Review Article

Unraveling the diagnostic enigma: laboratory diagnosis of sphingolipid activator protein deficiencies

Libin Yuan[1,2]^

[1]Department of Paediatric Laboratory Medicine, The Hospital for Sick Children, Toronto, Ontario, Canada; [2]Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada

Correspondence to: Libin Yuan, PhD, FACMG, FCCMG. Department of Paediatric Laboratory Medicine, The Hospital for Sick Children, Roy C. Hill Wing, 555 University Ave, Toronto, Ontario M5G 1X8, Canada; Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada. Email: libin.yuan@sickkids.ca.

Abstract: Sphingolipids are degraded by specific lysosomal acid hydrolases. Efficient degradation of glycosphingolipids with short carbohydrate chains by those enzymes relies on the assistance of sphingolipid activator proteins (SAPs), including GM_2 activator and four saposins (Saposin A, B, C, and D). Deficiencies in these enzymes or SAPs lead to the accumulation of specific sphingolipids. While enzyme activity assays are diagnostic for sphingolipidoses caused by enzyme deficiencies, the laboratory diagnosis of SAP deficiencies presents challenges due to the complexity of directly assessing these proteins' functions. Given the exceptional rarity of SAP deficiencies and the clinical similarities to the corresponding enzyme deficiencies, there is an increased risk of oversight and misdiagnosis. Biomarkers such as GM_2 ganglioside (34:1), sulfatide, glucosylsphingosine (lyso-Gb1), globotriaosylsphingosine (lyso-Gb3), and psychosine are important in addressing this diagnostic complexity, with molecular genetic analysis serving as the essential confirmatory test. In GM_2 activator, saposin B, and saposin C deficiencies, enzyme activity assays using regular synthetic substrates typically demonstrate normal enzyme activities. The discrepancy between an abnormal biomarker level and a normal enzyme activity may strongly suggest an activator defect. In cases of saposin A and prosaposin deficiencies, abnormal enzyme activities may be observed even when using regular synthetic substrates, potentially leading to the misdiagnosis of corresponding enzyme deficiencies. In such scenarios, if molecular genetic analyses of the genes encoding those enzymes return negative results, it may be necessary to conduct follow-up molecular testing targeting the *PSAP* gene for diagnostic purposes. This review paper delves into biochemical laboratory techniques to illuminate the intricacies involved in diagnosing SAP deficiencies. The aim is to streamline the laboratory diagnostic process and reduce diagnostic uncertainties.

Keywords: Lysosomal storage disorder; sphingolipid activator protein (SAP); GM_2 activator; prosaposin; saposin

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Introduction

Sphingolipids, named after the mythical creature Sphinx due to their initially mysterious function akin to the enigmatic riddle posed by Sphinx, are essential components of the eukaryotic cell membrane (1). They are primarily localized in the nervous system and play a critical role in various cellular processes, such as regulating membrane dynamics, modulating cell signaling, and mediating cell adhesion and recognition (2). Based on the structure, sphingolipids can be categorized into sphingoid bases (and their simple derivatives), ceramides, and complex sphingolipids (3). Glycosphingolipids, a common type of

^ ORCID: 0000-0002-2108-6769.

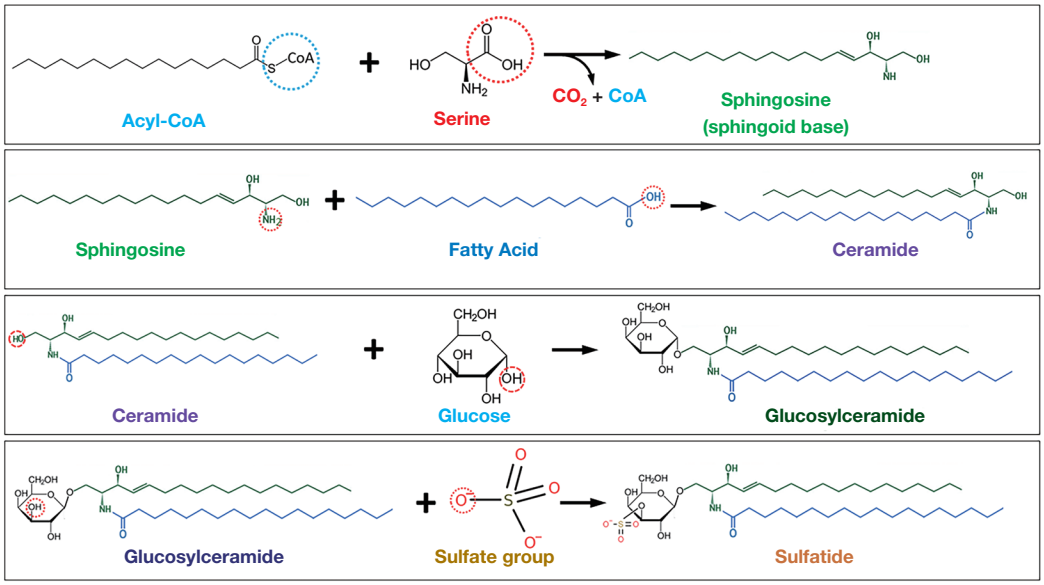

Figure 1 The synthesis and structures of representative sphingolipids. Sphingosine represents one of the most common sphingoid bases, synthesized by attaching an acyl group to serine. Ceramide is formed through the linkage of a fatty acid to the amine group of a sphingosine molecule. Glucosylceramide is a neutral glycolipid, with a glucose molecule attached to the head of ceramide. Sulfatide represents an acidic glycosphingolipid, which is a galactosylceramide molecule tagged with a sulfate group.

complex sphingolipids, are characterized by the presence of carbohydrate groups in their molecules (4). Neutral glycosphingolipids, such as glucosylceramides (GlcCer) and galactosylceramides (GalCer), contain an uncharged carbohydrate group. Acidic glycosphingolipids, represented by sulfatides and gangliosides, comprise negatively charged groups such as sulfate groups or sialic acid groups (5) (*Figure 1*).

Sphingolipids are transported to lysosomes through the membrane flow, where they are degraded into the basic units (6). The degradation of sphingolipids is accomplished through a stepwise process involving various specific lysosomal acid hydrolases (7,8) (*Figure 2*). These enzymes can easily access glycosphingolipids with long-chain carbohydrate groups and efficiently break them down into smaller molecules. However, these enzymes cannot access glycosphingolipids with short oligosaccharide chains and require activator proteins to facilitate the degradation process (9). To date, five known sphingolipid activator proteins (SAPs) have been identified, including GM_2 activator and four saposins (Saposin A, B, C, and D). SAPs exhibit membrane perturbing capabilities, act as physiological detergents, and/or engage in direct interactions with enzymes. These properties are indispensable for accessing and breaking down glycosphingolipids by lysosomal enzymes (10). Defects either in enzymes or SAPs lead to the inability to degrade certain sphingolipids. Consequently, these sphingolipids accumulate within lysosomes, causing sphingolipidoses, a subgroup of lysosomal storage disorders (11,12) (*Figure 3*).

The diagnosis of sphingolipidosis due to an enzyme deficiency can be accomplished by enzyme activity measurement (13). However, the diagnosis of SAP deficiencies remains challenging due to the complexities of SAP functional study in a clinical laboratory. Moreover, these disorders are exceptionally rare, increasing the likelihood of oversight and misdiagnosis. Because of the clinical similarities between SAP defects and corresponding enzyme deficiencies (14,15), physicians often encounter confusion caused by the discrepancy between clinical presentations and normal enzyme activities. This review paper focuses on laboratory methodologies and aims to provide insight into the enigmatic aspects surrounding the diagnosis of SAP deficiencies. By unraveling the complexities involved, it is hoped to streamline the laboratory diagnostic process, reducing diagnostic odysseys, and enhancing accuracy in identifying these defects.

GM_2 activator deficiency

GM_2 activator (GM2A or GM2AP), encoded by the

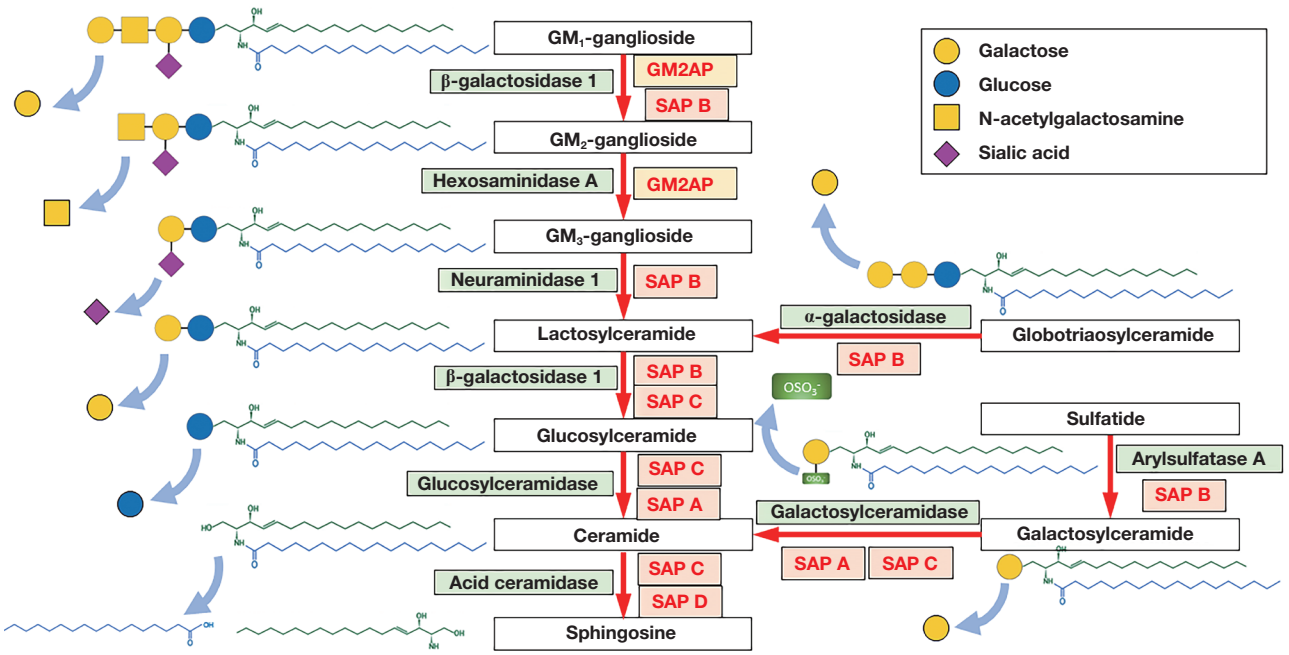

Figure 2 Representative metabolic pathway of sphingolipid degradation. This illustrative metabolic pathway highlights the stepwise degradation of sphingolipids. At each step, a monosaccharide or a sulfate group is sequentially removed from a sphingolipid molecule by a specific enzyme, ultimately resulting in the formation of ceramide. Ceramide is then further degraded by eliminating a fatty acid group to produce sphingosine. Each degradation step also relies on sphingolipid activator proteins. Among these activator proteins, saposin B and C exhibit broad specificity, while GM_2 activator, saposin A, and saposin D demonstrate narrower specificity. SAP, sphingolipid activator protein.

gene *GM2A*, is a glycoprotein with a molecular weight of 17.6 kDa (16,17). GM2AP functions as the activator protein mainly for the degradation of GM_2 gangliosides by hexosaminidase A (18). Its molecule possesses a hydrophobic cavity capable of harboring the ceramide moiety of GM_2 gangliosides, which plays a vital role in delivering GM_2 to hexosaminidase A (19). Any defect in hexosaminidase A or GM2AP results in the accumulation of GM_2 gangliosides in lysosomes, causing GM_2 gangliosidosis (19). Patients with GM_2 gangliosidosis commonly present with impaired development of the central nervous system, exaggerated startle response, hypotonia, and cherry red spots (19). The majority of GM_2 gangliosidosis cases are caused by hexosaminidase A deficiency, also known as Tay-Sachs disease (OMIM 272800), or combined hexosaminidase A and B deficiency, referred to as Sandhoff disease (OMIM 606873) (20). The diagnosis of Tay-Sachs disease or Sandhoff disease is established via enzyme assay, which reveals a significantly reduced activity (21). Sandhoff *et al.* observed an exceptional case of GM_2 gangliosidosis with clinical features resembling those found in Tay-Sachs disease patients, yet hexosaminidases appeared to be normal when using a synthetic substrate in the enzyme assay (22,23). This exceptional case was subsequently confirmed as the first reported case of GM_2 activator deficiency, also referred to as the AB variant of GM_2 gangliosidosis (OMIM 272750) (24). Given that synthetic substrates are usually small molecules readily accessible and digestible by hexosaminidases, thus not requiring GM2AP, such substrates are unsuitable for assessing GM_2 activator deficiency.

Functional study

O'Brien *et al.* demonstrated a significantly reduced hexosaminidase activity in fibroblasts from a patient with GM_2 activator deficiency using natural GM_2 ganglioside extracted from brain tissues as the substrate (25). It was evident that, in the absence of a functional GM_2 activator, the natural GM_2 gangliosides could not be degraded by the enzyme. Therefore, it is feasible to distinguish GM_2 activator deficiency from other types of GM_2 gangliosidoses by running parallel enzyme activity testing using synthetic

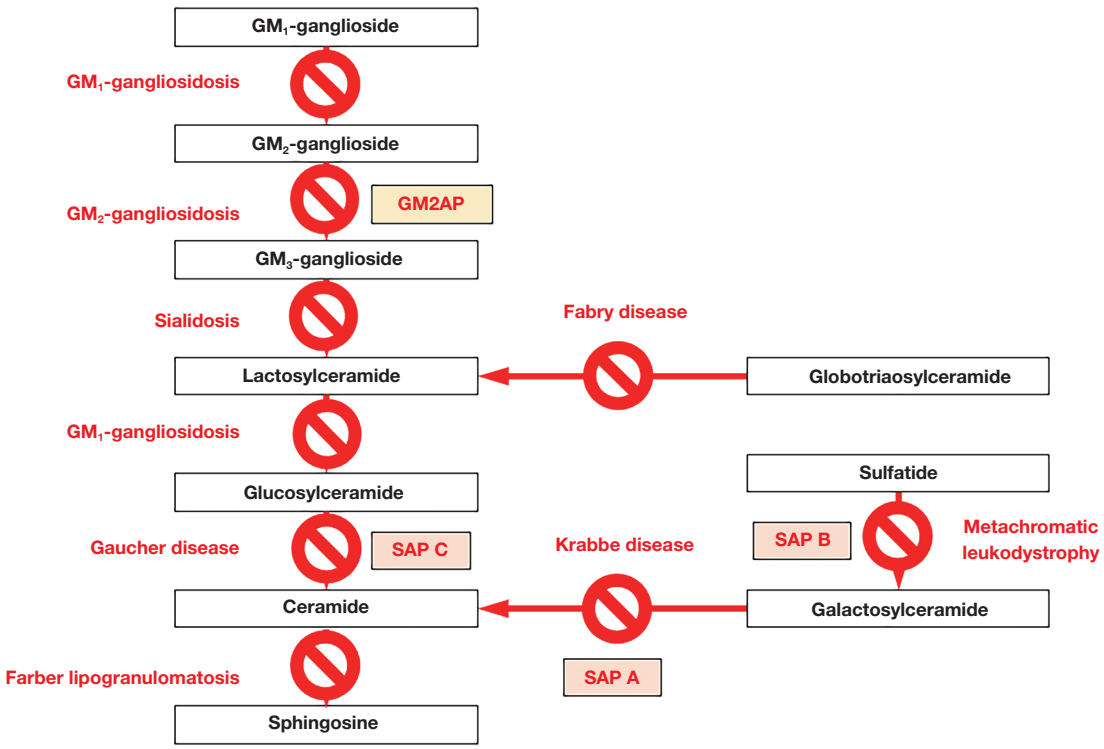

Figure 3 Representative disorders stemming from blockages in sphingolipid degradation pathway. Blockages in the degradation pathway can result from deficiencies in either enzymes or sphingolipid activator proteins, leading to the buildup of specific sphingolipids and respective disorders. Patients with GM_2 activator, saposin A, saposin B, and saposin C deficiencies typically present with GM_2 gangliosidosis, Krabbe disease, metachromatic leukodystrophy, and Gaucher disease, respectively. SAP, sphingolipid activator protein.

and natural substrates respectively. In cases of GM_2 activator deficiency, an abnormal activity result is obtained only when employing a natural substrate, while other types of GM_2 gangliosidoses present abnormal results in both tests using synthetic and natural substrates. It is worth noting that, while the presence of detergent such as sodium taurocholate is crucial for achieving optimal enzyme activity (25,26), detergent quantity should be carefully validated since an excessive amount can compensate for the defect in GM2AP and result in false-negative results for GM_2 activator deficiency. Tropak *et al.* established a fluorescence enzyme activity assay using a synthetic fluorescent GM_2 analog that was incorporated into liposomes to mimic the intra-lysosomal membrane (27). By utilizing the modified liposomes along with recombinant GM2AP, they provided an alternative strategy for distinguishing GM_2 activator deficiency from other GM_2 gangliosidoses. That is, in GM_2 activator deficiency, normal enzyme activity was observed in the presence of recombinant GM2AP, but abnormal activity was obtained when recombinant GM2AP was absent. In contrast, other GM_2 gangliosidoses exhibited reduced enzyme activity regardless of the presence or absence of recombinant GM2AP (27). Several other techniques can be used to quantitively measure the expression of GM2AP, such as Western blotting, enzyme-linked immunosorbent assay (ELISA), screen-printed carbon electrode (SPCE), and fluorescence-resonance energy transfer (FRET) assay (28-30). Nonetheless, these techniques are solely suitable for situations where mutations in the *GM2A* gene result in decreased expression or absence of GM2AP, or critical alterations in protein antigenicity.

Given the complex nature of GM2AP functional studies, the measurement of biomarkers becomes essential during the diagnostic journey for GM_2 activator deficiency. Abnormal levels of specific sphingolipid species along with typical clinical presentations in the setting of normal enzyme activity may strongly suggest an activator defect.

GM_2 gangliosides

Initially, GM_2 gangliosides were assessed using thin-layer chromatography (TLC), gas-liquid chromatography (GLC) and high-performance liquid chromatography (HPLC) (31-33). Tsuji *et al.* also established an ELISA method using an anti-GM2 monoclonal antibody to quantify GM_2 in fibroblasts (34). The application of mass spectrometry (MS) for GM_2 measurement offers an advantage due to its exceptional sensitivity. Tsui *et al.* developed a tandem mass spectrometry (MS/MS) strategy for the quantitative profiling of gangliosides in tissue lipid extracts, including GM_2 (35). They employed an aqueous partition method for ganglioside enrichment and proposed a three-step MS analysis strategy, including precursor ion scan of 290 m/z, product ion spectrum analysis, and ceramide moiety composition determination (35). Subsequently, a simple and accurate isotope dilution method was also established to quantify GM_2 human CSF samples with a linear range of 10–200 ng/mL (36). However, since GM_2 gangliosides are integral components of cell membranes and are particularly abundant in brain tissue, the quantitative measurement of GM_2 was mainly limited to cell or tissue extracts or CSF, significantly diminishing its clinical applicability (37,38). While an ultra-performance liquid chromatography (UPLC)-MS/MS method and an HPLC-MS/MS method were used to assess GM_2 concentrations in the plasma of GM_2 gangliosidosis patients, those studies suggested that GM_2 species in plasma have limited diagnostic value (39,40). Blondel *et al.* validated an LC-MS/MS method to measure multiple plasma biomarkers following an easy sample preparation step (protein precipitation) (41). They discovered that GM_2 (34:1) and the ratio of GM_2 (34:1) to GM_3 (34:1) were highly effective markers for distinguishing GM_2 gangliosidoses from normal controls with 100% sensitivity and 100% specificity (41). Those markers were also able to differentiate GM_2 gangliosidoses from other lysosomal storage disorders that may have secondary accumulation of gangliosides, including Niemann–Pick disease types A/B and C, GM_1-gangliosidosis, Fabry disease, Gaucher disease and Krabbe disease (41). Remarkably, they also observed elevated levels of GM_2 (34:1) in a patient with GM2 activator deficiency (41). Therefore, GM_2 (34:1) potentially can be used to streamline the diagnosis of GM_2 activator deficiency.

Other biomarkers

Welford *et al.* conducted an investigation into various biomarkers in plasma for gangliosidoses, including lysoGM$_2$ and lysoGM$_1$, neurofilament light chain (NF-L), and glial fibrillary acidic protein (GFAP) (42). LysoGM$_2$ and lysoGM$_1$ are deacylated derivatives of GM_2 and GM_1 gangliosides, respectively (43). NF-L is a neuron cytoskeletal protein and serves as a blood biomarker for neuronal damage (44). GFAP, on the other hand, is an astrocyte cytoskeletal filament as well as a biomarker for reactive astrocytosis (45). Using LC-MS/MS, Welford *et al.* observed elevated levels of lysoGM$_2$ and lysoGM$_1$ in almost all GM_2 gangliosidosis patients, as well as in those with GM_1 gangliosidosis (42). Additionally, through immunoassay, they also detected elevated NF-L and GFAP in those patients (42). However, although these biomarkers could be used for treatment monitoring, they are not sufficiently specific for the diagnosis of GM_2 gangliosidosis (42). GM_2 activator deficiency was not investigated in this study. With a multiplex LC-MS/MS, Pettazzoni *et al.* quantified several lysosphingolipids and found lysoGM$_2$ was elevated in 10 out of 13 patients of Tay-Sachs and Sandhoff diseases but not detected in other diseases (46). Yet, lysoGM$_2$ was reported to be normal in the plasma of a patient with a GM2 activator deficiency (47), indicating a limited clinical utility of lysoGM$_2$.

Molecular genetic analysis of the *GM2A* gene is an indispensable step in ultimately diagnosing GM_2 activator deficiency. To date, 26 cases of GM_2 activator deficiency have been reported, ascribed to a range of identified genetic variants including missense, nonsense, frameshift mutations, and deletions (24,48-66).

Saposin A deficiency

Saposins are four small non-enzymatic proteins localized within lysosomes, namely saposin A, B, C, and D (67). All four saposins derive from a common precursor protein, referred to as prosaposin, which is encoded by the *PASP* gene (67). Prosaposin molecule contains four distinct functional domains (68). Upon transport to lysosomes, prosaposin undergoes hydrolysis, releasing these domains as individual saposin proteins (69). Saposin A functions as an activator protein for galactosylceramidase, facilitating the breakdown of GalCer to ceramides (70). Saposin A is also involved in the degradation of GlcCer by β-glucosidase; thus, its function partially overlaps with that of saposin C, although the role of saposin A in the degradation of GlcCer is to a lesser extent (70). The inability to degrade GalCer can result in Krabbe disease, which is typically caused by a deficiency in galactosylceramidase (OMIM 245200) (71).

Saposin A deficiency (OMIM 611722) was first reported by Spiegel *et al.*, who described a case of a six-month-old girl with atypical Krabbe disease characterized by progressive encephalopathy and abnormal myelination (72). The significantly decreased galactosylceramidase activity in the leukocytes of this girl, which was around 1% of the average of the normal range, initially led to a misdiagnosis of Krabbe disease due to galactosylceramidase deficiency (72). Nevertheless, they observed that the galactosylceramidase activity in fibroblasts was normalized in the presence of detergents, which suggested an activator deficiency (72). Saposin A deficiency was subsequently confirmed by identifying a homozygous mutation within the saposin A domain of the *PSAP* gene (72). Although the authors stated that the unexpected decrease in galactosylceramidase activity could be attributed to compromised sample integrity (72), the significantly reduced enzyme activity does not seem to be solely due to the non-fresh sample, as non-fresh samples typically exhibit mild to moderate reductions. In the second documented case of saposin A deficiency, Kose *et al.* also noticed that galactosylceramidase activities in both dried blood spots (DBS) and leukocytes were decreased, although the activity in leukocytes was above the affected range for typical Krabbe disease (73). In the third case of saposin A deficiency reported by Calderwood *et al.*, galactosylceramidase enzyme activity in leukocytes was also slightly below the normal range but above the affected range (74). Those reports suggest that saposin A possibly plays a role in stabilizing the enzyme or increasing the maximal velocity of the enzyme (70). While galactosylceramidase activity in a freshly collected specimen might be less affected by saposin A defect, enzyme activity in a non-fresh specimen might be considerably compromised, significantly increasing the likelihood of being misdiagnosed as galactosylceramidase deficiency. While directly assessing saposin A functionality is currently not available in a clinical laboratory setting, quantification of saposin A amount in plasma can be achieved by time-delayed fluorescence immunoassays (75) or by immunoblotting (70). Nevertheless, these methods are only appropriate in the scenarios where genetic variants cause reduced or entirely absent protein expression or critical antigenicity alterations.

Due to the malfunction of saposin A, the impairment in the degradation of GalCer in skin fibroblasts was observed (72). Therefore, a prominent elevation of GalCer in fibroblast extractions was revealed using TLC, along with the elevations of GlcCer, lactosylceramides, and ceramides to a lesser extent (73). Considering the overlapping functionalities of saposin A and C but the relatively lesser role of saposin A in GlcCer degradation (70), GlcCer may lack distinctiveness as a biomarker. Moreover, an elevation of ceramides in saposin A deficiency became an enigmatic riddle, since as a product of GalCer and GlcCer degradation its elevation in saposin A deficiency is not anticipated. Unfortunately, this point was not discussed in the original report. Hence, more investigations were conducted on GalCer and its related metabolites as appropriate biomarkers for Krabbe disease, including saposin A deficiency.

GalCer

It was reported that GalCer primarily accumulated in localized globoid cells in the brain of Krabbe disease patients, but its total amount was generally low (76). While a slight elevation was detected in the kidney, no elevations were observed in the urine, CSF, and skin fibroblasts of Krabbe disease patients (77-79). To date, there have been no studies on GalCer levels in the blood of Krabbe disease patients that have yielded significant findings. Dawson reported a notable increase in the liver tissue, suggesting liver biopsy could be potentially valuable for diagnostic purposes (80). However, the invasive nature of biopsies has constrained the applicability of GalCer as a biomarker.

Psychosine

Psychosine, also known as galactosylsphingosine, is a cytotoxic byproduct resulting from GalCer deacylation and accumulates in the brain of Krabbe disease patients (81). The elevation of psychosine in the DBS from a saposin A deficiency patient was also reported (74). Several techniques were employed to measure psychosine levels in human and animal tissues, including TLC, HPLC, MS, and NMR spectroscopy (82-85). LC-MS and LC-MS/MS methods were also developed to quantify psychosine levels in mouse CSF and serum (86,87). Chuang *et al.* utilized HPLC-MS/MS to quantitatively detect psychosine in DBS from newborns and observed elevated psychosine levels in infantile patients with Krabbe disease (88). Subsequently, Turgeon *et al.* further validated this method and affirmed the significant potential of psychosine in DBS as a newborn screening biomarker (89). The role of psychosine in newborn screening for Krabbe disease was further confirmed through the analysis of longitudinal data

and improved LC-MS/MS methods (90-92). LC-MS/MS methods for simultaneously quantifying psychosine and glucosylsphingosine (lyso-Gb1) in plasma, serum and CSF were also developed (93,94). It is worth noting that the lower limit of quantitation (LLOQ) for psychosine in CSF was 0.1 pg/mL, compared to the LLOQ of 0.03 ng/mL in plasma (94). It suggests that CSF has the potential to detect even minor fluctuations in psychosine levels, rendering it a more sensitive specimen type than plasma (94). Nonetheless, the invasive collection procedure makes CSF unsuitable for treatment monitoring. The utilization of psychosine in plasma and serum as a biomarker for diagnosing and monitoring Krabbe disease still requires further investigations.

When evaluating patients suspected of Krabbe disease, it is essential to proceed with caution to prevent misdiagnosis, as both galactosylceramidase and saposin A deficiencies can exhibit reduced galactosylceramidase activity. Particularly in cases where a molecular genetic analysis of *GALC*, the gene encoding galactosylceramidase, returns negative findings, the subsequent testing should involve molecular analysis of the *PSAP* gene. The diagnosis of saposin A deficiency is confirmed when molecular analysis reveals homozygous or compound heterozygous pathogenic variants within the saposin A domain of the *PSAP* gene. Alternatively, the presence of one allele carrying a heterozygous pathogenic variant within the saposin A domain, along with another deleterious allele affecting the entire prosaposin protein, can also establish the diagnosis. In the three reported patients, homozygous pathogenic or likely pathogenic variants in the coding region of the saposin A domain, including the single base pair deletion c.207_209del, the missense variant c.209T>G, and the missense variant c.257T>A, were identified (72-74).

Saposin B deficiency

Saposin B was initially discovered by Mehl *et al.* and demonstrates broad-ranging specificity (10,95). It can extract sphingolipids of multiple types from the membrane and deliver them to their respective enzymes for degradation (10). Saposin B primarily stimulates the hydrolysis of GM_1 gangliosides, sulfatides, and globotriaosylceramide (Gb3) (96-98). Sulfatides are digested by arylsulfatase A, the deficiency of which causes metachromatic leukodystrophy (MLD) (OMIM 250100) (99). Gb3 is a substrate for α-galactosidase, a defect which leads to Fabry disease (OMIM 301500) (100). GM_1 ganglioside degradation relies on β-galactosidase 1, and its deficiency underlies GM_1-gangliosidosis (OMIM 230500, 230600, 230650) and mucopolysaccharidosis type IVB (OMIM 253010) (101). The accumulation of these sphingolipids and their derivatives concurrently would strongly indicate saposin B deficiency (OMIM 249900). Shapiro *et al.* reported that two siblings with characteristic symptoms of MLD exhibited a defect in sulfatide degradation, despite having nearly normal arylsulfatase A activity (102). Subsequent studies confirmed that those siblings were indeed affected by saposin B deficiency (103,104). Fujibayashi *et al.* developed a simple immunoblotting method using an anti-saposin B antibody to screen for saposin B deficiency (105). This method applies exclusively to patients with deleterious mutations that lead to reduced protein expression or critical antigenicity alterations. Fischer *et al.* set up an enzyme assay using purified arylsulfatase A and radiolabeled sulfatide to evaluate the activating effect of saposin B (106). This assay relies on the principle that the degradation of sulfatides by the purified arylsulfatase A requires the presence of either a detergent like taurodeoxycholate or functional saposin B protein (106). To eliminate the use of hazardous radioactive materials and laborious preparation procedure, Norris *et al.* also developed an ESI-MS/MS method to quantify saposin B activity based on the same strategy and demonstrated this nonradioactive method was reliable, fast and specific (107). These initiatives have made it possible to measure saposin B activity in human specimens for the diagnosis of saposin B deficiency. However, there have been no reports of establishing such a diagnostic test in a clinical laboratory setting yet.

Sulfatides

Urinary excretion of sulfatides is a characteristic biochemical feature of MLD (108). Sulfatides can be measured by TLC, spectrophotometry, HPTLC, or HPLC methods (109-112). Hsu *et al.* developed an ESI-MS/MS method to measure sulfatides in brain and pancreatic tissues (113). Whitfield *et al.* expanded an MS/MS method for urine sulfatide measurement and detected substantially increased levels of multiple hydroxy and non-hydroxy species of sulfatides in the urine samples from MLD patients (114). Cui *et al.* made further enhancements to the MS/MS method by synthesizing a new non-physiological sulfatide as an internal standard (115). Through MS/MS coupled with solid-phase extraction, Kuchař *et al.* identified five sulfatide isoforms, namely C22:0, C22:0-OH, C24:0, C24:1-OH, and C24:0-

OH sulfatides, which exhibited the most significant distinctions between urine samples from MLD patients and the control group (116). LC-MS/MS methods on DBS and dried urine spots (DUS) were also established (117,118). Barcenas *et al.* demonstrated that the sulfatide levels in the DUS from MLD patients did not overlap with those from non-MLD patients, whereas such an overlap existed in the DBS from MLD and non-MLD patients, indicating that DUS is a better specimen type compared to DBS (118).

Lysosulfatides

Lysosulfatides, the deacylation product of sulfatides, were detected by HPLC and reported to be elevated in the tissues of MLD patients (119). Several methods based on matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS methods were also developed to measure lysosulfatides (120-122). Mirzaian *et al.* established a LC-MS/MS method, by which they could quantify a wide range of sulfatide and lysosulfatide species in urine and plasma (123). Their findings suggested that sulfatides in plasma might lack sufficient sensitivity to reliably distinguish all MLD patients from healthy individuals, and that lysosulfatides might not be reliably quantifiable due to a potential interfering ion from sulfatides (123). Cao *et al.* also developed an LC-MS/MS method to quantify sulfatides and lysosulfatides in CSF (124). While there are various specimen types available for MLD biomarker measurement, urine sulfatides measurement, instead of lysosulfatides, likely remains the most clinically valuable test.

Gb3

As a biomarker for the X-linked disorder Fabry disease (OMIM 301500) (125), Gb3 is anticipated to also serve as a potential discriminative marker for distinguishing saposin B deficiency from arylsulfatase A deficiency. Methods such as TLC, gas chromatography (GC), and HPLC were initially developed to measure Gb3 at an earlier time (126-128). Zeidner *et al.* established a rapid and sensitive ELISA method to quantify Gb3 levels in plasma using an antibody with a distinct affinity for Gb3 (129). MS/MS method was also established by Boscaro and collaborators to measure Gb3 in human plasma and urine (130). Auray-Blais *et al.* utilized LC-MS/MS to quantify Gb3 on filter paper and found that it remained stable for 7 weeks in DBS (131). A new strategy of internal standard synthesis potentially expanded the capacity to measure various forms of Gb3 through MS (132). While Gb3 can be reliably measured in plasma and urine, Young *et al.*'s study revealed that plasma Gb3 levels were elevated exclusively in male patients with the classic form of Fabry disease, and the levels exhibited a wide range overlapping with those in the normal control group (133). Therefore, plasma Gb3 was not deemed an ideal marker for Fabry disease and urine Gb3 may possess greater diagnostic potential as a biomarker (133). Kuchař *et al.* reported that urinary Gb3 was significantly elevated in a saposin B deficiency patient, in addition to elevations of sulfatides (134).

Lyso-Gb3 and methylated Gb3

Lyso-Gb3 is a deacylation product of Gb3 by acid ceramidase (135). Togawa *et al.* reported that plasma lyso-Gb3 levels were elevated in both male and female Fabry patients, whereas plasma Gb3 levels in male patients with variant forms of Fabry disease and female patients were indistinguishable from those in the control group, suggesting that lyso-Gb3 may serve as a better biomarker compared to Gb3 (136). Auray-Blais *et al.*'s study demonstrated that urinary lyso-Gb3 is a reliable independent biomarker for Fabry disease (137). With UPLC-MS/MS, Gold *et al.* reported that both lyso-Gb3 and lyso-ene-Gb3 can be reliable biomarkers (138). Lavoie *et al.* developed a multiplex LC-MS/MS method to analyze lyso-Gb3 analogs in urine and identified the most abundant analogs with m/z 802, 820, and 836 as promising urinary biomarkers for Fabry disease (139). Metabolomic profiling with a time-of-flight (TOF)-MS unveiled more potential novel biomarkers for Fabry disease, including methylated Gb3-related isoforms (140). Abaoui *et al.*'s study suggested that methylated Gb3 isoforms are the intermediate metabolites between Gb3 and lyso-Gb3, thereby expanding the biomarker panel for Fabry disease (141). Regarding saposin B deficiency, further investigations into lyso-Gb3 are necessary to assess its potential clinical utility as a biomarker.

GM1 gangliosides

It has been reported that saposin B deficiency does not lead to the accumulation of keratan sulfate, which is a hallmark of mucopolysaccharidosis type IVB (142). Since saposin B is necessary for the degradation of GM_1 gangliosides by β-galactosidase 1, it was hypothesized that saposin deficiency may result in the accumulation of GM_1

gangliosides (143,144). However, only a slight elevation of GM_1 gangliosides was observed in the skin fibroblasts from a patient with saposin B deficiency, while a higher elevation of GM_3 gangliosides was detected instead (145). Due to limited research on GM_1 gangliosides and the related metabolites as biomarkers for saposin B deficiency, it is not recommended to include them in clinical diagnostic testing currently.

Saposin B deficiency is the most common sphingolipid activator deficiency, with more than 30 cases have been reported (146-148). The diagnosis of saposin B deficiency is established through molecular genetic analysis of the *PSAP* gene, which identifies either pathogenic variants within the saposin B domain on each allele, or one allele with a pathogenic variant in the saposin B domain and another allele with a pathogenic variant located outside of saposin B domain but damaging the entire prosaposin protein.

Saposin C deficiency

Saposin C protein was first isolated by Ho *et al.* (149). Similar to saposin B, saposin C also exhibits a broad specificity. Saposin C can stimulate the degradation of GlcCer by acid β-glucosidase, the degradation of GalCer by galactosylceramidase, and the degradation of sphingomyelins by sphingomyelinase (150-152). Saposin C deficiency (OMIM 610539) was initially reported by Christomanou *et al.*, who demonstrated that a variant form of Gauche disease presented by a patient stemmed from the absence of Saposin C (153). In contrast to typical Gaucher disease (OMIM 230800, 230900, 231000) caused by acid β-glucosidase deficiency, patients with saposin C deficiency exhibited a normal β-glucosidase activity when a synthetic substrate such as 4-methylumbelliferyl-β-D-glucopyranoside was used for enzyme assay (154,155). When a natural substrate such as GlcCer was used, saposin C deficiency patients had reduced β-glucosidase activity (153). Chang *et al.* introduced a fluorescence immunoassay for quantifying saposin C concentration in plasma (75). Meikle *et al.* utilized this method to assess the amount of saposin C protein in DBS (156). Those methods are convenient in cases where saposin C deficiency is due to mutations leading to reduced expression or the absence of saposin C or antigenicity alterations. Yoneshige *et al.* directly quantified the activity of synthetic saposin C using imiglucerase (Cerezyme®), which is a recombinant analogue of β-glucosidase, and artificial fluorescent substrates embedded into the liposome to mimic intra-lysosomal membrane (157). This strategy can be employed by a clinical lab to determine saposin C activity in patient samples. Nonetheless, it's worth noting that the clinical use of this strategy as a diagnostic tool for saposin C deficiency has not been reported yet.

A variety of biomarkers have been discovered for Gaucher disease, including tartrate-resistant acid phosphatase (TRAP), angiotensin-converting enzyme (ACE), cathepsin K, Chitotriosidase, pulmonary and activation-regulated chemokine (PARC/CCL18), chemokines macrophage inflammatory protein (MIP)-1α and MIP-1β, ferritin, GlcCer, GM_3 gangliosides and lyso-Gb1 (158,159). Elevations of chitotriosidase activity, chemokine CCL18, GlcCer, and lyso-Gb1 were also observed in the plasma of patients with saposin C deficiency (154,159,160). Since many of these biomarkers are associated with secondary abnormalities and lack sufficient specificity and sensitivity (158), only GlcCer and its related metabolites are discussed in the following section.

GlcCer

GlcCer was initially detected using methods such as GC and HPLC (161-163). Samuelsson *et al.* developed a GC-MS method for GlcCer detection (164). Murata *et al.* also reported a direct inlet-chemical ionization MS that was better for the determination and structural studies of cerebroside species including GlcCer (165). Hillborg *et al.* first reported the elevation of GlcCer in plasma from Gaucher patients (166). Multiple studies further confirmed that its plasma levels were elevated in Gaucher disease (161,162). Through an ESI-MS/MS method, Whitfield *et al.* observed a general increase in all species of GlcCer in the plasma of Gaucher patients; however, there was a substantial overlap between the levels detected in the affected group and those in the normal group (167). They also noticed that the ratio of GlcCer (C16:0)/lactosylceramide (C16:0) was approximately four times higher in the patient than in normal individuals, indicating its potential as a valuable diagnostic biomarker (167). When exploring newborn screening biomarkers with LC-MS/MS, Meikle *et al.* also reported that GlcCer (C16:0) was elevated only in 3 of 5 Gaucher patients with sensitivity and specificity of 60 and 100, respectively (156). Therefore, those studies suggest that the ratio of GlcCer (C16:0) to lactosylceramide (C16:0), instead of GlcCer (C16:0) alone, holds the potential of greater clinical value and is worthy of further investigations.

Lyso-Gb1

Lyso-Gb1 is a product of GlcCer deacylation by acid ceramidase (135). The elevations of lyso-Gb1 in the spleen, hepatic, and brain tissues of Gaucher patients, as well as in human fetuses affected with type 2 Gaucher disease, were reported (168-171). In those studies, the detection methods included TLC, GLC, or HPLC (168-170). Oshima reported using a GC-MS method to identify lyso-Gb1 in the spleen tissue of Gaucher patients (172). An HPLC-FAB-MS method was also established, which enabled accurate quantification of lyso-Gb1 due to its ability to effectively separate lyso-Gb1 from psychosine (173). Dekker *et al.* utilized an LC-ESI-MS/MS technique to quantify lyso-Gb1 and revealed a significant elevation of more than 200 folds in the plasma of Gaucher patients, without an overlap with the control group (159). Comparable elevations of lyso-Gb1 were also observed in the plasma of two patients with saposin C deficiency (159). Rolfs *et al.* also detected increased lyso-Gb1 levels in the plasma of patients with Gaucher disease using HPLC-MS/MS and determined an optimal cut-off of 12 ng/mL with both sensitivity and specificity of 100% (174). Their study provides strong evidence that lyso-Gb1 is an exceptionally sensitive and specific biomarker for Gaucher disease (174). Mirzaian *et al.* optimized the LC-ESI-MS/MS method by incorporating an isotope-labelled lyso-Gb1 standard to improve test sensitivity and enable the measurement of lyso-Gb1 in unconcentrated urine samples (175). They further demonstrated elevated lyso-Gb1 levels in the urine of symptomatic type 1 GD patients (175). Zhang *et al.* developed a hydrophilic interaction liquid chromatography (HILIC)-MS/MS method to reliably detect lyso-Gb1 in DBS, thus streamlining the utilization of lyso-Gb1 in newborn screening as a biomarker for Gaucher disease (176). Quantification of lyso-Gb1 in CSF was also reported (94). Hence, lyso-Gb1 stands as a highly sensitive and convenient biomarker for the diagnosis of Gaucher disease, as well as saposin C deficiency, as systematically reviewed by Revel-Vilk *et al.* (177). Moreover, metabolomics studies have unveiled related lyso-Gb1 analogs as potential biomarkers (178). Subsequent investigations have explored their role in the diagnosis of Gaucher disease (179-181). Nevertheless, those analogs will require further assessments in future research.

To date, 15 cases of saposin C deficiency have been reported (14,153-155,160,182-184). The diagnosis of saposin C deficiency is confirmed through molecular analysis of the *PSAP* gene, which reveals the presence of two pathogenic variants *in-trans*, with at least one affecting the saposin C domain, while the other impacting either saposin C or the entire protein.

Saposin D deficiency

Saposin D, as an activator for sphingomyelinase and ceramidase, facilitates the breakdown of sphingomyelins and ceramides (185,186). Matsuda *et al.* reported that a mouse model of saposin D deficiency presented with the accumulation of ceramides in the brain tissues (187). However, saposin D deficiency has not been reported in humans, potentially due to the broad specificity of other saposin proteins, particularly saposin C, which can compensate for the dysfunction of saposin D in the degradation of sphingomyelins and ceramides (150,188). Although it has been reported that pathogenic variants within the saposin D domain of the *PSAP* gene are associated with Parkinson's disease (189), this discovery remains a topic of controversy (190,191).

Prosaposin deficiency

Prosaposin deficiency (OMIM 611721), also known as combined saposin deficiencies, is attributed to the impairment of the entire prosaposin protein. Harzer *et al.* reported two siblings presented with an atypical form of Gaucher disease that was characterized by more severe symptoms, the accumulation of GlcCer and ceramides in liver tissue, as well as the partially reduced activities of galactosylceramidase, β-glucosidase, and ceramidase (192). Those two siblings possessed homozygous mutations in the initiation codon of the *PSAP* gene, which could have led to either the absence of prosaposin protein or mistargeting of prosaposin into medium instead of transport to lysosomes (193). Additional biochemical findings in those siblings included the absence of both saposin B and C in lysosomes, as well as the elevations of various neutral glycolipids in multiple tissues, including mono-, di-, tri-, and tetrahexosylceramides such as GlcCer, GalCer, lactosylceramides, and digalactosylceramides, in addition to sulfatides, ceramides, GM_3 and GM_2 gangliosides (194,195).

There have been 10 reported cases of prosaposin deficiency (134,192,196-200). Summarizing those ten cases reveals that patients with prosaposin deficiency typically present with more severe clinical symptoms, including hepatosplenomegaly, microcephaly, hypotonia, seizures, optic atrophy, and other manifestations (198,199). Multiple

types of sphingolipids accumulate in various tissues, including lyso-Gb1, lyso-Gb3, psychosine, ceramides, GlcCer, lactosylceramides, GalCer, Gb3, sulfatides and GM_3 gangliosides (198,199). Therefore, quantitative analysis of plasma lysosphingolipids by LC-MS/MS demonstrated a specific pattern of combined saposin deficiency, particularly elevations of both lyso-Gb1 and lyso-Gb3 (199). The enzyme assays revealed varying degrees of reduced activities in galactosylceramidase, β-glucosidase and ceramidase in leukocytes or fibroblasts, whereas the activities of other enzymes such as sphingomyelinase and neuraminidase remained within the normal range (192,199). Hence, a plasma or urine lysosphingolipid panel test that incorporates lyso-Gb1 and lyso-Gb3 could serve as a valuable tool for the early diagnosis of prosaposin deficiency. The diagnosis is confirmed through molecular genetic analysis, which identifies two *in-trans* pathogenic variants resulting in the malfunction of the entire prosaposin protein (199). The reported pathogenic variants caused frameshift, premature stop, or a splicing error in the *PSAP* gene, leading to the absence or loss of function of prosaposin protein and affecting multiple saposin proteins (134,198,199). Similar to saposin A deficiency, it is crucial to proceed with caution when discrepancies exist between reduced enzyme activities and negative molecular genetic analysis of the relevant enzyme genes. This is especially important when elevated levels of multiple biomarkers associated with different activator proteins are detected. In such cases, it is appropriate to pursue molecular analysis of the *PSAP* gene to confirm the diagnosis of prosaposin deficiency.

Conclusions

Lysosomal degradation of glycosphingolipids relies on the presence of SAPs. It is challenging to diagnose SAP deficiencies as affected individuals usually exhibit normal enzyme activities despite presenting with typical clinical features. Biomarkers such as GM_2 ganglioside (34:1), sulfatides, lyso-GB1, lyso-Gb3, and psychosine are valuable for addressing this diagnostic complexity, and molecular genetics analysis serves as the essential confirmatory test. In cases of saposin A and prosaposin deficiencies, it's noteworthy that abnormal enzyme activities can be observed. This could potentially result in the misdiagnosis of corresponding enzyme deficiencies. In such scenarios, discrepancies between reduced enzyme activities and negative findings in molecular genetic analysis of the relevant enzyme genes may indicate the necessity of a follow-up molecular test targeting the *PSAP* gene.

Ongoing research in this field may further advance our understanding of SAP deficiencies and improve the current diagnostic and therapeutic approaches.

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Footnote

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