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1 **Quantitative determination of plasma cholesteryl ester levels in Japanese preadolescents**
2 **from the Hokkaido study using liquid chromatography/tandem mass spectrometry**

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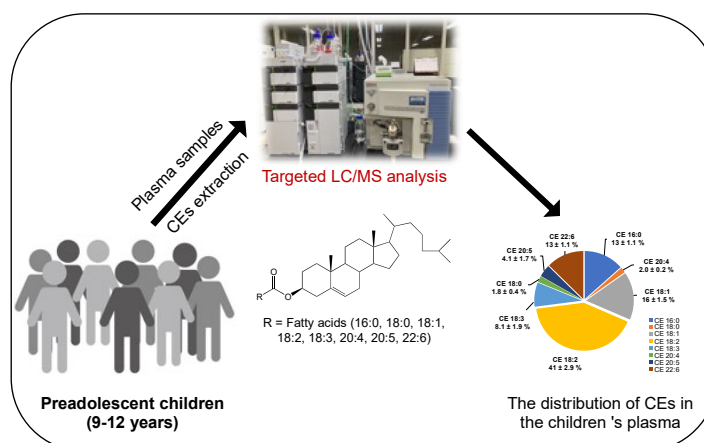
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Abstract

Cholesteryl esters (CE) are sterols comprising various fatty acyl chains attached to a cholesterol hydroxyl moiety. CEs are often considered plasma biomarkers of liver function; however, their absolute concentrations in the plasma of Japanese preadolescents have not been well explored. This study aimed to determine the plasma CE levels in Japanese preadolescents of different sexes, ages, and body weights living in Hokkaido, Japan using targeted liquid chromatography/tandem mass spectrometry. The analysis was performed on the non-fasting plasma of preadolescents aged 9–12 years ($n = 339$ healthy volunteers; 178 boys and 161 girls) from Sapporo, Hokkaido, Japan. The analysis results showed that the total CE levels in boys and girls were 871 ± 153 and 862 ± 96 pmol/ μ L, respectively. CE 18:2 ($41 \pm 2.9\%$) was found to be the most abundant species followed by CE 18:1 ($16 \pm 1.5\%$) and CE 16:0 ($13 \pm 1.1\%$). The ω -3 fatty acid-containing CEs such as CE 18:3 and CE 20:5 were significantly lower in girls than in boys. Despite the different ages, CEs were tightly regulated in the plasma of children's, and the total CEs ranged between 844 and 906 pmol/ μ L in boys and 824 and 875 pmol/ μ L in girls. The participants were further classified into three groups based on their body mass index underweight ($n = 237$), normal weight ($n = 94$), and overweight ($n = 8$). Most of the quantified CEs were accumulated in the overweight group. Interestingly, CE 18:3 was significantly upregulated in the overweight group compared to that in the normal range, and the area under the receiver operating characteristic curve was 0.73, suggesting that it could be a possible marker for obesity. This study marks the initial investigation of absolute CE levels in the plasma of children and can help elucidate the relationship between CEs and childhood obesity.

Graphical abstract



Keywords: Cholesterol, Cholesteryl esters, Hokkaido study, Liquid chromatography, Mass spectrometry, and Childhood obesity.

1. Introduction

Cholesterol is a major sterol lipid in mammals and an important structural component of cell membranes. It plays a crucial role in the maintenance of various cellular activities via its metabolism to various derivatives [1]. Cholesterol metabolism plays a crucial role in the regulation of its circulating levels, which in turn has a significant impact on human heart health. Free cholesterol accumulates in proteins, and is, involved in signal transduction and intercellular transport [2]. Cholesterol in the blood is transformed into cholesteryl ester (CE) by lecithin cholesterol acyltransferase (LCAT) [3]. CE comprises various long-chain fatty acids linked to the hydroxyl group of the cholesterol moiety. Both cholesterol and CEs in plasma are integrated into circulating lipoproteins, and abnormalities in their metabolism are linked to several diseases [4].

CE is a type of lipid used for storage and transport, and 70-80% of cholesterol in blood exists in an ester form [3,5]. In humans, the normal plasma concentrations of cholesterol + CE were determined to be in the range of 1.3-2.6 g/L. CEs are the primary components of high-density lipoprotein (HDL) and low-density lipoprotein (LDL) particles [6,7]. LDL transfers CEs to the peripheral tissues for storage whereas HDL transports CEs from peripheral cells to the liver for excretion [8]. Increased plasma concentrations of CE are important risk factors for cardiovascular diseases [9]. Thus, a decrease in the plasma concentrations of CE owing to LCAT deficiency can cause severe liver damage. Hence, monitoring of blood CE is very important because it plays a significant role as a biomarker for various diseases [10].

CEs are often measured by using enzymatic, gas chromatography (GC) and liquid-chromatography/mass spectrometry (LC/MS) based methods [11-13]. Efforts have been made to determine blood CE levels in both adults and children. In our previous study, we applied the targeted LC/MS method to determine the CE levels in the serum samples of healthy Japanese volunteers [57 ± 14 yrs., mean age $\pm$ standard deviation (SD)] and patients with renal disease (49 males and 15 females; 54 ± 17 years, mean age $\pm$ SD). In both groups, CE 18:2 (1.57 ± 0.32 mmol/L, mean $\pm$ SD) was found to be the most abundant in the serum samples followed by CE 18:1 (1.17 ± 0.23 mmol/L) and CE 16:0 (0.68 ± 0.16 mmol/L) [13]. In a study involving Japanese adults from Hokkaido ($n = 545$, aged 35-79 years), serum analysis revealed similar abundances: CE 18:2 (1.04 ± 0.38 μ mol/L) $>$ CE 18:1 (0.79 ± 0.31 μ mol/L) $>$ CE 16:0 (0.61 ± 0.24 μ mol/L) [12].

Plasma CEs were measured in 3- and 12- year-old Finnish children ($n = 226$) using GC, and the authors found an abundance of CE 18:2 (49.2%), CE 18:1 (22.6%), CE 16:0

(10.9%), and CE 20:4 (5.4%); however, their absolute levels were not reported in the study [14]. In a German study involving 115 healthy subjects aged 1 to 26 years, the plasma CEs were analyzed by using GC, and CE 18:1 was found to be the most abundant in the plasma at birth (28.01%) but which decreased to 20.28 % in 20 to 26-year-old adults(In contrast, CE 18:2 (6.82% at birth) drastically increased with age as follows: 1 to 5 years (47.04%), 5 to 10 years (50.34 %), 10 to 15 years (49.06 %), and 20 to 26 years (50.44 %) respectively [15]. Efforts were also made to measure the variation in CEs in Finnish infants (n = 591; 7- and 13-months old) with various dietary interventions, and CE 18:2 was found to be the most abundant followed by CE 18:1. Formula-fed infants exhibited significantly higher CE 18:2 levels than breastfed infants. [16, 17].

Although several studies being conducted to determine CE levels in adults and children of different populations, to the best of our knowledge, no studies on Japanese children have been reported. Moreover, a study involving Japanese children aged 6-15 years showed that plasma. CE transfer activity was significantly increased in obese children compared to normal-weight children [18]. Therefore, it is important to determine the absolute levels of CE in Japanese children. In this study, we aim to obtain the absolute amounts of eight molecular species of CEs in the plasma of the general population from a birth cohort study of preadolescents aged 9-12 years using targeted LC/MS and compare the variations in the values with respect to sex, age, and body weight.

2. Materials and methods

2.1. Samples

Participants were selected and plasma samples from non-fasting individuals were obtained from the Hokkaido birth cohort study on environment and children's health (The Hokkaido study), and stored at -80 °C [19-24]. The study included 339 preadolescents (178 boys and 161 girls) from the general population of Sapporo City, Hokkaido, Japan. These children were aged of 9-12 years and were part of a birth cohort study conducted from 2017 to 2019. This study was approved by the ethical review board (ERB 16 |71, 2017, Sept 27). The height and weight of the children were measured at pediatric clinics on the same day as blood collection. The research objectives and methods were explained to the participants, and a written informed consent was obtained from the parents, as well as from all children.

2.2. Statistical analysis

GraphPad Prism 8.0.1 (GraphPad Software Inc., La Jolla, CA, USA) was used for the statistical analysis. The Student's *t*-test was used to comprehensively analyze the CE levels in boys and girls. A one-way analysis of variance (ANOVA) with Tuckey's multiple comparison test or Dunnett's test was used for comparisons involving three or more groups. Statistical significance was set as $p < 0.05$. All results were expressed as mean $\pm$ SD.

2.3. Analytical methods

LC/MS-grade methanol (MeOH) and hexane were purchased from Kanto Chemical Co, Inc. (Tokyo, Japan). Ammonium acetate (1M) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Cholesteryl palmitate (CE 16:0), cholesteryl stearate (CE18:0), cholesteryl oleate (CE 18:1), cholesteryl linoleate (CE 18:2), cholesteryl linolenate (CE 18:3), cholesteryl arachidonate (CE 20:4), cholesteryl eicosapentaenoate (CE 20:5), cholesteryl docosahexaenoate (CE22:6) and their $^2\text{H}_3$ substituted analogs were synthesized in our laboratory [25]. The quantification method for the CEs in the serum, urinary supernatant, and urinary sediment of patients with renal disease has been published previously by our research group [13]. A TSQ Quantum Access MAX mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and an ultra-high-performance LC system (Shimadzu, Kyoto, Japan) were used for quantification. The Accucore C18 column (50 $\times$ 2.1 mm ID, 2.6 μm particle size; Thermo Fisher Scientific Inc., Waltham, MA, USA) was used for the separation, and the column oven was set at 40 $^\circ\text{C}$. The mobile-phase gradients were 5 mM ammonium acetate in methanol/water = 1/1 (pump A) and isopropanol (pump B). The gradients were set as follows: 0-1 min (65% A, 35% B); 1-3 min (gradually changed to 35 % A, and 65% B); 3-5 min (10% A, 90% B); 5-7 min (gradually changed to 35 % A, 65% B); and 7-10 min (65% A, 35% B) at a flow rate of 0.2 mL/min. Ionization was performed using atmospheric pressure chemical ionization and detected by selective reaction monitoring. Method validation has been previously published [13]. To evaluate the linearity of this method, a series of diluted CE standard solutions were prepared with final concentrations of 0.005, 0.05, 0.5, 1, 5, and 10 pmol/ μL using a respective deuterated internal standard; (final concentration: 0.05 pmol). The CE concentrations in the sample were calculated from the constructed linearity curves.

3. Results and discussion

In the Hokkaido study, a total of 339 children participated 178 boys and 161 girls. A comparison of the preadolescents' characteristics in terms of height, weight, and body mass index (BMI) was performed according to sex and age (Table 1). The results are expressed as

mean $\pm$ SD. Individuals were further classified into three groups based on their BMI [which was calculated as weight (kg) divided by height squared (m^2): underweight = $<18.5 \text{ kg/m}^2$, normal weight = $18.5 - 25.0 \text{ kg/m}^2$, and overweight = $>25.0 \text{ kg/m}^2$ [18]. The mean BMI of boys ranged from 17.4 ± 2.5 to 19.0 ± 3.3 , whereas, that of girls, ranged from 16.2 ± 2.2 to 18.4 ± 2.6 . In the case of both boys and girls, a slight increase in the BMI was observed in 9- and 12-year-old children. The number of underweight children was the highest (boys ($n = 117$, 34.5%) and girls ($n = 120$, 35.3%)), whereas that of overweight children was the lowest [boys ($n = 6$ 1.8 %) and girls ($n = 2$, 0.6%)].

The mean concentrations of individual CEs and total CE levels in the plasma of children of different sexes and ages are shown in Table 2. The concentration ranges for the plasma of children (irrespective of sex and age) were: 109-121 pmol/ μL for CE 16:0, 14.4-16.9 pmol/ μL for CE 18:0, 137-141 pmol/ μL for CE 18:1, 343-375 pmol/ μL for CE 18:2, 61.8-79.2 pmol/ μL for CE 18:3, 17.3-18.2 pmol/ μL for CE 20:4, 32.1-41.9 pmol/ μL for CE 20:5, and 106-113 pmol/ μL for CE 22:6. A comparison of these results with the CE levels measured in the serum samples of 23 adults' (57 ± 14 years) revealed significantly higher levels in the adult's serum samples (CE 16:0, 680 pmol/ μL ; CE 18:0, 40 pmol/ μL ; CE 18:1, 1170 pmol/ μL ; CE 18:2, 1570 pmol/ μL ; CE 18:3, 70 pmol/ μL ; CE 20:4, 250 pmol/ μL ; and CE 22:6, 50 pmol/ μL). Notably, increased plasma concentration of CE is an important risk factor for cardiovascular diseases [9].

The CE data clearly showed that although a small variation was observed at the molecular species level between different age groups, the total CE levels were tightly regulated in the plasma. The total CEs ranged from 844-906 pmol/ μL in boys and from 824-875 pmol/ μL in girls. The percentage distribution of the CE molecular species in the plasma of children is represented using a pie chart, as shown in Figure 1. All eight targeted CEs were detected in the plasma samples of the children; the results showed that CE 18:2 was the most predominant species which contributed 41% to the total CEs, followed by CE 18:1 with a contribution of approximately 16%. CE 18:0 was the least abundant molecular species in the children's plasma. In the 1980s, a study was conducted in Finland to determine the plasma CEs in 407 children aged 3-12 years, and the results showed an abundance of CE 18:2 (approximately 49-51%), which is consistent with our results [14]. Further, the authors reported a positive correlation between the dietary polyunsaturated fatty acid to saturated fatty acid ratio and the high levels of plasma CE 18:2. A clinical study involving the determination of CEs in serum samples of 566 volunteers (aged 19-79 years) from Beijing, China also revealed the abundance

of CE 18:2 with a mean percentage of more than 50%. However, CE 20:5 and CE 22:6 were reported to be the minor species, which is contradictory to our results. This possibly could be due to the lower consumption of fish in their diet [26].

The differences in the CE levels according to sex are evaluated as shown in Figure 2. The CE levels in boys and girls ranged from 15 ± 3 pmol/ μ L to 359 ± 34 pmol/ μ L and from 15 ± 3 pmol/ μ L to 358 ± 61 pmol/ μ L, respectively. The total CE represents the sum of the concentrations of each CE species analyzed in this study. The total CE levels for boys and girls were 871 ± 153 pmol/ μ L and 862 ± 96 pmol/ μ L, respectively. A significant difference was observed in the levels of CE 18:3, CE 20:5, and the total CEs for boys and girls, with a lower level observed in girls. No significant sex-specific differences were observed in the levels of CE 18:2, CE 16:0, CE 18:0, CE 18:1, CE 20:4, and CE 22:6 molecular species. Although there is a lack of data in the literature regarding the sex-specific differences in the plasma CE levels, a Finns study on adults showed no difference between the serum CEs of men and women [27]. Another Finns study involving 3- and 12-year-old children showed a sex-specific difference in plasma CEs [14]. These results are consistent with our findings in the case of Japanese preadolescents.

The differences in CE levels according to age are shown in Figure 3. The total CE levels in all children belonging to the each age groups 9, 10, 11, and 12 years were 894 ± 104 , 876 ± 107 , 840 ± 71 , and 855 ± 98 pmol/ μ L, respectively. Significant differences were observed in the CE 18:0 and CE 18:3 levels of children aged 9, 10, and 12 years, with a slight decrease observed for those aged 12 years. The CE 18:2 and total CE levels exhibited significant differences in children aged between 9 and 11 years, with a slight decrease in those aged 11 years. The remaining species exhibited no significant differences in the different age groups. The living area and age-dependent changes in the plasma CE concentrations of Finnish children were investigated, and mixed variations among each CE species were observed. For example, CE 18:2 increased in the plasma of 12-year-old children's compared to that in 3-year-old children [14]. A German study involving 116 healthy subjects aged 1 to 26 years old showed clear evidence of age-specific changes in the plasma CE levels [15]. Although the variations observed were different for different CE molecular species an increased CE 18:2 level was observed at an early age (47.04% in children aged 1 to 5 years) compared to the at birth level (15.2%).

The relationship between the plasma CEs levels and childhood obesity is shown in Figure 4A. Children were characterized as underweight, normal weight, or overweight based

on their BMI. The total CE level in the plasma samples was found to be 2108 ± 799 pmol/ μ L in underweight children, 2578 ± 962 pmol/ μ L in normal weight children, and 1940 ± 615 pmol/ μ L in overweight children. CE 18:3 and CE 20:4 exhibited a significant ($p < 0.05$) increase in the plasma levels of overweight children compared to those in underweight children. The area under the curve (AUC) obtained from the receiver operating characteristic (ROC) curve for CE 18:3 of normal and overweight children was 0.73 (Figure 4B), suggesting that CE 18:3 could be a possible marker for obesity. The total CEs were significantly lower in the plasma of underweight children's than that in normal-weight children. In a Norwegian study, it was revealed that maternal prenatal cholesterol levels are important in predicting the offspring's weight trajectories during childhood [28]. A previous study suggested that 75% of obese children maintain or face an increase in their obesity in adult life, and lipids including CEs are positively associated with obesity in children [29].

In a Finnish study involving 444 children aged 6-9 years (232 girls and, 247 boys), the total saturated fatty acid and mono-unsaturated CEs in the plasma were higher in overweight/obese children; however, the total polyunsaturated fatty acid CEs were lower [30]. Another clinical study involving adult Tunisian volunteers showed accumulation of CEs in adipose cells, suggesting that increased CEs are a phenotype of obesity. Moreover, increased serum CEs are also associated with the risk of high blood pressure and cardiovascular disease [31]. In a study involving Japanese children aged 6-15 years (31 obese children; 14 boys and 17 girls) and normal-weight children (12 boys and 9 girls), it was observed that the plasma CE transfer activity was increased significantly in obese children [18].

This evidence suggest that increased CEs levels in plasma have a negative impact on health. It is important to monitor plasma levels in children from an early age. This study was limited to eight CE molecular species, and the influence of diet and physical exercise on CEs was not monitored. Overall, this was the first investigation of plasma CEs in Japanese preadolescents using LC/MS. Age, sex, and BMI-related changes in the plasma CE levels of children were measured and compared. Although girls exhibited lower total CEs than boys, most CEs were tightly regulated in the plasma, with CE 18:2 as the most abundant species. Increased plasma levels of CE 18:3 and CE 20:4 in overweight children may also be associated with childhood obesity. This study highlights the significance of monitoring CEs from an early age for controlling lifestyle-associated disorders.

4. Conclusions

Cholesterol and CEs are believed to be linked to cardiovascular diseases because of their abundance in biological systems and important roles in cell structure and function. This study investigated the absolute levels of CE in the plasma of Japanese children and their variations according to concerning sex, age, and BMI. The predominant species in the plasma of children's plasma was CE 18:2, followed by CE 18:1, and the total CE levels were significantly lower in the plasma of underweight children's than in the normal-weight children. No significant differences were observed in the CE molecular species of CE between different ages, groups, or sexes. Increased CE 18:3 levels in overweight children may be a promising marker of childhood obesity. This study revealed the plasma CE levels in Japanese preadolescents. Future studies should explore the role of CE in various lifestyle-associated diseases.

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Authors contributions

Conceptualization: D.G., S.G.B.G., A.I., H.C, and S.-P.H.; methodology: D.G., validation: D.G.; formal analysis: D.G.; investigation: D.G.; and S.G.B.G., and resources: D.G., S.G.B.G., A.I., Y.A.B., R.M.K., R.K., H.C, and S.-P.H.; data curation: D.G., and S.G.B.G.; writing-original draft preparation: D.G., and S.G.B.G.; writing-review and editing: D.G., S.G.B.G., A.I., Y.A.B., R.M.K., R.K., H.C, and S.-P.H.; supervision: S.G.B.G., A.I., H.C, and S.-P.H.; funding acquisition: D.G., S.G.B.G., A.I., and S.-P.H. All authors have read and agreed to the published version of the manuscript.

Data availability:

Data will be available from the corresponding author upon a request made.

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Declaration of competing interests

The authors declare no conflict of interest relevant to this article.

Ethics approval

This study was approved by the review board of the Faculty of Health Sciences. Hokkaido University (approval number: 22-23) and Center for Environmental Science and Health Sciences, Hokkaido University (approval number: 21-136).

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	Age (years old (n))	Height (cm)	Weight (Kg)	BMI (Kg/m ²)	BMI (n)		
					Under weight	Normal weight	Over weight
Boys (n=178)	9 (n = 68)	134 ± 5.0	31.8 ± 6.1	17.4 ± 2.5	117	55	6
	10 (n = 60)	139 ± 6.4	35.0 ± 8.3	17.9 ± 3.3			
	11 (n = 20)	148 ± 6.5	41.8 ± 8.1	19.0 ± 3.3			
	12 (n = 30)	150 ± 9.4	44.2 ± 9.8	19.4 ± 3.3			
Girls (n= 161)	9 (n = 46)	136 ± 5.7	30.2 ± 5.9	16.2 ± 2.2	120	39	2
	10 (n = 70)	140 ± 7.0	34.6 ± 8.1	17.4 ± 2.8			
	11 (n = 16)	151 ± 5.0	39.5 ± 5.8	17.3 ± 2.0			
	12 (n = 29)	151 ± 6.5	42.6 ± 8.4	18.4 ± 2.6			

Table 1: Characteristics of preadolescents aged 9-12 years.

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Molecules	9 years		10 years		11 years		12 years	
	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls
CE 16:0	121 ± 19.8	120 ± 18.1	119 ± 18.4	119 ± 19.7	112 ± 13.3	109 ± 12.9	114 ± 20.1	112 ± 15.4
CE 18:0	16.9 ± 5.1	15.9 ± 3.20	16.5 ± 3.2	16.7 ± 5.3	15.3 ± 2.50	15.1 ± 2.10	15.1 ± 2.20	14.4 ± 2.0
CE 18:1	140 ± 7.4	140 ± 6.90	141 ± 7.4	139 ± 6.6	138 ± 6.30	137 ± 4.50	139 ± 6.90	138 ± 5.7
CE 18:2	375 ± 43.0	364 ± 30.3	357 ± 42.9	357 ± 42.6	355 ± 22.3	343 ± 19.8	357.8 ± 24.9	358 ± 19.1
CE 18:3	79.2 ± 21.8	70.5 ± 20.5	77.7 ± 22.9	72.1 ± 20.2	68.4 ± 21.4	63.1 ± 16.4	69.7 ± 20.6	61.8 ± 25.2
CE 20:4	18.2 ± 1.3	18.1 ± 1.20	18.1 ± 1.9	17.7 ± 1.4	17.6 ± 1.30	17.3 ± 1.40	17.6 ± 1.40	17.8 ± 1.6
CE 20:5	41.2 ± 19.7	33.1 ± 15.7	41.9 ± 21.0	34.3 ± 17.0	36.8 ± 17.6	32.1 ± 13.6	35.8 ± 15.5	34.2 ± 17.2
CE 22:6	112 ± 24.0	111 ± 21.5	113 ± 26.1	109 ± 24.3	107 ± 21.3	106 ± 15.9	112 ± 21.3	110 ± 20.9
Total CE	906 ± 108	875 ± 93.6	844 ± 216	867 ± 104	852 ± 68.0	824 ± 72.3	861 ± 95.0	847 ± 82.0

401
402 Table 2: Average concentration of the CE molecules in the plasma of preadolescents
403 (pmol/ μ L, values are expressed as mean $\pm$ SD).
404

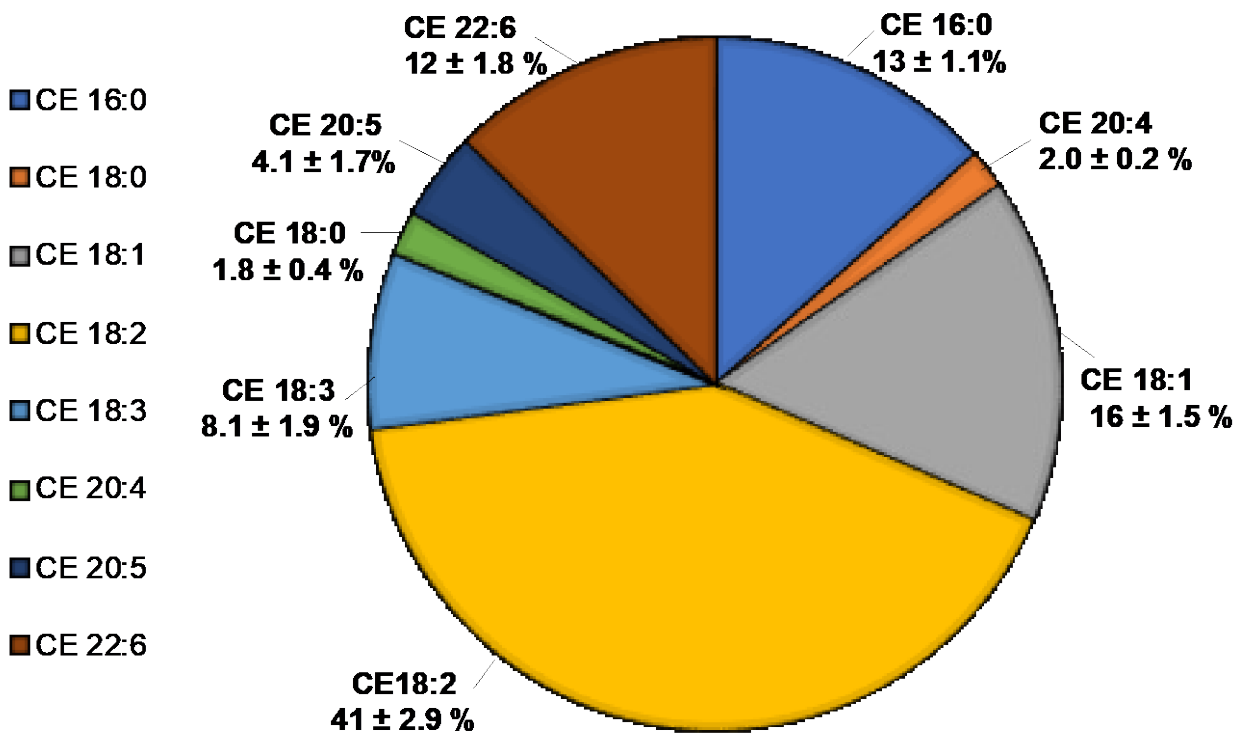


Fig 1. Relative abundance (concentrations) of CE species in the plasma of preadolescents. Results are expressed as mean $\pm$ SD (n = 339)

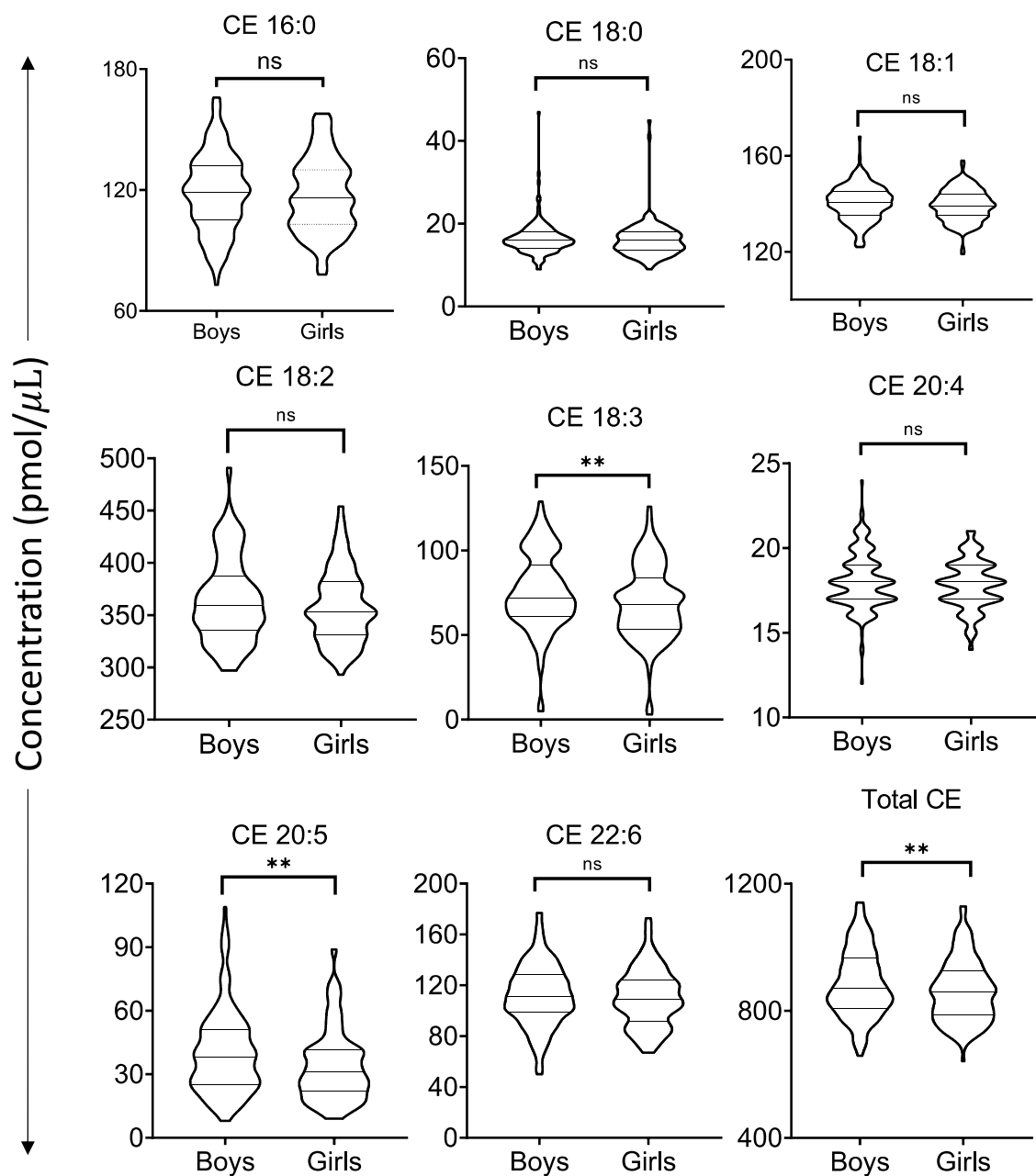


Fig 2. Violin plots of each CE level in the plasma of preadolescent girls and boys according to sex; t-test, n.s., = not significant; * $p < 0.05$; and ** $p < 0.005$.

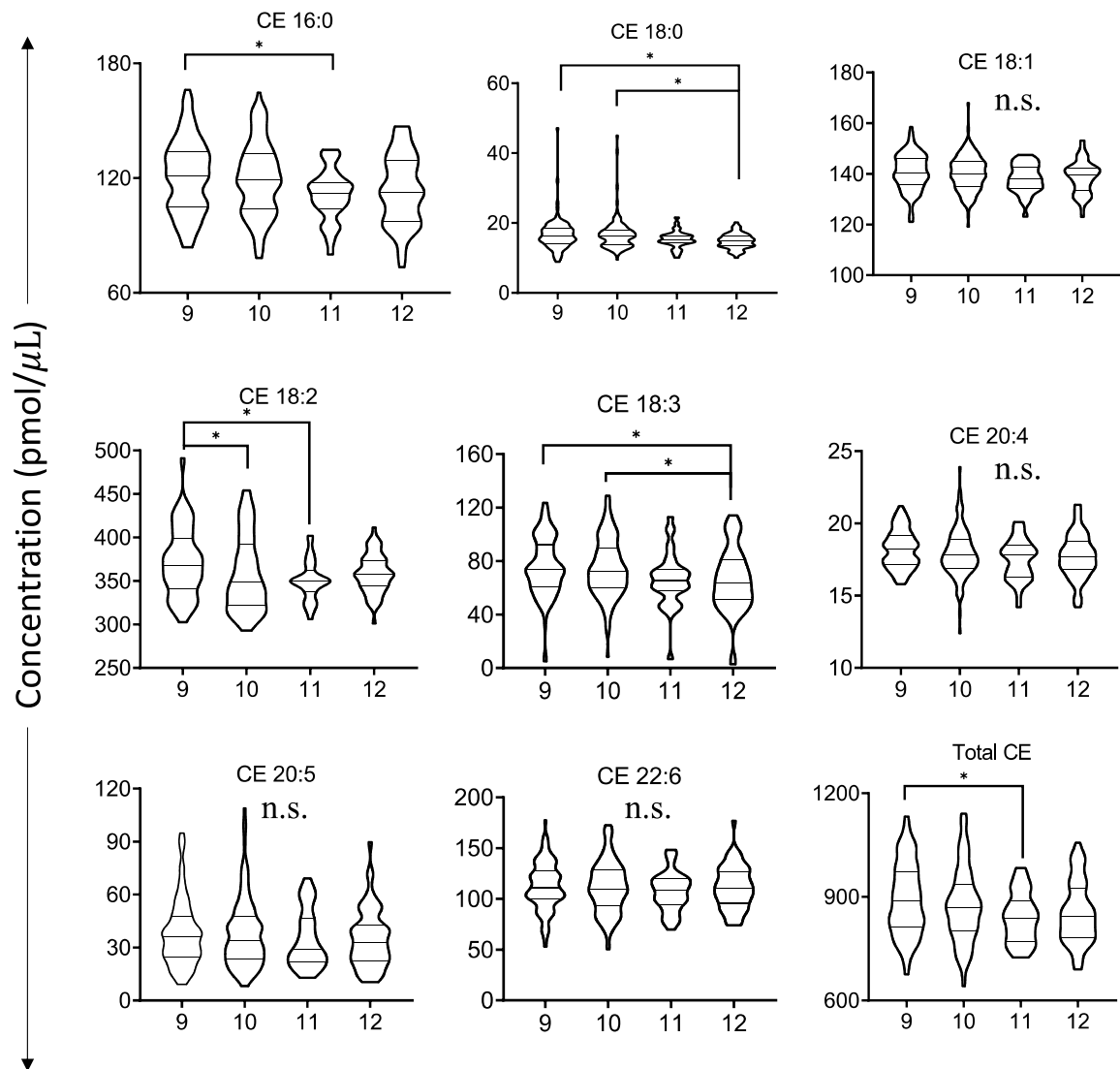
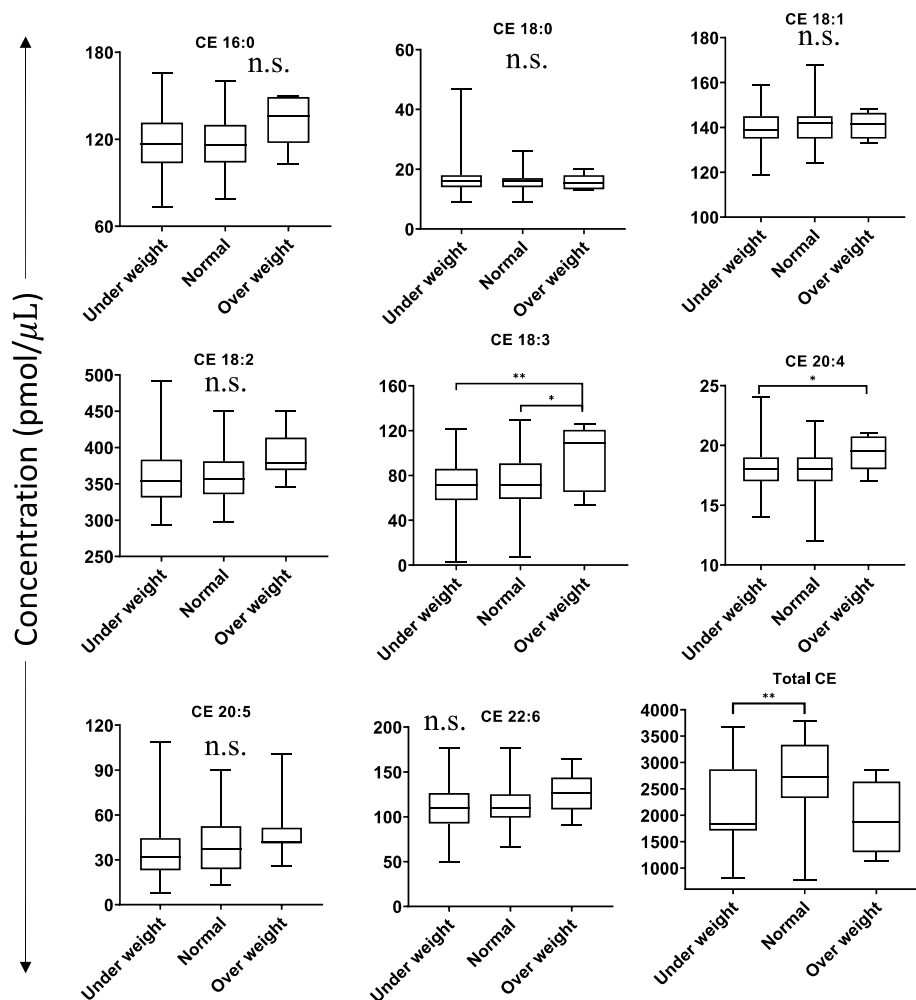


Fig 3. Violin plots of each CE level in the plasma of preadolescents aged 9-12 years old according to age. One-way ANOVA using the multiple comparisons test, n.s.; * $p < 0.05$; and ** $p < 0.005$.

A)



B)

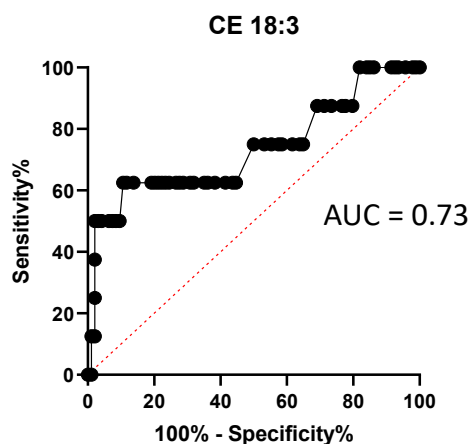


Fig 4. A) Box plots for each CE level in the plasma of preadolescents according to the BMI. BMI < 18 = underweight (n = 237);, BMI = 18.1 25 = normal weight (n = 94), and BMI > 2.5 = overweight (n = 8). One-way ANOVA using the multiple comparisons test, n.s.; * $p < 0.05$ and; ** $p < 0.005$. B) ROC curve analysis of CE 18:3 of normal-weight and overweight children.