rafts of the glycosynapse enriched in galcer and sulfatide interact with each other and thereby regulate processes such as differentiation and process extensions. Galactolipid interaction might also be important for the compaction of myelin by disruption of the cytoskeleton, which is necessary for the cytoplasmic surfaces to adhere and to produce compacted myelin. This process involves the myelin-specific proteins MBP and PLP, which mediate adhesion between cytosolic and extracellular surfaces, respectively (Rasband and Macklin, 2012). (Palavicini et al., 2016) showed, using $Cst^{-/-}$ mice, that sulfatide promotes the interaction between adjacent PLP extracellular domains, which might account for the non-compacted myelin formation in this specific animal model.

Sulfatide has also been associated with the highly hydrophobic myelin and lymphocyte protein (MAL) in cell culture experiments and myelin structures (Frank et al., 1998; Frank, 2000; Saravanan et al., 2004). It was hypothesised that MAL is involved in the vesicular transport of sulfatide and other myelin components to the myelin membrane. MAL might also be involved in organising GSLs into lipid rafts, stabilising and supporting signalling through these domains (Frank, 2000). Because myelin is a fluid structure, lipids can move laterally and thereby facilitate interaction with different myelin components, as well as reaching cytoplasmic areas where endocytosis and vesicular transport are possible (Olsen and Faergeman, 2017). Furthermore, the oligodendroglial isoform of neurofascin (NF-155), a critical molecule for the paranodal axo-glial junction (Charles et al., 2002), localises with sulfatide to the paranodal region of non-compacted myelin. NF-155 together with Caspr and contactin are absent at the paranodal region of the $Cgt^{-/-}$ and $Cst^{-/-}$ mouse models lacking sulfatide (Dupree et al., 1999; Ishibashi et al., 2002; Palavicini et al., 2016). (Palavicini et al., 2016) further proposed that the aggravated tremor and ataxia phenotype observed in the $Cst^{-/-}$ mouse model are consequences of the diminished NF-155–PLP interactions resulting in disruption of myelin–axon junctions. These results support that sulfatide is not a significant contributor to myelin formation in rodents, but it is essential for the proper formation of the axo-glial junctions at the paranode.

Finally, the importance of both glial sulfatide and neuronal gangliosides (sialic acid containing GSLs) in the coordination of axo-glial adhesion and paranodal organisation was recently addressed (McGonigal et al., 2019). By taking advantage of transgenic mice in which GSLs are selectively depleted ($Cst^{-/-}$ and $GalNAcT^{-/-}$), the functional interaction between sulfatide and gangliosides could be studied specifically in neurons and glial cells. The mouse models show prominent disorganisation of proteins at the nodes of Ranvier (NF-155, Caspr and myelin-associated protein [MAG], proteins involved in axo-glial adhesion) and age-dependent neurodegeneration, where the $Cst^{-/-}$ model, which lack sulfatide, shows a more severe phenotype. However, the strongest phenotype was observed in mice lacking both sulfatide and gangliosides in oligodendrocytes and neurons, respectively, where mice developed normally up to postnatal day 10 and then degenerated and died between the age of 20 and 25 days at the peak of myelination. Histological examination of $Cst^{-/-} \times GalNAcT^{-/-}$ mice revealed

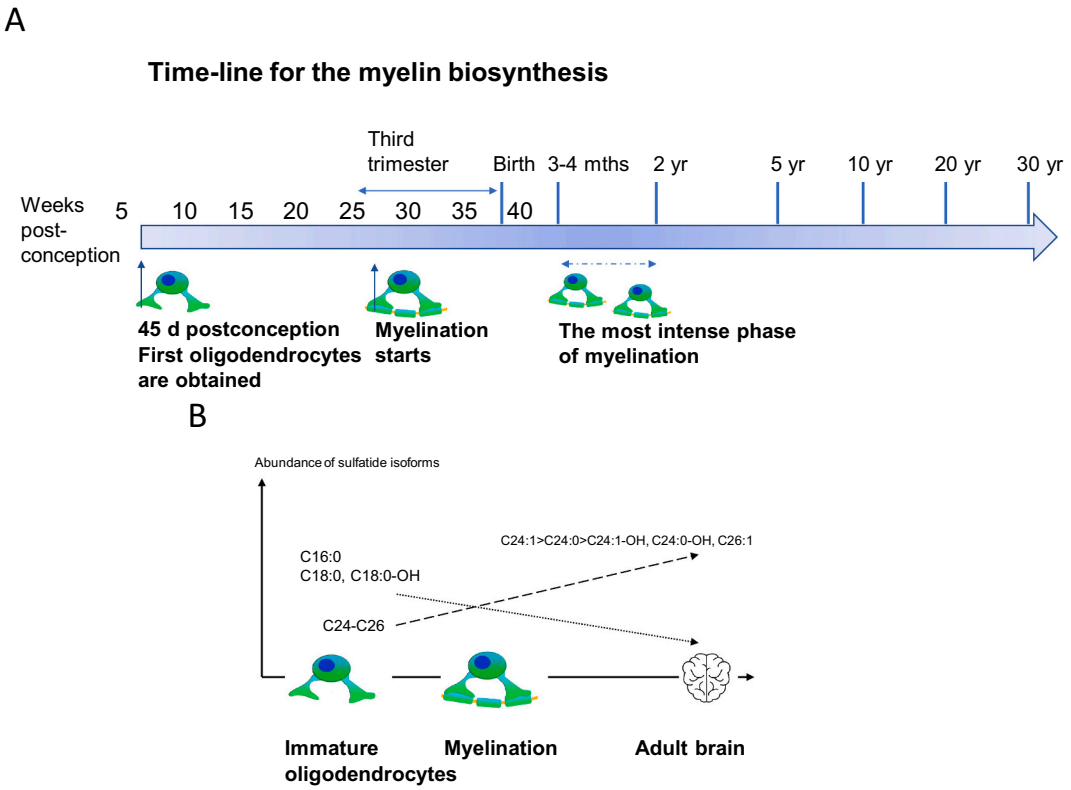

Fig. 4. Myelin biosynthesis. A. Time-line of myelin biosynthesis in humans. The first oligodendrocytes are detected already by 45 days post-conception, however, myelination is considered to start about 20 weeks later at the beginning of the third trimester. The most intense phase of myelination begins after birth, around 3–4 months of age, in which oligodendrocytes and Schwann cells produce enormous amounts of lipids, such as galactosylceramide and sulfatide, in a relatively short period of time (first 2 years of postnatal life). Subsequently, the accumulation rate of galactosylceramide and sulfatide in the mammalian brain can be used as a measure of myelin production. Myelination occurs for another 2–3 decades in the human cerebral white matter B. Developmental expression of fatty acid sulfatide isoforms in the mammalian brain. The short chain fatty acid sulfatide isoforms (C16–C18) seem to dominate in the immature brain, whereas longer acyl chains (C22–C24) are upregulated from when the active phase of myelination begins. In the adult mammalian brain, C24:1 > C24:0 > C24:1-OH, C24:0-OH, C26:1 sulfatide isoforms dominate, with C24:1 being most abundant.

widespread axonal degeneration and disruption of the axo-glial junctions at the nodes of Ranvier. Mice furthermore exhibited a sulfatide-dependent loss of NF-155 and a significant reduction in MAG levels of CNS myelin. These results suggest that glial sulfatide and neuronal gangliosides act together in the myelination process involving compartmentalisation and stabilisation of NF-155 and MAG to the nodal regions for maintaining proper axo-glial interaction (McGonigal et al., 2019). The authors also highlighted the importance of GSLs for membrane protein localisation, essential for proper axo-glial function.

Direct interaction of GSLs with nodal proteins has, to the best of our knowledge, not been described. However, the ability of lipids to alter membrane features such as fluidity, curvature, as well as organisation into lipid raft structures, and thereby influence protein localisation and subsequently the function of the protein, is an attractive explanation (for sulfatide, see reviews (Aureli et al., 2015, Grassi et al., 2016, Ozgen et al., 2016). In fact, the lateral diffusion of myelin-associated MBP and PLP depends on the galactolipid environment (Ozgen et al., 2014; Baron et al., 2015). Aberrant myelin biogenesis might not only occur during depletion of selected GSLs; alterations in GSL isoforms might also be of importance. These aspects will be covered in the next section summarising different pathological aspects of sulfatide in disorders affecting myelin biogenesis.

3. Sulfatide in disorders with myelin pathology

The unique lipid-rich composition of myelin reflects the importance of lipids in this specific membrane structure. Alterations in the complex lipid/protein organisation most likely contribute to the myelin defects seen in disorders affecting the CNS, such as hypomyelination (decrease in myelin production), dysmyelination (abnormally formed myelin) and demyelination (loss off myelin by degenerative processes). The lipid composition of myelin may change during disease development of, for example, multiple sclerosis (MS) and leukodystrophies, which in the end may contribute to myelin destabilisation and degradation. Furthermore, to understand the axonal pathology in demyelinating diseases it is important to emphasise that the myelin sheath and the underlying axon are a closely connected functional unit and try to understand how the damage of one component affects the function and survival of the other. Because sulfatide is a highly multifunctional GSL – possibly affecting the properties of membrane proteins such as ion channels, receptors, and transporters – altered expression of sulfatide could be linked to various kind of neurological symptoms.

Sulfatide alterations in conditions with myelin pathology are summarised in Table 3, divided into animal models and human disorders.

3.1. Altered sulfatide isoform expression in myelin pathology – animal studies and case reports

3.1.1. Deficiency of ceramide synthase(s)

The specific fatty isoform of sulfatide is important to consider when studying the specific role of sulfatide in myelin biogenesis and disease. As stated above, the fatty acid composition of sulfatide is developmentally regulated in the brain; this expression correlates with the expression of genes involved in GSL synthesis. A family of enzymes involved in the generation of ceramide by N-acylation of the sphingosine base are the ceramide synthases (CerS) (Fig. 2). Six mammalian ceramide

synthases have been described and each gene shows a characteristic tissue-specific expression. In addition, each CerS isoform has a substrate specificity towards specific fatty acid residues (Park et al., 2014). The CerS2 activity has been shown to be specific towards very-long-chain fatty acids (C22–C24) and in the *Cers2*$^{-/-}$ mouse model, there is a reduction in very-long-chain fatty acids (C22:0–C24:0) alongside a massive decrease in sulfatide and galactosylceramide in the myelin (Imgrund et al., 2009; Ben-David et al., 2011). The mice can form relatively normal myelin, but from early adulthood myelin stainability is progressively lost and the myelin sheaths are structurally defective. The results support that CerS2 is important for the maintenance of myelin as well as stabilisation of cerebellar histological architecture in the adult mouse brain. Furthermore, reduced levels of CerS2 may cause progressive myoclonic epilepsy in humans (Mosbech et al., 2014). Another rare genetic disorder, autosomal recessive progressive myoclonus epilepsy, results from mutations in *CERS1* (Vanni et al., 2014). Inactive CerS1 in neurons might thus result in elevated substrates, that is, sphinganine and sphingosine. Unfortunately, lipid data from affected patients were not available in this study.

3.1.2. Fatty acid 2-hydroxylase deficiency

Another enzyme involved in the synthesis of ceramide species containing hydroxylated fatty acids is fatty acid 2-hydroxylase (FA2H) (Eckhardt et al., 2005). By studying the *Fa2h*$^{-/-}$ mouse model, lacking 2-hydroxylated GSL, researchers made similar observations as for the *CerS2*$^{-/-}$ mouse; mice can generate normal myelin but exhibit myelin degeneration as they age (Zoller et al., 2008). These findings are in line with the suggestion that sulfatide (and galcer) are myelin stabilisers. In addition, mutations in the *FA2H* gene are associated with leukodystrophy in patients with spastic paraparesis and dystonia (Edvardson et al., 2008), which underscores the importance of 2-hydroxylated fatty acids of galactolipids for proper myelin function. In a recent paper using the *Fa2h*$^{-/-}$ mouse model, the authors suggested that 2-hydroxylated sphingolipids affects the turnover of the oligodendrocytic myelin paranodal and inner loop protein (Opalin), possibly involved in the pathogenesis of spastic paraplegia (Hardt et al., 2020).

3.1.3. Fatty acid elongase deficiency

A *de novo* mutation in a gene involved in the elongation of fatty acids, *ELOVL1* – responsible for the synthesis of C22–C26 fatty acids – was recently described in patients with neurocutaneous disorder involving skin ichthyosis, hypomyelination, spastic paraplegia and high-frequency deafness (Kutkowska-Kazmierczak et al., 2018; Mueller et al., 2019). In patient fibroblasts, there were reduced amounts of $\geq$ C24 ceramides and sphingomyelins. Subsequently the *Elolv1*$^{-/-}$ mouse model was created, with symptoms of hypomyelination, subtle impairment of motor and auditory functions and decreased postnatal survival (Isokawa et al., 2019). Of note, the chain lengths of brain sphingolipids, including sulfatide, were markedly shortened, further supporting the importance of very long chain fatty acid sphingolipids for myelin function. In line with these findings, accumulation of sulfatide containing the C18:0 fatty acids in neurons has been associated with lethal audiogenic seizures in mice (van Zyl et al., 2010).

3.2. Sulfatide in immune-mediated demyelination – multiple sclerosis

Studies of sulfatide as a modulator of immune functions have mainly focused on inflammation and demyelination in the CNS. Enhanced antibody response to sulfatide has been closely associated with CNS inflammation and demyelination in humans (Fredman et al., 1991; Kanter et al., 2006; Nobile-Orazio and Giannotta, 2011). MS is the prototype of an immune-mediated demyelination disorder, in which lymphocytes and monocytes invade the brain and cause inflammation and immune attacks destroying myelin sheaths (Steinman, 2014). In patients affected by MS, myelin is initially formed in a correct manner but deficient remyelination over time results in persistent loss of myelin (Podbielska et al., 2013). The demyelination in MS is initially focal and per definition the lesions disseminate in space and time (Thompson et al., 2018). After years of relapsing-remitting MS, patient may enter a second phase called progressive MS, showing increased disability over time in the absence of acute attacks (Steinman, 2014). The exact cause of demyelination in MS is unknown. Although MS is not a 'classical genetic disease', involvement of genetic predisposition is well documented, where certain human leucocyte antigen (*HLA*) genotypes give the strongest association. In patients with MS, both altered levels of sulfatide and anti-sulfatide antibodies have been found (CSF and/or serum) (Marbois et al., 2000; Ilyas et al., 2003; Kanter et al., 2006; Brennan et al., 2011; Haghighi et al., 2012; Moyano et al., 2013). In our more recent study, we could not reproduce the findings of increased CSF sulfatide concentrations in patients with MS (newly diagnosed relapsing-remitting MS and progressive MS) compared with healthy controls (Novakova et al., 2018). Furthermore, sulfatide CSF levels of patients with MS correlate with neither degenerative CSF biomarkers (neurofilament light [NF-L], glial fibrillary acidic protein [GFAP]) nor magnetic resonance imaging (MRI) measures (T2 contrast-enhancing lesions and brain volume) nor clinical assessment of neurological disability (Expanded Disability Status Scale [EDSS] score). Instead, in line with (Moyano et al, 2013), our study suggests variations in sulfatide isoforms in relation to different disease stages of MS (Moyano et al., 2013; Novakova et al., 2018).

3.2.1. Animal models of MS

In EAE, a murine model for MS, anti-sulfatide T cell responses improve the course of the disease (Jahng et al., 2004; Maricic et al., 2014). In another MS mouse model – named the cuprizone model – researchers found that vitamin K is involved in myelination and remyelination, coupled to increased production of individual brain sulfatide isoforms (C18–C24) (Popescu et al., 2018). In a more recent paper, using this reversible demyelination model and the Shiverer (Shi) mouse (mutant MBP resulting in myelin destabilisation), (Maganti et al., 2019) showed that lipid distribution changes (sulfatide in particular) correlate with local morphological changes. These data further highlight the role of sulfatide in preservation of the myelin structures.

3.3. Sulfatide in leukodystrophies – metachromatic leukodystrophy

The importance of membrane transport for the maintenance of the myelin sheath suggests that proper turnover of myelin components, including lipids, is crucial. This is dramatically demonstrated in neurodegenerative diseases where accumulation of GSL in myelinating glial cells leads to cytotoxicity and demyelination. Metachromatic leukodystrophy (MLD) is such a disorder caused by the deficiency of the enzyme arylsulfatase A (ASA) (van Rappard et al., 2015). In our review, we use MLD to represent the leukodystrophy group of disorders. Due to the enzyme deficiency, sulfatide is accumulated in the lysosomes of myelinating cells, as well as astrocytes and neurons, resulting in severe neurological symptoms. A classic hallmark of late infantile MLD is progressive demyelination. Reduced ASA protein expression in MLD is confounded by the presence of an ASA pseudodeficiency in up to 20% of the population. These individuals can present with as little as 5% of residual enzyme activity *in vitro*, a value in the range of patient with MLD. Thus, genetic analysis and analysis of sulfatide storage in body fluids are of great importance for correct diagnosis of MLD. Interestingly, the *ARSA*-pseudo-deficiency allele correlates with a more severe clinical phenotype in patients with MS (Baronica et al., 2011). This finding suggests that pseudodeficiency alleles, as well as heterozygosity in *ARSA*, could be involved in the pathogenicity of complex genetic disorders.

Analysis of sulfatide in urine has long been used as a diagnostic tool for MLD. More recently, this approach has also been applied to dry urine spots (Kuchar et al., 2013; Spacil et al., 2016). However, normalisation for different urine volumes is problematic because sulfatide is mainly

located in the urine sediment and the traditional corrections for hydrophilic analytes based on creatinine is not optimal for hydrophobic lipids like sulfatide. Because urine sulfatide comprises of many isoforms, conversion of these into a single sulfatide specie (lyso-sulfatide without fatty acids) has also been described in an attempt to enhance the sensitivity of this biomarker (Spacil et al., 2016).

Sulfatide levels in CSF and the sural nerve have furthermore been correlated to the severity of neuropathy in patients with MLD (Dali et al., 2015), and in a recent publication, increased levels of CSF sulfatide and the cytotoxic compound lyso-sulfatide correlated with worse motor function in MLD (Cao et al., 2020). Interestingly, plasma C16:0/C16:0-OH and C18:0 have been addressed as possible biomarkers for MLD (Mirzaian et al., 2015; Saville et al., 2017). Lumbar puncture is an invasive procedure, and thus the usage of suitable plasma biomarkers for the diagnosis and stratification of patients with MLD appear more adequate. These findings further highlight the importance of carefully evaluating specific sulfatide isoforms in biomarker studies.

3.3.1. Animal models of MLD

A mouse model for MLD was produced more than two decades ago; sulfatide storage was detected with a similar pattern as compared to the human disease (Hess et al., 1996). *Arsa*$^{-/-}$ mice develop symptoms in line with the early phase of the human disease – for example gait disturbances. However, these animals do not develop demyelination. Some important consequences of sulfatide storage could be addressed by studying this mouse model. As in human MLD, sulfatide storage is also detected in neurons and astrocytes in the mouse model (Molander-Melin et al., 2004). The C18:0 isoform of sulfatide increases in the brain of *Arsa*$^{-/-}$ mice, possibly reflecting the storage in neurons and astrocytes suggesting that other cells might be more affected by the storage over time (Isaac et al., 2006). Furthermore, lipid raft fractions isolated form *Arsa*$^{-/-}$ mouse brain show increased levels of C18:0 sulfatide compared with non-lipid raft fractions (Moyano et al., 2014). This result is in agreement with previous findings of lipid raft associated GSL containing mainly C18–20 fatty acids (Ramstedt and Slotte, 2006). We also showed accumulation of the cytotoxic compound lyso-sulfatide in these mice (Blomqvist et al., 2011). Subsequently, another mouse model was created that overexpressed sulfatide on the *Arsa*$^{-/-}$ background (Ramakrishnan et al., 2007). The *Cst*-transgenic *Arsa*$^{-/-}$ mouse model shows the typical hallmarks of late infantile MLD and seems to be a more suitable model to study the pathomechanism of the disease, as well as the effects of therapeutic intervention.

3.4. Sulfatide in neurodegenerative disorders associated with demyelination

3.4.1. Alzheimer's disease

With few exceptions, dementia is an acquired syndrome of cognitive impairment. The most common condition causing dementia is Alzheimer's disease (AD), a progressive neurodegenerative disorder with characteristic extracellular amyloid-beta (Aβ), plaques and neurofibrillary tangles composed of hyperphosphorylated tau (Blennow et al., 2006). White matter changes, including demyelination and oligodendrocyte loss, are observed in patients with late-onset AD with concomitant cerebrovascular disease (Blennow et al., 1991; Benitez et al., 2014; Nasrabady et al., 2018). Substantial reductions in sulfatide levels have been found in AD brains (most severe in grey matter, with >90% reduction) compared with cognitively normal controls, and researchers have suggested these changes to be related to white matter pathology (Svennerholm and Gottfries, 1994; Han et al., 2002; Cheng et al., 2013; Gonzalez de San Roman et al., 2017). In line with these findings, (Han et al., 2002) showed CNS sulfatide depletion in patients with mild cognitive impairment due to AD. In an accompanying paper, these authors showed a corresponding reduction in the CSF sulfatide levels (Han et al., 2003b). However, we were not able to reproduce these findings in a cohort of patients selected by their pathological levels of AD biomarkers (CSF Aβ42, total and phosphorylated tau, cut-off values 90% sensitive and specific for AD) (Blomqvist et al., 2017) nor in a second cohort of patients with AD recruited in the Gothenburg MCI study, a monocentre study performed at the memory clinic at Sahlgrenska University Hospital (Wallin et al., 2016; Svensson et al., 2021). Possible explanations for our inability to obtain congruent biomarker results with previous studies in AD are discussed below.

Loss of CerS2 activity, responsible for the synthesis of very long-chain fatty acid forms of ceramide described in Section 3.1.1, was found in multiple brain regions of subjects with preclinical AD pathology, possible preceding neurofibrillary tangle pathology (Couttas et al., 2016). In line with this result, depletion of sulfatide and galactosylceramide could be observed in AD brain tissue. The authors postulated that aberrant galactolipid biosynthesis over time contributes to myelin deterioration, synaptic dysfunction, and neurological decline in AD.

3.4.1.1. Studies of AD pathology using animal models. White matter changes have also been observed in AD transgenic mouse models (Mitew et al., 2010; Schmued et al., 2013). Interestingly, in a recent study using imaging mass spectrometry and the tgArcSwe transgenic mouse model, the authors showed an association between Aβ plaques and myelin-specific sulfatide isoforms, namely C18:0, C22:0, C22:0-OH, C24:0 and C24:0-OH, which were depleted in the vicinity of the Aβ plaques (Kaya et al., 2018). The human *APOE* ε4 allele is the major genetic risk factor for AD and sulfatide depletion is linked to apoE lipoprotein metabolism in mouse models of AD (Cheng et al., 2010; Han, 2010). In line with these studies, sulfatide depletion, disruption of the myelin sheath and apoE deposition are localised to Aβ plaques in several brain regions of a familial AD mouse model (Kaya et al., 2020). The authors further highlighted the importance of lipid homeostasis in AD. Another protein involved in lipoprotein metabolism, low-density lipoprotein (LDL) receptor-related protein 1 (LRP1), has been shown to play a critical role in synaptic integrity. LRP1 levels are reduced in the brains of patients with AD and other animal and *in vitro* cell studies support a role for LRP1 in Aβ metabolism and clearance (Kang et al., 2000; Liu et al., 2017). LRP1 depletion in mice results in a global amelioration of lipid metabolism in the brain including decreased levels of sulfatide, possibly associated with neurodegeneration (Liu et al., 2010).

3.4.2. Vascular dementia

Vascular dementia (VaD) is associated with white matter lesions, including subcortical changes (leukoaraiosis) and demyelination. (Wallin et al., 1989) demonstrated a substantial reduction in myelin lipids, including sulfatide, in brains from patients with VaD as compared with healthy controls. A few years later, the same group reported increased CSF sulfatide concentration in patients with VaD compared with age-matched controls (Fredman et al., 1992). These findings are suggestive of demyelination. In addition, the proinflammatory cytokine tumor necrosis factor alpha, previously described to be involved in mediating myelin damage, correlated well with the CSF sulfatide concentration from patients with subcortical VaD (Tarkowski et al., 2003). Furthermore, there was a weak correlation between CSF sulfatide concentrations and white matter lesions in non-demented elderly individuals in the Leukoaraiosis And DISability (LADIS) study (Jonsson et al., 2010). However, in another patient cohort with leukoaraiosis, CSF sulfatide levels did not differ compared with age-matched controls (Tarvonen-Schroder et al., 1997). In a follow-up of the LADIS study, the authors showed that low levels of CSF sulfatide predicted the progression of white matter lesions (Jonsson et al., 2012).

We recently investigated CSF sulfatide concentrations in patients with subcortical small vessel type of dementia (SSVD) as well as patients with mixed dementia, combined AD and SSVD (Svensson et al., 2021). There were no significant differences in CSF sulfatide (total amount and isoform expression) in these patient groups compared with age-matched healthy controls. Furthermore, the CSF sulfatide concentration did not

correlate with the CSF NF-L concentration, further supporting that CSF sulfatide is a poor biomarker of demyelination in dementia disorders. However, we found a strong positive correlation between CSF sulfatide and the CSF/serum albumin ratio, which reflects blood-brain barrier (BBB) damage. Reduced BBB function may result in increased passage of sulfatide into the CNS but the isoform pattern of sulfatide was very similar in all individuals included in the study, suggesting a CNS origin of the sulfatide. Thus, further studies are needed to explore the mechanisms underlying the marked association between CSF total sulfatide level and CSF/serum albumin ratio.

3.4.3. HIV-1

Another condition where myelin degeneration in the CNS is commonly observed is HIV infection, especially in patients with HIV-associated dementia. *In vitro* binding studies have shown that surface glycoprotein gp120 on HIV-1 binds to surface glycolipids, including sulfatide and its precursor galcer, and sulfatide seems to play a role as a functional receptor (Cook et al., 1994; McAlarney et al., 1994). The CSF sulfatide concentration was elevated in asymptomatic HIV-infected patients compared with HIV negative controls, and in the same cohort of patients, there were positive titres of anti-sulfatide antibodies in the serum of HIV-infected patients with or without CNS involvement (Gisslen et al., 1999). No positive titres of anti-sulfatide antibodies could be detected in CSF. The authors interpreted the findings indicative of increased metabolic turnover of myelin during HIV-1 infection, but anti-sulfatide antibodies do not seem to be a major factor contributing to the CNS myelin damage in HIV-1 infection.

3.5. Sulfatide in diseases with white matter changes – miscellaneous

3.5.1. Human disorders

Sulfatide CSF levels have also been studied in other patient cohorts expressing white matter damage, such as normal pressure hydrocephalus (NPH) and subcortical arteriosclerotic encephalopathy (SAE) (Tullberg et al., 2000). The results showed that sulfatide levels differed between patients with NPH and SAE. However, the same research group could not replicate these results in another cohort of patients with SAE and idiopathic SAE (Agren-Wilsson et al., 2007). Furthermore, increased CSF sulfatide levels have been observed in children with white matter abnormalities of different origin (Kristjansdottir et al., 2001). In conditions with peripheral neuropathy, such as Guillain–Barré syndrome (GBS), autoimmune reactions, including anti-sulfatide antibodies, might be of relevance (Fredman et al., 1991; Fredman, 1998; Souayah et al., 2007; Schirmer et al., 2016). By using a lipidomic approach, (Peter et al., 2020) recently showed that the CSF sulfatide concentration increases in patient with GBS.

3.5.2. Animal studies

Recently, lipids – and in particular sphingolipids – have been suggested to contribute as mediators in the pathogenesis of addictive states, such as drug dependence and alcohol abuse, as reviewed by (Kalinichenko et al., 2018). The investigation of sulfatide involvement in chronic drug consumption is of specific interest because addictive states are known to cause neurodegeneration with structural impairment of myelin. Animal studies have shown downregulation of CST and thereby sulfatide isoforms in brains of rats receiving alcohol, a finding that perhaps reflects neurodegeneration (Yalcin et al., 2015; Yalcin et al., 2017). Administration of nicotine has been applied to examine the potential co-factor role of smoking for heavy drinkers, showing a larger effect on sulfatide isoforms in the brain compared with alcohol alone (Yalcin et al., 2015). Furthermore, this reduction in sulfatide isoforms correlated with white matter changes. These data suggest a contribution of sulfatide to the toxic effects of smoking and alcohol abuse. An earlier study also implicated the role of sulfatide in opiate and endorphin receptor mechanisms (Craves et al., 1980). Finally, neuropathy in obesity-induced diabetes in mice has been associated with early loss of myelin-associated lipids, including sulfatide, starting in the PNS and further progressing to CNS at advanced diabetic stages (Palavicini et al., 2020).

4. Sulfatide in CSF – a relevant biomarker for neurodegeneration?

The proper balance of sphingolipids is essential to sustain neuronal and glial function in the brain. This fact has led researchers to investigate possible correlations between sphingolipid disturbances and neurodegenerative disorders. It is now accepted that a widespread disruption of lipid composition occurs in diseases affecting the brain. Biofluids such as plasma, serum, urine and CSF are accessible sources of biomarkers. As CSF acts as a clearance mechanism for extracellular molecules in the CNS, this specific body fluid is commonly used as an indirect measure of brain metabolism. Thus, analyses of CSF lipids can potentially reflect the lipid disruption observed in the brain tissue of many neurodegenerative disorders. We summarise data from the literature on CSF sulfatide analysis in neurodegenerative disorders associated with dysmyelination/ demyelination, in an attempt to determine whether CSF sulfatide is a possible biomarker for neurodegeneration.

The broad definition of a biomarker is 'a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention' (Califf, 2018). A biomarker can be derived from biochemical, molecular, histologic, radiographic or physiologic characteristics. A diagnostic biomarker further detects or confirms the presence of a disease or identifies an individual with a subtype of the disease. The perfect biomarker should meet the following criteria: (1) discriminate between affected and non-affected probands; (2) reflect or be involved in the pathophysiology of the disease; (3) be specific for the disease; (4) change in parallel with the burden of the disease; (5) be readily quantifiable in accessible biological fluid or clinical samples; and (6) be economical, quick and consistent. Most clinically useful biomarkers do not meet all these criteria but ideally, they should.

4.1. CSF

Lumbar puncture was introduced by Dr. Heinrich Quincke in the 19th century; thus, for over a century, CSF has been studied for diagnostic and therapeutic purposes. CSF protects the brain inside the skull and plays an important role in the homeostasis and metabolism of the CNS. CSF is produced by the choroid plexuses, highly vascularised structures of epithelial cells in the brain ventricles, by using a combined process of diffusion, pinocytosis and active transfer, and is further absorbed in the arachnoid granulations (villi) or extracranial lymphatics (Khasawneh et al., 2018; Bothwell et al., 2019). CSF moves in a single outward direction from the ventricles, but multidirectional in the subarachnoid space. The classic view is that CSF flows due to the forces generated by cardiac pulsations and pulmonary respiration. Ciliary beating of ependymal cells lining the brain ventricles is also essential for normal CSF flow (Sawamoto et al., 2006). However, alternative pathways for CSF dynamics have been described that challenge the 'classical' description, as reviewed by (Bulat and Klarica, 2011; Khasawneh et al., 2018; Bothwell et al., 2019). CSF production in humans is about 430–530 ml per day; at any given moment, 150–160 ml circulates in the ventricular system (Khasawneh et al., 2018, Bothwell et al., 2019). Thus, CSF turns over at a rate of three to four times a day.

The BBB consists of the epithelial cells of the choroid plexus and these cells control the transfer of water and solutes into CSF (Brady and Tai, 2012). Fluid transport from the brain parenchyma into CSF across the BBB contributes to 10%–20% of the CSF volume. Because the remaining part is derived from plasma through filtration in the choroid plexus, there are similarities between CSF and plasma composition; however, CSF has a much lower protein content compared with plasma (0.3%–0.4% of the plasma protein concentration) and a higher fraction of CNS-derived molecules. In fact, CSF is mainly composed of water

(99%), with the remaining 1% comprising proteins, amino acids, lipids, ions, neurotransmitters and glucose (Brady and Tai, 2012; Khasawneh et al., 2018). Furthermore, from an analytical point of view, CSF is a challenging biological fluid due to its low lipid content (15 μg/ml in total, approximately 300-fold lower compared with plasma) (Irani, 2009). However, using increasingly sensitive techniques over the years, CNS-specific low-abundant molecules can now also be detected in CSF. At present, CSF is mainly used to diagnose infectious and inflammatory diseases of the CNS, including meningitis and encephalitis, and autoimmune disorders, such as MS and GB (Teunissen et al., 2018a). Common CSF biomarkers for clinical laboratory diagnostics are albumin (BBB function), oligoclonal bands IgG/IgM, β2-microglobulin (neuroinflammation, oligoclonal IgG bands serve as corner stones of MS diagnosis), AD biomarkers (total-tau, Aβ, phosphorylated tau), NF-L (large-calibre myelinated axons, neurodegeneration, inflammation), GFAP (activated astrocytes, traumatic head injury, glioma, intracranial haemorrhage), CXCL13 (neuroborreliosis), 14–3-3 and prion proteins (Creutzfeldt–Jakob disease), to give a few examples (Teunissen et al., 2018b).

4.2. CSF sulfatide in normal and pathogenic conditions

The question that remains to be answered – is sulfatide a reliable CSF biomarker for neurodegenerative disorders? The accumulated results so far do not support this hypothesis. As exemplified in Section 3.1, the results of individual studies with separate patient cohorts have been inconsistent. Even though several studies cited above showed increased CSF sulfatide concentration in patients with different kind of demyelination diseases, other studies could not reproduce these findings. Thus, two of the aforementioned central criteria of a biomarker – (1) discriminate between affected and non-affected probands and (3) be specific for the disease – are not fulfilled for CSF sulfatide based on the accumulated literature of patients with white matter lesions of different origin, including patients with MS as well as AD/SSVD/mixed dementia. The usage of mass spectrometry might, however, reveal changes at other levels when comparing specific sulfatide isoforms in CSF in different neurodegenerative disorders to those found in healthy individuals. In the case of MS, researchers have recently emphasised that the differences might involve sulfatide isoform distribution and not the total amount of sulfatide (Moyano et al., 2013; Novakova et al., 2018). Plausible explanations for the contradictory results reported in the literature are discussed in the following sections.

4.2.1. Methodological aspects

Thin-layer chromatography enzyme-linked immunosorbent assay (TLC-ELISA) was the first method with sufficient sensitivity to detect sulfatide in CSF (Davidsson et al., 1991). This technique has been used in many studies, including those described in previous sections (Wallin et al., 1989; Fredman et al., 1992; Tullberg et al., 2000; Molander-Melin et al., 2004; Haghighi et al., 2012; Jonsson et al., 2012). Liquid chromatography–tandem mass spectrometry (LC-MS/MS) is now the gold standard for CSF sulfatide quantification (Han et al., 2003b, Blomqvist et al., 2017, Novakova et al., 2018, Cao et al., 2020, Svensson et al., 2021). The introduction of mass spectrometry for lipid analysis has improved tremendously the sensitivity and enabled simultaneous analysis of lipid classes (lipidomic approach) as well as lipid molecular species (lipid isoforms). Is quantification based on TLC comparable to quantification based on LC-MS/MS? The CSF sulfatide concentrations obtained in healthy individuals in 2017 are surprisingly close to the levels observed with TLC-ELISA, published in 1991: 71 ± 18 nmol/l (mean ± standard deviation [SD]), ages 28–68 years *versus* 100 ± 46 nmol/l (mean ± SD), ages 25–80 years, respectively (Davidsson et al., 1991; Blomqvist et al., 2017). This similarity suggests that the methods are comparable when analysing total CSF sulfatide levels. However, the reproducibility of the TLC-ELISA methodology is rather poor over time and measuring CSF sulfatide levels in a disease state requires simultaneous analysis of the entire cohort, including controls. This is hard to achieve due to automation difficulties. Comparing different LC-MS/MS publications, the extraction method, interferences and reproducibility of the LC-MS/MS methods need to be evaluated carefully. Internal standards, preferably labelled with a stable isotope, are required; however, in the case of sulfatide quantification, sulfatide with C17:0 fatty acid is commonly used for all isoforms.

4.2.2. Patient material selection

A plausible explanation for the contradictory results reported in the literature is the heterogeneity in what inclusion criteria were used when selecting the patients for study entry. For some of the complex polygenetic disorders exemplified above, an even more meticulous subgrouping of patients might enable a better comparison among studies. The selection of patient material in the earlier studies (Wallin et al., 1989, Fredman et al., 1992, Tullberg et al., 2000, Molander-Melin et al., 2004, Haghighi et al., 2012, Jonsson et al., 2012) was perhaps not optimal based on the current pathophysiological knowledge, which has led to improved patient grouping criteria. The size of the patient material might be another confounding factor (some studies are very small, which results in a greater risk of chance findings and non-generalisable results). Another important aspect in studying biomarkers, in for example MS, is the time of sampling. Because demyelination is not a constant process in MS, but rather a relapsing-remitting feature, the precise definition of the MS stage at sampling is important for optimal detection of biomarkers during demyelination and subsequently comparison with previous findings.

Furthermore, recent advances creating ultrasensitive immunological and mass spectrometry assays has enabled quantification of CNS biomarkers in plasma, where specifically blood NF-L has been shown to reflect the pathophysiology of the CNS with negligible peripheral interference (Ashton et al., 2021). (Moyano et al., 2013) showed an increase in four major sulfatide isoforms in plasma of patients with relapsing-remitting MS, possibly reflecting demyelination. Because plasma (or serum) is easier to collect than CSF from a lumbar puncture, the possibility of using this matrix to explore sulfatide as a biomarker for demyelination should not be neglected. Indeed, galcer has been suggested to as a reliable serum index of demyelination in MS (Lubetzki et al., 1989), however, we were not able to show a similar increase in CSF galcer when comparing patients with MS (a mixture of patients with relapsing-remitting MS or progressive MS) with healthy controls (Haghighi et al., 2012).

4.2.3. CSF sulfatide in MLD

In the monogenic disorder MLD with impaired degradation of sulfatide caused by deficiency of the lysosomal enzyme ASA, patients develop severe dementia. Sulfatide is markedly increased in different body fluids, such as urine, plasma and CSF (Kaye et al., 1992; Malm et al., 1996; Dali et al., 2015; Mirzaian et al., 2015). This is most likely due to the fact that MLD is a monogenic disease with impaired lysosomal ASA activity resulting in a massive accumulation and subsequent exocytosis of sulfatide in an attempt to reduce the disease burden (Klein et al., 2005). The CSF sulfatide concentration might be correlated more specifically with the severity of this devastating disorder. In one study the authors showed a correlation between the CSF sulfatide concentration and the severity of neuropathy in patients with MLD (Dali et al., 2015). In addition, studying the CSF sulfatide isoform pattern of this specific disorder might give more information regarding the pathophysiology which is not fully understood. Thus, CSF sulfatide is a relevant biomarker for diagnosis in the primary storage disorder MLD with severe demyelination and might also function as a biomarker following disease severity and therapeutic intervention. Whether disease severity in MLD also correlates with plasma and/or urine sulfatide concentrations remains to be elucidated.

4.3. Aspects of CSF sulfatide-associated structures – extracellular membrane vesicles

Are there any explanations as to why myelin damage in general cannot be reflected by sulfatide release into CSF? There are two main points regarding this question.

First, we still have limited knowledge about the precise origin and transport of sulfatide in CSF. Most likely, sulfatide is associated with extracellular membrane vesicles (EVs) in biological fluids, suitable for transport of hydrophobic molecules. EVs are a general term for membrane structures outside a cell and are based on their origin divided into exosomes (endosomal compartment), microvesicles or ectosomes (cell membrane budding) and apoptotic bodies (Raposo and Stoorvogel, 2013; Urbanelli et al., 2016). EVs are released by most cell types and are important for cellular communication by transferring proteins, lipids and RNA among cells. EVs differ in their size, morphology and content; hence, they can be separated by different techniques such as ultracentrifugation, immunoadsorption and mass spectrometry. EVs contain lipids as well as proteins, and several studies have revealed the enrichment of cholesterol and sphingolipids mimicking lipid rafts (Record et al., 2014). Because EVs may traffic from specific tissue sites to different body fluids that are easily accessible, these vesicles might be suitable for diagnostic purposes. In cell culture systems, neurons and glia have been shown to secrete EVs (Record et al., 2014). Moreover, (Harrington et al., 2009) found that membranous nanostructures (30–200 nm) are naturally occurring in CSF of both healthy individuals and patients with different brain diseases. The finding of EVs in CSF, most likely mirroring the releasing cell types of the CNS, has further supported the idea that CSF mimic specific pathophysiological conditions in the brain.

EVs in body fluids such as CSF most likely maintain the biological features of the releasing cells and thereby include potential disease markers. In fact, several proteins involved in CNS disorders released from cells are associated with EVs in CSF (Colombo et al., 2012; Record et al., 2014). Could isolation and thereby the concentration of endosomal EVs from CSF reveal differences in biomarker content that is not observed in 'normal' CSF sampling? Could different isoforms of sulfatide associate with different kinds of EVs reflecting cell origin? Variations in CSF EVs will probably be of interest in disease studies. However, EVs require enrichment because they are diluted by more abundant fluid components. More accurate and standardised isolation and purification methods are needed before implementation of such biomarkers in a laboratory diagnostic setting. It should also be mentioned that shedding and occurrence of EVs in CSF might not in general represent the total membrane content in the brain, but rather the accessibility to CSF.

Second, looking further into EV structures, could metabolically active ASA be present and thereby affect the sulfatide composition during CSF circulation? Exosomes are autonomous units of active enzymes related to lipid metabolism (Record et al., 2014). For example, exosomes released from rat leukaemia cells have been described to contain active phospholipases (Subra et al., 2010). In another study, the authors analysed CSF nanoparticles by comparing patients with AD with normal individuals and showed the presence of sphingomyelin, ceramide and dihydroceramide in nanoparticles as well as supernatant fluid (representative of interstitial metabolism) with different distribution (Fonteh et al., 2015). These results further illustrate the complexity and compartmentalisation of sphingolipid metabolism.

4.4. Aspects of sulfatide transport and degradation mechanisms

Another question to be addressed is whether sulfatide associates with other cellular structures and/or transporters not released into CSF in pathological conditions affecting myelin. ApoE is mainly known for its involvement in lipid metabolism by interaction with the LDL receptor and LRP1 protein. Human apoE is heavily glycosylated (*O*-linked and sialylated) but the extent differs depending on the tissue/cell type origin, possibly linked to its interaction with lipids (Kockx et al., 2018). In the CNS, apoE is the main apolipoprotein involved in lipid transport; it is predominantly secreted by astrocytes and to a lesser extent by neurons and glia (Kockx et al., 2018). The transport of cholesterol and phospholipids from astrocytes to neurons is crucial in maintaining brain integrity and homeostasis. ApoE expression is increased by injury in astrocytes, microglia and neurons in transgenic mice (Van Dooren et al., 2006) and an apoE knock-out mice show the importance of apoE in restoring neuronal function after injury (Xu et al., 2006). ApoE of astrocyte origin is extensively glycosylated, which is reflected in the CSF (Yamauchi et al., 1999). Glycosylation of apoE does not seem to play an essential role for trafficking, but, it may act as a 'fine tuner' of apoE delivery in response to changes in extracellular lipids. Could individuals with different apoE glycosylation have different abilities of delivering apoE bound sulfatide in CSF? Furthermore, in an inflammatory environment, could substantial leakage of myelin-associated sulfatide be captured by apoE to be used for delivery of self-antigen contributing to inflammation in a CD1-dependent manner? Exogenous pathways for lipid antigen presentation mediated by apoE through receptor-mediated endocytosis have been shown by using apoE-deficient mice and has been suggested to have important implication for microbial immunity, autoimmunity and atherosclerosis (van den Elzen et al., 2005). ApoE released from astrocytes has also been shown to capture sulfatide from the myelin surface and enter neurons by the LDL receptor and LRP1 in transgenic mouse models (Cheng et al., 2010). In addition, the apoE4 isoform contains more sulfatide compared with the apoE3 isoform in human CSF (Han et al., 2003a); thus, the genetic distribution of individual apoE isoforms might influence the release of sulfatide into CSF.

The lysosomal enzyme ceramidase is bifunctional (Ferraz et al., 2016). In addition to the main function of degrading ceramide into fatty acid and sphingosine, the enzyme also deacetylates glycosphingolipids into their corresponding water-soluble lyso-compounds. In MLD and the corresponding *Arsa*$^{-/-}$ mouse model, lyso-sulfatide is accumulated to presumably cytotoxic levels (Toda et al., 1990; Blomqvist et al., 2011). This alternative pathway might explain the absence of sulfatide release into CSF during neurodegenerative conditions with myelin damage. To the best of our knowledge, CSF lyso-sulfatide in neurodegenerative conditions has not been extensively studied.

5. Conclusion and perspectives

Body fluid biomarkers are of importance for clinical diagnostics as well as for patient stratification in clinical trials and treatment monitoring. Excellent CSF biomarkers exist – as exemplified in this review – however, there is still a need for new CSF biomarkers to improve diagnosis (Teunissen et al., 2018a, 2018b). Implementation of new CSF biomarkers is hampered by limited knowledge of the cause of neurological disorders (especially during subclinical stages), limited success in transforming biomarker findings from screening platforms into high-throughput assays adapted for the clinical laboratory as well incorporation of CSF diagnostics into clinical practice.

CSF biomarkers for neurodegeneration involving white matter pathology have been studied extensively. One biomarker that has been found to reflect neurodegeneration and inflammation reliably is NF-L (axonal dysfunction). This ability has been shown in patients with MS (Thompson et al., 2018) as well as in other diseases such as amyotrophic lateral sclerosis. However, NF-L is not disease specific and could for some diseases only be applied in a correct diagnostic setting. Thus, more disease specific CSF biomarkers are needed for white matter disorders.

Sulfatide is not only an abundant structural component of myelin but also involved in the maintenance of the myelin sheath by establishing connections with other molecules such as gangliosides and myelin specific proteins and/or by signal modulation. Changes in sulfatide metabolism can lead to membrane rearrangements that are possibly involved in various neurological disorders. Thus, studying changes in sulfatide composition of the myelin sheath in demyelinating disorders might

provide important clues to disease pathology and thereby therapeutic options. However, analysis of CSF sulfatide does not seem to reflect consistently the lipid disruption observed in many of the demyelinating disorders, apart from MLD due to the genetically determined accumulation of non-metabolised sulfatide. The previous suggestion of CSF sulfatide being a marker of myelin damage has thereby been challenged by more recent studies using more sophisticated laboratory techniques as well as improved sample selection criteria due to increased knowledge of disease pathology.

CRediT authorship contribution statement

MB contributed to the conception and design as well as drafting of the manuscript. JEM contributed to conception and design as well as review and editing. HZ and KB contributed to review and editing. All authors have approved the final version of the manuscript.

Declaration of competing interest

HZ has served on scientific advisory boards for Denali, Roche Diagnostics, Wave, Samumed, Siemens Healthineers, Pinteon Therapeutics, Nervgen and CogRx; has given lectures in symposia sponsored by Fujirebio, Alzecure and Biogen; and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work).

KB has served as a consultant, on advisory boards, or on data monitoring committees for Abcam, Axon, Biogen, JOMDD/Shimadzu, Julius Clinical, Lilly, MagQu, Novartis, Roche Diagnostics and Siemens Healthineers, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program, all unrelated to the work presented in this paper.

MB and JEM have given lectures in symposia and expert meetings sponsored by Sanofi Genzyme, Takeda Pharmaceutical Company, Biomarin Pharmaceutical and Actelion.

Acknowledgements

HZ is a Wallenberg Scholar supported by grants from the Swedish Research Council (#2018-02532); the European Research Council (#681712); the Swedish State Support for Clinical Research (#ALFGBG-720931); the Alzheimer Drug Discovery Foundation (ADDF), USA (#201809-2016862); the AD Strategic Fund and the Alzheimer's Association (#ADSF-21-831376-C, #ADSF-21-831381-C and #ADSF-21-831377-C); the Olav Thon Foundation; the Erling-Persson Family Foundation; Stiftelsen för Gamla Tjänarinnor; Hjärnfonden, Sweden (#FO2019-0228); the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 860197 (MIRIADE); and the UK Dementia Research Institute at UCL.

KB is supported by the Swedish Research Council (#2017-00915); the Alzheimer Drug Discovery Foundation (ADDF), USA (#RDAPB-201809-2016615); the Swedish Alzheimer Foundation (#AF-742881); Hjärnfonden, Sweden (#FO2017-0243); the Swedish state under the agreement between the Swedish government and the County Councils, the ALF-agreement (#ALFGBG-715986); the European Union Joint Program for Neurodegenerative Disorders (JPND2019-466-236); and the National Institutes of Health (NIH), USA, (grant #1R01AG068398-01).

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