AMERICAN JOURNAL OF PHYSIOLOGY
ENDOCRINOLOGY AND METABOLISM

Am J Physiol Endocrinol Metab 330: E589–E605, 2026.
First published March 24, 2026; doi:10.1152/ajpendo.00049.2026

RESEARCH ARTICLE

Clinical Metabolism

The interaction between glucose levels and body mass index on the regulation of the circulating sphingolipidome in humans

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Abstract

Circulating sphingolipids have been associated with diabetes risk and chronic complications. This study characterized the sphingolipidome in a subset of the CA.ME.LI.A. cohort to identify lipid signatures related to sex, body mass index (BMI), and fasting glucose levels. Three hundred sixty-seven subjects (217 men) were stratified into six groups based on BMI (normal weight or overweight/obese) and fasting glucose levels (normal, impaired, or diabetes). Circulating sphingolipids were measured by LC-MS/MS. The effects of BMI, glucose levels, and their interaction on the sphingolipidome were analyzed using a two-way ANOVA model. Women showed higher circulating sphingolipid levels than men, except for ganglioside GM3. Glucose levels produced relevant changes on hexosyl- and lactosylceramides, which were significantly reduced in subjects with diabetes, independently of BMI. Some ceramide and sphingomyelin species also varied only according to glucose levels. Dihydroceramide 18:0 and 24:1 were higher in overweight/obese subjects, whereas sphingomyelin 18:1 and GM3 24:0 were higher in normal-weight individuals. Gangliosides GM3 were higher in normal body weight with normal glucose levels and impaired fasting glucose as compared with overweight obese individuals of the same categories. Sphingomyelin 18:1, GM3 24:1, sphingosine, and dihydrosphingosine-1-phosphate levels were significantly regulated by both BMI and glucose. In overweight/obese individuals, sphingosine-1-phosphate and dihydrosphingosine-1P levels were reduced in impaired fasting glucose and diabetes. The circulating sphingolipidome differs in men and women, being modulated by BMI and glucose levels. These data support the concept that sphingolipids could be novel biomarkers for obesity, diabetes, and associated complications.

NEW & NOTEWORTHY Sphingolipid glycosylation is an enzymatic process that does not follow the pattern of nonenzymatic hemoglobin glycosylation. Unexpectedly, hexosyl- and lactosylceramides decreased in impaired fasting glucose and diabetes, with and without obesity. On the other hand, dihydroceramides increased in overweight/obesity with prediabetes/diabetes. The circulating sphingolipidome is differentially regulated in humans according to sex, glucose, and BMI.

diabetes; glycosphingolipids; obesity; sphingolipids; sphingosine-1P

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Submitted 3 February 2026 / Revised 27 February 2026 / Accepted 18 March 2026

INTRODUCTION

The CA.ME.LI.A project (CArdiovascular risks, MEtabolic syndrome, Liver, and Autoimmune disease) is an epidemiological study conducted in the city of Abbiategrasso (MI, Italy) between 2009 and 2011. The study design and baseline characteristics of the population were described previously (1). This population was studied after stratification into subgroups according to fasting glucose levels and/or body mass index (BMI) (2), and the effects of the two variables on cardiovascular events/deaths and all-cause mortality at 7-yr follow-up were investigated either independently or in combination (2).

The number of scientific papers with the word "biomarkers" in the title has grown exponentially over the years, with over 768,500 papers indexed in PubMed alone (3–5). This growth reflects the increasing recognition of biomarkers as tools for diagnosis, prognosis, and monitoring treatment response. However, translating this potential into widespread clinical use remains a challenge due to difficulties in validation and implementation.

Indeed, cardiovascular disease (CVD) remains the leading cause of death globally, with T2 diabetes mellitus being one of the most important comorbidities, and obesity being an additive major risk factor for both diseases. World Health Organization (WHO) reports that obesity has nearly tripled since 1975, with >1 billion people living with obesity (650 million adults, 340 million adolescents, and 39 million children) in 2022 (6). The rapid and widespread increase in obesity rates across the world over the past few decades has led to the recognition of obesity as a global epidemic or even a "globesity" crisis (7–9).

Type 2 diabetes mellitus is a multifactorial disease characterized by insulin resistance and β-cell dysfunction. Chronic overnutrition and obesity promote a state of low-grade inflammation and metabolic stress that impairs insulin signaling in key metabolic tissues, including the liver, muscle, and adipose tissue. As the disease progresses from normoglycemia to impaired fasting glucose and overt diabetes, a number of metabolic processes is affected (10–15).

Despite the use of established traditional cardiovascular risk factors such as age, sex, prevalent hypertension, total, LDL, HDL-cholesterol, and glucose levels, the morbidity for CVD-associated pathologies and overall mortality is still rising. The discovery of novel biomarkers might improve prediction of CVD risk, to monitor disease progression, and potentially be used as therapeutic targets before clinical signs and symptoms appear (16–18).

Total, HDL, and LDL cholesterol are used to assess cardiovascular risk in addition to traditional risk factors (age, gender, smoking, blood pressure) (https://www.escardio.org/Education/Practice-Tools/CVD-prevention-toolbox/SCORE-Risk-Charts). As a matter of fact, cholesterol and its different forms represent only a small part of the entire lipidome circulating in plasma. So, it would be very useful to identify new lipid species that could be added as biomarkers in the calculation of the risk score.

Multi-omics, driven by artificial intelligence and machine learning, are emerging as powerful tools for the identification of biomarkers associated not only with diabetes but also with cardiovascular diseases and heart failure (19–21). Profiling of polar and nonpolar low-MW metabolites, in addition to proteomic analysis, has revealed many different altered biochemical mechanisms associated with vascular dysfunction, inflammation, myocardial remodeling, and progression toward heart failure (22, 23). Consequently, sphingolipidomics represents a new frontier for advancing precision medicine in metabolic and cardiovascular diseases.

Sphingolipids (SL) are a part of the entire lipidome, comprising a group of molecules characterized by a backbone composed of a sphingoid base, mainly sphingosine (d18:1) and dihydrosphingosine (d18:0) (24–26). They are cellular structural elements, being constituents of membranes and metabolism regulators as they take part in several biological processes with key functions; in particular, they are involved in essential cellular phenomena such as differentiation, proliferation, movement, and apoptosis (27, 28). In Fig. 1, a general overview of their metabolism is reported.

In recent years, many studies have suggested a crucial role of sphingolipids in the pathogenesis of the most common diseases, from type 2 diabetes mellitus to Alzheimer's disease, from cardiovascular diseases to hepatocellular carcinoma (29–40). In particular, plasma sphingolipids have been implicated in the development of metabolic and cardiovascular diseases (41, 42).

Altered sphingolipid metabolism could be involved in defective insulin signaling, vascular endothelial dysfunction, and atherosclerosis (43–46). Several studies have shown that ceramides and some sphingolipids could contribute to insulin resistance and, consequently, to the development of type 2 diabetes and heart disease (47–49). Moreover, it has been suggested that changes in plasma sphingolipid concentration could be of use for monitoring metabolic alterations in subjects with obesity with metabolic syndrome (50). The multiple roles of sphingolipids in maintaining homeostasis within the body suggest they could serve as both biomarkers in combination with clinical parameters for the prevention of metabolic/cardiovascular diseases (51–53), as well as therapeutic targets.

We aimed to characterize the circulating sphingolipidome in a subset of the CA.ME.LIA study subjects to identify novel "signatures" potentially associated with impaired fasting glucose levels/diabetes/overweight/obesity, and associated comorbidities (cardiovascular diseases and cancer) (1, 2).

MATERIALS AND METHODS

Study Sample

For the lipidomic analysis, we used a subset of participants in the CA.ME.LI.A study that has been described previously (1, 2). The CA.ME.LI.A study was approved by the Ethics Committee of the Legnano Hospital (Milan, Italy) on April 08, 2009 (Protocol Number 245/2009).

The population enrolled in the CA.ME.LI.A study had been previously subdivided into six groups according to fasting glucose and BMI levels: *1*) normal fasting glucose (NFG)/normal body weight (NBW); *2*) NFG/overweight/

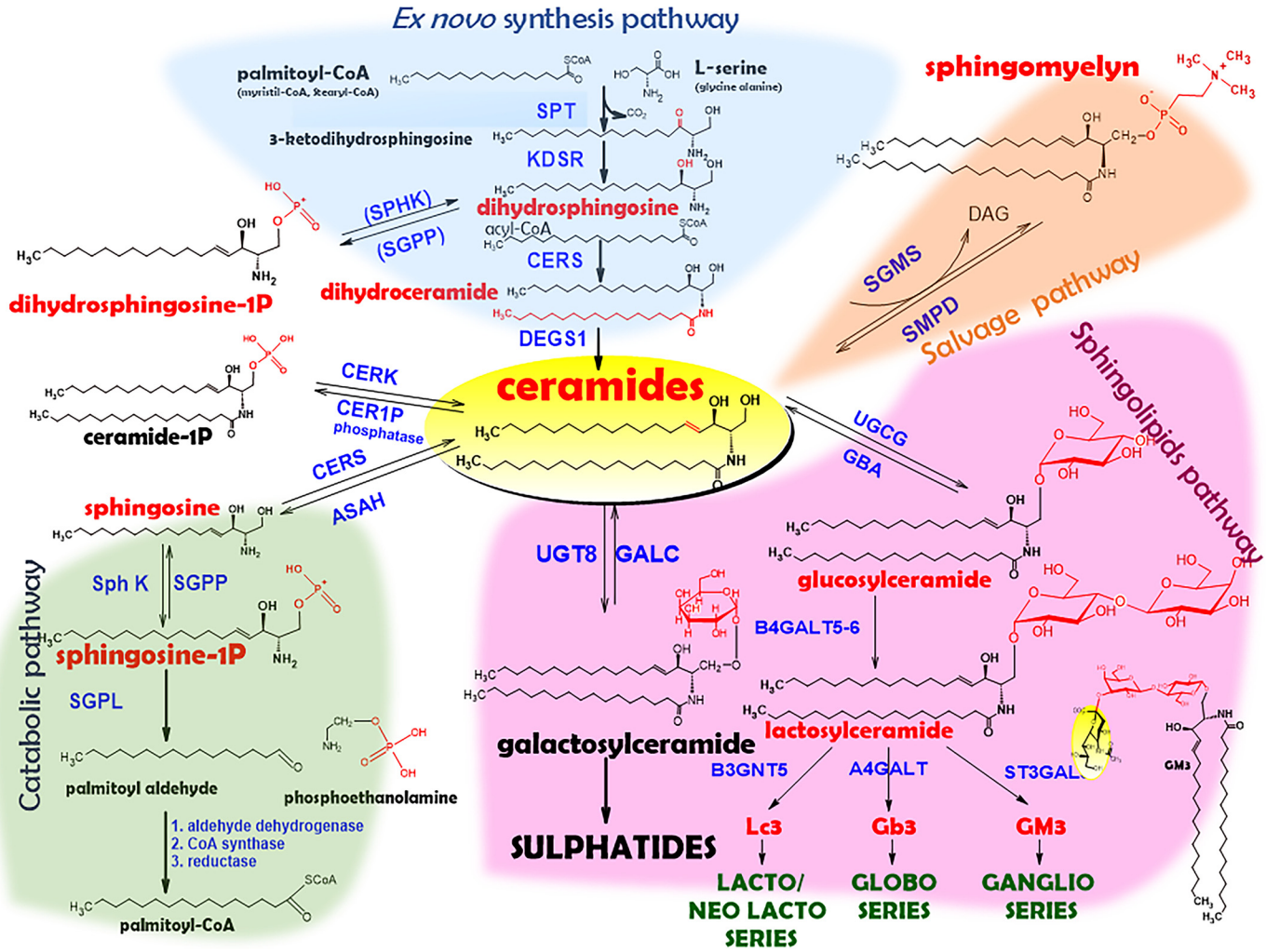

Figure 1. Schematic pathways of sphingolipids metabolism. Abbreviations of enzymes reported in the scheme are abbreviated following the Human Genome Organization (HUGO) gene nomenclature committee: SPT, serine palmitoyltransferase; KDSR, 3-ketodihydrosphingosine reductase; CERS1-6, dihydroceramide synthases; DEGS1, dihydroceramide desaturase 1; CERK, ceramide kinase; ASAH 1–2, acid ceramidase; Sph K1-2, sphingosine kinase; SGPP1-2, sphingosine 1P phosphatase; SGPL1, sphingosine 1P lyase; UGCG, glucosylceramide synthase; UGT8, galactosylceramide synthase; GBA1, glucocerebrosidase; B4GALT5-6, lactosylceramide synthase; ST3GAL5, GM3 synthase; B3GNT5, lactotriosylceramide synthase; A4GALT, globotriosylceramide synthase; SGMS1-2, sphingomyelin synthases; SMPD1-4, sphingomyelinases; GALC, galactocerebrosidase. Cer1P phosphatase lacks HUGO abbreviation.

obese (OWO); *3*) impaired fasting glucose (IFG)/NBW; *4*) IFG/OWO; *5*) diabetes mellitus (DM)/NBW; and *6*) DM/OWO, to study their association with cardiovascular events and all-cause mortality over a 7-yr follow-up (2).

In the present study, the lipidomic analysis was carried out on sera from subgroups of the original study (1, 2). As expected, the DM/NBW, the DM/OWO, and the IFG/NBW groups were underrepresented, accounting for only 1.1%, 5.6%, and 7.2% of the whole CA.ME.LI.A population, respectively, and had a different sex distribution (2). To limit the effect of such unbalanced distribution, we included the sera from all of the 28 subjects belonging to the smallest group (DM/NBW), and used a stratified, random sampling procedure to allocate the remaining sera into five groups while also retaining the same sex distribution of the three underrepresented groups (i.e., the DM/NBW, DM/OWO, and IFG/NBW groups). Main characteristics of the 367 sera selected for the lipidomic analyses are reported in Table 1.

Sphingolipid Extraction from Plasma

Ceramides (Cer) and dihydroceramides (DHCer), sphingomyelins (SM), hexosylceramides (HexCer), lactosylceramides (LacCer), gangliosides (GM3), and all the other sphingoid bases [Sphingosine (Sph), sphingosine-1-phosphate (S1P), dihydrosphingosine (DHSph), dihydrosphingosine-1-phosphate (DHS1P)] were extracted from frozen plasma and assessed using liquid chromatography–mass spectrometry (LC-MS/MS) (54–56).

Plasma (25 μL) was added with the internal standard (IS) mixture (10 μL, Cer 12:0, SM 12:0, HexCer 12:0, and Sph d17:0, 20 μM), diluted with water (75 μL), and mixed with a methanol/chloroform solution (850 μL, 2:1, vol/vol). The lipids were extracted by thermo-shaking (38°C for 1 h at 1,000 rpm), followed by alkaline methanolysis (75 μL of 1 M KOH in 90% methanol) with incubation under thermo-shaking for 2 h at 38°C. The reaction was then neutralized by the addition of 4 μL of 1 M acetic acid in methanol. The organic phase

Table 1. *CA.ME.LI.A study sample was selected for lipidomic analysis and stratified by gender, BMI, and fasting glucose levels*

	Overall $n = 367$	Women $n = 150$	Men $n = 217$	*P* Value*
NFG/NBW	63 (17.2%)	26 (17.3%) (7.1%)	37 (17.1%) (10.1%)	>0.9
NFG/OWO	56 (15.3%)	24 (16.0%) (6.5%)	32 (14.7%) (8.7%)	
IFG/NBW	54 (14.7%)	21 (14.0%) (5.7%)	33 (15.2%) (9.0%)	
IFG/OWO	98 (26.7%)	39 (16.0%) (10.6%)	59 (27.2%) (16.1%)	
DM/NBW	28 (7.6%)	13 (8.7%) (3.5%)	15 (6.9%) (4.1%)	
DM/OWO	68 (18.5%)	27 (18.0%) (7.3%)	41 (18.9%) (11.2%)	

In all columns, the first % was calculated on the total number of subjects in the column (Overall $n = 367$, Women $n = 150$, Men $n = 217$). For Men and Women columns, an additional % vs. the overall population is also reported on the left. BMI, body mass index; CA.ME.LI.A, CArdiovascular risks, MEtabolic syndrome, LIver and Autoimmune disease; DM, diabetes mellitus; IFG, impaired fasting glucose; NBW, normal body weight; NFG, normal fasting glucose; OWO, overweight/obese. *Pearson's Chi-squared test; Fisher's exact test.

was separated via centrifugation (25 min at 13,400 rpm), and 900 μL were transferred and evaporated in a SpeedVac vacuum concentrator. The residuals were dissolved in 100 μL of methanol + 0.5 mg/mL butylated hydroxytoluene (BHT), centrifuged again for 10 min at 13,400 rpm, withdrawn into a glass vial, and 5 μL of clear supernatant were directly injected into LC-MS/MS instrument for quantitative analysis. If the samples were cloudy, the tip-tip filtration already described was applied (57).

LC-MS/MS for Sphingolipidomic Target Analysis

Instrumentation.

The analytical system consisted of an LC Dionex 3000 UltiMate (Thermo Fisher Scientific, Waltham, MA) with autosampler, binary pump, and column oven coupled to a tandem mass spectrometer AB Sciex 3200 QTRAP (AB Sciex, Concord, ON, Canada) equipped with electrospray ionization TurboIonSpray source operating in positive mode (ESI+).

Chromatographic analysis of sphingolipids and glycosphingolipids.

To chromatographically isolate the complex SL (DHCer, Cer, SM, HexCer, LacCer, and GM3), we used a reverse-phase Acquity BEH C8 column 1.7 μm, 2.1 × 100 mm (Waters, Milford, MA) equipped with a pre-column, using as mobile phases (A) water + 0.2% formic acid + 2 mM ammonium formate and (B) methanol + 0.2% formic acid + 1 mM ammonium formate. The flow rate was 0.3 mL/min, and the autosampler and the column oven were kept at 20°C and 30°C, respectively. The elution gradient (%B) was set as follows: 0–3 min (80%–90%), 3.0–6.0 min (90%), 6.0–19.0 min (90%–99%), 19.0–20.0 min (99%–80%), held until 24 min. Due to the lack of authentic standards for every fatty acid chain, those that are not available were quantified as a reference to the closest sphingolipid subspecies.

Chromatographic analysis of free sphingoid bases.

A reversed-phase Cortecs C18 1.6 μm, 2.1 × 100 mm (Waters, MA) column, equipped with a pre-column, was used for sphingoid bases (Sph, S1P, DHSph, and DHS1P). The mobile phase was (A) water + 0.2% formic acid + 2 mM ammonium formate and (B) methanol + 0.2% formic acid + 1 mM ammonium formate. The elution gradient (%B) was set as follows: 0–12 min (70%–85%), 12.0–12.2 min (85%–99%), 12.2–15.0 min (99%), 15.0–15.2 min (99%–70%), held until 20 min. The flow rate was 0.2 mL/min, and the column temperature was set to 30°C.

Mass spectrometry conditions.

The instrument was managed with the proprietary manufacturer's software and according to the manufacturer's instructions. The analytical data were processed using Analyst software (v.1.2). The ion spray voltage was set at 5.5 kV, and the source temperature was set at 300°C. Nitrogen was used as a nebulizing gas (GS 1, 45 psi), turbo spray gas (GS 2, 50 psi), and curtain gas (25 psi). The collision-activated dissociation (CAD) was set to a medium level. The dwell time was set at 50 ms, and the MS scan was performed in positive ion modes (ESI+). MS/MS experiments were conducted using nitrogen as a collision gas. Compound-dependent parameters (CE, DP) have been optimized via direct infusion. Spectra were acquired by multiple reaction monitoring (MRM), scanning for each analyte, and the transitions are reported in Supplemental Table S1 (see, https://doi.org/10.6084/m9.figshare.31545361).

Quantitative Analysis

The lipidomic target analysis comprised 45 sphingolipid species, the most representative ceramides, dihydroceramides, sphingomyelins, hexosylceramides, lactosylceramides, GM3, plus four sphingoid bases [sphingosine, dihydrosphingosine, sphingosine-1P (S1P), and dihydrosphingosine-1P (DHS1P)]. Linearity was observed for each compound in the whole range ($R^2 > 0.99$), with a range between 0.01–5 μM for all sphingolipids except for sphingomyelins, which was 1–100 μM. Quantitative analysis was performed by interpolating each peak area of the analyte/area IS with the calibration curve slope for each sphingolipid. The sphingolipid amount was expressed in μmol/L.

Statistical Analysis

Descriptive statistics were computed for all study variables. Continuous variables were summarized as means ± standard deviation (SD) when normally distributed, or as median and interquartile range (IQR: Q1–Q3) when normality assumptions were not met. Categorical variables were reported as absolute frequencies and percentages.

For sphingolipid species with values under the limit of quantification (DHCer 18:0, DHCer 18:1, LacCer 20:0, GM3 18:0, GM3 18:1, GM3 20:0, GM3 24:0), a constant—defined as the square of the first quartile divided by the third quartile—was added to all observations to avoid computational artifacts (58). Continuous sphingolipid concentrations were subsequently transformed to improve adherence to normality assumptions required for parametric modeling. The most appropriate transformation for each metabolite was identified using the Box–Cox procedure (59).

Following the random selection of participants, as detailed in the sampling section, two-way analysis of variance (ANOVA) models were fitted to evaluate the main effects of

fasting glucose category and BMI category, as well as their interaction, on sphingolipid concentrations. All models were adjusted for age (continuous) and sex (categorical: male/female) to account for potential confounding. Factor-level comparisons for fasting glucose and BMI were coded using sum-to-zero contrasts. When significant omnibus effects were detected, post hoc contrasts were performed using Tukey's honest significant difference (HSD) procedure and estimated marginal means (emmeans) to characterize pairwise differences. In the presence of significant interaction terms, stratified analyses were conducted to elucidate effect modification (60).

All statistical analyses were performed using R, version 4.5.0, employing the packages *dplyr*, *car*, *MASS*, *emmeans*, and *multcomp*. Graphical representations were generated using GraphPad Prism 7.0, MetaboAnalyst 4.0, and BioRender (https://BioRender.com).

RESULTS

Sphingolipids in the Whole Study Sample

The anthropometric and ultrasonographic, biochemical, and hematological characteristics, family history, habits, and personal diseases of the 367 selected patients ($n = 367$; 150 women, 217 men) are reported in Supplemental Tables S2, S3, S4, and S5. When compared with men, women were slightly older. BMI was not different between sexes. Men had significantly higher visceral adipose tissue (VAT), interadventitia common carotid artery diameter (ICCAD), and intima-media thickness (IMT) (Supplemental Table S2). Women had significantly higher SAT as compared with men. Glucose, insulin, and HOMA index were not statistically different between sexes. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (gamma-GT), and homocystein (Hcys) were consistently higher in men. By contrast, as already observed (1, 2) C reactive protein (CRP) was higher in women. LDL-cholesterol did not differ between sexes, whereas HDL was significantly higher in women (Supplemental Table S3).

On this sample of 367 subjects, we performed targeted sphingolipidomic analysis, quantitatively analyzing 45 circulating sphingolipid species listed in Supplemental Table S1. The results of the quantification in the population sample, and partitioned by sex, are reported in Supplemental Tables S6 (ceramides and dihydroceramides), S7 (hexosyl and lactosyl-ceramides), and S8 (sphingomyelins and sphingoid bases). In Supplemental Fig. S1, typical distributions of circulating sphingolipids classes in the population sample are reported as histograms divided into deciles. A Gaussian distribution of Cer, SM, HexCer, and LacCer is evident, whereas DHcer and GM3 seem to be skewed toward the lower values. HexCers correspond to both GlcCer and GalCer together, since they cannot be easily distinguished by reversed-phase LC-MS/MS, although GlcCer is largely predominant in the blood due to the tissue-specific localization of GalCer in oligodendrocytes and Schwann cells (61). As already reported (62), there was approximately eight times the amount of GlcCer compared with GalCer. Distribution histograms of the 45 specific sphingolipid species are reported in Supplemental Figs. S2–S7. A pie diagram in the figures indicates the weight of each specific sphingolipid (calculated with the median) within the total sphingolipid family. The most represented species within the respective families were Cer 14:0, Cer 24.0, Cer 24:1, DHCer 24:0 and 24:1, SM 18:0 and 24:1, HexCer 22:0, 24:0, and 24:1, LacCer 16.0 and 24:1, and GM3 16:0, 22:0 and 24:1.

When the study sample was divided by sex, women ($n = 150$) and men ($n = 217$) exhibited significant differences in several sphingolipid species (Supplemental Tables S6, S7, and S8, Supplemental Figs. S8 and S9). The heatmap of Supplemental Fig. S8 shows a trend with higher sphingolipids in women as compared with men, except for GM3 and HexCer, which were higher in men. In particular, Cer 14:0, Cer 18:1, DHCer 18:1, CER18:0/24:0 ratio, LacCer 18:0, 18:1, 20:0, SM 16:0, 18:1, all summed SM species, and DHS1P were significantly higher in women ($P < 0.05$). Conversely, GM3 18:0 and GM3 24:0 were significantly lower in women.

Plasma Circulating Sphingolipids in the Study Sample Subdivided by Fasting Glucose Levels and BMI

In Supplemental Tables S9–S12, the anthropometric, ultrasonographic, biochemical, and hematological characteristics, together with family history, personal habits, and morbidity, are reported for the population sample divided into the six groups as described in Table 1 under MATERIALS AND METHODS.

In Tables 2, 3, 4, 5 and 6, the sphingolipid levels found in the six groups identified, as described in Table 1, are reported, while in Fig. 2, the heatmap of the scaled mean of each lipid species in the six groups is shown. The NBW groups are reported on the left side, whereas the OWO are shown on the right and arranged from left to right by increasing levels of fasting glucose (NFG, IFG, DM).

By studying the signature of Cer family in relation to glucose levels, it is interesting to observe a depletion of almost all circulating sphingolipids species in the DM groups, both NBW and OWO. Conversely, Cer 22:0, 24:0, and 24:1 appear increased in IFG associated with OWO. The Cer ratios (16:0/24:0, 18:0/24:0, and 24:1/24:0) that have been proposed for calculation of the CER score (63) are all increased when DM was associated with overweight or obesity (DM/OWO).

In the OWO patients, a similar trend was observed for DHCer, and, noticeably, they showed a trend to increased plasma levels early in the presence of IFG, probably, being the accumulation of these molecules, a marker of glucose levels alteration, exacerbated by the presence of the inflammatory state typical of obesity (Fig. 2 and Table 2).

Increased fasting glucose levels have striking effects on hexosyl- and lactosylceramide species (precursors of more complex species of glycosphingolipids), independently of BMI. Almost all species are lowered in DM groups versus NFG, both in NBW (e.g., median; IQR of summed HexCer species 17; 13–22 vs. 21; 16–24 μmol/L) and OWO patients (15; 10–21 vs. 20; 15–23 μmol/L). The same trend was shown by ceramide derivatives with lactose (Fig. 2 and Table 3).

SM showed a peculiar trend with decreased levels in relation to increased fasting glucose, but this trend was much more enhanced in the normal body weight subjects with a striking decrease in this group, especially of the SM18:1 specie (Fig. 2 and Table 4).

Table 2. *Ceramides and dihydroceramide species concentrations (μmol/L) in the plasma of the CA.ME.LI.A study sample stratified by BMI and fasting glucose levels*

Variable	NFG/NBW $n = 63$[1]	NFG/OWO $n = 56$[1]	IFG/NBW $n = 54$[1]	IFG/OWO $n = 98$[1]	DM/NBW $n = 28$[1]	DM/OWO $n = 68$[1]	Fasting Glucose *P* Value	BMI *P* Value	Interaction *P* Value
Cer 14:0	0.016 (0.013, 0.019)	0.015 (0.012, 0.019)	0.016 (0.013, 0.018)	0.015 (0.012, 0.019)	0.013 (0.011, 0.018)	0.014 (0.011, 0.017)	0.0414*	0.3702	0.6631
Cer 16:0	0.47 (0.39, 0.52)	0.42 (0.33, 0.49)	0.45 (0.40, 0.53)	0.44 (0.38, 0.50)	0.41 (0.37, 0.51)	0.41 (0.36, 0.54)	0.4378	0.2852	0.6293
Cer 18:0	0.08 (0.06, 0.10)	0.08 (0.05, 0.10)	0.08 (0.06, 0.10)	0.08 (0.06, 0.11)	0.07 (0.05, 0.10)	0.08 (0.05, 0.12)	0.2067	0.1887	0.4975
Cer 18:1	0.013 (0.010, 0.016)	0.011 (0.009, 0.015)	0.013 (0.010, 0.015)	0.012 (0.009, 0.015)	0.011 (0.008, 0.013)	0.011 (0.008, 0.014)	0.0414*	0.1958	0.1124
Cer 20:0	0.08 (0.06, 0.11)	0.08 (0.05, 0.10)	0.08 (0.05, 0.10)	0.08 (0.05, 0.11)	0.07 (0.04, 0.09)	0.08 (0.04, 0.11)	0.4580	0.9113	0.5549
Cer 22:0	0.57 (0.49, 0.69)	0.52 (0.39, 0.71)	0.58 (0.36, 0.76)	0.63 (0.40, 0.77)	0.48 (0.33, 0.77)	0.57 (0.32, 0.81)	0.6342	0.6986	0.3997
Cer 24:0	3.46 (2.90, 4.57)	3.41 (2.38, 4.21)	3.58 (2.41, 4.47)	3.81 (2.56, 4.63)	3.77 (1.82, 4.68)	3.18 (1.90, 4.56)	0.8959	0.7869	0.4167
Cer 24:1	0.81 (0.61, 0.99)	0.76 (0.58, 0.97)	0.76 (0.59, 1.03)	0.82 (0.58, 1.00)	0.75 (0.49, 1.05)	0.72 (0.52, 1.02)	0.6101	0.7127	0.7995
Cer Sum	5.52 (4.63, 7.04)	5.27 (3.77, 6.67)	5.93 (3.88, 6.71)	5.84 (3.99, 7.09)	5.43 (3.08, 7.32)	4.99 (3.28, 7.13)	0.8488	0.8844	0.4682
Cer16:0/Cer24:0	0.13 (0.10, 0.16)	0.12 (0.10, 0.16)	0.13 (0.10, 0.20)	0.12 (0.10, 0.16)	0.12 (0.10, 0.20)	0.13 (0.11, 0.23)	0.7088	0.7703	0.3179
Cer18:0/Cer24:0	0.022 (0.018, 0.029)	0.024 (0.018, 0.029)	0.027 (0.019, 0.032)	0.024 (0.020, 0.032)	0.023 (0.018, 0.029)	0.028 (0.021, 0.040)	0.3998	0.0604	0.0652
Cer24:1/Cer24:0	0.24 (0.20, 0.28)	0.24 (0.20, 0.30)	0.25 (0.21, 0.33)	0.23 (0.19, 0.28)	0.23 (0.18, 0.33)	0.26 (0.22, 0.35)	0.9789	0.6928	0.2002
DHCer 16:0	0.025 (0.019, 0.031)	0.024 (0.020, 0.028)	0.024 (0.019, 0.034)	0.026 (0.021, 0.034)	0.023 (0.018, 0.027)	0.025 (0.017, 0.034)	0.2070	0.6145	0.2826
DHCer 18:0	0.006 (0.004, 0.008)	0.006 (0.004, 0.009)	0.006 (0.004, 0.009)	0.008 (0.005, 0.011)	0.006 (0.005, 0.008)	0.008 (0.005, 0.012)	0.0977	0.0007*	0.4468
DHCer 18:1	0.003 (0.002, 0.005)	0.004 (0.002, 0.005)	0.003 (0.001, 0.005)	0.004 (0.002, 0.008)	0.004 (0.001, 0.007)	0.005 (0.002, 0.007)	0.6498	0.0820	0.5857
DHCer 24:0	0.16 (0.11, 0.20)	0.18 (0.11, 0.23)	0.13 (0.09, 0.22)	0.19 (0.09, 0.29)	0.14 (0.08, 0.22)	0.18 (0.07, 0.28)	0.8906	0.1091	0.5412
DHCer 24:1	0.07 (0.05, 0.09)	0.08 (0.05, 0.10)	0.07 (0.05, 0.10)	0.08 (0.05, 0.12)	0.07 (0.05, 0.09)	0.08 (0.05, 0.14)	0.5894	0.0278*	0.7284
Sum	0.27 (0.19, 0.34)	0.30 (0.18, 0.36)	0.25 (0.18, 0.36)	0.30 (0.19, 0.48)	0.26 (0.17, 0.34)	0.30 (0.15, 0.47)	0.7219	0.0547	0.5401

BMI, body mass index; CA.ME.LI.A, CArdiovascular risks, MEtabolic syndrome, LIver and Autoimmune disease; Cer, ceramides; DHCer, dihydroceramides; DM, diabetes mellitus; IFG, impaired fasting glucose; NBW, normal body weight; NFG, normal fasting glucose; OWO, overweight-obese. [1]Median (Q1, Q3). Two-way analysis of variance (ANOVA) models was applied to evaluate the main effects of fasting glucose levels and BMI category, as well as their interaction. *Significance for $P < 0.05$.

GM3 is one of the simplest gangliosides (sialic acid-containing glycosphingolipid) directly synthesized by GM3 synthase (ST3GAL5) from LacCer (Fig. 1). Overweight/obesity seems a condition that, by itself, impacts on GM3 levels, with a general trend to a decreased concentration of circulating GM3 species in the plasma of obese subjects, with normal fasting glucose (median; IQR: 3.0; 1.8, 5.5 vs. 1.8; 1.4, 3.5 μmol/L, NFG/NBW vs. NFG/OWO). The fasting glucose levels, however, appear to have an impact on GM3 levels depending on the fatty acid chain length (Fig. 2 and Table 5).

Sphingosine-1P (S1P) and dihydrosphingosine-1-phosphate (DHS1P), which are involved in sphingolipid catabolism but with a powerful signaling activity, showed a peculiar behavior in OWO patients, with a reduction of circulating levels in subjects with impaired glucose metabolism (IFG and DM) compared with normal obese subjects. On the other hand, they seem to be affected by the BMI alone, with the NFG/OWO group showing higher values than the counterpart with normal body weight (NFG/NBW). Sph and DHSph seem to have opposite trend in relation with fasting glucose in NBW and OWO patients (Fig. 2 and Table 6).

In Tables 2, 3, 4, 5 and 6, the median sphingolipid values are reported for every group of patients and their *P* values in the two-way ANOVA model including both BMI, fasting glycemia and their interaction, as described in *Statistical Analysis*. Figure 3 shows the dot plots of the sphingolipid species that, by two-way ANOVA analysis, displayed significant differences for at least one of the three variables considered. Several sphingolipid species exhibited significant differences ($P < 0.05$) according to glucose levels, independently of BMI. These included log-transformed Cer (14:0 and 18:1), SM 18:0, square-rooted SM 18:1, log-transformed SM 24:1, square-rooted HexCer (16:0, 18:0, 20:0, and 24:1), HexCer total, and square-rooted LacCer (16:0, 18:1, and their sum). Post hoc analyses were conducted using Tukey's honest significant difference (HSD) test on estimated marginal means to further explore the significant effects observed in the ANOVA models.

For Cer 14:0, the comparison between DM and IFG was marginally significant ($P = 0.0522$). Similarly, for Cer 18:1, borderline differences were observed in comparisons between DM and IFG ($P = 0.0622$) and between DM and NFG ($P = 0.0582$) (Table 2).

Interestingly, several hexosylceramides showed consistent and significant differences according to glucose levels groups. HexCer 16:0 was significantly lower in DM as compared with both NFG ($P = 0.0006$) as well as IFG ($P = 0.0025$). Similarly, DM exhibited significantly lower levels of HexCer 18:0 compared with NFG ($P = 0.0001$) and IFG ($P = 0.0016$). The same pattern was observed for HexCer 20:0 ($P = 0.001$ and $P = 0.0007$, respectively) and HexCer 24:1 ($P = 0.0001$ and $P = 0.0004$). For HexCer 22:0, the comparison between DM and NFG also reached statistical significance ($P = 0.0185$). In terms of the sum of HexCer species they were significantly lower in NBW DM and OWO DM ($P = 0.0057$ and $P = 0.0163$), indicating an impact of fasting glucose levels on total hexosylceramide concentrations (Table 3).

For LacCer 16:0, significant differences emerged among glucose levels groups. Specifically, individuals with diabetes showed significantly lower levels as compared with both normoglycemic individuals (NFG; $P = 0.0003$) and those

Table 3. *Glycosylated ceramides concentrations (µmol/L) in the plasma of the CA.ME.LI.A study sample stratified by fasting glucose levels and BMI*

Variable	NFG/NBW $n = 63$[1]	NFG/OWO $n = 56$[1]	IFG/NBW $n = 54$[1]	IFG/OWO $n = 98$[1]	DM/NBW $n = 28$[1]	DM/OWO $n = 68$[1]	Fasting Glucose *P* Value	BMI *P* Value	Interaction *P* Value
HexCer 16:0	1.54 (1.03, 1.84)	1.41 (1.20, 1.74)	1.42 (1.09, 1.77)	1.45 (1.13, 1.76)	1.17 (0.89, 1.59)	1.17 (0.94, 1.51)	0.0004*	0.7340	0.8023
HexCer 18:0	0.26 (0.17, 0.33)	0.25 (0.19, 0.31)	0.22 (0.17, 0.28)	0.23 (0.17, 0.27)	0.18 (0.14, 0.25)	0.18 (0.12, 0.26)	0.0001*	0.9648	0.7655
HexCer 18:1	0.054 (0.042, 0.062)	0.049 (0.042, 0.057)	0.053 (0.044, 0.063)	0.050 (0.044, 0.060)	0.049 (0.040, 0.056)	0.049 (0.042, 0.066)	0.4268	0.6108	0.2038
HexCer 20:0	0.37 (0.24, 0.48)	0.34 (0.23, 0.41)	0.35 (0.23, 0.42)	0.33 (0.24, 0.41)	0.25 (0.18, 0.36)	0.25 (0.16, 0.37)	0.0003*	0.6227	0.4318
HexCer 22:0	4.87 (3.55, 5.50)	4.44 (3.16, 5.26)	4.49 (2.71, 5.44)	4.09 (3.03, 5.39)	3.57 (2.53, 4.78)	3.02 (1.67, 4.76)	0.0159*	0.2822	0.9659
HexCer 24:0	7.7 (5.9, 9.1)	7.5 (5.7, 9.2)	7.3 (5.8, 9.9)	7.3 (5.1, 10.0)	7.4 (5.4, 8.9)	6.3 (3.7, 8.2)	0.1457	0.1662	0.4174
HexCer 24:1	5.97 (4.39, 7.11)	5.64 (4.03, 6.89)	6.01 (4.51, 6.76)	5.26 (4.07, 6.93)	4.39 (3.30, 5.58)	3.95 (2.93, 5.46)	0.0001*	0.3331	0.9762
HexCer Sum	21.4 (16, 24)	20.4 (15, 23)	19.9 (15, 24)	18.6 (14, 24)	17.2 (13, 22)	14.8 (10, 21)	0.0048*	0.2512	0.8764
LacCer 16:0	9.55 (7.42, 11.61)	9.48 (7.99, 11.40)	9.83 (8.16, 10.79)	8.86 (7.42, 11.04)	7.37 (6.14, 9.75)	7.49 (6.75, 9.58)	0.0001*	0.6066	0.7023
LacCer 18:0	0.28 (0.20, 0.34)	0.27 (0.21, 0.36)	0.28 (0.21, 0.36)	0.26 (0.20, 0.32)	0.20 (0.15, 0.29)	0.24 (0.15, 0.31)	0.1229	0.7764	0.8249
LacCer 18:1	0.065 (0.050, 0.079)	0.061 (0.049, 0.079)	0.067 (0.045, 0.079)	0.062 (0.049, 0.075)	0.049 (0.037, 0.061)	0.052 (0.041, 0.067)	0.0004*	0.3792	0.7817
LacCer 20:0	0.15 (0.11, 0.21)	0.14 (0.11, 0.18)	0.14 (0.09, 0.20)	0.13 (0.11, 0.18)	0.12 (0.08, 0.16)	0.12 (0.08, 0.17)	0.1220	0.7151	0.5146
LacCer 22:0	0.62 (0.38, 0.77)	0.51 (0.34, 0.62)	0.56 (0.38, 0.83)	0.51 (0.38, 0.66)	0.54 (0.35, 0.71)	0.47 (0.31, 0.68)	0.7485	0.3241	0.8558
LacCer 24:0	0.74 (0.29, 0.96)	0.54 (0.27, 0.83)	0.66 (0.32, 1.04)	0.57 (0.34, 0.84)	0.66 (0.35, 0.97)	0.61 (0.32, 0.87)	0.7993	0.2129	0.8694
LacCer 24:1	3.52 (2.27, 4.35)	2.78 (1.90, 4.03)	3.28 (2.25, 4.18)	3.00 (2.17, 3.79)	2.63 (1.87, 3.84)	2.65 (1.85, 3.49)	0.1581	0.4538	0.7030
LacCer Sum	15.2 (12.3, 18.2)	14.3 (11.5, 17.4)	14.9 (11.9, 17.4)	13.5 (11.7, 16.5)	11.4 (9.4, 15.4)	12.0 (9.8, 14.1)	0.0034*	0.4303	0.7401

BMI, body mass index; CA.ME.LI.A, CArdiovascular risks, MEtabolic syndrome, LIver and Autoimmune disease; DM, diabetes mellitus; HexCer, hexosylceramides; IFG, impaired fasting glucose; LacCer, lactosylceramides; NBW, normal body weight; NFG, normal fasting glucose; OWO, overweight-obese. [1]Median (Q1, Q3). Two-way analysis of variance (ANOVA) models was applied to evaluate the main effects of fasting glucose levels and BMI category, as well as their interaction. *Significance for $P < 0.05$.

with impaired fasting glucose (IFG; $P = 0.0002$). No significant difference was observed between IFG and NFG ($P = 0.9799$). Notably, no significant interaction was found between glucose levels and BMI, indicating that the observed differences in LacCer 16:0 are independent of BMI. Similarly, for LacCer 18:1, post hoc comparisons revealed significantly decreased levels between diabetic (DM) and normoglycemic individuals (NFG; $P = 0.0005$) as well as impaired fasting glucose (IFG; $P = 0.004$). These results suggested that LacCer 18:1 level is altered in diabetes, independent of BMI. Finally, the sum of LacCer species was significantly different between groups. DM had lower levels than NFG ($P = 0.009$) and also than IFG ($P = 0.0054$), highlighting a consistent pattern of impaired sphingolipid metabolism in individuals with diabetes as compared with those with normal or impaired fasting glucose.

For SM 18:0 ($P = 0.0046$), SM 18:1 ($P = 0.0024$), and SM 24:1 ($P = 0.047$), a significant difference was found between DM and IFG, indicating that the levels of these three species significantly discriminate individuals with diabetes with respect to those with prediabetes (Table 4).

With respect to BMI, significant differences, independently of glucose levels, were observed for log-transformed DHCer 18:0, square-rooted DHCer 24:1, square-rooted SM 18:1, and log-transformed GM3 24:0. A trend to higher levels of DHCer 18:0, DHCer 18:1, SM 18:1, and GM3 was observed in subjects with obesity (Table 4).

For a limited number of sphingolipid species, there was a significant interaction effect between BMI and glucose levels (Fig. 4): square-rooted SM 18:1, square-rooted GM3 24:1, square-rooted Sph, square-rooted DHS1P, as well as the ratios S1P/Sph and DHS1P/DHSph. For SM 18:1, a significant interaction between glucose levels and BMI was present ($P = 0.0053$). Among participants with normal body weight (BMI < 25), glucose levels had significant effects: comparisons between NFG and DM (diabetes) yielded $P = 0.0034$, and between IFG (prediabetes) and DM, $P = 0.0007$. These findings suggest that in normal-weight individuals, SM 18:1 levels are significantly associated with glucose levels. Furthermore, a significant difference between BMI categories (normal weight vs. overweight/obese) was observed only within the diabetic group (DM), with $P = 0.0001$, indicating a BMI-related modulation of SM 18:1 level among subjects with diabetes. Also, GM3 24:1 level showed a significant interaction between glucose level and BMI. In normal weight individuals, there was a significant decrease with increasing fasting glycemia, not observed in OWO patients. On the other hand, BMI affects GM3 24:1 level independently from glycemia, as NFG/NBW were significantly lower than NFG/OWO patients. Regarding sphingoid bases, DHS1P displayed a significant interaction ($P = 0.031$) bet-ween BMI and glucose levels. Among overweight/obese individuals (BMI ≥ 25), significant differences were found between NFG, IFG ($P = 0.0170$), and DM ($P = 0.0101$). These differences were not observed in the normal weight group. In addition, within the NFG category, a significant difference in DHS1P levels was found between BMI groups ($P = 0.0096$), suggesting that overweight/obesity modulates DHS1P levels in normoglycemic individuals. On the other hand, in subjects with diabetes, overweight/obesity has lower levels as compared with

Table 4. *Sphingomyelin species concentrations (μmol/L) in the plasma of the CA.ME.LI.A study sample stratified by fasting glucose levels and BMI*

Variable	NFG/NBW $n = 63$[1]	NFG/OWO $n = 56$[1]	IFG/NBW $n = 54$[1]	IFG/OWO $n = 98$[1]	DM/NBW $n = 28$[1]	DM/OWO $n = 68$[1]	Fasting Glucose *P* Value	BMI *P* Value	Interaction *P* Value
SM 16:0	120 (107, 126)	115 (108, 121)	115 (106, 124)	113 (104, 125)	114 (103, 122)	112 (102, 122)	0.3222	0.3964	0.6569
SM 18:0	39 (34, 44)	38 (33, 43)	38 (33, 47)	42 (35, 47)	37 (30, 40)	39 (30, 46)	0.0067*	0.1845	0.5112
SM 18:1	23.9 (21.2, 27.1)	24.1 (20.3, 27.8)	24.0 (20.4, 27.7)	25.2 (21.0, 28.3)	21.4 (17.4, 24.8)	25.0 (22.0, 28.5)	0.0037*	0.0021*	0.0053*
SM 24:0	41 (36, 48)	40 (35, 43)	40 (34, 46)	41 (35, 45)	40 (34, 47)	38 (31, 45)	0.6400	0.3112	0.3694
SM 24:1	60 (53, 66)	56 (52, 62)	59 (53, 66)	57 (52, 63)	53 (50, 60)	56 (48, 62)	0.0413*	0.4714	0.6244
SM sum	277 (254, 308)	270 (255, 292)	274 (249, 308)	278 (253, 307)	264 (246, 286)	267 (243, 291)	0.0610	0.9567	0.5874
Cer/SM	0.020 (0.017, 0.024)	0.019 (0.016, 0.024)	0.021 (0.014, 0.025)	0.022 (0.015, 0.025)	0.020 (0.013, 0.027)	0.021 (0.012, 0.026)	0.9159	0.7632	0.4853

BMI, body mass index; CA.ME.LI.A, CArdiovascular risks, MEtabolic syndrome, LIver and Autoimmune disease; Cer, ceramides; DM, diabetes mellitus; IFG, impaired fasting glucose; NBW, normal body weight; NFG, normal fasting glucose; OWO, overweight-obese; SM, sphingomyelins. [1]Median (Q1, Q3). Two-way analysis of variance (ANOVA) models was applied to evaluate the main effects of fasting glucose levels and BMI category, as well as their interaction. *Significance for $P < 0.05$.

NBW. In addition, also for Sph a significant interaction between BMI and glucose levels was present ($P = 0.0045$). Among normal-weight individuals, a significant difference was found between NFG and DM ($P = 0.0309$), whereas the same comparison was borderline significant among overweight/obese individuals ($P = 0.0899$). Within the DM group, a significant difference in Sph levels was observed between BMI categories ($P = 0.004$), further supporting the presence of a BMI-related modulation. The ratios DHS1P/DHSph and S1P/Sph displayed the same trend. Figure 4 shows the interaction plot of both variables in the species that displayed a significant interaction between BMI and glucose levels.

DISCUSSION

The objective of this work was to evaluate the regulation of circulating sphingolipids in relation to fasting glucose levels and BMI in a sample of the CA.ME.LI.A study population (1, 2). The results uncover characteristic alterations of the sphingolipidome signature in diabetes and also in prediabetes. When prediabetes/diabetes coexisted with overweight/obesity, these alterations were synergistic.

Since sphingolipid families have circulating concentrations that can vary >100 times, we also expressed (both in normal weight and in overweight/obese) the alterations due to impaired fasting glucose or overt diabetes as % of the value in normoglycemia. This allowed us to understand which family is more impacted, and to have a comparative view of the magnitude of the deregulation of different classes (Supplemental Fig. S10). To appreciate at a glance the impact of increasing fasting glycemia and/or overweight/obesity on sphingolipids interplay, Fig. 5 describes increases/decreases of the different classes under the different conditions analyzed in this paper.

Glycosylated Ceramides

Glucosylceramides (GlcCers) derive from the glycosylation of ceramides by glucosylceramide synthase (UGCG). Addition of a galactose residue by lactosylceramide synthases (B4GALT5-6) leads to the LacCer family, from which complex glycosphingolipids of Lacto-, Globo-, and Ganglioseries derive (Fig. 1). Ceramide accumulation in β-cells, liver, skeletal muscle, adipose tissue, and plasma is highly toxic and has been associated with insulin resistance, obesity, and type 2 diabetes by promoting lipotoxicity, mitochondrial dysfunction, reactive oxygen species (ROS) generation, and apoptosis (47, 64–66).

Ceramides' glycosylation has been proposed as a buffering mechanism to protect against ceramide cytotoxicity by converting it to a more hydrophilic and less harmful derivative. Despite the pathologically uncontrolled accumulation of GlcCers (Gaucher disease), which is known to lead to toxic storage overload and inflammation, some studies confirmed that blocking glycosylation worsens insulin signaling (66). However, several additional studies suggest that glycosphingolipids themselves are involved in insulin

Table 5. *Ganglioside species concentrations (μmol/L) in the plasma of the CA.ME.LI.A study sample stratified by fasting glucose levels and BMI*

Variable	NFG/NBW $n = 63$[1]	NFG/OWO $n = 56$[1]	IFG/NBW $n = 54$[1]	IFG/OWO $n = 98$[1]	DM/NBW $n = 28$[1]	DM/OWO $n = 68$[1]	Fasting Glucose *P* Value	BMI *P* Value	Interaction *P* Value
GM3 16:0	1.41 (0.70, 1.96)	0.90 (0.60, 1.48)	1.17 (0.61, 2.61)	1.04 (0.53, 1.99)	1.16 (0.42, 1.84)	1.22 (0.64, 1.77)	0.8318	0.4151	0.3426
GM3 18:0	0.28 (0.12, 0.76)	0.14 (0.07, 0.42)	0.23 (0.07, 0.95)	0.26 (0.09, 0.71)	0.39 (0.08, 0.72)	0.41 (0.12, 0.68)	0.7619	0.2071	0.1215
GM3 18:1	0.012 (0.000, 0.054)	0.006 (0.000, 0.030)	0.005 (0.000, 0.024)	0.006 (0.000, 0.042)	0.006 (0.000, 0.042)	0.006 (0.000, 0.024)	0.5754	0.5574	0.2799
GM3 20:0	0.07 (0.02, 0.16)	0.04 (0.01, 0.12)	0.05 (0.02, 0.17)	0.06 (0.02, 0.16)	0.08 (0.01, 0.12)	0.07 (0.02, 0.11)	0.8463	0.6291	0.4773
GM3 22:0	0.52 (0.28, 0.95)	0.41 (0.17, 0.62)	0.45 (0.18, 0.76)	0.44 (0.26, 0.73)	0.40 (0.26, 0.76)	0.39 (0.20, 0.70)	0.5431	0.5272	0.4729
GM3 24:0	0.11 (0.07, 0.52)	0.07 (0.03, 0.20)	0.09 (0.05, 0.67)	0.09 (0.05, 0.27)	0.15 (0.05, 0.50)	0.11 (0.05, 0.38)	0.8113	0.0448*	0.6834
GM3 24:1	1.01 (0.5, 1.3)	0.39 (0.24, 0.69)	0.54 (0.34, 1.36)	0.42 (0.28, 0.70)	0.40 (0.26, 0.83)	0.43 (0.26, 0.69)	0.38710	0.13635	0.0248*
GM3 sum	3.0 (1.8, 5.5)	1.8 (1.4, 3.5)	2.3 (1.5, 6.5)	2.2 (1.4, 4.8)	2.8 (1.2, 4.4)	2.6 (1.5, 4.1)	0.8689	0.3052	0.3244

BMI, body mass index; CA.ME.LI.A, CArdiovascular risks, MEtabolic syndrome, LIver and Autoimmune disease; DM, diabetes mellitus; GM3, gangliosides; IFG, impaired fasting glucose; NBW, normal body weight; NFG, normal fasting glucose; OWO, overweight-obese; SM, sphingomyelins. [1]Median (Q1, Q3). Two-way analysis of variance (ANOVA) models was applied to evaluate the main effects of fasting glucose levels and BMI category, as well as their interaction. *Significance for $P < 0.05$.

Table 6. *Sphingoid bases concentrations (μmol/L) in the plasma of the CA.ME.LI.A study sample stratified by fasting glucose levels and BMI*

Variable	NFG/NBW $n = 63$[1]	NFG/OWO $n = 56$[1]	IFG/NBW $n = 54$[1]	IFG/OWO $n = 98$[1]	DM/NBW $n = 28$[1]	DM/OWO $n = 68$[1]	Fasting Glucose *P* Value	BMI *P* Value	Interaction *P* Value
Sph	0.107 (0.087, 0.125)	0.094 (0.079, 0.118)	0.102 (0.081, 0.126)	0.105 (0.081, 0.123)	0.095 (0.073, 0.112)	0.104 (0.090, 0.130)	0.8025	0.2189	0.0045*
S1P	2.07 (1.59, 2.53)	2.41 (1.85, 2.85)	2.09 (1.55, 2.51)	1.96 (1.56, 2.36)	2.08 (1.33, 2.51)	1.98 (1.45, 2.40)	0.2254	0.5156	0.1502
DHSph	0.011 (0.007, 0.015)	0.010 (0.005, 0.015)	0.012 (0.009, 0.015)	0.011 (0.007, 0.015)	0.010 (0.006, 0.013)	0.011 (0.008, 0.016)	0.7987	0.4488	0.1216
DHS1P	0.41 (0.29, 0.54)	0.51 (0.37, 0.61)	0.40 (0.31, 0.56)	0.40 (0.29, 0.54)	0.44 (0.27, 0.55)	0.37 (0.31, 0.49)	0.1362	0.3734	0.0309*
S1P/Sph	18 (14, 25)	24 (18, 28)	21 (15, 27)	19 (15, 25)	21 (14, 28)	18 (14, 22)	0.664231	0.438280	0.0010*
DHS1P/DHSph	34 (24, 59)	46 (30, 97)	34 (25, 53)	37 (24, 62)	46 (31, 63)	35 (21, 50)	0.63393	0.60587	0.0312*

BMI, body mass index; CA.ME.LI.A, CArdiovascular risks, MEtabolic syndrome, LIver and Autoimmune disease; DHS1P, dihydrosphingosine-1-posphate; DHSph, dihydrosphingosine; DM, diabetes mellitus; GM3, gangliosides; IFG, impaired fasting glucose; NBW, normal body weight; NFG, normal fasting glucose; OWO, overweight-obese; S1P, sphingosine-1-phosphate Sph, sphingosine;. [1]Median (Q1, Q3). Two-way analysis of variance (ANOVA) models was applied to evaluate the main effects of fasting glucose levels and BMI category, as well as their interaction. *Significance for $P < 0.05$.

resistance and diabetes pathogenesis, perhaps with different mechanisms from ceramide (67). In an animal model of type 2 diabetes, it was demonstrated that inhibiting glycosphingolipid synthesis improved glycemic control and insulin sensitivity (68, 69). Glycosphingolipid derangement in cell membranes may affect microdomains (lipid rafts) and influence insulin receptors or GLUT4 trafficking (70). LacCer species have been hypothesized to be responsible for inflammatory signaling, oxidative stress, and mitochondrial dysfunction, and they can also worsen insulin resistance and tissue damage in diabetes (71).

By dividing the CA.ME.LI.A study sample by sex (Supplemental Table S7 and Supplemental Figs. S8 and S9), three LacCer species (18:0, 18:1, and 20:0) resulted significantly higher in women than in men, whereas very long chain LacCers 24:0 and 24:1 were higher (not significantly) in men. Similarly, HexCer species 18:0 and 18:1 were higher in women, whereas those with longer chains, 22:0, 24:0, and 24:1, were again higher in men, although all not significantly. How this observation could explain the higher mortality rate for CVD observed in men, also in this population, needs further studies and a longer follow-up (2).

By studying the CA.ME.LI.A study sample in relation to glucose and BMI, the sphingolipidomic signature revealed the most impressive impairment at the level of hexosylated (probably glucosylated more than galactosylated) and lactosylated ceramides (Fig. 2 and Table 3). Heatmap (Fig. 2) showed a decreased level of hexosyl- and lactosyl-ceramides species in diabetes, both in normal body weight and overweight/obese. This observation is in line with the work of Düsing et al. (72), which demonstrated that circulating HexCers were downregulated in patients with CAD and concomitant DM as compared with patients with non-diabetes. In particular, in addition to the sum of all circulating HexCers measured, we also found that several single species exhibited significant differences ($P < 0.05$) among glucose levels groups, independently of BMI. HexCer 16:0, 18:1, 20:0, 22:0, 24:1 were lower in DM as compared with NFG, both in the normal body weight group as well as in the overweight/obese. LacCer species displayed a similar trend to HexCers, with LacCer 16:0, LacCer 18:1, and the sum of total species significantly affected by glucose fasting levels (Table 3). None of these species was impacted by BMI. Our results suggest that the ceramide glycosylation is tightly regulated in humans. There is a strong relation between glucose levels and ceramide glycosylation, independently from BMI, with a trend toward a decrease in all HexCer and LacCer species in IFG groups, which became more overt in DM subjects both NBW and OWO, reaching a decline of ≈−20% as compared with NFG group (Supplemental Fig. S10).

From our results, it appears that glycosphingolipids play a complex role in insulin function and more generally in cell homeostasis. Glycosylation of ceramide may be seen as a protective response to reduce the pool of "free" available ceramide. Some animal model data support that reducing glycosphingolipid levels reduced insulin resistance (68). Glycosphingolipids, however, may themselves affect membrane microdomains (lipid rafts) and influence insulin receptor signaling or GLUT4 trafficking (70).

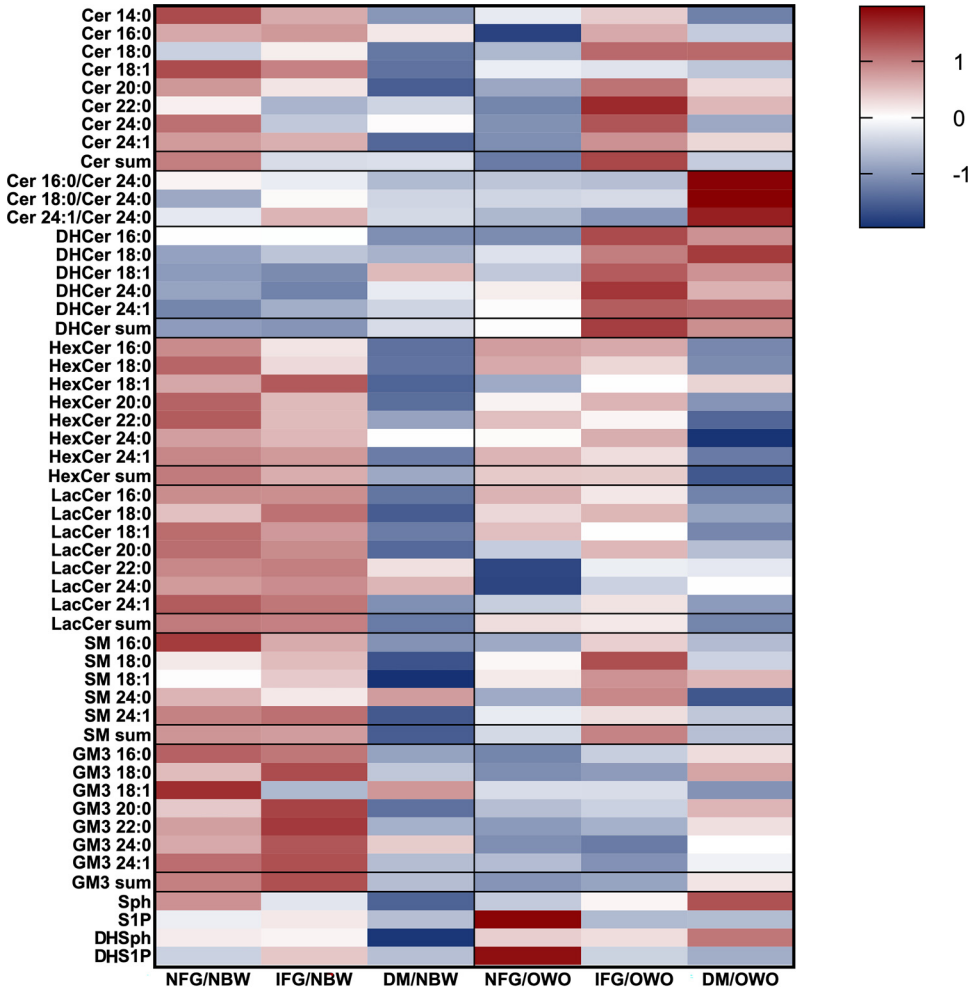

Figure 2. Heatmap representing the scaled mean of each lipid species in the six clustered groups. On the left, the three clusters are characterized by normal body weight (NBW), and on the right, the three clusters with overweight/obesity. DM, diabetes mellitus; IFG, impaired fasting glucose; NBW, normal body wight; NFG, normal fasting glucose; OWO, overweight/obese. Lipid Species: Cer, Ceramides; DHCer, dihydroceramides; DHS1P, dihydrosphingosine-1-phosphate; DHSph, dihydrosphingosine; GM3, gangliosides; HexCer, hexosylceramides; LacCer, lactosylceramides; S1P, sphingosine-1-phosphate; SM, sphingomyelin; Sph, sphingosine.

Our findings, although on a selected sample of the CA.ME.LIA population highlights a potential failure in ceramide buffering via "in vivo" glycosylation. Circulating glycosylated/lactosylated species significantly decrease with increasing fasting glycemia, suggesting a potential dysregulation of the membrane clusters associated also with the insulin receptor.

GlcCer and LacCer play a role in a complex metabolic process leading to gangliosides synthesis. Gangliosides are complex molecules, rich in carbohydrates and sialic acid, and they have a fundamental role in both cellular signaling and in the membrane lipid-raft structure, encompassing also the insulin receptor. It could be hypothesized that physiological glucose metabolism could be a consequence of the synthetic/catabolic balance of these molecules and from their proper insertion at the membrane level. The increase of GM3 in our pre- and diabetic subjects (NBW or OWO) suggests that these alterations could have pathological consequences.

Ganglioside Monosialyltetraosylceramide

It is well known that ganglioside monosialyltetraosylceramide (GM3) has a role in diabetes pathophysiology (73–75), contributing to maintaining the membrane lipid raft microdomains structure and modulating the activity of the insulin receptor (IR). TNF-α, typically higher in subjects with obesity, type 2 diabetes, or chronic low-grade inflammation, promotes expression of ST3GAL5, leading to excessively high GM3 levels. GM3 in excess breaks down lipid rafts harboring the insulin receptor and thus acts as an inhibitor of insulin signaling, in the progression to type 2 diabetes. In this context, a different behavior of long-chain fatty acids (LCFA) GM3 (16:0–20:0), very long-chain FA (22:0–24:0), and hydroxylated FA (22:1–24:1) has been proposed in relation to BMI and lipidemia, glycemia, or both (75). Our findings seem to confirm the relationship between glucose impairment and GM3 elevation in OWO subjects, with a general increase in all the summed species of ≈22% in IFG and more than ≈40% in DM obese patients, as compared with NFG, thus suggesting that this could be a putative biomarker for insulin resistance (Supplemental Fig. S10). In the NBW group, DM subjects showed an increase of the sum of GM3 species as compared with IFG levels (2.8 vs. 2.3 μmol/L, median). Surprisingly, the NFG/NBW subjects displayed higher levels (but not statistically different) than the corresponding obese group. The two-way ANOVA analysis evidenced only for GM3 24:0 species, a significant influence of BMI (Table 5 and Supplemental Fig. S9), and only for GM3 24:1, an interaction between BMI and glucose levels.

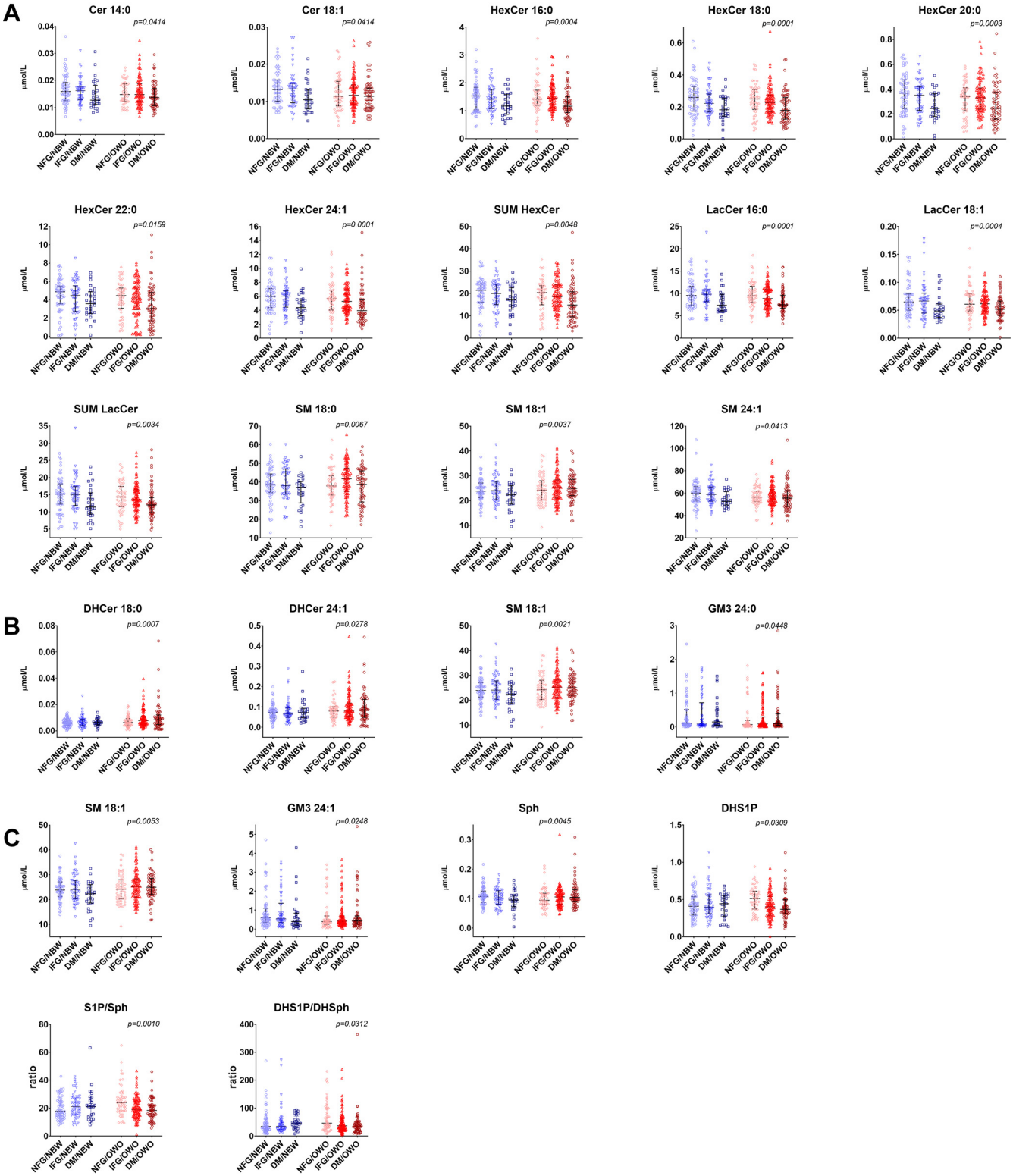

Figure 3. Dot plots representing sphingolipid species significantly discriminated against fasting glucose levels (*A*), body mass index (BMI) (*B*), or both (*C*). Middle line represents the median value; upper and lower lines indicate the 75th and 25th percentiles, respectively. DM, diabetes mellitus; IFG, impaired fasting glucose; NBW, normal body weight; NFG, normal fasting glucose; OWO, overweight/obese. Lipid Species: Cer, ceramides; DHCer, dihydroceramides; DHS1P, dihydrosphingosine-1-phosphate; DHSph, dihydrosphingosine; GM3, gangliosides; HexCer, hexosylceramides; LacCer, lactosylceramides; S1P, sphingosine-1-phosphate; SM, sphingomyelin; Sph, sphingosine.

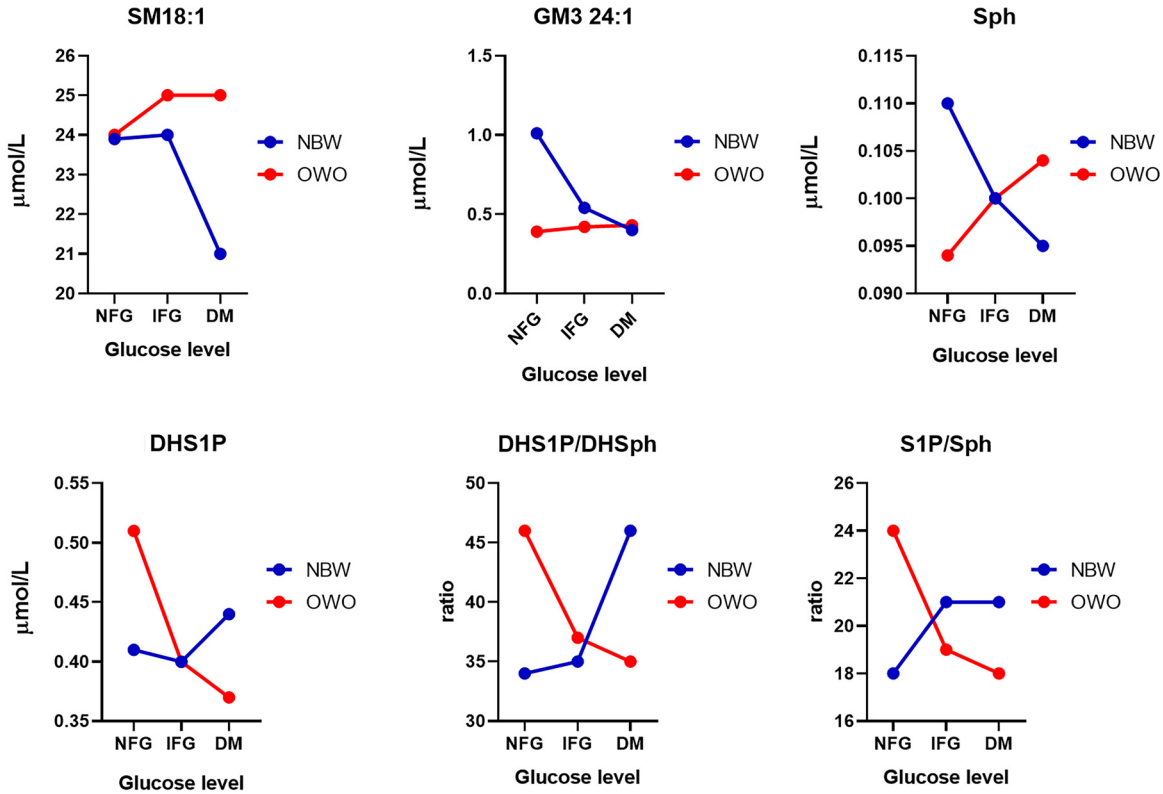

Figure 4. The interaction plot illustrates the trend of the three sphingolipids influenced by both glucose levels and BMI. DHS1P, dihydrosphingosine; DM, diabetes mellitus; GM3 24:1, ganglioside 24:1; IFG, impaired fasting glucose; NBW, normal body weight; NFG, normal fasting glucose; OWO, overweight/obese; SM 18:1, sphingomyelin 18:1; Sph, Sphingosine.

Several GM3 species (18:0, 24:0, 24:1) are significantly higher in men than in women. Interestingly, men displayed an almost doubled number of cardiovascular events and deaths in the 7-yr follow-up of CA.ME.LI.A study (2), suggesting the potential involvement of some GM3 species in this phenomenon (Supplemental Table S8 and Supplemental Figs. S8 and S9).

Our findings suggest a complex scenario regarding the derangement of sphingolipid pathways in human pathologies, highlighting a central role of ceramide glycosylation within the sphingolipid metabolism. HexCers, LacCers, and GM3 play a central role as putative mediators and/or prognostic markers for CAD events and deaths, in prediabetes/diabetes, but this deserves further exploration and confirmation in larger clinical trials. Of interest is the observation of the different behavior of many species between sexes, even more strengthening the need for personalized medicine. Identification of one or more suitable glycosylated ceramide species (chain lengths, saturation, subcellular localization) that correlate well with insulin resistance could be proposed as a novel diagnostic marker for the prediction/prognosis of diabetes and its complications, with the aim of finding potential therapeutic targets for novel pharmacological approaches.

S1P and Sphingoid Bases

Sphingosine-1-phosphate (S1P) and dihydrosphingosine-1-phosphate (DHS1P) derive from sphingosine and dihydrosphingosine by the action of sphingosine kinase (SPHK1/2) (Fig. 1). They are part of the catabolic pathway of ceramide, leading to palmitoyl aldehyde and phosphoethanolamine, and then to their exit from the sphingolipid metabolism. Their role is, however, much more complex and nuanced for cell metabolism as they both act as potent cell signaling lipids secreted extracellularly (e.g., in circulation) to affect cell surface S1P receptors (S1PRs 1–5), or they act on intracellular targets (HDAC1/2, PHB2, etc.) (76, 77) and play important roles in obesity and in glucose homeostasis, from insulin resistance to diabetes (64, 77, 78). S1P receptors are heterogeneous in functions and intracellular targets, sometimes with opposite effects, making the role of this molecule in diabetes development very complex. In vitro experiments suggested that low S1P levels contribute to heightened vascular risk due to impaired anti-inflammatory properties (79).

Although most of the literature focuses on ceramides and S1P, the "dihydro" moieties (including DHSph, DHS1P) are also increasingly recognized as relevant. Elevated DHS1P may reflect increased flux through the sphinganine → DHS1P pathway, possibly due to increased ex novo sphingoid base synthesis (which is seen in obesity/insulin resistance) or altered enzyme activities of the downstream sphingolipid pathway (e.g., sphingosine kinases, dihydroceramide desaturase) (Fig. 1). As in pancreatic β-cells, sphingosine/S1P pathways regulate cell survival and insulin secretion, it is plausible that altered DHS1P may reflect a β-cell stress or maladaptation in early T2D. Higher circulating DHS1P and DHS1P/DHSph ratio were associated with ∼53%–54% increased risk of incident T2D per SD increase (80).

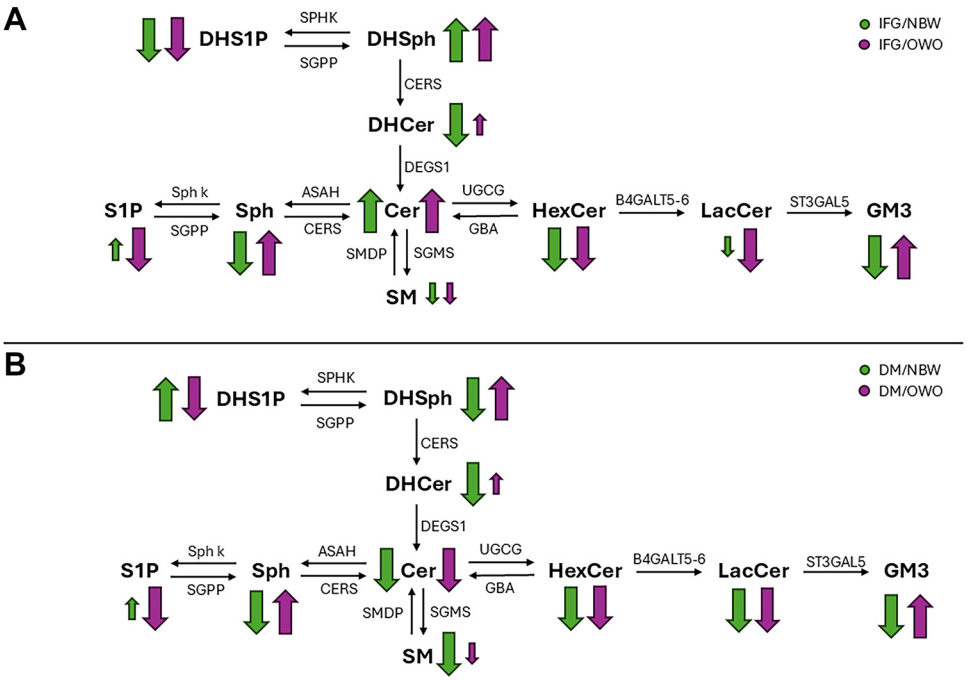

Figure 5. *A*: impaired fasting glucose (IFG): schematic representation of sphingolipids biosynthesis and degradation pathways, with arrows illustrating changes of different lipid classes in impaired fasting glucose as compared with normal fasting glucose. Green arrows represent normal body weight subjects, purple arrows represent overweight/obese subjects. *B*: diabetes (DM): schematic representation of sphingolipids biosynthesis and degradation pathways, with arrows illustrating changes of different lipid classes in diabetes as compared with normal fasting glucose. Green arrows represent normal body weight subjects, purple arrows represent overweight/obese subjects. Abbreviations of enzymes names in the scheme follow the HUGO (Human Genome Organization) gene nomenclature committee: CERS1-6, dihydroceramide synthases; DEGS1, dihydroceramide desaturase 1; ASAH 1–2, acid ceramidase; Sph K1-2, sphingosine kinase; SGPP1-2, sphingosine 1P phosphatase; UGCG, glucosylceramide synthase; GBA1, glucocerebrosidase; B4GALT5-6, lactosylceramide synthase; ST3GAL5, GM3 synthase; SGMS1-2, sphingomyelin synthases; SMPD1-4, sphingomyelinases. Lipid Species: Cer, ceramides; DHCer, dihydroceramides; SM, sphingomyelin; HexCer, hexosylceramides; LacCer, lactosylceramides; GM3, gangliosides; Sph, sphingosine; S1P, sphingosine-1-phosphate; DHSph, dihydrosphingosine; DHS1P, dihydrosphingosine-1-phosphate.

In our study sample, both sphingosine and dihydrosphingosine, together with their phosphorylated derivatives, were affected by fasting glucose and even just by BMI. Differences were observed in NBW versus OWO groups, independently of glucose levels (NFG), suggesting a possible role of VAT adipose tissue (which is almost doubled in OWO as compared with NBW) as an important potential actor in the etiology of insulin resistance, and possibly supporting the use of these molecules as predictive markers. It is possible that increased levels of inflammatory cytokines with associated more severe inflammation and insulin resistance could directly affect sphingoid alterations in patients with overweight/obesity, though new data from more severely obese populations may be required to address this issue. In this study, neither S1P nor DHS1P seem to be significantly altered by hyperglycemia in NBW patients, whereas in OWO patients, we observed a trend to a decrease of both molecules as compared with normoglycemic subjects, either in the presence of poor glycemic control (S1P $\approx -18\%$, DHS1P $\approx -21\%$) and more with overt diabetes (S1P $\approx -18\%$, DHS1P $\approx -27\%$) (Supplemental Fig. S10). By applying the two-way ANOVA model, Sph and DHS1P were found to be significantly regulated by glucose and BMI interaction (Fig. 4 and Table 6), as well as the ratio S1P/Sph and DHS1P/DHSph, confirming a role for both molecules as early biomarkers in disease development (79).

A recent work (81) reported an inverse correlation between circulating S1P levels and disease severity in patients with COVID-19. Our observation could explain the increased susceptibility of the diabetic patient to COVID-19. Sph, DHS1P, DHS1P/DHSph, and S1P/Sph ratio were significantly impacted by the interaction of glucose and BMI. Larger studies are needed to confirm the role of sphingoid bases in different human pathologies and, on tissue-specific localization of dihydro series, the functional consequences of their elevation, receptor signaling, and intracellular actions, and their causal role in the pathophysiology of diabetes.

DHCer and Cer.

Dihydrosphingolipids are part of the ex novo sphingolipid pathway as a ceramide precursor (Fig. 1). DHCers accumulate when the conversion to ceramide by dihydroceramide desaturase is impaired, and/or when the flux in the ex novo sphingolipid synthesis is increased due to saturated fatty acids, nutrient overload, or lipotoxicity. Reduced clearance of sphingolipids may also affect DHCer levels. Thus, DHCer could act as an early biomarker of obesity-related metabolic dysfunction before the onset of overt diabetes and may represent the "metabolic memory" associated with impaired lipid metabolism. Plasma DHCer levels are elevated in individuals who later develop type 2 diabetes, and in humans with T2D, as compared with non-diabetic controls (82, 83). Higher levels of long-chain (C14:0–C20:0) and very long-chain DHCer (C22:0–C25:0) have been found in women, whereas, very higher and ultra-long chain ceramides have been reported in men (84).

In this study, although DHCer levels were constantly higher in women, we did not find major differences, and only DHCer 18:1 was significantly higher in women. Concerning Cers species, Cer 14:0 and Cer 18:1 were otherwise higher in women, whereas very long chain Cer species as Cer 24:0 and 24:1, were higher (but not significantly) in men (Supplemental Table S6) in line with Pina et al. (84). When the population was subdivided according to BMI and fasting glucose, DHCers showed a trend to higher values, especially in IFG/OWO patients, confirming that the levels of such molecules are potential biomarkers of early metabolic dysfunction. Notably, DHCer 18:0 and 24:1 appeared significantly affected by BMI, confirming their role even in obesity-driven glucose derangement (Figs. 2 and 3 and Table 2).

Sphingomyelins.

Sphingomyelins, one of the main lipid components of plasma membranes, especially in lipid rafts, are quantitatively the most abundant sphingolipids circulating in plasma (≈250 μmol/L). They can be intracellularly converted by sphingomyelinase into ceramide and phosphorylcholine. Therefore, sphingomyelins may serve as reservoirs to modulate increases in ceramide o toxic levels, as it occurs in stress responses and apoptosis (Fig. 1). SM 16:0, 18:1, and 24:1 are increased in the plasma of individuals with type 2 diabetes as compared with controls (85), and elevated SM 16:1 and 18:0 predicted the onset of diabetes up to 10 yr before diagnosis (86). In addition, SM 18:0 and 20:0 were found to be elevated in insulin-resistant subjects, independent of BMI (87).

We found that both the total amount of sphingomyelins and SM16:0 and SM18:1 species were significantly higher in women than in men (Supplemental Table S8 and Supplemental Figs. S8 and S9). These results are in line with recently published findings (84), which found more elevated sphingomyelin levels in women with different degrees of insulin derangement. This suggests that distinct lipid signatures associated with altered lipid metabolism can be accentuated by sex differences (84). By partitioning the population by BMI and glucose levels, we noted a decrease in the circulating SMs pool in DM groups versus NFG, and, in addition, a difference between OWO and NBW even in normoglycemia. The two-way ANOVA model revealed three species significantly affected by glucose levels (SMs 18:0, 18:1, 24:1) regardless of BMI, and one significantly affected by the interaction of both BMI and glucose levels (SM 18:1), thus confirming the relevance of very long-chain unsaturated sphingolipid species in the pathophysiology of glucose homeostasis (Table 4 and Figs. 2, 3, and 4).

Omics approaches, particularly sphingolipidomics, are emerging as powerful tools for the identification of biomarkers associated not only with diabetes but also with cardiovascular disease and heart failure. Quantitative profiling of sphingolipid species, such as ceramides and sphingomyelins, has revealed strong associations with vascular dysfunction, inflammation, myocardial remodeling, and progression toward heart failure. These lipid signatures reflect underlying cardiometabolic alterations and provide prognostic information beyond traditional risk factors, supporting earlier detection and improved risk stratification of cardiovascular complications. Consequently, sphingolipidomics represents a promising strategy for advancing precision medicine in cardiovascular disease and heart failure.

Conclusions

These data indicate that there are characteristic alterations of sphingolipids in the presence of both diabetes and prediabetes. When prediabetes/diabetes are associated with overweight or obesity, the alterations are synergistic. This study could pave the road to larger trials on sphingolipid metabolism to identify novel and beneficial biomarkers for the pathogenesis of the metabolic syndrome, diabetes, and their increased cardiovascular complications and deaths. Future mechanistic studies employing different mixtures of sphingolipids may directly test the possibility that they may cause or protect from insulin resistance by impairing proximal insulin receptor signaling.

Limitations of the Study

In a previous study, the CA.ME.LI.A population was stratified based on BMI and fasting to study the relationship with cardiovascular events and all-cause mortality in a 7-yr follow-up. In this study, the sphingolipid determination was conducted only on a selected number of samples (367/2,554) within the six subgroups already identified.

Significance of the study

This study provides evidence of the synergic impact of overweight/obesity and prediabetes/diabetes on sphingolipid metabolism. Increased blood glucose levels produce a decline in circulating glycosylated sphingolipids in NBW. When hyperglycemia is associated with overweight/obesity, this trend is even more marked, also in IFG subjects. Interestingly, SM18:1, GM3 24:1, and DHS1P emerged as lipid species that are synergistically influenced by both BMI and glucose levels.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding authors, R.P. and F.F.

SUPPLEMENTAL MATERIAL

Supplemental Figs. S1–S10 and Supplemental Tables S1–S12: https://doi.org/10.6084/m9.figshare.31545361.

ACKNOWLEDGMENTS

Lucia Centofanti is a PhD student in “Scienze della nutrizione—Università degli Studi di Milano”. She is supported by PNC-PNRR project: D3_4 Health Digital Driven Diagnostics, prognostics and therapeutics for sustainable Health care (Project PNC 0000001 D3_4 Health, CUP B53C22006080001, The National Plan for Complementary Investments to the NRRP, Funded by the European Union-NextGeneration EU). Graphical abstract created with a licensed version of BioRender.com.

GRANTS

The CA.ME.LI.A project was supported by Regione Lombardia Grant DG Sanità 08/07/2008 No. 7364, Italian Ministry for Education Grant MIUR, GR-2011 02350447; by Dipartimento di Scienze della Salute, Università degli Studi di Milano, Grant: Linea 2 del Piano di Sostegno alla Ricerca 2020: Looking for

Novel Biomarkers of Diabetes and Cardiovascular Diseases, "NODICARDIO"- LINEA 2 DEL PIANO DI SOSTEGNO ALLA RICERCA PSR2020_DIP_013_PARONI; Grant: Linea 2 del Piano di Sostegno alla Ricerca 2025 "Digging a little deeper into the CA.ME.LI.A. population: impact of dietary habits on circulating metabolome, BMI, glucose, liver and kidney metabolism. Relation with the incidence of cardiovascular events"—LINEA 2 DEL PIANO DI SOSTEGNO ALLA RICERCA PSR2020_DIP_013_PARONI; Grant: "IncentivoAllaRicerca2025" "DEleterious MEtabolic effects of high-fat and high-fructose diet on MIce Endocrine pancreas and Liver"—F_DOTAZIONE_2025_DIP_013 (to F.F.).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

P.M.B., F.F., and R.P. conceived and designed research; C.M., L.C., M.D.C., P.Z., S.P., I.P., I.G., and R.P. performed experiments; C.M., L.C., M.D.C., R.Z., G.D.P., E.B., F.S., G.S., L.B., F.B., A.R., P.M.B., and M.F. analyzed data; C.M., L.C., M.D.C., M.B., M.T., F.F., and R.P. interpreted results of experiments; C.M., L.C., M.D.C., R.Z., G.D.P., I.P., P.S., G.S., and F.B. prepared figures; M.B., M.F., F.F., and R.P. drafted manuscript; M.T., P.M.B., M.F., F.F., and R.P. edited and revised manuscript; P.M.B., M.F., F.F., and R.P. approved final version of manuscript.

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