ORIGINAL

Molecular species profiles of plasma ceramides in different clinical types of X-linked adrenoleukodystrophy

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Abstract : X-linked adrenoleukodystrophy (X-ALD) is a genetic disorder associated with peroxisomal dysfunction. Patients with this rare disease accumulate very long-chain fatty acids (VLCFAs) in their bodies because of impairment of peroxisomal VLCFA β-oxidation. Several clinical types of X-ALD, ranging from mild (axonopathy in the spinal cord) to severe (cerebral demyelination), are known. However, the molecular basis for this phenotypic variability remains largely unknown. In this study, we determined plasma ceramide (CER) profile using liquid chromatography-tandem mass spectrometry. We characterized the molecular species profile of CER in the plasma of patients with mild (adrenomyeloneuropathy ; AMN) and severe (cerebral) X-ALD. Eleven X-ALD patients (five cerebral, five AMN, and one carrier) and 10 healthy volunteers participated in this study. Elevation of C26:0 CER was found to be a common feature regardless of the clinical types. The level of C26:1 CER was significantly higher in AMN but not in cerebral type, than that in healthy controls. The C26:1 CER level in the cerebral type was significantly lower than that in the AMN type. These results suggest that a high level of C26:0 CER, along with a control level of C26:1 CER, is a characteristic feature of the cerebral type X-ALD. J. Med. Invest. 70 : 403-410, August, 2023

Keywords **:** *ceramide, liquid chromatography-tandem mass spectrometry, X-linked adrenoleukodystrophy, peroxisomes*

INTRODUCTION

X-linked adrenoleukodystrophy (X-ALD) is a peroxisomal disorder characterized by progressive demyelination in the central and peripheral nervous systems and adrenal insufficiency (1, 2). X-ALD is attributed to a mutation in *ABCD1*, which encodes an ATP-binding cassette half-transporter (ALD protein) (3). Functional deficiency of ABCD1 results in the failure to transport very long-chain fatty acids (VLCFAs) into peroxisomes, where oxidative degradation of VLCFAs occurs. Consequently, this deficiency leads to the accumulation of VLCFAs, such as C24 : 0 FA and C26 : 0 FA, in the patient's body (3). Because the *ABCD1* gene is located on chromosome X, men with the mutated gene show apparent symptoms of X-ALD from childhood to adulthood, whereas half of female individuals with the mutated gene show mild neurological symptoms in old age. In men, the prevalence of X-ALD is estimated to be between 1 per 20,000–30,000 individuals (4).

Several clinical types of X-ALD, ranging from mild (axonopathy in the spinal cord) to severe (cerebral demyelination), have been known (3). The clinical severity of X-ALD is unrelated to the genotype of *ABCD1* mutation (3). Furthermore, the correlation between the extent of VLCFA accumulation in a patient's body and the clinical severity of X-ALD symptoms is not clear (5). In fact, there are patients with X-ALD with mild or no neurological symptoms despite elevated plasma VLCFA levels (6). At present, the mechanisms by which accumulated VLCFAs are involved in the development of neurological degeneration in X-ALD have not been elucidated, although VLCFA accumulation in the blood is known to be a robust diagnostic biomarker for X-ALD (4). Furthermore, the molecular mechanisms underlying phenotypic variability also remain unclear.

Ceramides (CERs) are intermediates of sphingolipid metabolism (7-9), and their *N*-acyl residues are predominantly occupied by VLCFAs in mammalian cells including neural cells (10, 11). CERs are biosynthesized from an acyl-CoA and a sphingoid base by the enzymatic activity of six CER synthases, each of which produces CERs with different *N*-acyl chain length (8). Among them, CER synthase 2 which produces CERs containing a VLCFA is highly expressed in many tissues including neural tissues (12, 13). Thus, the VLCFA-accumulating condition in cells of patients with X-ALD could cause the accumulation of VLCFA-containing CERs in many cells.

Intracellularly produced CER plays a signaling role for growth

Abbreviations :
AdolC, adolescent cerebral ; AMN, adrenomyeloneuropathy ; CC, childhood cerebral ; CER, ceramide ; FA, fatty acid ; LC-MS/MS, liquid chromatography-tandem mass spectrometry ; VLC, very long-chain ; X-ALD, X-linked adrenoleukodystrophy.

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arrest, differentiation, and apoptosis (14-19). Interestingly, CERs of different chain lengths have different physiological and pathophysiological roles (20). In fact, studies that forcibly alter the cellular CER composition by overexpression or silencing of ceramide synthases show that long-chain fatty acid-containing CERs are pro-apoptotic, while VLCFA-containing CERs are anti-apoptotic (21-28). A part of them are also released outside of cells as the component of lipoprotein particles (29) and exosomes (30, 31). The released CER-containing particles/vesicles can be uptaken by cells and affect their functions (32, 33). Considering the chain length-specific role of CER, clarification of the molecular species profiles of CER in patients with X-ALD is worth investigating to understand the pathology of X-ALD.

It has been shown that FA profile of plasma reflects those of body cells in X-ALD (34) to some extent. Based on this, the VLCFA in plasma is used as a robust biomarker for diagnosis of X-ALD (4). Here, we determined plasma CER levels using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and characterized the molecular profile of CER in the plasma of patients with X-ALD with mild (AMN) and severe (cerebral) symptoms. Our results suggest that a high level of C26 : 0 CER, along with a control level of C26 : 1 CER, is a characteristic of the cerebral type of X-ALD.

PATIENTS AND METHODS

Collection of human blood samples

The study protocol, which involved healthy participants, was approved by the Ethics Committee of Tokushima University Hospital (No. 2653). All methods were performed in accordance with the guidelines of the Declaration of Helsinki. The influence of sex on CER concentration was evaluated using fasting plasma samples from five men and three women. We found no significant differences in the concentrations of each CER species between groups. Thus, the analytical data were grouped without sex discrimination.

To evaluate the influence of fasting, blood samples were collected from five healthy participants (four men and one woman) after overnight fasting. Non-fasting blood samples were collected on another day from six healthy participants (four men and two women) who consumed breakfast. To evaluate the influence of sample preparation, fasting plasma and serum samples were collected on separate days from the members in the same group described above. It should be noted that there was no arbitrary manipulation when we recruited participants for each experiment. Venous blood from healthy volunteers was transferred to a blood collection tube containing EDTA-2K (NP-EK0405 ; Nipro Medical Co., Ltd., Tokyo, Japan) for plasma preparation, or with a serum-separating agent (NP-SP1016 ; Nipro Medical Co., Ltd., Tokyo, Japan) for serum preparation. Tubes for plasma preparation were immediately centrifuged (1,500 × g, 5 min, 3 °C), while the serum preparation was incubated first for 30–60 min at room temperature before centrifugation. The supernatants of each tube were collected and stored at −80 °C until lipid extraction and analysis.

Plasma samples were collected from patients who underwent X-ALD at the Graduate School of Medicine, Gifu University. The study protocol was approved by the Ethics Committees of the Graduate School of Medicine, Gifu University (No. 29-286) and the Ethics Committees of Tokushima University Hospital (No. 2726). All methods were performed in accordance with the guidelines of the Declaration of Helsinki. Informed consent was obtained from all participants or their legal guardians. Table 1 shows the sample size and mean age of patients with X-ALD in each group as well as those of healthy volunteers. Patients' characteristics were summarized in the Supplementary Table 1. Plasma from 11 patients with X-ALD and 10 healthy volunteers were analyzed in a blinded manner, in which the specimen information was withheld from the investigator until the experiment was completed.

Materials

N-lauroyl-D-*erythro*-sphingosine (C12 : 0 CER), *N*-palmitoyl-D-*erythro*-sphingosine (C16 : 0 CER), and *N*-lignoceroyl-D-*erythro*-sphingosine (C24 : 0 CER) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). CER from bovine brain was obtained from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of reagent, HPLC, or LC-MS grade.

Extraction of lipids from blood samples

In a typical analysis, 0.1 mL plasma or serum was subjected to lipid extraction using the Bligh and Dyer method (35) for LC-MS/MS. Briefly, 0.1 mL of plasma or serum were mixed with 1.0 mL of chloroform, 2.0 mL of methanol, and 0.7 mL of KCl solution (40 mg/mL) on ice, and then sonicated for 10–20 s. After adding 50 pmol of C12 : 0 CER as an internal standard, 1.0 mL each of chloroform and water were added to the mixture for phase separation. After acidification of the aqueous layer to pH 2–3 using 5 M HCl, the mixture was centrifuged at 1,100 × *g* for 5 min. The chloroform layer was collected and the remaining upper layer was mixed with 2.0 mL of chloroform. After centrifugation, the chloroform layer was collected again. The combined chloroform layers were evaporated under a stream of nitrogen. The recovered lipids were dissolved in 0.8 mL of methanol and then filtered through a non-polar filter (Chromatodisc 4 N, 0.2 μm, Kurabo, Osaka, Japan). The filtrate was evaporated again and the resultant lipids were dissolved in 0.1 mL of methanol/formic acid (99 : 1, v/v) containing 5 mM ammonium formate for LC-MS/MS analysis.

Analyses of CERs using LC-MS/MS

CER analyses were performed using a quadrupole-linear ion trap hybrid mass spectrometer 4000 Q TRAP (Applied Biosystems/MDS Sciex, Concord, ONT, Canada) operating in multiple reaction monitoring positive ionization mode with an Agilent 1100 liquid chromatograph (Agilent Technologies, Wilmington DE, USA) and an HTSPAL autosampler (CTC Analytics AC, Zwingen, Switzerland). CER species were detected using the multiple reaction monitoring mode and the detection condition of

Table 1. Sample size, mean age, and clinical type of each X-ALD patient group and healthy volunteers

	Healthy		X-ALD			
	Male	Female	CC (male)	AdolC (male)	AMN (male)	Carrier (female)
n	6	4	4	1	5	1
Age (years)	32.0 ± 11.4	24.3 ± 2.2	6.8 ± 1.3	14	50.6 ± 19.5	79

Age values are mean ± S.D. CC, childhood cerebral ; AdolC, adolescent cerebral ; AMN, adrenomyeloneuropathy.

each CER species is summarized in Table 2. Lipids were separated using a Cadenza CD-C18 column (50×2 mm, 3μm, Imtakt Corp., Kyoto, Japan) at 42 °C and eluted with methanol/formic acid (99 : 1, v/v) containing 5 mM ammonium formate as the mobile phase at an isocratic flow of 300 μL/min. The ratios of peak areas of objective CER species to internal standard (*d*18 : 1/12 : 0 CER) were used to determine the amount of each CER species. Standard curves for correcting the ionization efficiency were prepared by measuring the CER mixtures consisting of several molar ratios of C12 : 0 CER and C16 : 0 or C24 : 0 CER (Fig. 1C). Reciprocal values of slopes of standard curves prepared with C16 : 0 (1.00) and C24 : 0 CER (1.47) were used as correction factors of ionization efficiency tentatively for long-chain (< C22) and very long-chain (≥ C22) CER (VLC-CER), respectively.

Statistical analysis

Statistical analyses were performed using the Student's *t*-test (to compare two groups) and one-way ANOVA with a post-hoc Tukey test (to compare more than two groups). In all analyses, a *p*-value of < 0.05 was considered statistically significant.

RESULTS

Plasma or serum CER levels in healthy participants

The structures of the precursor and product ions selected for CER detection using LC-MS/MS are shown in Fig. 1A. Retention times of each CER species measured in this study were determined by analyzing CER mixture from bovine brain (data not shown). Good separation of each CER species in human plasma was achieved under these conditions (Fig. 1B). Standard curves were prepared by measuring the CER mixtures consisting of several molar ratios of C12 : 0 CER and C16 : 0 or C24 : 0 CER (Fig. 1C). Peak area ratios of long-chain C16 : 0 and very long-chain C24 : 0 CERs to those of the internal standard were increased lineally in a concentration-dependent manner.

Table 2. Detection condition of each CER species

CER species	Precursor ion $[M + H]^+$	Product ion	DP (V)	CE (V)	CXP (V)
C12 : 0	482.6			35	
C16 : 0	538.5			40	
C18 : 0	566.7			42	
C20 : 0	594.7			45	
C22 : 0	622.8	264.4	40	48	16
C24 : 0	650.9			50	
C24 : 1	648.6			50	
C26 : 0	678.9			52.5	
C26 : 1	676.9			52.5	

DP, declustering potential ; CE, collision energy ; CXP, collision cell exit potential.

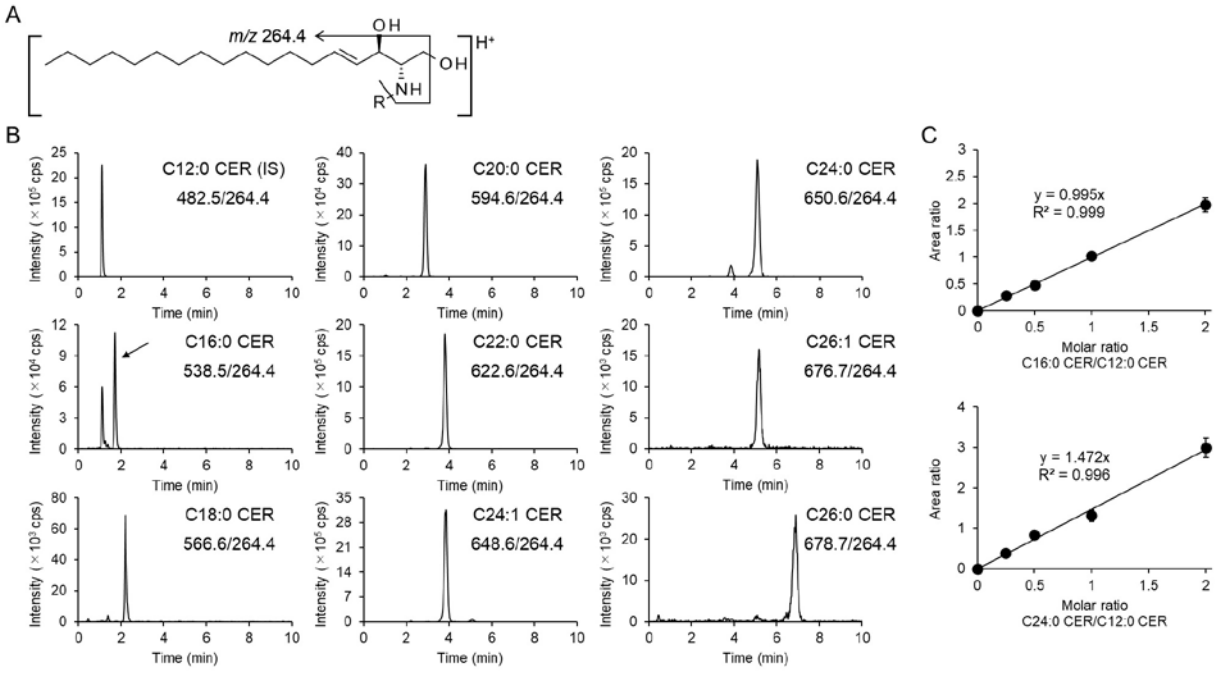

Fig 1. Determination of CER molecular species in human plasma using LC-MS/MS.
A) Structure of the precursor and product ions used for detecting CER. B) Plasma lipids from healthy participants were extracted and analyzed using LC-MS/MS with C12 : 0 CER as an internal standard. Typical liquid chromatograms of CER in plasma are shown. C) Standard curves were prepared using varying concentrations of C16 : 0 CER or C24 : 0 CER and fixed amount of C12 : 0 CER.

We found that the dominant plasma CER species in human participants were C24 : 0 CER (approximately 4.5 μM), followed by C24 : 1 CER (2.0 μM) and C22 : 0 CER (1.0 μM) (Fig. 2A). No significant difference was observed in the levels of CER species between overnight-fasted and non-fasted plasma samples. The concentrations and compositions of plasma CER species in male participants were similar to those in female participants (Fig. 2B). Furthermore, CER levels in the plasma and serum were not significantly different (Fig. 2C), which agrees with previous reports (36, 37).

Plasma CER levels in patients with X-ALD

Patients with X-ALD exhibit an accumulation of VLCFA, specifically C24 : 0 and C26 : 0 FAs, in their blood and tissues because of genetic defect of transporting system of VLCFA into the peroxisome (3). Fig. 3A shows the levels of CER species in individual plasma samples from healthy controls ($n = 10$, open columns) and patients with X-ALD ($n = 11$, filled columns). The plasma concentrations of C24 : 0 (1.5-fold), C26 : 0 (4.3-fold), and C26 : 1 CERs (3.3-fold) in patients with X-ALD were significantly higher than those in healthy participants (Fig. 3B). In contrast, the concentrations of C20 : 0 (0.66-fold), C22 : 0 (0.67-fold), and C24 : 1 CERs (0.62-fold) in the plasma of patients with X-ALD were slightly but significantly lower than those in healthy controls. The ratio of C24 : 0 FA/C22 : 0 FA or C26 : 0 FA/C22 : 0 FA in total plasma lipids was used for the diagnosis of X-ALD (4). When we applied this methodology to the CER, the variation of individual values in each group was reduced (Fig. 3C), and VLC-CER accumulation in patients with X-ALD became more apparent. The levels of C24 : 0, C26 : 0, and C26 : 1 CERs in patients with X-ALD were 2.4-, 6.3-, and 4.7-fold higher, respectively, relative to those in healthy controls (Fig. 3D).

In an extended analysis, the patients were divided according to clinical severity (3) (Fig. 4). The childhood cerebral type (CC) and adolescent cerebral type (AdolC) are characterized by progressive demyelination/neurodegeneration in the central nervous system. Here, we classified patients with CC and AdolC into the "cerebral" (severe) type. Adrenomyeloneuropathy (AMN) is characterized by relatively mild symptoms with neurodegeneration limited to the spinal cord. Patients with AMN and a female carrier were classified into the "AMN" (mild) type. We observed a significant increase in the level of C24 : 0 CER in patients with AMN compared to that in healthy participants. The levels of C26 : 0 CERs were significantly higher in both the cerebral and AMN groups than in healthy participants (Fig. 4A). Analysis of the data as values relative to C22 : 0 CER showed a significant elevation in C24 : 0 CER and C26 : 0 CER levels in both types of patients with X-ALD (Fig. 4B). In contrast, CERs with unsaturated VLCFAs such as C24 : 1 and C26 : 1 CER, seemed to change in a clinical type-dependent manner. The C24 : 1 CER level of the cerebral group was significantly lower than that of healthy participants. The C26 : 1 CER level in the cerebral group was similar to that in healthy participants and significantly lower than that in the AMN group (Fig. 4A). The tendency unsaturated VLC-CER levels in the cerebral group are lower than those in the AMN group is consistent with the findings of a recent investigation conducted in the Netherlands (38).

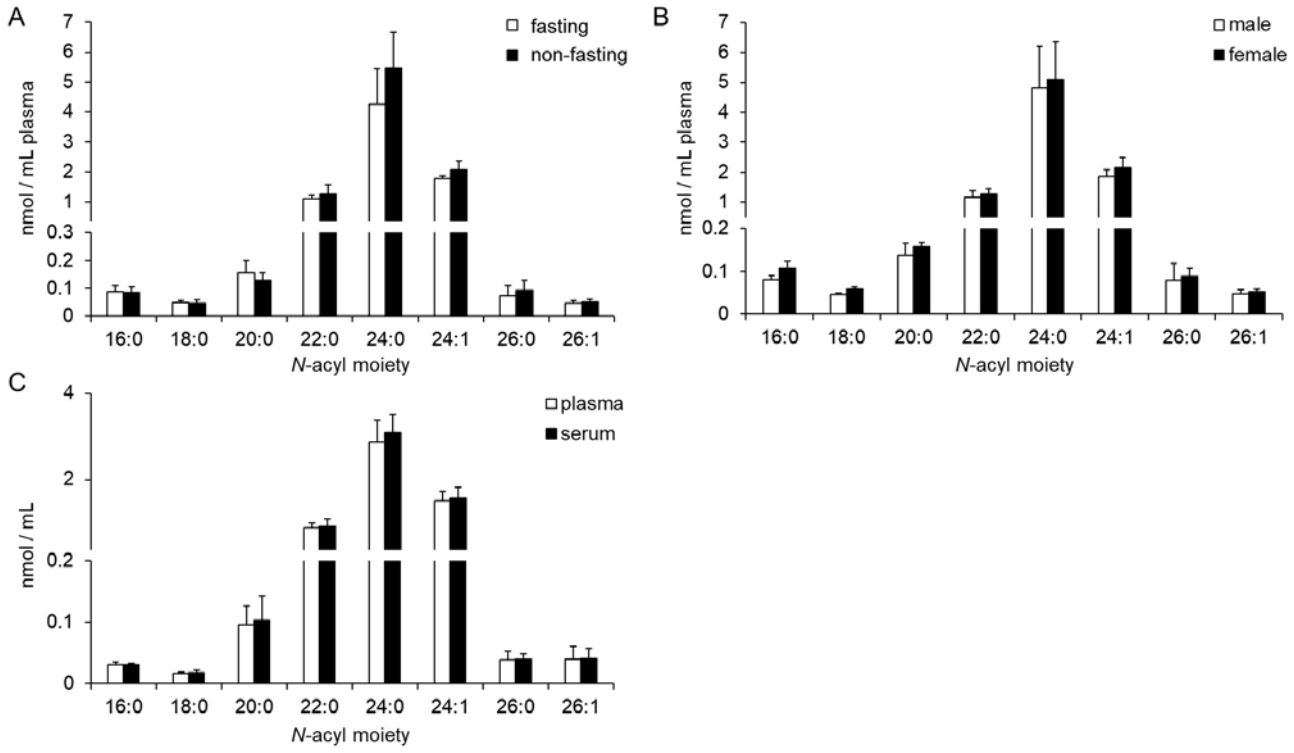

Fig 2. CER profiles in human plasma or serum.
Plasma or serum lipids from healthy participants were extracted and analyzed using LC-MS/MS. A) CER profiles in the plasma of healthy participants with ($n = 5$) or without overnight fasting ($n = 6$). B) Plasma CER profiles in men ($n = 7$) and women ($n = 3$). C) CER profile in plasma or serum ($n = 5$ for each group). Values are presented as the mean ± S.D.

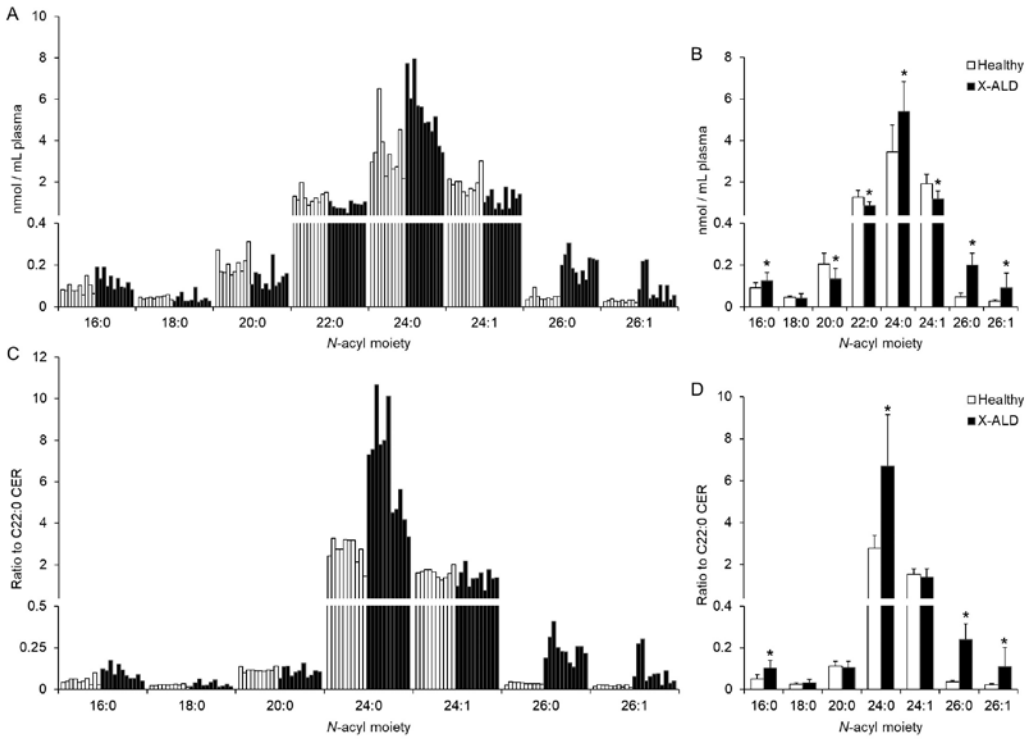

Fig 3. CER profiles in plasma of patients with X-ALD and healthy controls.
Plasma lipids of patients with X-ALD and healthy controls were extracted and subjected to LC-MS/MS. A) CER concentrations in individual plasma samples. $n = 10$ (healthy controls, open columns) and 11 (X-ALD, filled columns). B) Mean values of (A). C) Ratio of the concentration of each CER to that of C22 : 0 CER. D) Mean values of (C). Values are presented as mean ± S.D. $^{*}p < 0.05$, compared to healthy controls (Student's t-test).

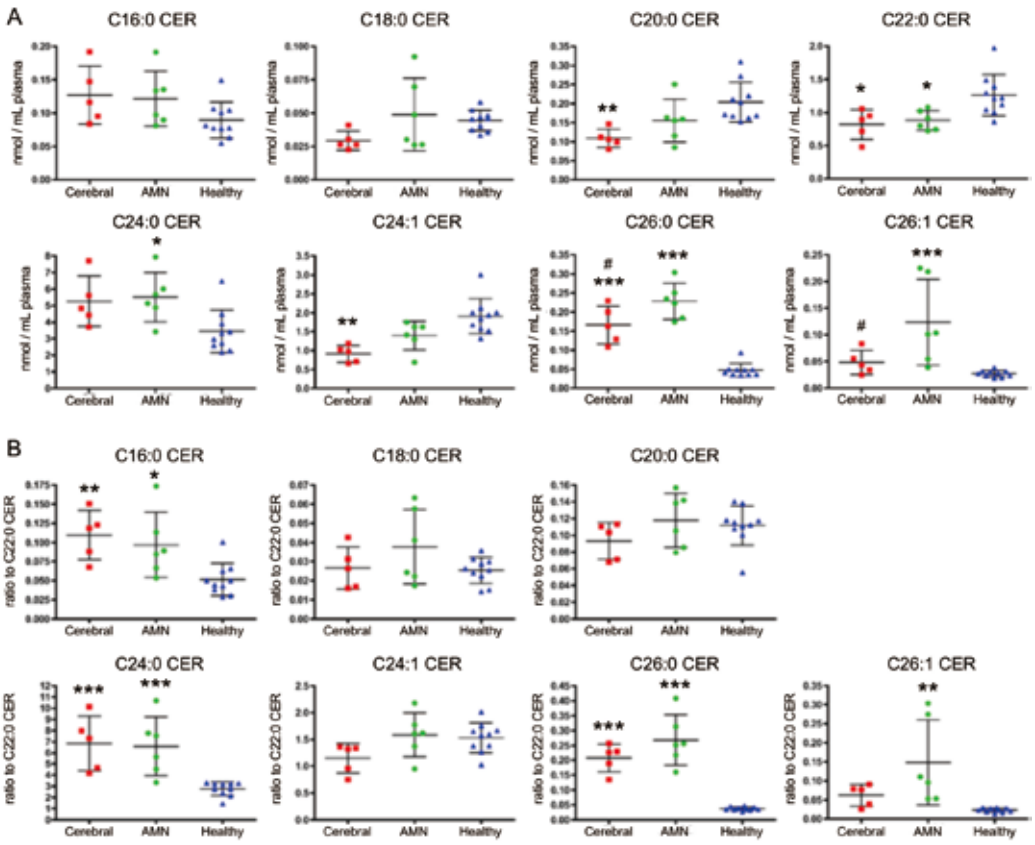

Fig 4. Comparison of plasma CER profiles of patients with X-ALD with different clinical severity.
A) Concentrations of CER species in the cerebral group, AMN group, and healthy controls. B) Ratio of concentration of each CER to that of C22 : 0 CER in the cerebral group, AMN group, and healthy controls. Values are presented as mean ± S.D. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$ vs. healthy controls and $^{\#}p < 0.05$ vs. the AMN group (one-way ANOVA with a post-hoc Tukey test).

DISCUSSION

In this study, we determined the molecular species profile of CER in plasma and serum using LC-MS/MS. We found that the plasma CER profiles in male and female participants were similar, and that C24 : 0, C24 : 1, and C22 : 0 CERs were the major molecular species in the plasma. We also found that the plasma and serum CER profiles were not significantly different, as shown in previous studies (36, 37).

X-ALD is a demyelinating disease characterized by the systemic accumulation of VLCFA, particularly C24 : 0 and C26 : 0 FAs, due to the inability to import VLCFA into peroxisomes that carry out the oxidative degradation of VLCFA (3). The clinical phenotypes of patients with X-ALD are highly variable and there is no apparent correlation between the severity of X-ALD symptoms and the degree of VLCFA accumulation (3). We determined the CER in the plasma of patients with mild (AMN) and severe (cerebral) X-ALD using the LC-MS/MS-based method developed in this study. We found that lower levels of unsaturated VLC-CER, such as C24 : 1 CER and C26 : 1 CER, are features of cerebral X-ALD that distinguish it from AMN. This was unexpected, because our recent study confirmed that unsaturated VLCFAs with chain lengths greater than C20 are also peroxisomal β-oxidation substrates (39). Similar results have been observed in recent European (38) and Korean (40) cohort studies. Richmond *et al*. reported that a CER with an unsaturated VLCFA in the plasma was significantly lower in the cerebral group than in the non-cerebral group (38). Lee *et al*. have shown that CER with C26 : 1 in fibroblasts is considerably low in cerebral group as compared to AMN group (40). Findings of these reports and our results suggest that synthesis of unsaturated VLCFA-CER is down-regulated in cerebral group, as hypothesized by Lee *et al*. (40). Intriguingly, it has been reported that pharmacological induction of stearoyl-CoA desaturase 1, which produces C16 : 1 and C18 : 1 FAs from C16 : 0 and C18 : 0 FAs and thereby increases levels of monounsaturated VLCFAs, reduces the toxicity of VLCFAs in fibroblasts derived from patients with X-ALD (41). The accumulation of saturated VLC-CER without the accumulation of unsaturated VLC-CER may have a stressful effect on cellular homeostasis in neuronal tissues.

In conclusion, elevation of C26 : 0 CER level was found to be a common feature of X-ALD, regardless of the severity of the clinical type. C26 : 1 CER levels were significantly lower in the cerebral group than in the AMN group. Although plasma CER composition of healthy participants in this study is similar to those in previous reports that analyzed CER composition in children (42, 43) and adults (44, 45), there is a limitation that ages of healthy controls were not matched completely to those in patients with X-ALD. Small sample size in patients with X-ALD is also the limitation in this study. Further studies with a larger number of patient samples as well as the age-matched healthy controls are needed to confirm clinical type-specific CER profiles.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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Supplementary Table 1. Summary of patients' characteristics

Clinical phenotype	Sex	Age (years)	Adrenal insufficiency	Administration of Steroid	Administration of Lorenzo's oil
CC	M	8	-	-	-
	M	7	-	-	-
	M	7	-	-	-
	M	5	+ (high ACTH level)	-	-
AdolC	M	14	-	-	-
AMN	M	61	-	-	-
	M	39	-	-	-
	M	68	+	+	-
	M	63	-	-	-
	M	22	-	-	-
Carrier	F	79	-	-	-

M, male ; F, female.