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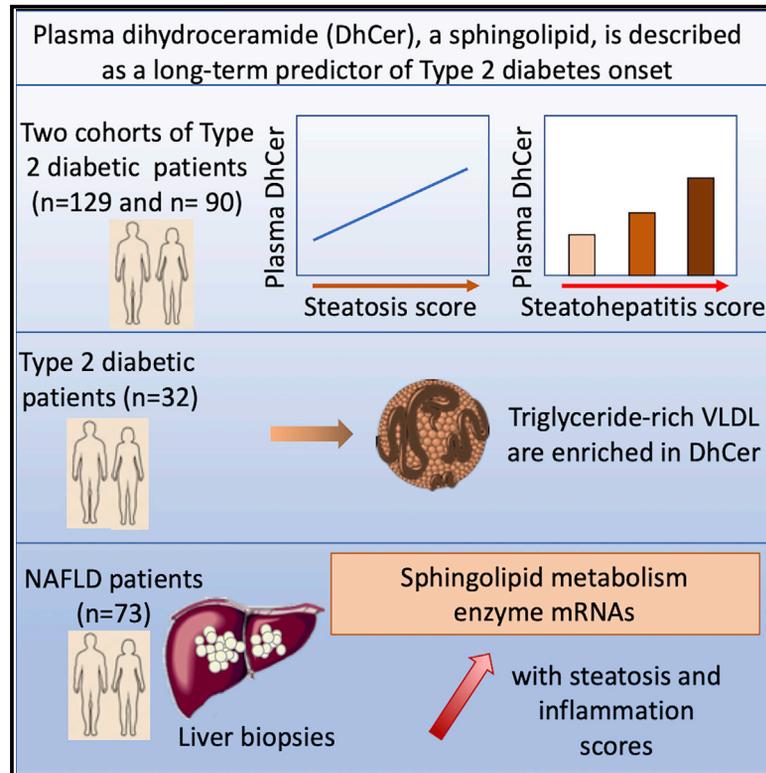
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Dihydroceramides in Triglyceride-Enriched VLDL Are Associated with Nonalcoholic Fatty Liver Disease Severity in Type 2 Diabetes

Graphical Abstract



Authors

Aurélie Carlier, Franck Phan, Anaïs Szpigel, ..., Pascal Ferré, Fabienne Foufelle, Olivier Bourron

Correspondence

pascalfr@gmail.com

In Brief

Plasma dihydroceramides are long-term predictors of type 2 diabetes onset. Carlier et al. discovered that plasma dihydroceramides are associated with non-alcoholic fatty liver disease severity in diabetic patients and are found in triglyceride-enriched VLDL. It strongly suggests that dysregulated hepatic fat metabolism is an early and major component of diabetes onset.

Highlights

- Plasma dihydroceramides are associated with NAFLD severity in type 2 diabetic patients
- Plasma dihydroceramides are found in triglyceride-enriched VLDL
- A role for dihydroceramide in triglyceride-rich VLDL synthesis/secretion is suggested
- Expression of enzymes of hepatic sphingolipid synthesis increases with NAFLD severity



Article

Dihydroceramides in Triglyceride-Enriched VLDL Are Associated with Nonalcoholic Fatty Liver Disease Severity in Type 2 Diabetes

Aurélie Carlier,^{1,2,9} Franck Phan,^{1,2,9} Anaïs Szpigiel,^{1,9} Eric Hajduch,^{1,3} Joe-Elie Salem,^{3,4} Jérémie Gautheron,^{3,5} Wilfried Le Goff,^{3,6} Maryse Guérin,^{3,6} Floriane Lachkar,¹ Vlad Ratziu,^{1,3,7} Agnès Hartemann,^{1,2,3} Pascal Ferré,^{1,3,8,9,10,11,*} Fabienne Foufelle,^{1,3,9} and Olivier Bourron^{1,2,3,9}

¹Centre de Recherche des Cordeliers, INSERM, Sorbonne Université, Université de Paris, 75006 Paris, France

²Sorbonne Université, Assistance Publique-Hôpitaux de Paris, Diabetes Department, Hospital Pitié-Salpêtrière, 75013 Paris, France

³Institute of Cardiometabolism and Nutrition, ICAN, Assistance Publique-Hôpitaux de Paris, Paris, France

⁴Sorbonne Université, Assistance Publique-Hôpitaux de Paris, CIC Paris-Est, Hospital Pitié-Salpêtrière, 75013 Paris, France

⁵Centre de Recherche Saint-Antoine, INSERM, Sorbonne Université, 75012 Paris, France

⁶UMR ICAN, INSERM, Sorbonne Université, 75013 Paris, France

⁷Sorbonne Université, Assistance Publique-Hôpitaux de Paris, Hepatology Department, Hospital Pitié-Salpêtrière, 75013 Paris, France

⁸Assistance Publique-Hôpitaux de Paris, Oncology and endocrine biochemistry Department, Hospital Pitié-Salpêtrière, 75013 Paris, France

⁹These authors contributed equally

¹⁰Twitter: @FerrPascal2

¹¹Lead Contact

*Correspondence: pascalfr@gmail.com

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SUMMARY

Plasma dihydroceramides are predictors of type 2 diabetes and related to metabolic dysfunctions, but the underlying mechanisms are not characterized. We compare the relationships between plasma dihydroceramides and biochemical and hepatic parameters in two cohorts of diabetic patients. Hepatic steatosis, steatohepatitis, and fibrosis are assessed by their plasma biomarkers. Plasma lipoprotein sphingolipids are studied in a sub-group of diabetic patients. Liver biopsies from subjects with suspected non-alcoholic fatty liver disease are analyzed for sphingolipid synthesis enzyme expression. Dihydroceramides, contained in triglyceride-rich very-low-density lipoprotein (VLDL), are associated with steatosis and steatohepatitis. Expression of sphingolipid synthesis enzymes is correlated with histological steatosis and inflammation grades. In conclusion, association of plasma dihydroceramides with nonalcoholic fatty liver might explain their predictive character for type 2 diabetes. Our results suggest a relationship between hepatic sphingolipid metabolism and steatohepatitis and an involvement of dihydroceramides in the synthesis/secretion of triglyceride-rich VLDL, a hallmark of NAFLD and type 2 diabetes dyslipidemia.

INTRODUCTION

In obesity, lipid ectopic storage is a major cause of metabolic complications and particularly of type 2 diabetes, although their severity obviously depends upon genetically based individual susceptibility. Once accumulated in tissues, lipids can generate species that will lead to insulin resistance, endoplasmic reticulum (ER) stress, oxidative stress, and inflammation and thus contribute to the onset of type 2 diabetes. The sphingolipid ceramides are one of these bioactive lipid species that were shown to induce insulin resistance, ER stress, and inflammation.^{1–6}

Ceramides can be synthesized *de novo*. The first step is the condensation of palmitate with serine, which is catalyzed in the ER by serine palmitoyl transferase (SPT) to form dihydrosphingosine; the latter is then acylated into dihydroceramide (DhCer) by ceramide synthases (CerS). There are several isoforms of CerS (CerS 1 to CerS 6) that confer the specificity of DhCer and cer-

amide species, according to the class of acylated fatty acids (chain length and unsaturation).⁷ Finally, dihydroceramide desaturase (DEGS) desaturates DhCer to produce ceramides, which are exported to the Golgi and converted into various sphingolipids such as sphingomyelin (sphingomyelin) or glucosylceramides, essential components of membranes. The liver is an important site of sphingolipid synthesis, and hepatic sphingolipids can be exported into the plasma mainly through lipoprotein.⁸

Liver-produced ceramides can play a role in hepatic steatosis. In obese rodents, decreasing *de novo* hepatic ceramide synthesis reduces steatosis and plasma aminotransferases.⁹ Secreted hepatic ceramides may even be harmful for other peripheral tissues such as the muscle, heart, and adipose tissue.¹⁰

DhCer differ from ceramides by the absence of a double bond and hence different fluidity and packing behaviors.¹¹ Unlike ceramides, they are far less abundant and were hitherto deemed



Table 1. Participant Characteristics in the CERADIAB and DIACART Cohorts

	CERADIAB Cohort n = 90	DIACART Cohort n = 149	Statistical Significance
Men n (%)	46 (51%)	119 (79%)	a
Age years	60 (53–66)	67.3 (61–73)	a
BMI kg/m ²	29.1 (26.5–34)	29.2 (25.1–32)	NS
Diabetes duration (years)	7 (2–14)	15.8 (8–22)	a
Waist circumference cm	–	103 (99–111)	
HbA1c % mmol/mol	7.8 (7.1–9.1)	7.7 (6.8–8.3)	NS
	62 (54–76)	61 (51–67)	
Fasting plasma glucose mmol/L	7.8 (6.4–9.9)	9.0 (6.6–10.8)	NS
HOMA-IR	3.4 (1.8–5.3)	5.2 (2.0–7.0)	a
	n = 67	n = 78	
GFR Cockcroft	101 (79.3–129)	81.5 (61–98)	a
Creatinine μmol/L	77 (65–88.5)	93 (76–103)	a
Microalbuminuria mg/24h	21.9 (7.4–63)	169 (8.6–99.8)	NS
Coronary artery disease n (%)	12 (13.3%)	126 (84%)	a
Hypertension n (%)	54 (60%)	99 (66%)	NS
Past or active smoking n (%)	7 (7.8%)	100 (67%)	a
Insulin treatment n (%)	23 (25.6%)	71 (48%)	a
Metformin n (%)	69 (76.7%)	119 (80%)	NS
Hypoglycemic sulfonylureas n (%)	57 (63.3%)	69 (46%)	NS
Dipeptidyl peptidase-IV inhibitor n (%)	21 (23.3%)	36 (24%)	NS
Glucagon-like peptide-1 receptor agonist n (%)	11 (12.2%)	10 (7%)	NS
Statin n (%)	44 (48.9%)	131 (88%)	a
Antiplatelet agent n (%)	30 (33.3%)	123 (82%)	a
Antihypertensive drugs n (%)	55 (61.1%)	118 (79%)	NS
hsCRP mg/L	1.73 (0.9–3.42)	2.12 (0.66–2.96)	NS
Total cholesterol mmol/L	4.45 (3.7–5.17)	3.92 (3.35–4.36)	a
Triglycerides mmol/L	1.46 (1.1–1.96)	1.59 (0.88–1.87)	NS
HDL-cholesterol mmol/L	1.06 (0.95–1.37)	1.09 (0.87–1.26)	NS
LDL-cholesterol mmol/L	2.53 (1.86–3.16)	2.06 (1.60–2.39)	a
ASAT U/L	24 (20–29)	27 (21–31)	NS
ALT U/L	25 (17–35)	29 (18–32)	NS
GGT U/L	32 (24–50)	46 (24–50)	NS
FT	0.14 (0.08–0.25)	0.37 (0.20–0.53)	a
F0 n (%)	64 (71.1%)	42 (28.2%)	
F0-F1 n (%)	8 (8.9%)	19 (12.8%)	
F1 n (%)	3 (3.3%)	11 (7.4%)	
F1-F2 n (%)	9 (10%)	29 (19.5%)	
F2 n (%)	2 (2.2%)	21 (14%)	
F3 n (%)	4 (4.5%)	16 (10.7%)	
F3-F4 n (%)	0 (0%)	2 (1.3%)	
F4 N%	0 (0%)	9 (6%)	

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Table 1. Continued

	CERADIAB Cohort n = 90	DIACART Cohort n = 149	Statistical Significance
ST	0.58 (0.46–0.70)	0.54 (0.38–0.73)	NS
S0 n (%)	12 (13.3%)	22 (14.7%)	
S0-S1 n (%)	3 (3.3%)	16 (10.8%)	
S1 n (%)	14 (15.6%)	21 (14.2%)	
S1-S2 n (%)	14 (15.6%)	21 (14.2%)	
S2 n (%)	20 (22.2%)	23 (15.3%)	
S2-S3 n (%)	2 (2.2%)	5 (3.3%)	
S3 n (%)	25 (27.8%)	41 (27.5%)	
NT	0.5 (0.5–0.5)	0.45 (0.25–0.5)	NS
N0 n (%)	21 (23.6%)	49 (32.9%)	
N1 n (%)	55 (61.8%)	78 (52.2%)	
N2 n (%)	13 (14.6%)	22 (14.8%)	
FLI	ND	64.9 (43.6–90.9)	
<30 n (%)		24 (16%)	
>60 n (%)		89 (60%)	

Data are presented as median and interquartile for quantitative data or as frequency for qualitative data. NS, not significant; ASAT, aspartate aminotransferase; ALT, alanine aminotransferase; BMI, body mass index; HDL-cholesterol, high-density lipoprotein-cholesterol; hsCRP, high sensitivity C reactive protein; GFR, glomerular filtration rate; GGT, gammaglutamyl transferase; LDL-cholesterol, low-density lipoprotein-cholesterol; ST, SteatoTest; FT, FibroTest; NT, NASHTest; fatty liver index, FLI.

^aDifference statistically significant.

metabolically inert. Plasma DhCer concentration was recently proposed as a biomarker of metabolic dysfunction.¹² Circulating levels of DhCer were increased together with ceramides in a small cohort of young patients (10–17 years) with type 2 diabetes¹³ and were associated with the severity of insulin resistance in primates¹⁴ and with waist circumference in Mexican families.¹⁵ Higher hepatic DhCer concentrations were found in the liver of insulin-resistant human subjects.¹⁶ Recently, our group has shown that DhCer is the most increased sphingolipid subspecies in plasma of patients with type 2 diabetes compared to control individuals and type 1 diabetic patients.⁶ Importantly, lipidomic analysis of the plasma of individuals from population-based prospective cohorts revealed that elevated specific DhCer species in the plasma were predictors of diabetes up to 9 years before disease onset.^{16,17}

The major aim of the present study was to identify factors associated with plasma levels of DhCer in type 2 diabetic patients to better understand their predictive value for the occurrence of type 2 diabetes and their relationship with concurrent metabolic disorders.

RESULTS

Characteristics of Type 2 Diabetic Patients in the CERADIAB and DIACART Cohorts

The main clinical and biochemical characteristics for the CERADIAB and DIACART cohorts are shown in Table 1 (see also the STAR Methods). Briefly, in the DIACART cohort, the percentage of male subjects was higher and the diabetes duration longer than in the CERADIAB cohort. The number of patients with cor-

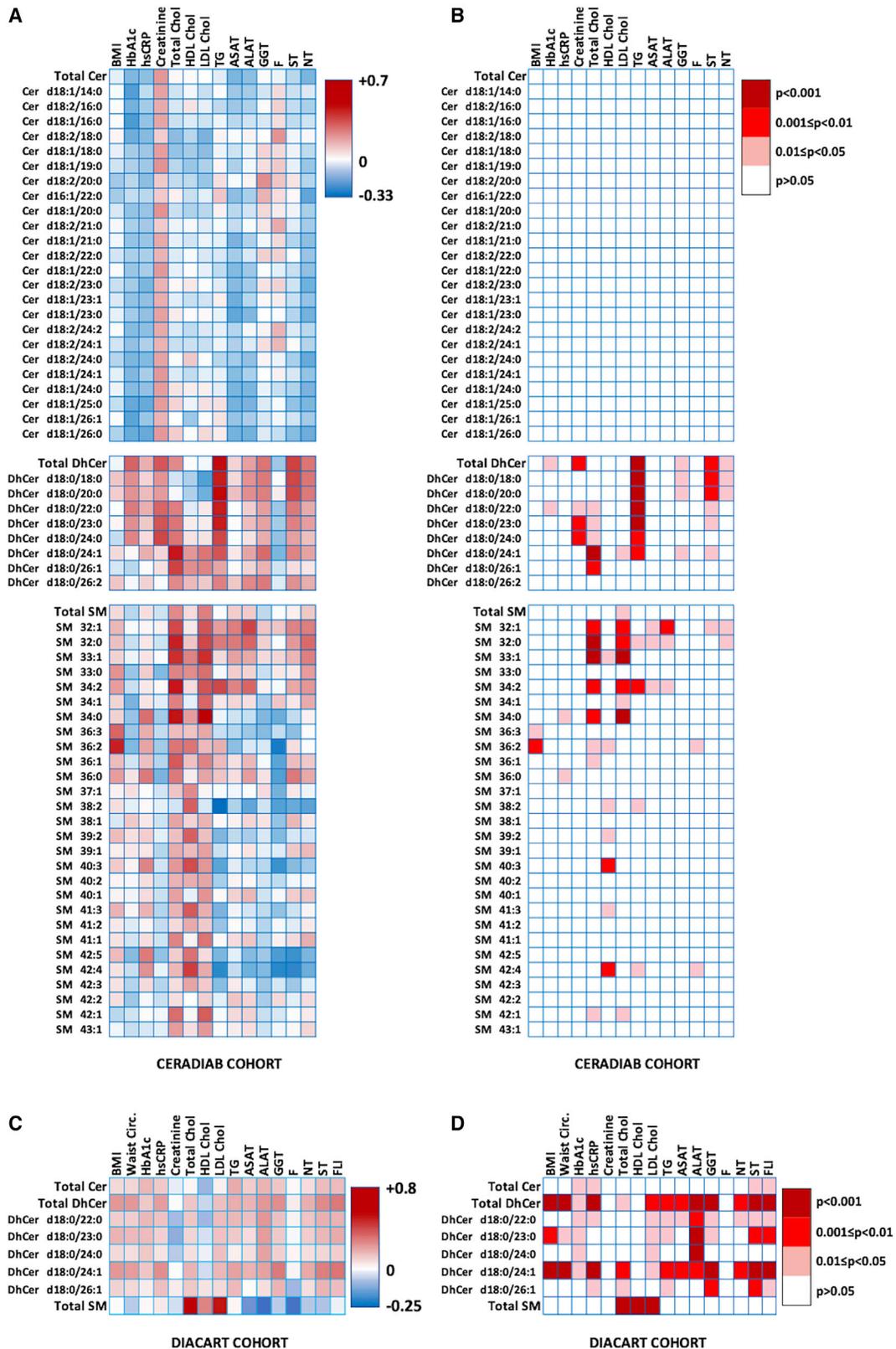
onary artery disease was higher in the DIACART cohort (84% versus 13.3%) as well as the treatment with insulin (48% versus 25.6%), the HOMA-IR, the severity of renal dysfunction, total and LDL cholesterol, and the fibrosis score.

Plasma DhCer Correlates with Hepatic Biomarkers of Non-Alcoholic Fatty Liver Disease in the CERADIAB Cohort

All the biological variables were included in the initial analyses either as quantitative or qualitative variables. The clinical characteristics shown in Figure 1 including SteatoTest, NASHTest, and Fibrotest are those positively correlated with at least one sphingolipid species. Figure 1A shows the correlation coefficients and Figure 1B the corresponding statistical significance. Of note, HOMA-IR was not significantly associated with ceramides or DhCer.

In the CERADIAB cohort, plasma total or individual ceramide concentrations were correlated with no variable. Sphingomyelin species were correlated with total, HDL or LDL cholesterol, and plasma total concentrations of sphingomyelin were positively correlated with LDL cholesterol (Figures 1A and 1B).

In contrast, plasma total DhCer concentration was associated positively with HbA1c, creatinine, triglycerides, GGT, SteatoTest, and NASHTest but not with FibroTest (Figures 1A and 1B). We then performed a multivariate analysis in order to correct for confounding factors and select the most influential variable. In multivariate analysis total plasma DhCer concentration was associated with SteatoTest ($\beta = 0.343$, $p = 0.004$). Plasma DhCer concentrations increased in parallel with SteatoTest (Figure 2A)



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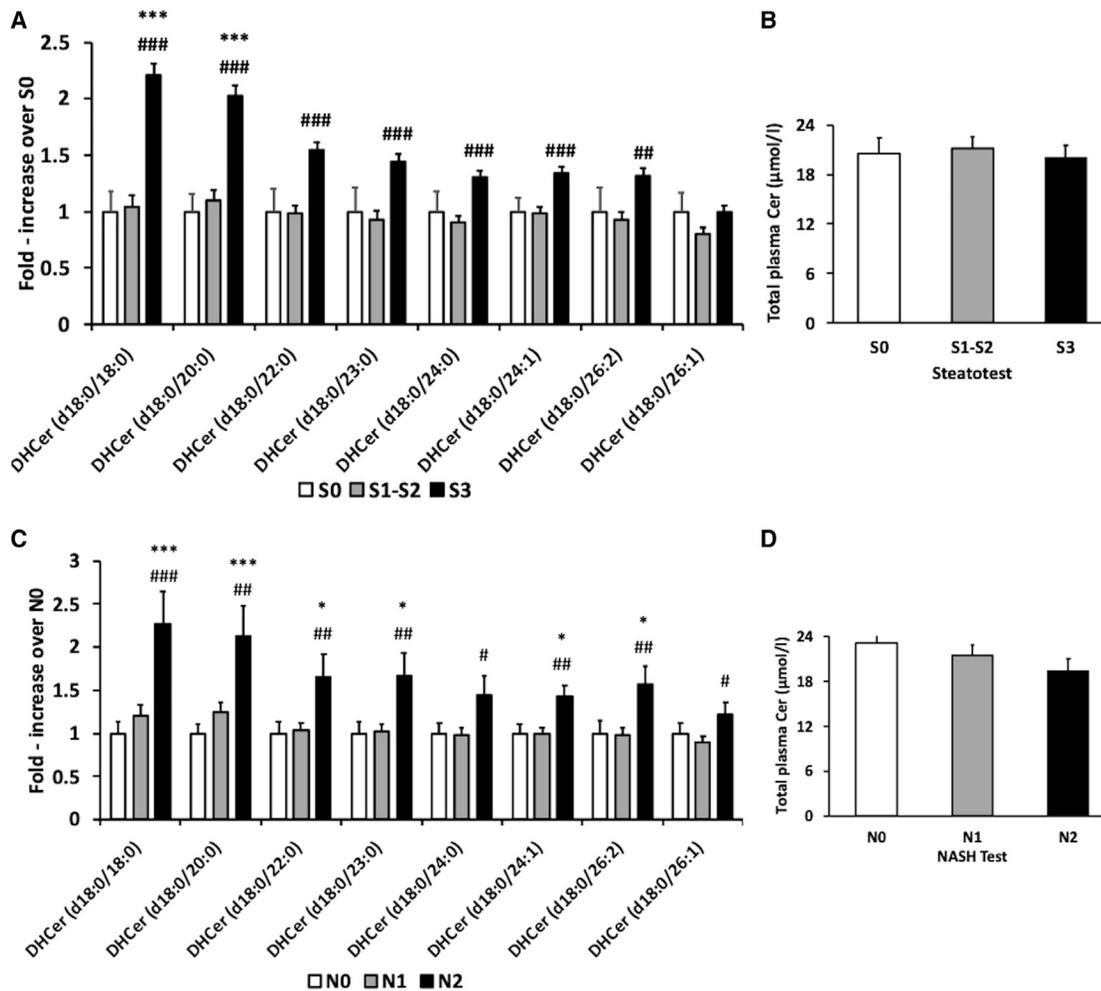


Figure 2. Plasma DhCer and Total Cer Concentrations According to SteatoTest and NASH Test in the CERADIAB Cohort

(A) Plasma DhCer concentrations according to SteatoTest (ST) values, S0: $0 < ST \leq 0.3$, S1-S2: $0.3 < ST \leq 0.70$, S3: $ST > 0.70$.

(B) Total plasma ceramide concentrations according to SteatoTest scores.

(C) Plasma DhCer concentrations according to NASH Test scores.

(D) Total plasma ceramide concentrations according to NASH test scores.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ difference statistically significant when compared to S0 or N0. S0: $n = 12$, S1-S2: $n = 52$, S3: $n = 26$. N0: $n = 21$, N1: $n = 55$, N2: $n = 14$. DhCer, dihydroceramide; Cer, ceramide.

and NASH Test scores (Figure 2C), whereas total plasma ceramides did not (Figures 2B and 2D).

Principal-component analysis allowed to aggregate the DhCer species in two principal components (Figure 3A): PC1, explaining 55.3% of the total phenotypic variance and associated with DhCer 18:0 to DhCer 24:0 concentrations, $r \geq 0.89$, and PC2,

explaining 36% of the total phenotypic variance and associated with unsaturated long-chain DhCer concentration, DhCer 24:1 DhCer 26:1 and DhCer 26:2, $r \geq 0.92$. PC1 was more strongly associated with BMI, HbA1c, TG, GGT, SteatoTest, and NASHT-test than PC2, whereas PC2 was more strongly associated with total cholesterol than PC1 (Figure 3B).

Figure 1. Correlations between Plasma Sphingolipids and Clinical Characteristics in the CERADIAB and DIACART Cohorts Shown as a Heatmap

(A) Correlation coefficient based on univariate regression was calculated between the sum of each class of sphingolipid species or individual sphingolipid species and biological characteristics for the CERADIAB cohort ($n = 90$).

(C) Similar calculations were made for the DIACART cohort ($n = 149$), but, for ceramide and sphingomyelin, only the sum of the different species is presented. (B and D) Positive correlations are depicted in red and negative correlations in blue. Statistical significance of the correlations is indicated in (B) for the CERADIAB cohort and in (D) for the DIACART cohort. A Benjamini-Hochberg correction procedure for multiple comparisons was used.

BMI, body mass index; Cer, ceramide; Chol, cholesterol; DhCer, dihydroceramide; F, fibrotest; FLI, fatty liver index; hsCRP, high sensitivity C-reactive protein; NT, NASH test; sphingomyelin, sphingomyelin; ST, steatostest; TG, triglyceride.

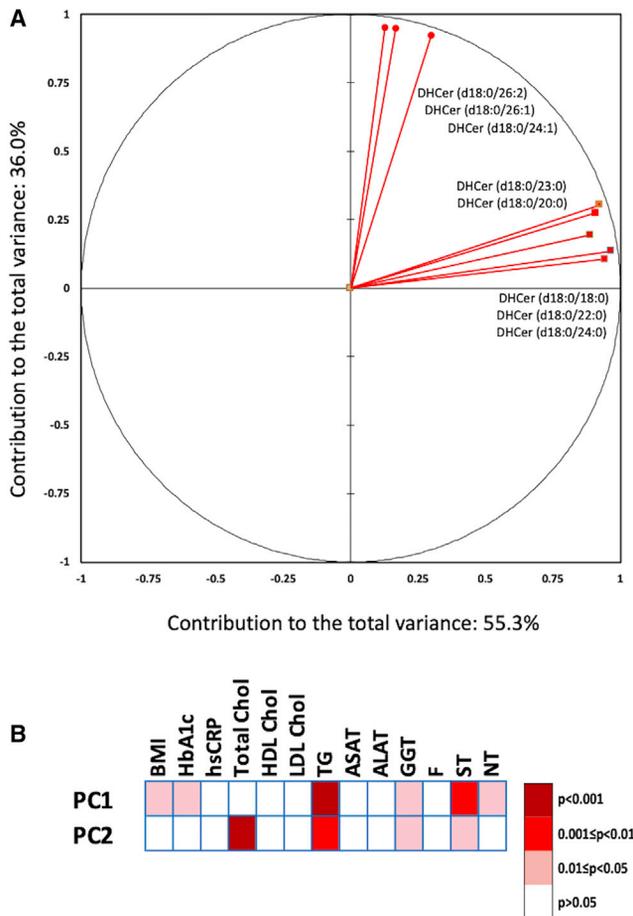


Figure 3. Principal-Component Analysis of DhCer Species in the CERADIAB Cohort

A principal-component analysis was applied to the DhCer data of the CERADIAB cohort ($n = 90$).

(A) Variable correlation plot.

(B) Statistical significance of the correlations between PCs and clinical variables shown as a heatmap. Statistical significances were corrected by the Benjamini-Hochberg procedure for multiple comparisons.

Plasma DhCer Correlate with Hepatic Biomarkers of Non-Alcoholic Fatty Liver Disease in the DIACART Cohort

The analyses performed in the CERADIAB cohort were replicated in the DIACART cohort composed of 149 type 2 diabetic patients of which characteristics are described in Table 1. Since waist circumference was available in this cohort, Fatty Liver Index (FLI) was calculated as well as SteatoTest. For the sphingolipid analysis, we could only reliably quantify the DhCer species: 22:0, 23:0, 24:0, 24:1, 26:1, and we used the sum of their concentrations as total plasma DhCer. The correlation coefficient in univariate analyses between the different sphingolipid species and relevant clinical characteristics is shown in Figure 1C and the statistical significance in Figure 1D. Plasma total ceramide shown is composed of the sum of ceramides, 18:1 14:0, 18:1 16:0, 18:1 17:0, 18:1 18:1, 18:2 18:0, 18:1 18:0, 18:2 20:0, 16:1 22:0, 18:1 14:0, 18:1 16:0, 18:1 22:0, 18:2 23:1, 18:1 23:1, 18:2

23:0, 18:1 23:0, 18:2 24:2, 18:2 24:1, d18:1 24:1, 18:2 24:0, 18:1 24:0, 18:1 25:0, 18:1 26:1, 18:1 26:0; plasma total sphingomyelin shown is composed of sphingomyelins, 18:1 14:0, 16:1 17:0, 18:1 16:1, 18:1 16:0, 18:0 16:0, 18:1 17:0, 18:2 18:0, 18:1 18:0, 18:0 18:2, 18:2 20:0, 18:1 20:0, 18:2 22:0, 18:1 22:0, 18:2 24:1, 18:1 24:1, 18:1 24:0.

In univariate analysis, plasma total ceramide concentration was associated positively with HbA1c, hsCRP, SteatoTest, and FLI. In multivariate analysis, plasma total ceramide concentration was associated with HbA1c ($\beta = 0.176$, $p = 0.032$). In univariate analysis, plasma total sphingomyelin concentration was associated positively with total, HDL, and LDL cholesterol. In multivariate analysis plasma total sphingomyelin concentration was associated positively with total cholesterol ($\beta = 0.75$, $p < 0.0001$) and HDL cholesterol ($\beta = 0.191$, $p = 0.0001$).

In univariate analysis, plasma total DhCer concentration was associated positively with BMI, waist circumference, HbA1c, hsCRP and liver biomarkers, GGT, SteatoTest, and FLI (Figure 1D). Among the 5 DhCer species analyzed, DhCer 24:0 was the least correlated with SteatoTest and FLI. In multivariate analysis (not including SteatoTest to avoid collinearity), plasma total DhCer was associated with FLI ($\beta = 0.405$, $p < 0.0001$). If FLI was omitted from the multivariate analysis and SteatoTest included, plasma total DhCer was associated with SteatoTest ($\beta = 0.335$, $p < 0.0001$).

When shown as a scatterplot, total plasma DhCer concentration was significantly correlated with FLI (Figure 4A) and SteatoTest (plot not shown, $r = 0.376$, $p < 0.0001$). Plasma DhCer concentrations increased in parallel with FLI (Figure 4C) and NASH Test values (Figure 4D). Interestingly, there was a strong correlation between FLI and SteatoTest biomarkers in the DIACART cohort (Figure 4B), strengthening our confidence in the use of SteatoTest in the CERADIAB cohort.

These results obtained in two independent cohorts confirm the strong relationship between plasma DhCer and particularly saturated forms plus d18:0 24:1 and nonalcoholic fatty liver disease (NAFLD) in diabetic patients.

VLDL DhCer Are Correlated with Hepatic Steatosis and Inflammation

Plasma sphingolipids are mainly transported in the plasma by lipoproteins.⁸ In order to identify the plasma DhCer pool associated with steatosis and NASH biomarkers, we isolated the various lipoprotein fractions in a group of 32 type 2 diabetic patients described in Table S1. As can be seen biochemical parameters and biomarkers in these patients are similar to those in the CERADIAB and DIACART cohorts. HDL, LDL, and very low density lipoprotein (VLDL) biochemical data are described in Table S2. The DhCer species that could be reliably measured in this experiment were 16:0, 18:0, 24:0, 24:1, 26:0, and 26:1. A comparison of HDL, LDL and VLDL sphingolipid concentrations is given in Figure S1 as fold changes versus HDL values.

The concentrations of DhCer and Cer increased from HDL to LDL and VLDL, whereas for sphingomyelin and according to the sphingomyelin species there is a decrease, no change or a decrease from HDL to LDL and VLDL. As expected, triglyceride content was highest in VLDL (Table S2). The amount of Apo B

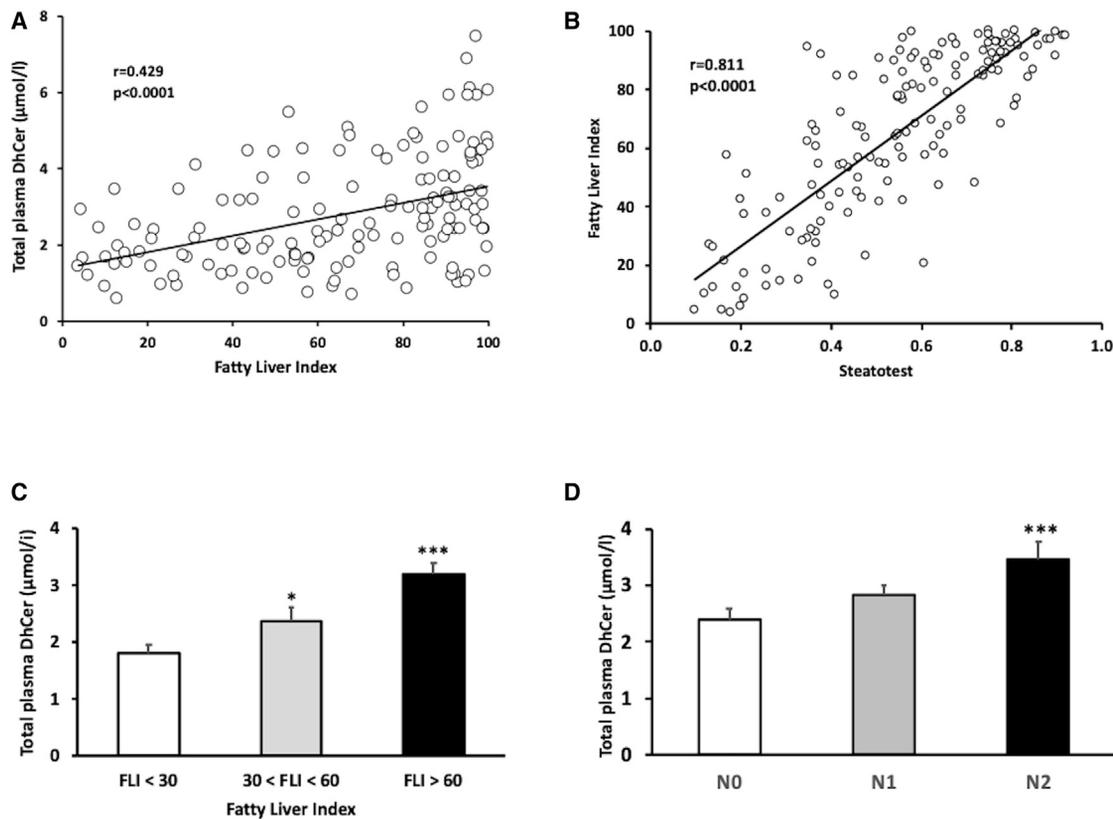


Figure 4. Correlations between Plasma DhCer Concentrations, Fatty Liver Index, and NASH Test in the DIACART Cohort

(A) Correlation of total plasma DhCer concentration with Fatty Liver Index. The coefficient r is shown as well as the p value; $n = 149$.

(B) Correlation of the Fatty Liver Index with SteatoTest. The coefficient r is shown as well as the p value; $n = 149$.

(C) Total plasma DhCer concentration (mean \pm SEM, $n = 149$) according to the fatty liver index scores.

(D) Total plasma DhCer concentration (mean \pm SEM, $n = 149$) according to the NASH Test scores. * $p < 0.05$ and *** $p < 0.001$ difference statistically significant when compared to FLI < 30 or N0. For the DhCer species included in the plasma total concentration, see Figure 1C. FLI < 30 : $n = 25$, 30 $<$ FLI < 60 : $n = 38$, FLI > 60 : $n = 86$. N0: $n = 48$, N1: $n = 77$, N2: $n = 24$.

(Apo B-100) in VLDL particles (one Apo B per particle) was similar regardless of the degree of steatosis (Figure 5A). The ratio triglyceride/Apo B of VLDL increased in parallel with the degree of steatosis (Figure 5B) implying that steatotic patients produce larger, triglyceride enriched VLDL particles.

Total VLDL DhCer concentrations were correlated with SteatoTest (Figure 5C), NASHTest ($r = 0.38$, $p = 0.003$), and with VLDL triglycerides (Figure 5E), whereas total VLDL ceramide concentration was not (Figure 5F; results not shown). The ratio total VLDL DhCer/total VLDL Cer concentrations was correlated with SteatoTest (Figure 5D). Total DhCer concentrations of LDL or HDL were not correlated with SteatoTest or NASHTest (Figure 5G and results not shown). Finally, Total VLDL DhCer was not correlated with the fibrotest values ($r = 0.051$, $p = 0.215$), in agreement with the results presented in Figure 1.

Collectively, these data indicate that the pool of DhCer in VLDL, i.e., lipoproteins directly originating from the liver, is related to NAFLD severity and that larger triglyceride-rich VLDL secreted in steatotic patients are enriched in DhCer when compared to ceramide.

Hepatic Expression of Sphingolipid Metabolism Enzymes Increases with the Severity of Steatosis and Activity Determined Histologically

We then hypothesized that patients with NAFLD could have altered hepatic *de novo* sphingolipid biosynthesis. We explored 73 individuals including 24 type 2 diabetic patients with histological documentation of hepatic steatosis, activity, and fibrosis (Table S3) and measured the hepatic expression of enzymes involved in *de novo* sphingolipid synthesis, serine palmitoyl transferase (SPTLC1), ceramide synthase 2 and 4 (CERS2, CERS4), dihydroceramide desaturase 1 (DEGS1), and sphingomyelin synthase (SGMS1).

SPTLC1, DEGS1, and SGMS1 expressions increased with the severity of steatosis and activity determined histologically (Figure 6) and independently of the presence or absence of type 2 diabetes (results not shown). In contrast, CERS2 and 4 expressions were not correlated with the severity of steatosis and activity (results not shown). Finally, fibrosis did not correlate with the expression of any of the enzymes tested (Figure 6; results not shown). This corroborates the absence of correlation of plasma sphingolipids with fibrosis biomarkers (Figure 1). However,

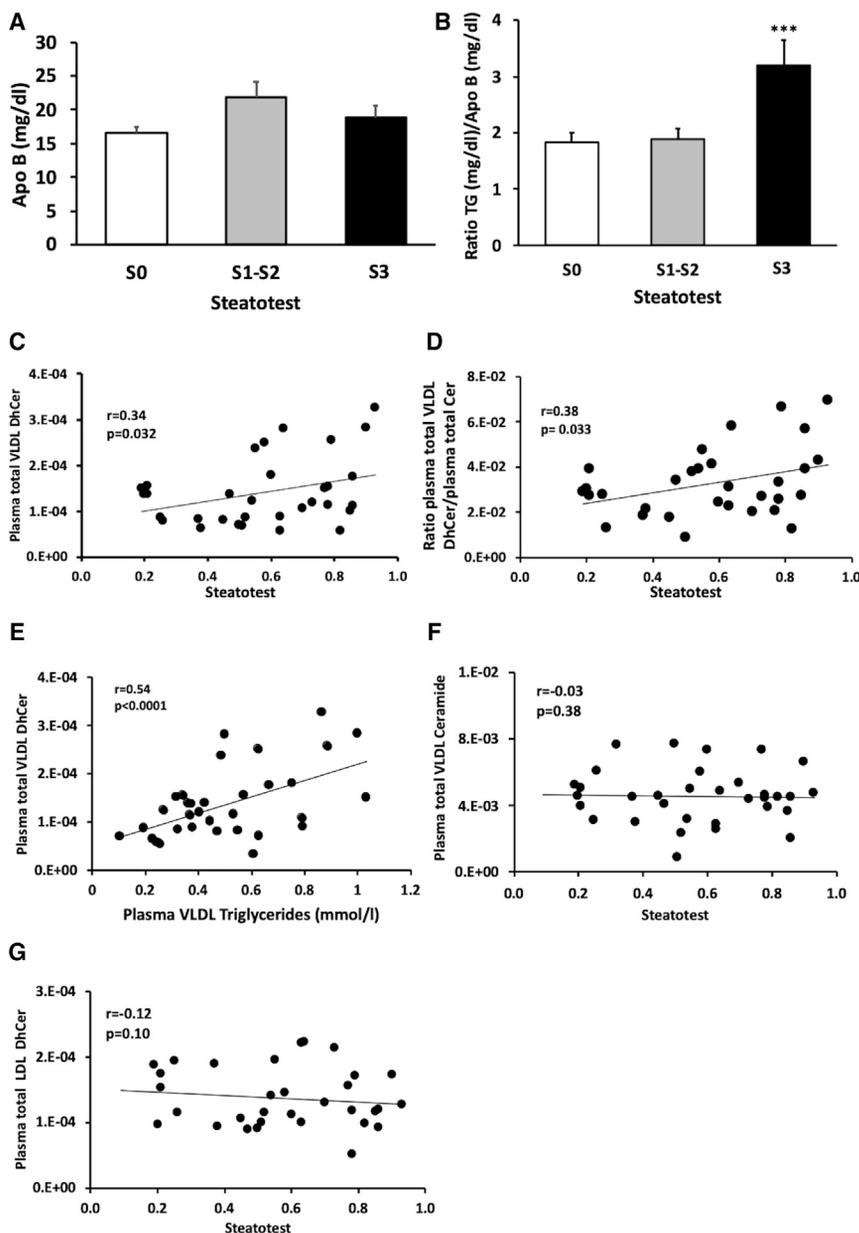


Figure 5. Analysis of VLDL Sphingolipids and Their Relation with SteatoTest and NASH Test

Analysis were performed on 32 type 2 diabetic patients. For (A) and (B), S0: n = 7, S1-S2: n = 13, S3: n = 12.

(A) Apo B concentration in the VLDL fraction according to the degree of estimated steatosis. S0: $0 < ST \leq 0.3$, S1-S2: $0.3 < ST \leq 0.70$, S3: $ST > 0.70$.

(B) Ratio triglyceride (TG) concentration/Apo B concentration in the VLDL fraction according to the degree of estimated steatosis.

(C) Scatterplot showing the correlation between VLDL total dihydroceramide (DhCer) concentration and SteatoTest.

(D) Scatterplot showing the correlation between the ratio VLDL total DhCer/VLDL total ceramide concentration and SteatoTest.

(E) Scatterplot showing the correlation between VLDL total DhCer concentration and VLDL triglycerides.

(F) Scatterplot showing the correlation between VLDL total ceramide concentration and SteatoTest.

(G) Scatterplot showing the correlation between LDL total DhCer concentration and SteatoTest. In the scatterplots, concentration units are given as $\mu\text{mol/L}$ plasma. *** $p < 0.001$ difference significant when comparing the S0 group with S3. Total plasma concentration of sphingolipid is the sum of species described in Figure S1.

advanced fibrosis (stage >2) was rare in this cohort and might have limited the ability to detect an association.

These results suggest that NAFLD severity (steatosis and inflammation) is associated with an increased capacity of sphingolipid synthesis that can potentially induce quantitative changes in DhCer, ceramide, and sphingomyelin species.

DISCUSSION

We demonstrate here (1) a positive relationship between plasma DhCer and NAFLD severity and (2) that an elevated plasma concentration of specific DhCer species is related to their presence in triglyceride-enriched VLDL. Triglyceride-enriched VLDLs are a major component of the atherogenic dyslipidemia associated

with metabolic syndrome, type 2 diabetes,^{18,19} and NAFLD.²⁰ Since the same dihydroceramides species were significantly elevated in the plasma of individuals who will progress to diabetes up to 9 years before disease onset,¹⁷ the current study strongly suggests that dysregulated hepatic fat metabolism is an early and major component of type 2 diabetes onset. NAFLD is often present in metabolic diseases characterized by insulin resistance such as the metabolic syndrome, pre-diabetes, diabetes, and visceral obesity.

Hepatic fat accumulation is an early sign of insulin resistance and can pre-date the occurrence of diabetes or arterial hypertension.²¹ Longitudinal studies have shown that metabolic steatosis is associated with a 2.4–3.5 increased risk of incident diabetes,²² while steatohepatitis has a 3-fold increased risk of developing T2D compared to simple steatosis.

However a limitation of the current data, which are not part of a longitudinal follow-up study, is that the demonstration that NAFLD mediates the association between DhCer and the risk of type diabetes is at best, indirect.

Our findings can also potentially explain why plasma DhCer 18:0 18:0, 18:0 20:0, and 18:0 22:0 are significantly associated with waist circumference,¹⁵ a clinical marker of visceral obesity, a condition associated with steatosis²³ and that plasma DhCer

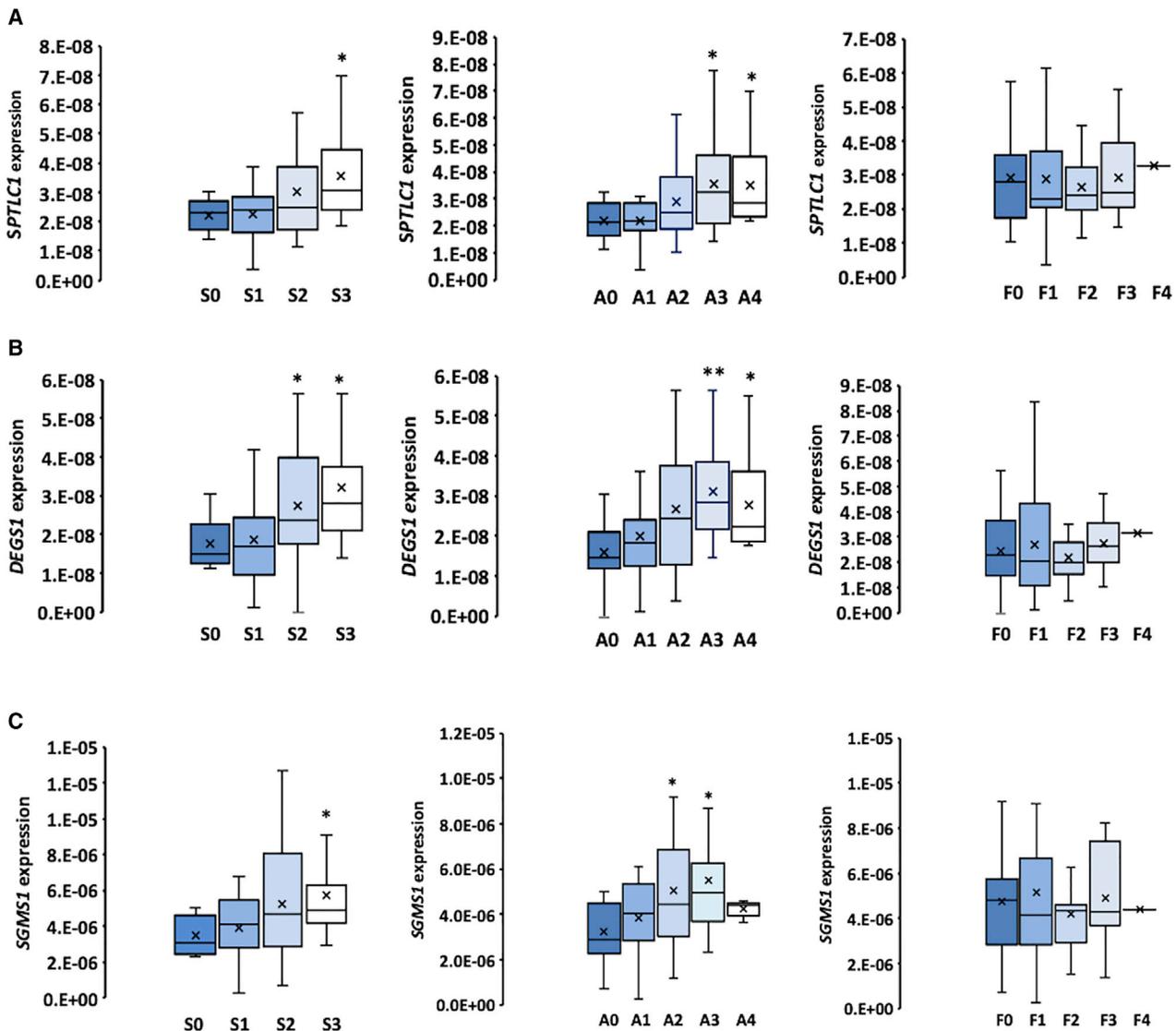


Figure 6. Analysis of the Expression (mRNA) of Enzymes of Sphingolipid Metabolism in Liver According to the NAFLD State

Box-and-whisker plot showing *SPTLC1* (A), *DEGS1* (B), and *SGMS1* (C) gene expressions according to steatosis (S0–S3), activity (A0–A4), and fibrosis (F0–F4) grades determined histologically. * $p < 0.05$ and ** $p < 0.01$ difference significant when comparing a given grade to grade 0. mRNA expression is given as arbitrary units. S0: $n = 9$, S1: $n = 20$, S2: $n = 26$, S3, $n = 18$. A0: $n = 10$, A1: $n = 13$, A2: $n = 30$, A3: $n = 15$, A4: $n = 5$. F0: $n = 30$, F1: $n = 19$, F2: $n = 15$, F3: $n = 8$, F4, $n = 1$.

18:0 16:0, 18:0 22:0, and 18:0 24:0 are associated with prediabetes and diabetes in high-fat-high fructose fed non-human primates.¹⁴

Unexpectedly, there was no correlation of DhCer or ceramides with HOMA-IR, an index of insulin resistance. Most studies that found an association between elevated plasma ceramide or DhCer and insulin resistance compared control subjects or patients with a low HOMA-IR with overtly insulin resistant subjects.^{16,18,24} Here, we studied a population of type 2 diabetic subjects, which might explain the discrepant results.

The reason why triglyceride-rich VLDLs are enriched in DhCer is unknown. Since VLDL have a very short half-life (4–6 h) precluding major membrane rearrangement outside of the liver, the increased DhCer concentration is most probably linked to VLDL

formation/secretion. The increase in DhCer concentrations in patients with type 2 diabetes could then reflect an overall increase in sphingolipid synthesis due to an enhanced VLDL secretion in the steatotic liver.²⁵ The increased expression of some of the enzymes involved in sphingolipid synthesis with NAFLD severity is compatible with this hypothesis. Although we have no direct measurement of sphingolipid concentrations in the liver, it was shown previously that patients with a high fat content have higher hepatic ceramide and DhCer concentrations.²⁶

In this study, we show that VLDL DhCer but not ceramide concentration correlated with SteatoTest and VLDL triglycerides. This suggests that DhCer and ceramide can be involved in different metabolic pathways and argue for a specific role of DhCer during the synthesis/secretion of large triglyceride-enriched VLDL. One

hypothesis is related to the structural characteristics of DhCer in terms of hydrophobicity, membrane rigidifying properties, negative curvature and membrane fusion,^{11,27} and how they could impact the membrane assembly/function of large VLDL. For instance, in cancer cells, the DhCer/Ceramides ratio is increased by $\Delta 9$ -Tetrahydrocannabinol treatments and induces changes in ER membrane properties resulting in a rigidification of the autophagosome membrane²⁸ and its permeabilization.

Alterations of hepatic sphingolipid metabolism and particularly ceramide accumulation due to *de novo* synthesis have been suspected to play a role in the progression from steatosis to NASH,²⁹ by inducing cell stress and cell death. Since DhCer are precursors of ceramides, an increased plasma DhCer concentration reflects a stimulation of the ceramide synthesis flux.²⁶ Interestingly, a study with a small number of NASH patients has shown an increase in hepatic ceramide concentrations contrary to the plasma where levels were unchanged.³⁰ Plasma DhCer thus could be a better biomarker than plasma ceramides for hepatic sphingolipid metabolism. Whether circulating DhCer is a marker of progression from steatosis to NASH needs to be tested in prospective studies.

In conclusion, specific DhCer species in the plasma are associated with hepatic steatosis and with NASH in type 2 diabetic patients. This could be related to their abundance in triglyceride-rich VLDL, which are increased as a consequence of hepatic steatosis.

The association between steatosis, DhCer, and VLDL production suggests a specific requirement for these sphingolipids in the process of triglyceride-enriched VLDL synthesis or secretion. Since VLDL is a major component of the atherogenic dyslipidemia associated with metabolic syndrome, type 2 diabetes,^{18,19} and NAFLD,²⁰ these data may bring a better understanding of specific mechanisms of dyslipidemia in these atherogenic conditions. In addition, NAFLD severity is associated with altered sphingolipid synthesis raising the question of the role of sphingolipid metabolism in NAFLD progression. This will require further prospective studies.

Limitations of Study

Since this study was not initially designed for studying liver metabolism, one limitation is the use of biomarkers to indirectly evaluate NAFLD severity rather than MRI-based techniques, fibroscan, or the gold standard, histological characterization of liver biopsies. Nevertheless, biomarkers have been validated against histology for the diagnosis of steatosis and steatohepatitis,^{31,32} including in a subset of patients with type 2 diabetes.²⁴ They are acceptable alternatives for the diagnosis of steatosis when imaging tools are not available or feasible, according to current guidelines.³³ We have used here two surrogates for hepatic steatosis, SteatoTest and FLI, which are based on different parameters. Their concordance in terms of steatosis severity strengthens their use in this study.

Another limitation of this study is the relatively low number of patients analyzed for their lipoprotein sphingolipids, and this analysis must be strengthened in further studies.

A final limitation of this study is the fact that we have used a different group of patients in order to study hepatic sphingolipid enzyme expression in which we could not measure both the expression of hepatic sphingolipid enzymes and the concentra-

tion of sphingolipids because of insufficient biological material. Nevertheless, we show a clear relationship between histologically defined steatosis and inflammation with sphingolipid enzyme expression independently of the presence or absence of type 2 diabetes.

STAR★METHODS

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Supplemental Information can be found online at <https://doi.org/10.1016/j.xcrm.2020.100154>.

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AUTHOR CONTRIBUTIONS

Conceptualization, P.F., F.F., and O.B.; Formal Analysis, J.-E.S. and P.F.; Investigation, A.C., F.P., A.S., W.L.G., M.G., E.H., and F.L.; Resources, F.P., A.H., J.G., O.B., and V.R.; Writing – Original Draft, P.F., F.F., and O.B.; Writing – Review and Editing, P.F., F.F., O.B., E.H., A.H., V.R., J.G., and F.P.; Supervision, F.F.; Funding Acquisition, F.F. and O.B. All authors gave final approval of the present manuscript. O.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
Total cholesterol	Diasys	Cat#113009910026
Free cholesterol	Diasys	Cat#178909910025
Triglycerides	ThermoFisher diagnostics	Cat#981786
Phospholipids	Diasys	Cat#157419910930
apolipoprotein B	ThermoFisher diagnostics	Cat#981930
RNeasy Mini Kit	QIAGEN	Cat#74106
Oligonucleotides		
<i>CERS2, CERS4, DEGS1, SGMS1, SPTLC1, 18S</i>	This paper	Table S4
Software and Algorithms		
XLSTAT	Addinsoft	https://www.xlstat.com/fr/
SteatoTest	Poynard et al. ³¹	https://www.biopredictive.com/fr/
NASHTest	Poynard et al. ³⁴	https://www.biopredictive.com/fr/
FibroTest	Halfon et al. ³⁵	https://www.biopredictive.com/fr/
Fatty Liver Index	Bedogni et al. ³²	N/A
Other		
HPLC Agilent column Poroshell 120, EC-C8, 2.1 × 100mm	Chromoptic	Cat# 695775-906

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Pascal Ferré (pascalfr@gmail.com)

Material Availability

This study did not generate new unique reagents.

Data and Code Availability

The published article includes all datasets generated or analyzed during the study.

EXPERIMENTAL MODELS AND SUBJECT DETAILS

CERADIAB is a cross-sectional study, that included over a 7 month period 90 adult patients with type 2 diabetes (inclusion criteria) from the Diabetes Department of the Pitié-Salpêtrière Hospital (Paris, France) for assessing the plasma concentrations of sphingolipids. Exclusion criteria were: Diabetes without overweight, diabetes with autoimmune markers of type 1 Diabetes, post-transplantation diabetes mellitus, Maturity Onset Diabetes of the Young (MODY), diabetes diagnosed before 35 years old, patients taking medications such as glucocorticoids, HIV medications and atypical antipsychotics, immunosuppressive therapy patients with type 1 and type 2A dyslipidemia, non-metabolic hepatopathy such as chronic hepatitis B or C, genetic hemochromatosis, autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, α 1-antitrypsin deficiency, Wilson's disease, cirrhosis, severe renal failure defined by an estimated glomerular filtration rate by the modification of diet in renal disease equation (MDRD) < 30 ml/min. Clinical assessment included comorbidities, personal disease history (type and duration of diabetes), retinopathy, kidney function (estimated glomerular filtration rate and microalbuminuria), coronary artery disease, anthropometric measures (weight, height, BMI) and treatment.

DIACART is a 5 year prospective observational study (NCT02431234) that included 198 patients with type 2 diabetes from the Diabetes and Cardiology Departments of the Pitié-Salpêtrière Hospital (Paris, France) for assessing the factors associated with lower limb arterial calcification³⁶. Inclusion criteria were type 2 diabetes with at least 1 of the following factors: coronary artery disease defined by history of myocardial infarction, coronary stent, coronary bypass or peripheral arterial occlusive disease or age > 50 years

for men and > 60 years for women. Coronary calcium score alone was not considered as an inclusion criteria. Exclusion criteria were an estimated glomerular filtration rate < 30 mL/min, a history of lower limb angioplasty and/or bypass, immunodeficiency, type 1 diabetes. All patients with infectious disease at inclusion with clinical and/or biological signs of inflammation were excluded. During the study follow-up (mean 31.2 ± 3.9 months; median 30.5 months and range 26–44.8), 18 patients were lost and 11 patients died before the end of the study. Serum biomarkers of NAFLD measurements were missing in 20 patients. Thus at the end of the study, 149 patients were screened for NAFLD using serum biomarkers.

Blood samples for lipoprotein analysis were obtained from 32 individuals with type 2 diabetes over a period of six months, seen consecutively for their annual diabetes assessment in the diabetes department of the Pitié-Salpêtrière Hospital (Paris, France), with inclusion/exclusion criteria similar to the ones of the CERADIAB cohort.

Liver samples were obtained from 73 patients from the Hepatology Department of La Pitié-Salpêtrière Hospital investigated for unexplained, persistently elevated aminotransferases. Steatosis, activity and fibrosis were scored according to the FLIP-SAF histological classification³⁷. Part of the liver biopsies were frozen at -80°C for RNA extraction.

In the various cohorts, no individuals were related.

METHOD DETAILS

Biochemical measurements

Biochemical measurements were made on venous blood and urine samples collected after an overnight fast. Insulin resistance was assessed by the calculation of HOMA-IR in patients not treated with insulin. Serum biomarkers of fibrosis, FibroTest®³⁵, steatosis, SteatoTest®³¹, Fatty Liver Index (FLI)³², and steatohepatitis, NASHTest®,³⁴ were measured. FibroTest uses an algorithm based on serum α_2 -macroglobulin, apo A1, haptoglobin, total bilirubin and gamma glutamyl transferase (GGT). Fibrosis severity is categorized as: minimal (F0-F1), moderate (F2) or severe (F3-F4). SteatoTest is a continuous linear biochemical assessment of steatosis grade using an algorithm based on serum α_2 -macroglobulin, apo A1, haptoglobin, total bilirubin, GGT, body mass index (BMI), cholesterol, triglycerides and fasting plasma glucose. It ranges from 0.00 to 1.00, as an equivalent of the histological grade of steatosis: S0, no steatosis; S1: minimal steatosis; S2: moderate steatosis; and S3: marked or severe steatosis. FLI is a continuous linear biochemical assessment of steatosis grade using an algorithm based on BMI, waist circumference, triglycerides and GGT. It gives a quantitative estimate of liver steatosis ranging from 0.00 to 100. A FLI < 30 rules out and a FLI > or = 60 rules in fatty liver. NASHTest uses an algorithm based on serum α_2 -macroglobulin, apo A1, haptoglobin, total bilirubin, GGT, ASAT, cholesterol, triglycerides, fasting plasma glucose and BMI. The diagnosis of non-alcoholic steatohepatitis (NASH) is expressed in three classes (N0 (N = 0.25): no NASH; N1 (N = 0.5): NASH possible; N2 (N = 0.75): NASH).

Isolation and characterization of lipoproteins

Major lipoprotein fractions namely Triglyceride-Rich Lipoprotein, Low Density Lipoprotein (LDL) and High Density Lipoprotein (HDL) were prepared from plasma by sequential ultracentrifugation at 120,000 rpm at 15°C using a Beckman Optima Max-TL centrifuge. By this method we successively isolated Triglyceride-Rich Lipoprotein (VLDL+IDL d < 1.019 g/ml, abbreviated as VLDL), LDL (d: 1.019–1.063 g/ml) and HDL (d: 1.063–1.21 g/ml) fractions using periods of centrifugation of 1h, 3h and 5h respectively. After isolation, lipoprotein fractions were analyzed for their lipid and protein content. Commercial kits (Diasys, France) were used for total cholesterol, free cholesterol and phospholipids. Triglycerides (TG) and apolipoprotein B were quantified with a commercial kit (Thermo Electron, France). Lipoprotein fractions were dialyzed extensively against PBS before use for lipidomic analyses.

Plasma lipid analysis by LC-MS/MS

Three-hundred microliters of plasma or lipoprotein fractions were used to quantify sphingolipids. The lipid subspecies were extracted and analyzed, as described in³⁸, at the Lipidomic Core Facility of the Université of Bourgogne (Dijon, France) or at the lipidomic facility of the ICAN Institute (Sorbonne Université, Paris).

Sphingolipids were extracted according to the Bligh and Dyer method as described by Reis et al.³⁹. Briefly, plasma aliquots (50 μL of plasma + 150 μL of saline) were mixed with d18:1–17:0 Cer, used as an internal standard, and extracted with 750 μL of 2:1 chloroform/methanol for 10 min. Chloroform (250 μL) was then added and extraction followed for 10 min. Distilled water (250 μL) was added and extraction continued for 10 more min. After centrifugation (10,000 $\times g$, 10 min, 4°C), the organic phase was collected. The aqueous phase was acidified with hydrochloric acid (8 μL , 3 mol/liter) and further extracted with 600 μL of chloroform for 10 min. After centrifugation (10,000 $\times g$, 10 min, 4°C) the organic phase was collected and combined with the previous sample. Pooled organic phases were washed with 800 μL of the upper phase from a chloroform/methanol/water (96.7:93.3:90) mixture. The organic phase was evaporated under vacuum. Extracts were finally dissolved with 200 μL of 60:30:4.5 chloroform/methanol/distilled water, and 3 μL were injected on a 1200 6460-QqQ LC-MS/MS system equipped with an ESI source. Separation was achieved on a Poroshell C8 2.1 \times 100 mm, 2.7- μm column (Agilent Technologies). Calibration curves were obtained for each molecule using authentic standards extracted by identical methods as used for plasma samples. Quadratic regression was applied to calculate plasma sphingolipid concentrations.

RNA extraction and Real-time qPCR

Frozen hepatic samples were processed for RNA extraction using the RNeasy Mini Kit (QIAGEN, Venlo, the Netherlands) according to the manufacturer's protocol. Expression of hepatic genes was analyzed as described⁶. Ribosomal RNA 18S was used for normalization and for relative quantification of gene expression using the $2^{-\Delta\Delta C_t}$ method. Sequences of primers used are indicated in Table S4.

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantitative data are expressed as mean \pm standard deviation (SD) or as median and interquartile when distribution was normal or non-normal respectively or as frequency for qualitative data. Comparisons of quantitative variables were analyzed by Student's t test or Mann-Whitney tests when distribution was normal or non-normal respectively. Comparison of frequencies was performed by the XLSTAT test for two proportions (XLSTAT software, Addinsoft®). A $p < 0.05$ was considered statistically significant. For multiple comparisons, the Benjamini-Hochberg false discovery rate procedure was used. The correlation between linear variables and the corresponding p value were assessed by calculating Pearson's or Spearman's coefficient r when distribution was normal or not normal respectively (XLSTAT software, Addinsoft®). Concerning the covariates used in the analyses, all biological variables were initially entered in the univariate analysis. Then only variables associated significantly at univariate analysis with total ceramide, total dihydroceramide or total sphingomyelin were entered in a multivariate analysis. The variables used in the multivariate analysis were for the CERADIAB cohort, BMI, HbA1c, hsCRP, creatinine, total cholesterol, HDL and LDL cholesterol, triglycerides ASAT, ALAT, GGT, fibrotest, steatotest, NASH test. The same variables were used for the DIACART cohort plus waist circumference and FLI. Multivariable analysis was performed by use of ANCOVA (XLSTAT software, Addinsoft®). For multivariable analysis, beta-coefficients (β) were calculated to allow for direct comparison of the relative influence of the explanatory variables (biological characteristics) on the dependent variable (sphingolipid concentrations), and their significance was tested ($p \leq 0.05$ considered significant). A principal component analysis (PCA) was applied to the DHCer species of the CERADIAB cohort to further identify clusters of distribution (XLSTAT software, Addinsoft®).

ADDITIONAL RESOURCES

The DIACART study (Arterial Calcification in the Diabetes) is a 5 year prospective observational study (NCT02431234) for assessing the factors associated with lower limb arterial calcification³⁶. A complete description is given at <https://clinicaltrials.gov/ct2/show/NCT02431234>