# Ceramide profiling of stratum corneum in Sjögren–Larsson syndrome

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## Abstract

*Background:* Sjögren–Larsson syndrome (SLS) is a neurocutaneous disorder whose causative gene is the fatty aldehyde dehydrogenase *ALDH3A2* and of which ichthyosis is the major skin symptom. The stratum corneum contains a variety of ceramides, among which ω-*O*-acylceramides (acylceramides) and protein-bound ceramides are essential for skin permeability barrier formation.

*Objectives:* To determine the ceramide classes/species responsible for SLS pathogenesis and the enzymes that are impaired in SLS.

*Methods:* Genomic DNA was collected from peripheral blood samples from an SLS patient and her parents, and whole-genome sequencing and Sanger sequencing were performed. Lipids were extracted from stratum corneum samples from the SLS patient and healthy volunteers and subjected to ceramide profiling via liquid chromatography coupled with tandem mass spectrometry.

*Results:* A duplication (c.55_130dup) and a missense mutation (p.Lys447Glu) were found in the patient's *ALDH3A2* gene. The patient had reduced levels of all acylceramide classes, with total acylceramide levels at 25% of healthy controls. Reductions were also observed for several nonacylated ceramides: ceramides with phytosphingosine or 6-hydroxysphingosine in the long-chain base moiety were reduced to 24% and 41% of control levels, respectively, and ceramides with an α-hydroxy fatty acid as the fatty acid moiety were reduced to 29%. The fatty acid moiety was shortened in many nonacylated ceramide classes.

*Conclusion:* These results suggest that reduced acylceramide levels are a primary cause of the ichthyosis symptoms of SLS, but reductions in other ceramide classes may also be involved.

Key words: Acylceramide, Ceramide, Ichthyosis, Mass spectrometry, Sjögren–Larsson syndrome, Skin permeability barrier.

*Abbreviations:* SLS, Sjögren–Larsson syndrome; FALDH, fatty aldehyde dehydrogenase; FA, fatty acid; SC, stratum corneum; LCB, long-chain base; DS, dihydrosphingosine; S, sphingosine; P, phytosphingosine; H, 6-hydroxysphingosine; SD, 4,14-sphingadiene; N, nonhydroxy; A, α-hydroxy; O, ω-hydroxy; EO, esterified ω-hydroxy; P-O, protein-bound ω-hydroxy; acylceramide, ω-*O*-acylceramide; TLC, thin-layer chromatography; LC, liquid chromatography; MS/MS, tandem mass spectrometry; ER, endoplasmic reticulum.

## 1. Introduction

Sjögren–Larsson syndrome (SLS) is an autosomal recessive neurocutaneous disorder whose major symptoms are ichthyosis in the skin and mental retardation and spastic paraplegia in the nervous system [1,2]. The ichthyosis is accompanied by reduced permeability barrier function, hyperkeratosis, dry skin, and desquamation [3,4]. The causative gene for SLS is *ALDH3A2* [5]. *ALDH3A2* encodes fatty aldehyde dehydrogenase (FALDH), which converts fatty aldehydes to fatty acids (FAs) [2]. Since aldehydes are highly reactive in general, it is thought that the pathology in SLS patients is caused by the accumulated fatty aldehydes attacking proteins that play important roles in the skin and nervous system.

The lipid lamellae, which are multilayered lipid structures that fill the intercellular spaces between corneocytes, in the stratum corneum (SC) of the epidermis play an important role in the formation of the skin permeability barrier (hereafter referred to as the skin barrier) [6,7]. One of the major components of lipid lamellae are ceramides, which are composed of a long-chain base (LCB) and an FA (Fig. 1A). Humans have five species of LCBs, which differ in the number and/or position of their hydroxyl groups and double bonds: dihydrosphingosine (DS), sphingosine (S), phytosphingosine (P), 6-hydroxysphingosine (H), and 4,14-sphingadiene (SD) (Fig. 1A) [8-10]. In ceramides, nonhydroxy (N), α-hydroxy (A), or ω-hydroxy (O) FAs are attached to LCBs via an amide bond (Fig. 1A). Most of the ω-hydroxyl groups of the O-type FA moiety in ceramides are not free but are bound to linoleic acid or proteins, and these FAs are called esterified ω-hydroxy (EO) FAs or protein-bound ω-hydroxy (P-O) FAs, respectively [8,10,11]. Combinations of these five types of LCBs (DS, S, P, H, and SD) and five types of FAs (N, A, O, EO, and P-O) yield 25 classes of ceramides, and each of these is represented by a combination of the FA and LCB abbreviations (e.g., NS) (Fig. 1B). The EO-

type ceramides (EODS, EOS, EOP, EOH, and EOSD) and P-O-type ceramides (P-ODS, P-OS, P-OP, P-OH, and P-OSD) are called ω-*O*-acylceramides (acylceramides) and protein-bound ceramides, respectively, and both are essential for skin barrier formation [8,11,12]. Mutations of the genes involved in the production of acylceramides or protein-bound ceramides cause congenital ichthyosis [3,12].

It has been reported that levels of the acylceramide EOS (ceramide 1 in the old nomenclature) and the nonacylated ceramides AP (ceramide 6) and AH (ceramide 7) are reduced in the SC of SLS patients [13,14]. However, in those analyses, ceramides were separated using thin-layer chromatography (TLC), in which free (non-protein-bound) ceramides and protein-bound ceramides were only separated into four or five bands and two bands, respectively. Furthermore, the quantitativeness of these analyses was low. Thus, the detailed ceramide profile of the SC of SLS patients remains unknown. We have recently established a ceramide-measurement system that can separate and quantify not only all ceramide classes but also the different ceramide species based on FA chain length and degree of unsaturation via liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) [10,15]. In the present study, we collected SC samples by tape stripping and performed comprehensive ceramide analyses on them via LC-MS/MS to reveal the detailed ceramide composition of the SC of an SLS patient.

## 2. Material and methods

### *2.1. Ethics*

This study was approved by the ethics committees of Nagoya University Graduate School of Medicine (Permit Numbers: 2013-0279 and 2016-0412) and Hokkaido University (Permit Number: 2020-002). Informed consent was obtained from all volunteers and the parents of an SLS patient, and the research was conducted in accordance with the principles expressed in the Declaration of Helsinki.

### *2.2. Sequencing of ALDH3A2 genomic DNA*

Genomic DNA was extracted from peripheral blood samples taken from an SLS patient and her parents and subjected to whole-exome sequence analysis, as described previously [16]. Exons 1 and 9 of the *ALDH3A2* gene of the patient, her parents, and one healthy volunteer for comparison were amplified via polymerase chain reaction using the primers described previously [17], followed by Sanger sequencing.

### *2.3. Preparation and quantification of SC ceramides*

We collected SC samples from the forearms of the patient and three healthy volunteers (two males and one female), who were used as controls, by tape stripping. Tape stripping was performed as previously described [10]. Briefly, a 25 mm × 50 mm strip of film masking tape 465#40 (Teraoka Seisakusho, Tokyo, Japan) was stuck to the inner forearm and peeled off. This procedure was repeated three times, using a new tape strip each time. Because the first tape strip may have picked up unwanted substances such as dust, detergents, and lotions, we used the second tape strip for the lipid extraction. To do this, we cut two small pieces of tape

(5 mm × 10 mm) from the second tape strip and extracted the lipids from them by adding 400 μL of methanol. The third tape strip was taken as a spare. Quantification of ceramides via LC-MS/MS were performed as described previously [10]. The MS/MS was performed in multiple reaction monitoring mode using optimized collision energies and Q1 and Q3 settings specific to each ceramide species (Tables S1 and S2) [10]. From the ceramides containing C18 LCBs, we selected (based on our previous report [10]) and measured 115 free ceramide species (Table S1) and 36 protein-bound ceramide species (Table S2), which covered more than 95% of the total quantity of each of these categories. We quantified the ceramides by calculating the ratio of the corrected peak area (peak area minus the blank that was obtained for the tape without SC) for each ceramide species to that of the internal standard corresponding to each ceramide class.

## 3. Results

### *3.1. SLS symptoms of the patient*

The patient was a 5-year-old Japanese female who was born uneventfully to nonrelated parents at 38 weeks and 3 days of gestation. The patient began to sit unaided at 9 months and to walk independently at 1 year and 4 months. She began to speak meaningful words at 2 years and 3 months. At that age, she was brought to our outpatient clinic because of an unsteady gait and a tendency to fall. A physical examination revealed strabismus and mild ichthyosis on her extremities (Fig. 2A–C). Thereafter, it was noticed that she had bilateral increased tendon reflexes. Magnetic resonance imaging of her central nervous system revealed no abnormalities. An ophthalmological examination revealed no abnormal findings in the ocular fundus. The Wechsler Preschool and Primary Scale of Intelligence Test (WPPSI-III) yielded a full-scale IQ of 65.

### *3.2. ALDH3A2 mutations in the patient*

The patient had a normal G-banding karyotype. Whole-exome sequencing of trio analysis and subsequent Sanger sequencing revealed that the patient was compound heterozygous for a novel 76 bp duplication mutation (c.55_130dupCCTCTGCGGTTTCGGCTGCAGCAGCTGGAGGCCCTGCGGAGGATGG TGCAGGAGCGCGAGAAGGATATCCTGACGG) and a previously reported missense mutation, c.1339A>G [17], in *ALDH3A2* (Fig. 2D and E). The duplication mutation was of paternal origin and was located in exon 1, causing a premature stop codon (p.Ile45Serfs*34). The mutant protein contains only the first 45 amino acids at the N-terminal end of the full protein, which contains 485 amino acids, and is expected to be inactive. The maternal missense

mutation was located in exon 9 of *ALDH3A2* and caused substitution of the lysine residue with glutamine (p.Lys447Glu). It has been reported that this missense mutation reduces ALDH3A2 activity to 1% of that of the wild-type protein [17]. Based on the guidelines of the American College of Medical Genetics and Genomics [18], both variants were assessed as pathogenic (c.55_130dup: PVS1+PM2+PM3+PP4 and c.1339A>G: PS1+PM1+PM2+PP3+PP4).

*3.3. Decreased acylceramide levels in the SLS patient*

Acylceramides are essential for skin barrier formation [8,12]. It has been reported, based on TLC analysis, that levels of EOS are lower in SLS patients than in healthy individuals [13,14], but no quantitative analysis has yet been performed. It is unknown whether there are differences in the levels of other acylceramide classes. In this study, we collected SC samples from the SLS patient and three healthy volunteers (controls) via tape stripping and subjected them to acylceramide analysis via LC-MS/MS. EOH, EOS, and EOP were the most abundant acylceramide classes in the controls, in that order, while EODS and EOSD were barely detectable (Fig. 3A, Table S1). The quantities of EOH, EOS, and EOP were lower in the patient than in the controls (23.4%, 41.7%, and 11.2% of the control levels, respectively). The total quantity of acylceramides was approximately 25% of that of the controls. Each acylceramide class contains several molecular species with different chain lengths (C28–34) of the ω-hydroxy FA portion. The levels of all acylceramide species were lower in the patient than in the controls (Fig. 3B, Table S1).

O-type ceramides are precursors of acylceramides. To investigate the cause of the decrease in acylceramide levels, we assessed the quantities of O-type ceramides via LC-MS/MS. We detected OS, OH, and OP at low levels in the controls, and ODS and OSD were barely

detectable (Fig. 3C, Table S1). We found that OS, OH, and OP were present at higher levels in the patient than in the controls (6.5-, 3.0-, and 1.6-fold, respectively, and 3.8-fold in total). These results suggest that the reduced quantities of acylceramides result from impaired conversion of O-type ceramides to acylceramides.

*3.4. Reduced A-, H-, and P-type ceramide levels in the SLS patient*

It has been reported, based on TLC analysis, that levels of AP and AH ceramides are reduced in SLS patients [13,14]. However, these studies lacked quantitative analysis and did not include measurement of individual AP/AH species with specific FA chain lengths, nor were many other ceramide classes analyzed. Therefore, we used LC-MS/MS to analyze nonacylated ceramides other than O-type ceramides. There were substantial quantities of NP, NH, AH, AP, NS, NDS, AS, and ADS ceramides in the controls, in that order of abundance, but the abundance of SD-type ceramides was low (Fig. 4A, Table S1). The levels of NH, NP, ADS, AH, and AP ceramides were much lower in the SLS patient than in the controls. Of these, AP was the most reduced relative to the controls (~11% of the controls), and AH, NP, ADS, and NH followed in that order (23–52% of the controls). The levels of NDS and AS were similar in the controls and the patient. In contrast, the quantity of NS was 2.5-fold that in the controls. Comparing the total quantities of N-type (NS, NDS, NH, and NP) and A-type (AS, ADS, AH, and AP) ceramides in the patient, the A-type ceramides were more strongly reduced (29% of the controls) than the N-type ceramides (58% of the controls). This suggests that the activity of FA 2-hydroxylase, which produces A-type ceramides, was reduced in the patient.

The quantities of almost all ceramide species in the NH and NP classes were lower in the SLS patient than in the controls (Fig. 4B, Table S1). This decrease was stronger for ceramide

species with longer chain lengths. For example, the levels of NH and NP with a C24:0 FA in the patient were 65% and 44%, respectively, of those in the controls, whereas for NH and NP with a C26:0 FA these proportions were 51% and 24%, respectively. The weighted average of the FA chain lengths of NH and NP were 25.7 and 25.4, respectively, in the controls, and 25.4 and 24.7 in the patient (Fig. 4C). Shortening of the FA portion was more pronounced in NDS, AS, and ADS, with weighted-mean differences of 1.5, 1.0, and 2.2, respectively, between the controls and the patient. The weighted mean FA chain length of all N- and A-type ceramides was 25.2 in the controls and 24.2 in the patient: a difference of 1.0. These results suggest that the activity of the FA elongase was reduced in the patient.

The total quantities of S-type ceramides (NS, AS, OS, and EOS) were higher in the patient than in the controls, while those of DS-, H-, and P-type ceramides were lower (Fig. 4D). The reductions in the H- and P-type ceramides were especially pronounced: their levels in the patient were 41% and 24% of those in the controls, respectively. The total quantity of all free ceramides in the patient was 47% of that in the controls.

### *3.5. Normal levels of protein-bound ceramides in the SLS patient*

We next measured the quantities of protein-bound ceramides via LC-MS/MS. Although two models have been proposed for the structure of protein-bound ceramides (Fig. 1A) [19,20], in our measurement system they are indistinguishable by measuring the O-type ceramides that are released by alkali treatment. In the controls, P-OS was the most abundant type of protein-bound ceramides, followed by P-OH and P-OP (Fig. 5A, Table S2). No P-ODS and only trace amounts of P-OSD were detected. P-OS levels were higher and P-OH and P-OP levels were

lower in the patient, but these differences were small. Overall, the quantity of protein-bound ceramides was slightly higher in the patient than in the controls.

The most abundant ω-hydroxy FA in the protein-bound ceramides in the controls was C30:0 for both P-OS and P-OH, followed by C32:1, C32:0, and C34:1 (Fig. 5B, Table S2). All four of these major P-OS species were more abundant in the patient than in the controls. Almost all P-OH species were reduced in the patient compared to the controls, except the C34:1 species, which was slightly more abundant. In summary, the quantities and composition of protein-bound ceramides were not greatly affected in the patient, in contrast to those of free ceramides.

## 4. Discussion

Lower levels of the acylceramide EOS and the nonacylated ceramides AP and AH in SLS patients than in healthy controls have previously been reported, based on TLC analysis [13,14], and we confirmed these findings in the present study (Fig 3 and 4, Table S1). In addition to these ceramides, we have revealed here that levels of the acylceramides EOH and EOP and the nonacylated ceramides NH, NP, and ADS were also reduced in our SLS patient. Acylceramides are important for the formation of the lipid lamellae in the SC [21-23], and mutations in genes involved in their production cause congenital ichthyosis [3,12,24]. Reduced acylceramide levels have also been observed in a mouse model of SLS and in *ALDH3A2*-deficient human immortalized keratinocytes [25]. Thus, among the ceramide classes that are reduced in SLS patients, reduced acylceramide levels at least must be associated with the ichthyosis pathology. However, there is no clear evidence of the importance of the other ceramide classes (AP, NH, NP, ADS, and AH) that were reduced in this SLS patient for skin barrier formation. All of these ceramide classes have one or two more hydroxyl groups than NS ceramides, which are the most abundant ceramides in most other tissues. These additional hydroxyl groups may enhance the interaction between lipids in the lipid lamellae via hydrogen bonds. The loss or reduction of one of these particular ceramide classes may be compensated for by other ceramide classes, but the reduction of some or all of them may exacerbate the ichthyosis pathology that is caused by reduced quantities of acylceramides.

In the final step of the acylceramide synthesis pathway, transacylase PNPLA1 transfers the linoleic acid from a triglyceride to an O-type ceramide (Fig. 6) [26]. This reaction is facilitated by the α/β-hydrolase family member ABHD5 [27]. *PNPLA1* and *ABHD5* are the causative genes of autosomal recessive congenital ichthyosis and one of the ichthyosis

syndromes, Chanarin–Dorfman syndrome, respectively [28-31]. In our SLS patient, the abundance of O-type ceramides, which are precursors of acylceramides, was higher than in healthy controls, and that of acylceramides was lower (Fig. 3). We have obtained similar results in a mouse model of SLS [25]. These findings suggest that the transacylation reaction is impaired in SLS. In SLS, accumulated fatty aldehydes are thought to attack certain enzymes and reduce their activity. In particular, the function of enzymes involved in lipid metabolism may be impaired in SLS, since these enzymes use lipids as substrates and therefore have hydrophobic cavities in their substrate-binding pockets, which allow fatty aldehydes to enter and react with the amino acid residues that are important for the enzyme's activity. We speculate that either PNPLA1 or ABHD5 is the target of the fatty aldehydes that accumulate in SLS (Fig. 6).

We observed higher levels of NS ceramides and lower levels of NP and NH ceramides in the SLS patient than in the controls (Fig. 4). In the ceramide metabolic pathway, NDS undergoes unsaturation between C4 and C5 of the LCB moiety to form NS, or hydroxylation at C4 to form NP (Fig. 6). These reactions are catalyzed by the dihydroceramide Δ4 desaturase DEGS1 and the dihydroceramide 4-hydroxylase DEGS2, respectively [32]. NH is thought to be produced via the hydroxylation of NS at C6 of the LCB moiety, but the 6-hydroxylase has not been identified. The reduction in NP and increase in NS levels in the SLS patient suggest that the conversion of NDS to NP was hampered, and instead the metabolic flow from NDS to NS increased. In addition, it is likely that the NS–NH conversion was reduced. Thus, DEGS2 and an unidentified 6-hydroxylase may also be attacked by the accumulated fatty aldehydes in SLS (Fig. 6).

Levels of A-type ceramides, i.e., ceramides containing α-hydroxy FAs (2-hydroxy FAs), were reduced in the SLS patient (Fig. 4). In the brain, the 2-hydroxy FA in A-type ceramides is produced by the FA 2-hydroxylase FA2H [33]. We previously revealed that the quantities of 2-hydroxy galactosylceramides are reduced in the brain of the mouse model of SLS due to lowered FA2H function [34]. Since 2-hydroxy galactosylceramides are important for myelin function [35-37], it is likely that reduced levels of 2-hydroxy galactosylceramides are one of the causes of the neurological symptoms of SLS. The involvement of FA2H in the production of A-type ceramides in the epidermis is unclear and still needs to be investigated. However, it is possible that FA2H or another unidentified 2-hydroxylase is attacked by the accumulated fatty aldehydes in the epidermis, as it is in the brain, resulting in reduced levels of A-type ceramides in the skin of SLS patients (Fig. 6).

We observed shortening of the FA chain lengths of N- and A-type ceramides in the SLS patient (Fig. 4). Acyl-CoAs are elongated via the FA-elongation cycle [38]. The FA elongases ELOVL1 and ELOVL4 are important for FA elongation in the epidermis, and their mutations cause ichthyosis [39-42]. ELOVL1 elongates C20 and C22 acyl-CoAs to C24 or C26 [21,43]. In SLS, the accumulated fatty aldehydes may attack ELOVL1, thereby reducing its activity (Fig. 6).

We speculate that *trans*-2-hexadecenal, the metabolite of S-type ceramides, is the major fatty aldehyde causing SLS [44]. First, because S-type ceramides are abundant in the epidermis [10,45,46], their degradation product, *trans*-2-hexadecenal, may also be produced in large quantities. Second, *trans*-2-hexadecenal is an α,β-unsaturated carbonyl compound and is therefore more reactive than other fatty aldehydes. While most aldehydes form Schiff bases with a lysin residue, α,β-unsaturated carbonyl compounds can also react with nucleophilic

amino acid residues (histidine, cysteine, and lysine) via the Michael addition reaction. Among these nucleophilic amino acids, *trans*-2-hexadecenal has been reported to form a stable adduct with histidine [47]. S-type ceramides are converted to *trans*-2-hexadecenal via three reactions. First they are converted to sphingosine by ceramidases, then to sphingosine 1-phosphate by sphingosine kinases, and finally to *trans*-2-hexadecenal by sphingosine 1-phosphate lyase [44]. Inhibitors of these enzymes can reduce *trans*-2-hexadecenal and are thus expected to be therapeutic agents for SLS.

We consider that the enzymes impaired in SLS meet the following three criteria: 1) they are endoplasmic reticulum (ER) membrane proteins; 2) their substrates are structurally similar to fatty aldehydes (i.e., lipid-metabolizing enzymes); and 3) their histidine residues are important for enzyme activity. As described above, the major target of the accumulated *trans*-2-hexadecenal is histidine residues. However, simply attacking histidine residues on the surface of the proteins is unlikely to inhibit enzyme activity. Rather, the activity is inhibited only when *trans*-2-hexadecenal attacks the histidine residue(s) in the active center of the enzyme. For *trans*-2-hexadecenal to enter the active center, the enzyme should have a substrate pocket that can accommodate hydrophobic substrates (lipids). Since the sphingosine 1-phosphate lyase SGPL1 is an ER membrane protein [48], *trans*-2-hexadecenal is produced in the ER. Therefore, its target proteins must also be ER-localized proteins. Among the proteins we have predicted to have reduced activity in SLS, most of these criteria are met by ABHD, ELOVL1, FA2H, and DEGS2: all of these have lipid substrates; one of the catalytic triad residues of the α/β hydrolase family to which ABHD belongs is histidine [29]; ELOVL1, FA2H, and DEGS2 all have histidine boxes that are important for their activity [32,49,50]; and ELOVL1, FA2H, and DEGS2 are localized to the ER [43].

In this study, we have revealed the detailed ceramide profile of the SLS pathogenic condition. The low invasiveness of SC sampling by tape-stripping makes it less burdensome for patients and is useful in the diagnosis of SLS, as well as of other ichthyosis or skin disorders. In the future, ceramide profiling of the SC of patients with skin disorders will clarify the relationship between alterations in ceramide composition and skin pathology.

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## Figure legends

**Fig. 1.** Structures and nomenclature of human ceramides. (A) Structures of a ceramide (box) and its component LCB (upper part) and FA (lower part). Two models have been proposed for the R portion of a P-O FA (dotted box): (1) the carboxy group of glutamic acid is esterified with an O-type FA [19]; or (2) a modified linoleic acid moiety (epoxy–enone) binds to a nucleophilic amino acid residue of a protein via a Schiff base formation or a Michael addition reaction [20]. (B) Nomenclature of ceramide classes. Each ceramide class is represented by the combination of the abbreviations for its constituent LCB and FA. Ceramides are classified into free (non-protein-bound) ceramides and protein-bound ceramides (P-O-type ceramides), and the former is further classified into nonacylated ceramides (N-, A-, and O-type ceramides) and acylceramides (EO-type ceramides).

**Fig. 2.** Clinical features and *ALDH3A2* mutations in the SLS patient. (A–C) Photographs of the patient taken at five years of age. Mild, whitish scales are seen on her right fingers (A), right leg (B), and the dorsum of her right foot (C). (D and E) Exon 1 (D) and exon 9 (E) of *ALDH3A2* were amplified via PCR using genomic DNA prepared from peripheral blood samples taken from the patient (D and E), her mother (D and E), and her father (E). DNA fragments were separated via agarose gel electrophoresis (D) or subjected to Sanger sequencing (E).

**Fig. 3.** Reduced levels of acylceramides and increased levels of O-type ceramides in the SLS patient relative to controls. SC was collected from the SLS patient and three healthy volunteers (controls) by tape stripping. The lipids were extracted and acylceramides (A, B) and O-type

ceramides (C) were analyzed via LC-MS/MS. (A) Quantities of each acylceramide class (EOS, EOH, and EOP) and their total quantity (left). The structure of acylceramides and the modification site of each LCB (right). (B) Quantities of EOS and EOH species. The chain length and degree of unsaturation of the ω-hydroxy FA moiety are shown on the *x*-axis. (C) Quantities of each O-type ceramide class (OS, OH, and OP) and their total quantity (left). The structure of O-type ceramides and the modification site of each LCB (right). The values for the controls are the mean + SD of one measurement each from the three volunteers, whereas those for the single patient are the mean of three measurements.

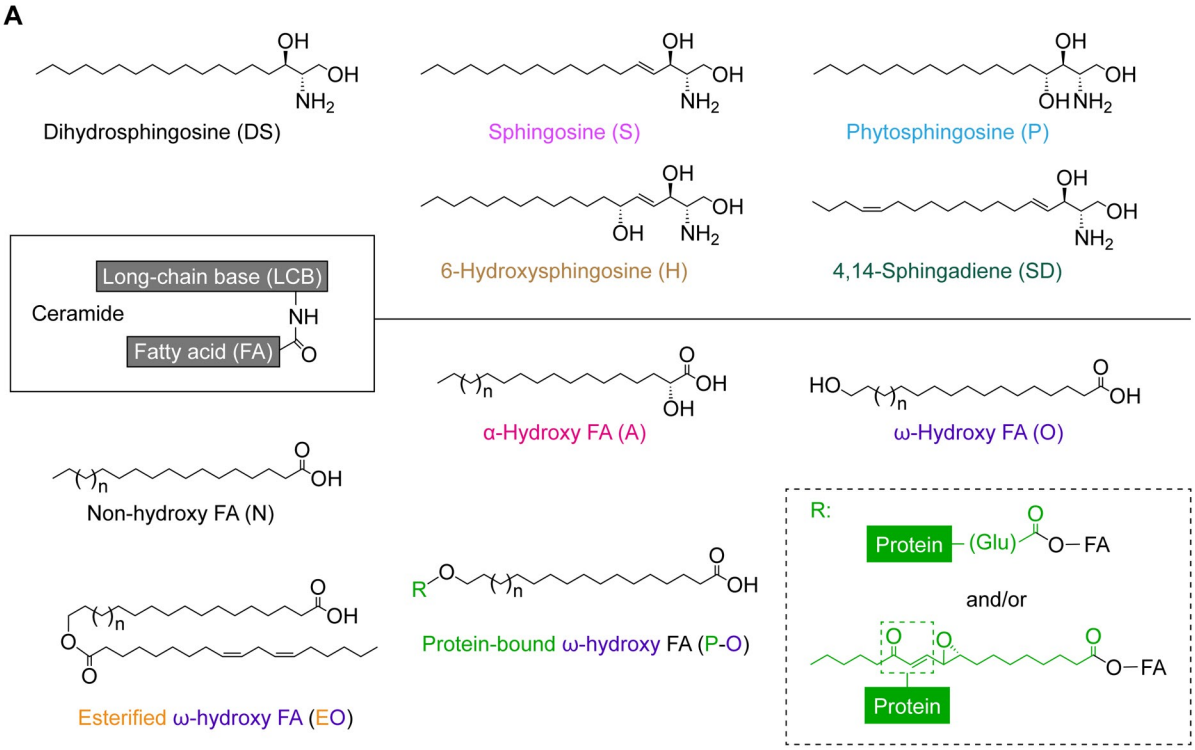
**Fig. 4.** Reduced levels of A-, H-, and P-type ceramides in the SLS patient. SC was collected from the SLS patient and three healthy volunteers (controls) by tape stripping. The lipids were extracted and N- and A-type ceramides were analyzed via LC-MS/MS. (A) Quantities of each N- and A-type ceramide class and the total quantities of N- and A-type ceramides (left). The structure of N/A-type ceramides and the modification site of each LCB (right). (B) Quantities of each NH and NP species. The chain length and degree of unsaturation of the FA moiety are shown on the *x*-axis. (C) Weighted averages of FA chain lengths of each N- and A-type class and of all N/A-type ceramides combined. (D) Total quantities of S-, DS-, H-, and P-type ceramides and the total quantity of all free ceramides combined, calculated based on the quantitative results for N- and A-type ceramides shown here and for EO- and O-type ceramides shown in Fig. 3. The values for the controls are the mean + SD of one measurement each from the three volunteers, whereas those for the single patient are the mean of three measurements.

**Fig. 5.** Protein-bound ceramide levels in the SLS patient. SC was collected from the SLS patient and three healthy volunteers (controls) by tape stripping. The lipids were extracted and protein-bound ceramides were analyzed via LC-MS/MS. (A) Quantities of each protein-bound ceramide class (P-OS, P-OH, and P-OP) and the total quantity of these ceramides (left). The structure of protein-bound ceramides and the modification site of each LCB (right). Cer, ceramide. (B) Quantities of each P-OS and P-OH species. The chain length and degree of unsaturation of the ω-hydroxy FA moiety are shown on the *x*-axis. The values for the controls are the mean + SD of one measurement each from the three volunteers, whereas those for the single patient are the mean of three measurements.

**Fig. 6.** Ceramide metabolic pathway and enzymes that are presumably affected in SLS. The pathways of FA elongation and ceramide metabolism, types of reactions in these pathways, and the names of the enzymes involved are shown. Enzymes marked in yellow are those predicted to be attacked by fatty aldehydes and to exhibit reduced activity in SLS as a result. The effects on ceramides caused by the inhibition of the respective enzymes are shown in boxes with dashed borders. N-type FAs undergo α- or ω-hydroxylation to become A- or O-type FAs. After conversion to acyl-CoAs, they are combined with the LCB dihydrosphingosine (DS) by ceramide synthases to form DS-type ceramides. DS-type ceramides are then converted to S- or P-type ceramides via unsaturation between C4 and C5 or via hydroxylation at C4, respectively. H-type ceramides are thought to be generated from S-type ceramides via C6-hydroxylation. O-type ceramides are metabolized to acylceramides (EO-type ceramides) via transacylation of the linoleic acid in triglycerides. Some acylceramides are thus converted to protein-bound ceramides via several reactions.

**Fig. 1**

**A**




**B**

| LCB \ FA | N | A | O | EO | P-O |
|---|---|---|---|---|---|
| DS | NDS | ADS | ODS | EODS | P-ODS |
| S | NS | AS | OS | EOS | P-OS |
| P | NP | AP | OP | EOP | P-OP |
| H | NH | AH | OH | EOH | P-OH |
| SD | NSD | ASD | OSD | EOSD | P-OSD |

Nonacylated ceramides

Acylceramides

Protein-bound ceramides

Free (non-protein-bound) ceramides

Fig. 2

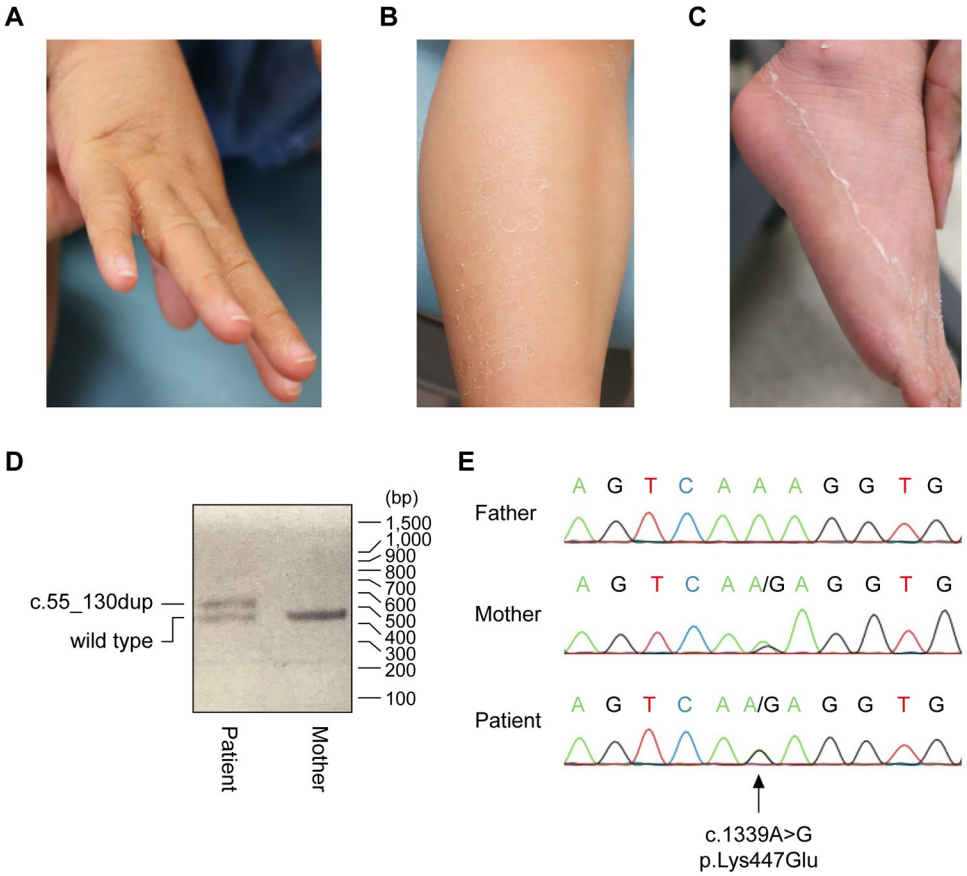

A
B
C
D
(bp)
1,500
1,000
900
800
700
600
500
400
300
200
100
c.55_130dup
wild type
Patient
Mother
E
Father
Mother
Patient
A G T C A A A G G T G
A G T C A A/G A G G T G
A G T C A A/G A G G T G
c.1339A>G
p.Lys447Glu

**Fig. 3**

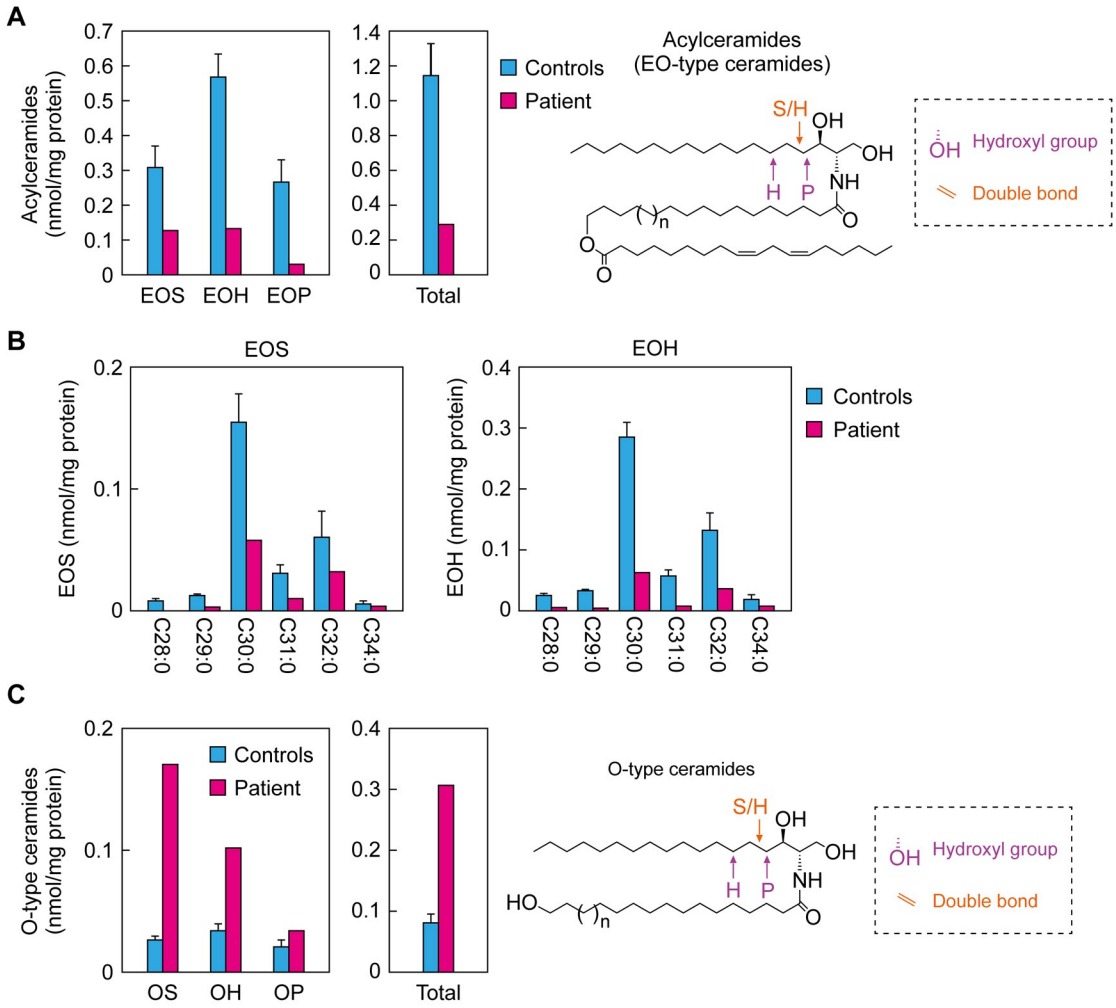

A
Acylceramides (nmol/mg protein)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
EOS
EOH
EOP
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Total
Controls
Patient
Acylceramides
(EO-type ceramides)
S/H
OH
OH
H
P
NH
O
n
O
O
OH Hydroxyl group
Double bond
B
EOS
EOS (nmol/mg protein)
0.2
0.1
0
C28:0
C29:0
C30:0
C31:0
C32:0
C34:0
EOH
EOH (nmol/mg protein)
0.4
0.3
0.2
0.1
0
C28:0
C29:0
C30:0
C31:0
C32:0
C34:0
Controls
Patient
C
O-type ceramides (nmol/mg protein)
0.2
0.1
0
OS
OH
OP
Controls
Patient
0.4
0.3
0.2
0.1
0
Total
O-type ceramides
S/H
OH
OH
H
P
NH
HO
n
O
OH Hydroxyl group
Double bond

Fig. 4

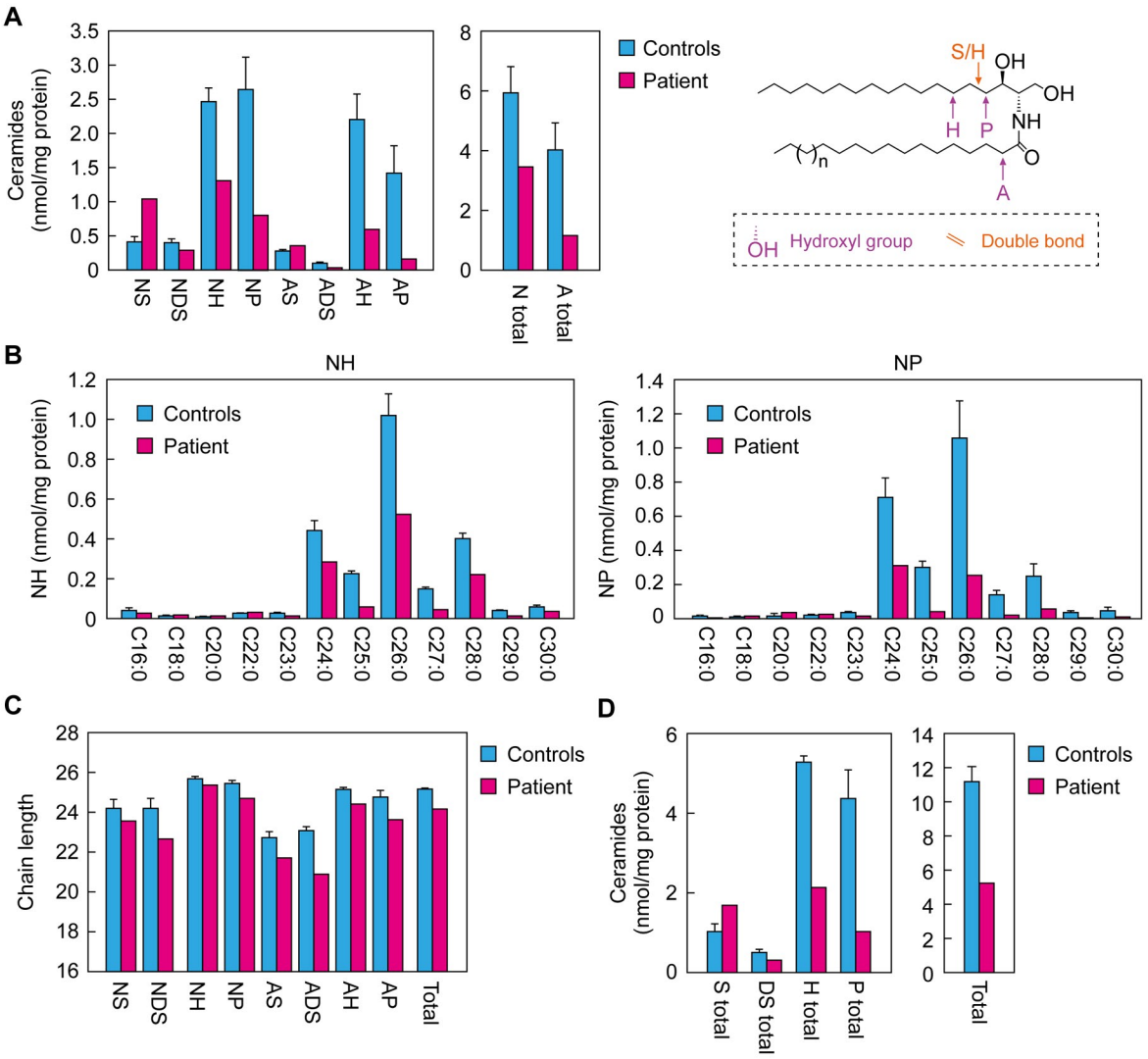

A
Ceramides (nmol/mg protein)
NS
NDS
NH
NP
AS
ADS
AH
AP
N total
A total
Controls
Patient
S/H
OH
OH
H
P
NH
O
A
OH Hydroxyl group
Double bond
B
NH
NH (nmol/mg protein)
NP
NP (nmol/mg protein)
C16:0
C18:0
C20:0
C22:0
C23:0
C24:0
C25:0
C26:0
C27:0
C28:0
C29:0
C30:0
C
Chain length
Total
D
Ceramides (nmol/mg protein)
S total
DS total
H total
P total
Total

**Fig. 5**

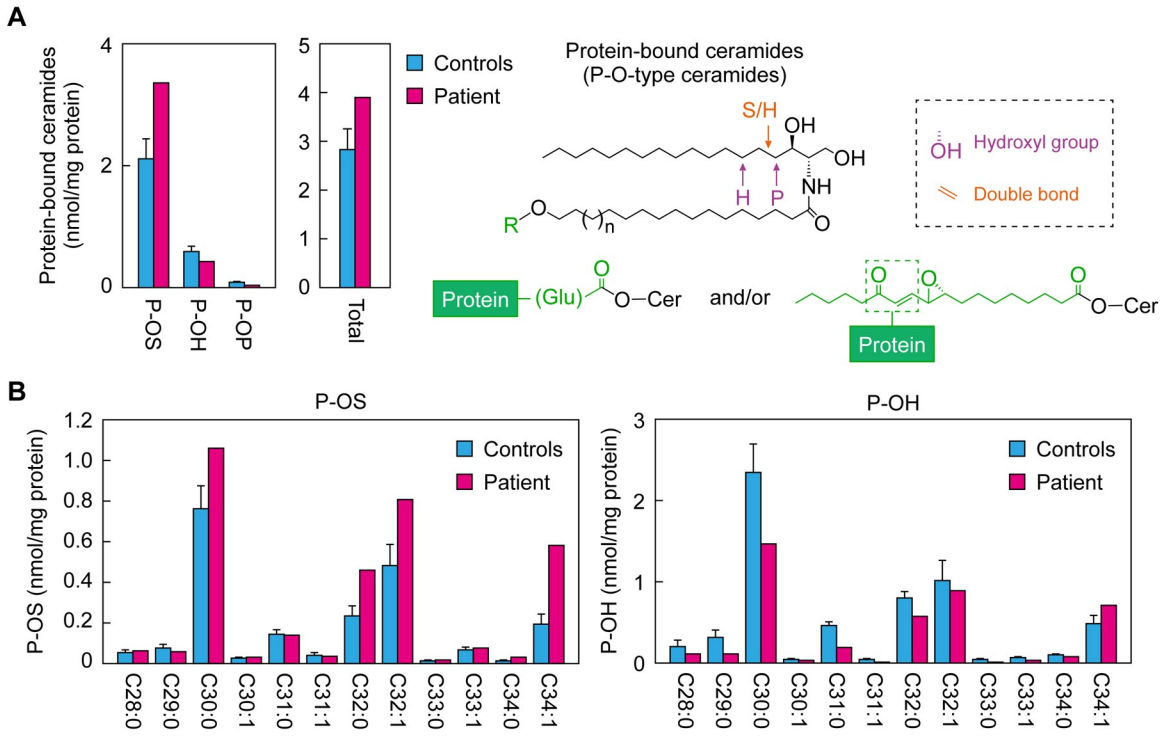
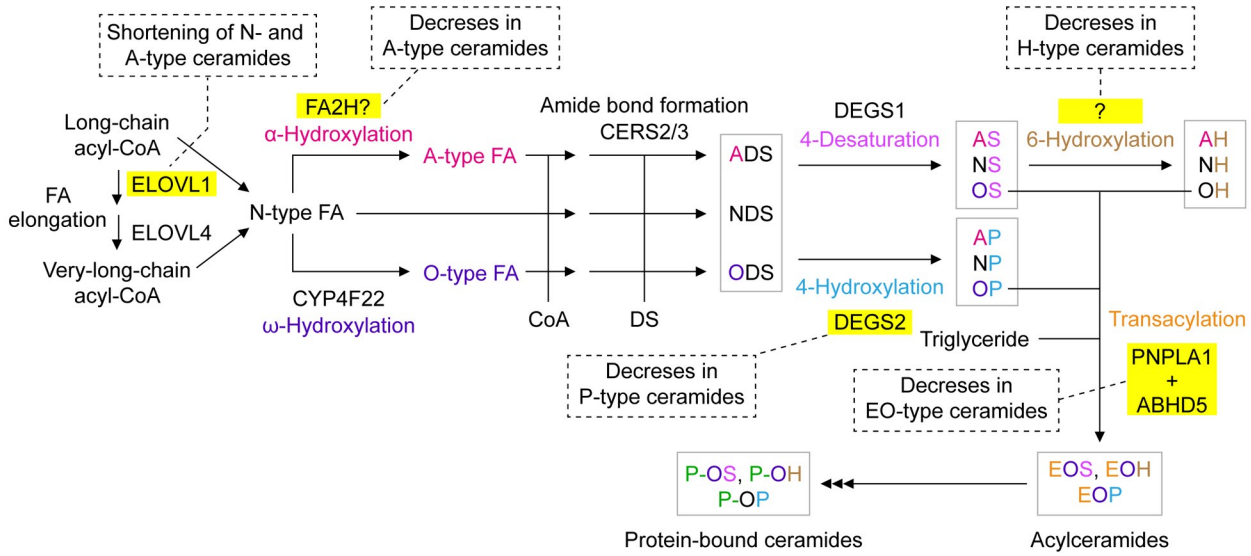

A
Protein-bound ceramides (nmol/mg protein)
P-OS
P-OH
P-OP
Total
Controls
Patient
Protein-bound ceramides
(P-O-type ceramides)
S/H
OH
NH
H
P
R
O
n
Hydroxyl group
Double bond
Protein
(Glu)
O–Cer
and/or
B
P-OS
P-OH
P-OS (nmol/mg protein)
P-OH (nmol/mg protein)
C28:0
C29:0
C30:0
C30:1
C31:0
C31:1
C32:0
C32:1
C33:0
C33:1
C34:0
C34:1

**Fig. 6**


Shortening of N- and A-type ceramides
Decreses in A-type ceramides
Decreses in H-type ceramides
Long-chain acyl-CoA
ELOVL1
FA elongation
ELOVL4
Very-long-chain acyl-CoA
N-type FA
FA2H?
α-Hydroxylation
A-type FA
CYP4F22
ω-Hydroxylation
O-type FA
Amide bond formation
CERS2/3
CoA
DS
ADS
NDS
ODS
DEGS1
4-Desaturation
AS
NS
OS
?
6-Hydroxylation
AH
NH
OH
AP
NP
OP
4-Hydroxylation
DEGS2
Triglyceride
Transacylation
PNPLA1
+
ABHD5
Decreses in P-type ceramides
Decreses in EO-type ceramides
P-OS, P-OH
P-OP
EOS, EOH
EOP
Protein-bound ceramides
Acylceramides