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Plasma signatures of Congenital Generalized Lipodystrophy patients identified by untargeted lipidomic profiling are not changed after a fat-containing breakfast meal

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ABSTRACT

Background: The incapacity to store lipids in adipose tissue in Congenital Generalized Lipodystrophy (CGL) causes hypoleptinemia, increased appetite, ectopic fat deposition and lipotoxicity. CGL patients experience shortened life expectancy. The plasma lipidomic profile has not been characterized fully in CGL, nor has the extent of dietary intake in its modulation. The present work investigated the plasma lipidomic profile of CGL patients in comparison to eutrophic individuals at the fasted state and after a breakfast meal.
Method: Blood samples from 11 CGL patients and 10 eutrophic controls were collected after 12 h fasting (T0) and 90 min after an *ad libitum* fat-containing breakfast (T90). The lipidomic profile of extracted plasma lipids was characterized by non-target liquid chromatography mass spectrometry.
Results: Important differences between groups were observed at T0 and at T90. Several molecular species of fatty acyls, glycerolipids, sphingolipids and glycerophospholipids were altered in CGL. All the detected fatty acyl molecular species, several diacylglycerols and one triacylglycerol species were upregulated in CGL. Among sphingolipids, one sphingomyelin and one glycosphingolipid species showed downregulation in CGL. Alterations in the glycerophospholipids glycerophosphoethanolamines, glycerophosphoserines and cardiolipins were more complex. Interestingly, when comparing T90 versus T0, the lipidomic profile in CGL did not change as intensely as it did for control participants.
Conclusions: The present study found profound alterations in the plasma lipidomic profile of complex lipids in CGL patients as compared to control subjects. A fat-containing breakfast meal did not appear to significantly influence the CGL profile observed in the fasted state. Our study may have implications for clinical practice, also aiding to a deeper comprehension of the role of complex lipids in CGL in view of novel therapeutic strategies.

1. Introduction

Congenital Generalized Lipodystrophy (CGL), also known as Berardinelli-Seip congenital lipodystrophy, is an autosomal recessive disorder characterized by severe loss of adipose tissue, approaching total tissue absence since birth or in early infancy [1]. Life expectancy is significantly shortened in CGL [2].

Four types of CGL have been described, all leading to significant disturbances in triacylglycerol (TAG) and phospholipid synthesis, lipid droplet formation and lipid deposition. In Type 1 CGL, deficiency of 1-

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acyl-sn-glycerol-3-phosphate acyltransferase beta (AGPAT2) leads to impaired TAG synthesis at the bioconversion step from lysophosphatidic acid to phosphatidic acid. In Type 2 CGL, deficiency of seipin impairs the fusion of lipid droplets and thus adipocyte maturation. Type 3 CGL is characterized by deficiency of caveolin 1, an adipocyte membrane protein involved in translocation of fatty acids into lipid droplets. In Type 4 CGL, deficiency of caveolae-associated protein 1 (cavin 1) impairs caveolae biogenesis [3,4].

The impaired capacity to store lipids in adipocytes observed in CGL results in ectopic fat deposition, which in turn leads to cellular lipotoxicity, mitochondrial dysfunction and endoplasmic reticulum (ER) stress [4,5]. Moreover, CGL patients suffer with severe dyslipidemia, insulin resistance and type II diabetes mellitus (T2D), liver steatosis, cardiomyopathy, hypoleptinemia and hypoadiponectinemia [6].

Impaired suppression of neural activity by food intake has been reported in several brain areas of CGL patients, with leptin administration attenuating this deficiency [7]. CGL patients have increased appetite [8,9]. Our group has recently identified a reduced satiation time in CGL patients, and a lack of physiological response to ghrelin after the intake of a fat-containing meal, as compared to healthy matched controls [10].

Dietary fats appear to be involved in the regulation of food intake. Oral infusion of medium-chain triglyceride emulsion reduced subsequent calorie intake, in comparison to long-chain triglyceride emulsion, in rats [11]. The orexigenic effect of ghrelin has been shown to depend on the increased hypothalamic levels of C18:0 ceramide, induced by ghrelin, in a knockout mouse model [12]. Phosphatidic acid and phosphatidylethanolamine inhibited food intake after their intraperitoneal administration in mice [13]. Studies in humans have corroborated rodent studies, confirming a role for fat intake [14], and more specifically medium-chain triglyceride intake [15], in decreasing appetite.

In view of the above, the present work aimed to evaluate the plasma lipidomic profile of CGL patients at the fasted state and after a fat-containing breakfast meal. Matched healthy eutrophic individuals participated in the research as controls. Our study suggests that in CGL the influence of circulating plasma lipids on appetite regulation is not associated with a very short-term dietary intervention.

2. Method

The present study was approved by the Ethics Committee of the Walter Cantídio University Hospital (Fortaleza, CE, Brazil) under registration CAAE 51,022,215.5.0000.5045, and approved by the Research Ethics Committee of the Universidade Federal de São Paulo (São Paulo, SP, Brazil) under registration CAAE 14,167,219.0.0000.5505. All individuals invited to join this research received explanation in full regarding the study and its risks and had the opportunity to have their questions clarified in full. The consenting participants signed a consent form.

The experimental design employed in the present study is the one recently published by our group [10]. Briefly, the 11 CGL consenting patients recruited for this study were patients of the Reference Center for Inherited and Acquired Lipodystrophies of the Walter Cantídio University Hospital (Fortaleza, CE, Brazil). All patients had a medically confirmed diagnosis of CGL prior to participation in this study. The patients' ongoing health management strategies include endocrinological, with pharmacotherapy to manage their T2D, dyslipidemia and hypertension, and nutritional advice, consisted of low-fat low-calorie dietary advice.

Four CGL patients presented the AGPAT2 gene mutation, and 2 presented the BSCL2 mutation. The remaining 5 patients did not present any of these two mutations but did not have their specific mutation identified. Two patients suffered with hypertension, and 8 suffered with T2D.

The control group consisted of 10 healthy eutrophic volunteers (EUT group) with no familial connection with the patients. CGL and EUT subjects were matched for age (19.6 ± 2.6 and 21.5 ± 2.7 years, respectively), gender (64% female/36% male and 60% female/40% male), and Body Mass Index (21.69 ± 0.79 and 20.49 ± 0.77 kg/m^2).

2.1. Blood collection

All participants arrived at the Reference Center clinic at around 7:00 AM after 12 h fasting. A qualified nurse inserted a catheter in the cubital vein and collected the first blood sample (T0). All participants were then offered a breakfast buffet, consisted of 19 breakfast items typically consumed by Brazilians, according to the Brazilian Survey of Familial Budget [19] (Supplementary Table 3). The breakfast buffet offered consisted globally of 815 g carbohydrates, 216.92 g protein, 225.14 g fat, with a total energy content of 6333.85 kcal. All participants were instructed to eat freely until full, and instructed not to eat the buffet items they did not like. The leftovers for each participant were weighted and recorded. The energy and macronutrient intake for both groups are presented in supplementary Table 4.

The EUT group ate in average for 12 min, while the CGL group ate in average for 8 min. Ninety minutes after the meal was finished, a second blood sample was collected (T90). Plasma samples (T0 and T90) were obtained by centrifugation (20 min, 1125 rcf, 4 °C) and kept at −80º C until analysis.

2.2. Lipid extraction

Lipid extraction was based on the Folch's method [16]. Briefly, 0.8 mL of plasma were added to 5 mL chloroform-methanol (2:1) and 0.5 mL 0.1 N NaCl, homogenized and centrifuged at 448 rcf for 10 min. The lipid phase was collected, dried in a vacuum concentrator centrifuge (Eppendorf, Hamburg, Germany) and stored at −8 °C until analysis.

2.3. Liquid chromatographic mass spectrometric analyses

Data generation and analyses were performed as previously standardized by our group [17,18]. Briefly, dried lipid extracts were resuspended in 1 mL isopropanol acetonitrile water solution (2:1:1 v/v/v) prior to untargeted liquid chromatography mass spectrometry (LC–MS) analyses. Five μL volume samples were injected in a ACQUITY FTN liquid chromatograph coupled to a XEVO G2-XS QToF mass spectrometer (Waters, Milford, MA, USA), equipped with an Agilent ZORBAX RRHP Eclipse Plus (C18, 95 Å, 1,8 μm, 2,1 × 100 mm) column.

Mobile phase A consisted of 5 mM ammonium acetate with 0.1% acetic acid in isopropanol methanol water (50:10:40, v/v/v). Mobile phase B consisted of 5 mM ammonium acetate with 0.1% acetic acid in isopropanol water (99:1, v/v). The flow rate was 0.25 mL/min with a linear gradient (in% B) as follows: 0 – 1.5 min: 0 –20%; 1.5 – 6.0 min: 20 – 45%; 6 – 9.0 min: 45 – 100%; 9.0 – 13.0 min: 100%; 13 – 13.2 min: decrease to 0%. Further 2.8 min were added for column re-equilibration, resulting in a 16-minute run time.

For the electrospray ionization source, the positive ion mode acquisition parameters were set as follows: capillary voltage 3.5 kV; sampling cone 40 V; source temperature 140 °C; desolvation temperature 550 °C; cone gas flow 50 L/h; desolvation gas flow 900 L/h. The negative ion mode acquisition parameters were set as follows: capillary voltage 2.4 kV; sampling cone 40 V; source temperature 140 °C; desolvation temperature 400 °C; cone gas flow 50 L/h; desolvation gas flow 900 L/h.

The acquisition scan ranged from 50 to 1700 Da. The data processing employed a high energy collision dissociation mass spectrometric (MS^E) approach. Leucine enkephalin (molecular weight = 555.62; 200 pg/μL in 1:1 ACN:H_2O) was used as reference compound for mass spectrometric fragmentation and measurement standards. The instrument was calibrated with 0.5 mM sodium formate. Data acquisition was conducted using MassLynx 4.1 software (Waters Corporation). CGL and EUT samples were injected at random.

2.4. MS data processing and statistical analysis

MS data analyses were performed as recently standardized by our group [18]. Briefly, raw data were processed with Progenesis QI software (Waters Corporation) for peak detection, alignment, integration, deconvolution, data filtering, ion annotation and MS^E-based putative identification of compounds. LIPID MAPS (https://www.lipidmaps.org/) database was used for compound identification employing the following search parameters: precursor mass error $\leq$ 5 ppm, fragment tolerance $\leq$ 10 ppm. Compounds were identified based on their fragmentation score, mass accuracy and isotope similarity.

Raw data for each sample in each ionization mode were analyzed separately using MetaboAnalyst 4.0 (https://www.metaboanalyst.ca/). Results were processed as follows: variables with missing values were excluded from the analysis, data filtering was based on interquartile range, samples were normalized by sum, data were log-transformed and submitted to range scaling.

Results were separately subjected to multivariate analysis (Principal Component Analysis, PCA) followed by Discriminant Analysis by Partial Least Squares (PLS-DA), and to univariate analysis (Student's *t*-test). Discriminant ions were selected according to their Variable Importance in Projection values (VIP $>$ 2). False Discovery Rate was decreased by the Benjamini-Hochberg test, with significance set at FDR $<$ 0.05.

3. Results

No differences in calorie intake or macronutrient intake were observed between CGL and EUT after the *ad libitum* fat-containing breakfast meal (supplementary Table 4). We have recently reported the subjective appetite outcomes and acylated ghrelin levels on fasting and after the breakfast meal for the CGL and EUT participants enrolled in the present study [10]. Demographic, anthropometric, clinical and serum biomarker data have also been reported [10].

3.1. Meal intake showed no effect on lipidomic profile

The PLS-DA score plots (Fig. 1) show no statistically significant differences in the lipidomic profiles of EUT and CGL between T0 and T90.

3.2. Lipidomic profile differs between EUT and CGL

Noticeable differences in lipidomic profile were observed between groups at T0 and at T90. Representative total ion chromatograms and mass spectra are presented in Supplementary Figure S1 for T0, and in Supplementary Figure S2 for T90.

At T0, the PLS-DA analysis of the first two principal components (PC) showed clearly defined clusters between CGL and EUT both in negative (R2 = 0.452 and Q2 = 0.182) and positive (R2 = 0.748 and Q2 = 0.474) ionization modes (Fig. 2).

At T0, 67 ions contributed significantly to group discrimination, 9 ions detected in negative ionization mode and 58 ions detected in positive ionization mode (Supplementary Table S1). Among the discriminant ions, 18 were identified through ion fragmentation (Table 1).

The main lipid categories showing differences between CGL and EUT at T0 include fatty acyls (3 ions), glycerolipids (7 ions), glycerophospholipids (7 ions), and sterol lipids (1 ion). The relative intensities of the 18 identified ion classes are shown as heatmaps in Fig. 3.

Same as in T0, the PLS-DA analysis at T90 also revealed a clear discrimination between CGL and EUT individuals, both in negative (2 PCs, R2 = 0.818 and Q2 = 0.655), and positive (2 PCs, R2 = 0.868 and Q2 = 0.645), ionization modes (Fig. 4).

At T90, 75 ions contributed significantly to group discrimination, 26 ions detected in negative ionization mode and 49 ions detected in positive ionization mode (Supplementary Table S2). Twenty-nine of these ions were identified through ion fragmentation (Table 2).

The main lipid categories showing alterations between CGL and EUT at T90 included fatty acyls (6 ions), glycerolipids (10 ions), glycerophospholipids (11 ions), and sphingolipids (2 ions). The relative intensities of the 29 identified ion classes are shown as heatmaps in Fig. 5.

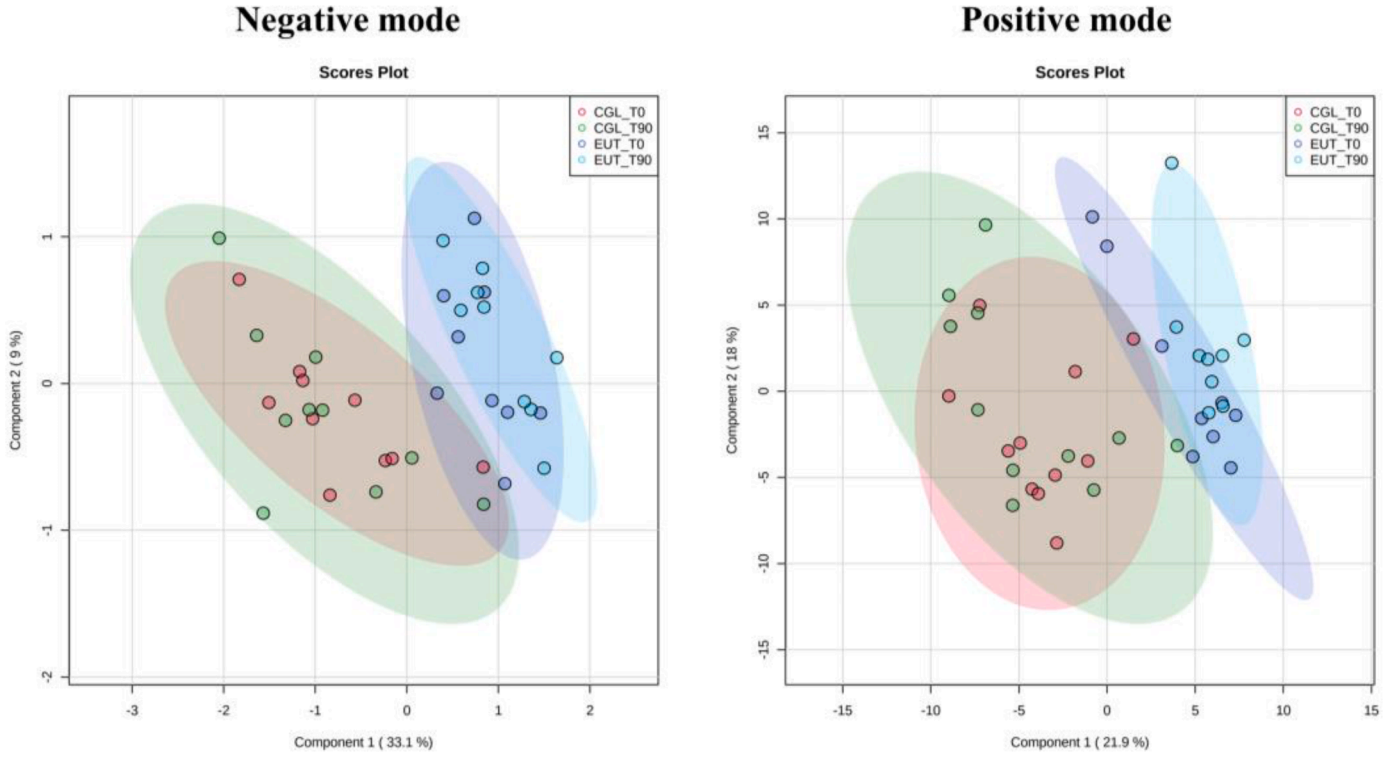

Fig. 1. Score plots of PLS-DA model for serum lipidomic profile data in negative and positive ionization modes for EUT and CGL at T0 *versus* T90. PLS-DA: discriminant analysis by partial least squares.

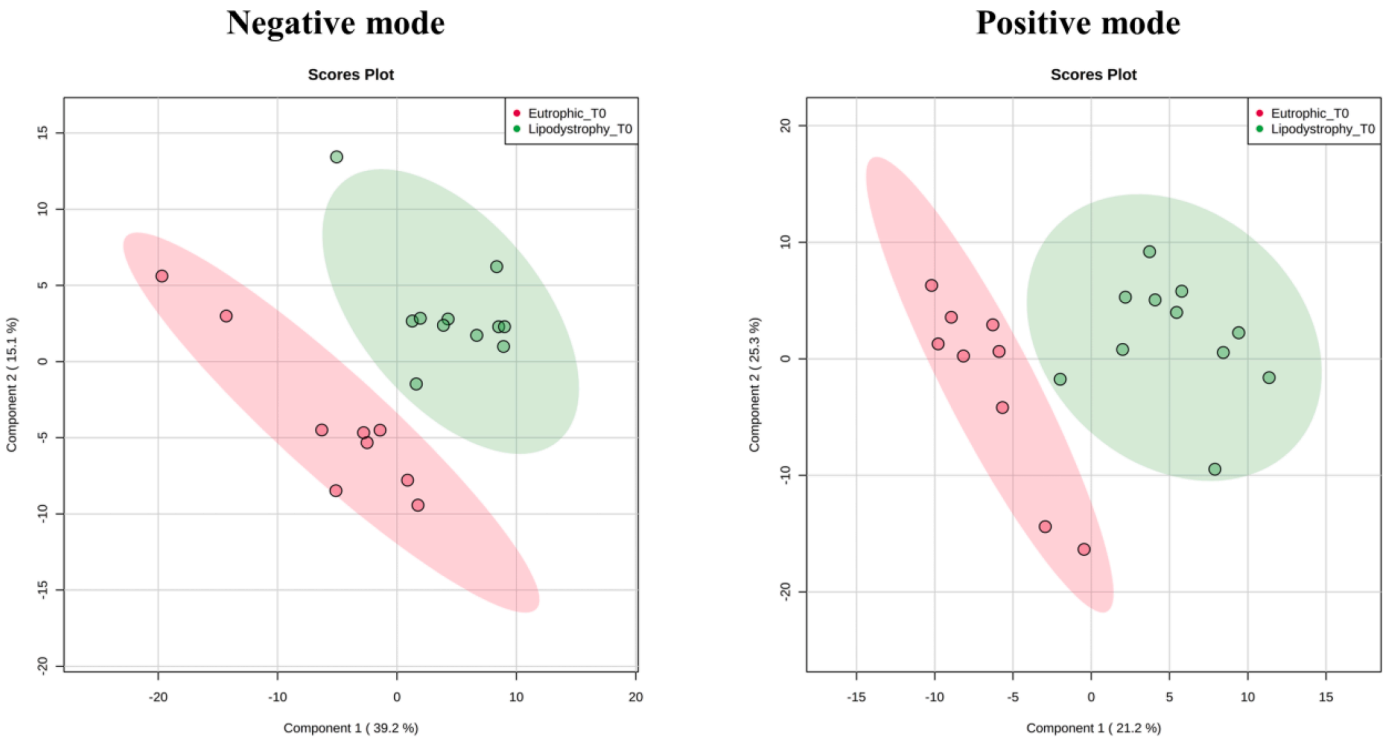

Fig. 2. Score plots of PLS-DA model for serum lipidomic data on negative and positive ionization modes at T0. PLS-DA: discriminant analysis by partial least squares.

Among the molecular species relevant for group discrimination, 5 had their systematic names identified: a) (3S,4R,9R,11S)-29-(4-hydroxyphenyl)-3-methoxy-4-methylnonacosane-9,11-diol (a fatty alcohol showing increased levels at both T0 and T90); b) 1,2-di-(9Z,12Z-octadecadienoyl)-sn-glycerol (a diacylglycerol showing increased levels at T0); c) 1-(1Z-eicosenyl)-2-(13Z,16Z-docosadienoyl)-glycero-3-phosphoethanolamine (a glycerophosphoethanolamine showing decreased levels at both T0 and T90); d) N-(hexadecanoyl)-1-β-glucosyl-4E,6E-tetradecasphingadienine (a neutral glycosphingolipid showing decreased levels at T90); e) N-(9Z-octadecenoyl)-4E,14Z-sphingadienine-1-phosphocholine (a ceramide showing decreased levels at T90). The compound structure and their relative intensities between EUT and CGL at the level of systematic name are shown in Fig. 6.

4. Discussion

In the present study, the plasma lipidomic profile of CGL patients was examined at the fasted state and after an *ad libitum* fat-containing breakfast meal. We aimed to further explore the lipid-associated molecular manifestations observed in CGL patients, and how the transition from the fasted to the fed state could influence the lipidomic profile in those patients.

The CGL patients recruited for this study are currently receiving medical and dietetic care provided by the Brazilian Public Health System at the Reference Center for Inherited and Acquired Lipodystrophies of the Walter Cantídio University Hospital (Fortaleza, Brazil). In view of the metabolic derangements observed in CGL, and due to their current hypertriglyceridemia and hepatic steatosis more particularly, our patients are advised to follow a low-fat low-calorie diet. Our patients receive instructions on how to replace simple for complex carbohydrates, and to increase their fiber and omega-3 polyunsaturated fat intake as additional strategies to control their T2D and inflammatory biomarkers. Additionally, our patients receive tailored pharmacological therapies with metformin, fibrates, and hydrochlorothiazide, prescribed and followed-up by their clinicians.

Paramount to the present study, all participants were advised to fast for 12 h overnight, and subsequently feed *ad libitum* on a breakfast buffet representative of the Brazilian diet [19]. Several of the breakfast items offered are characterized by a moderate to high fat content, including yellow cheese, mortadella, and margarine. The participants were advised to eat as much as they wanted out of the 19 breakfast items offered, and advised not to eat what they did not like. We have recently reported the participants' food intake and satiety time [10]. Briefly, no significant differences in calorie or macronutrient intake were found between CGL and EUT, but the CGL group showed shorter satiation and satiety times compared to EUT.

Our plasma lipidomic profile investigation revealed significant differences between CGL and EUT, with notable differences in fatty acyls (FAs), glycerolipids (GLs) and glycerophospholipids (GPs). Interestingly, the time elapsed between the two blood sample collections – immediately before the breakfast meal (T0) and 90 min after the end of the breakfast meal (T90) – did not appear to equally modulate the plasma lipidomic profile between groups.

FAs are synthesized by elongation of acetyl-CoA with malonyl-CoA and constitute a diverse lipid category of molecules, including fatty acids and eicosanoids [20]. Among FAs, we found that three molecular species were increased in CGL at T0, as compared to EUT. At T90, those three species remained high, and three more were increased.

Leptin stimulates mitochondrial fatty acid oxidation and decreases fatty acid synthesis through activation of adenosine 5-monophosphate-activated protein kinase (AMPK), decreasing the activity of acetyl-Coa carboxylase and reducing malonyl-CoA [5]. Leptin deficiency, a common finding in CGL, may be involved in the raised FA levels observed here. Adipose tissue transplantation in lipodystrophic mice increased leptin levels and restored beta oxidation and lipogenesis in the liver [21]. Leptin replacement therapy in lipodystrophic patients has been shown to increase the breakdown of fatty acids [22].

One FA molecular species showing increased levels in CGL patients was the fatty alcohol (3S,4R,9R,11S)-029-(4-hydroxyphenyl)-3-methoxy-4-methylnonacosane-9,11-diol, also known as phenolic phthiocerol. Increased levels of phenolic phthiocerol have been observed in the valproic acid-induced dyslipidemia in epileptic patients

Table 1
Discriminant ions (PLS-DA, VIP > 2.0) with statistically significant differences (*t*-test, FDR < 0.05) between groups at T0.

Ion	Chemical formula	Lipid category	Main class	Subclass	Systematic name	Mean VIP[a]	FDR[b]	Observed change[c]
Negative ionization mode								
8.76_856.5296 m/z	$C_{48}H_{76}NO_{10}P$	Glycerophospholipids	Glycerophosphoserines	Diacylglycerophosphoserines		2.17	0.0164	High
Positive ionization mode								
9.61_577.5115 m/z	$C_{37}H_{68}O_4$	Fatty acyls	Fatty alcohols	–	(3S,4R,9R,11S)-29-(4-hydroxyphenyl)-3-methoxy-4-methylnonacosane-9,11-diol (phenolic phthiocerol)	2.41	0.0006	High
9.37_287.2280 m/z	$C_{16}H_{30}O_4$	Fatty acyls	Fatty acids and Conjugates	Dicarboxylic acids		2.63	0.0025	High
9.22_335.2494 m/z	$C_{19}H_{36}O_2$	Fatty acyls				2.12	0.0030	High
9.03_653.4532 m/z	$C_{39}H_{66}O_5$	Glycerolipids	Diradylglycerols	Diacylglycerols	1,2-di-(9Z,12Z-octadecadienoyl)-sn-glycerol (DG (18:2/18:2/0:0))	2.48	0.0006	High
6.50_670.5555n	$C_{43}H_{74}O_5$	Glycerolipids	Diradylglycerols	Diacylglycerols		2.13	0.0006	High
9.61_594.5187n	$C_{37}H_{70}O_5$	Glycerolipids	Diradylglycerols	Diacylglycerols		2.44	0.0006	High
9.37_616.5041n	$C_{39}H_{68}O_5$	Glycerolipids	Diradylglycerols	Diacylglycerols		2.50	0.0007	High
9.36_592.5016n	$C_{37}H_{68}O_5$	Glycerolipids	Diradylglycerols	Diacylglycerols		2.35	0.0006	High
8.27_625.5157 m/z	$C_{39}H_{70}O_4$	Glycerolipids	Diradylglycerols	1-acyl,2-alkylglycerols		2.12	0.0152	High
8.22_810.7516 m/z	$C_{50}H_{96}O_6$	Glycerolipids	Triradylglycerols	Triacylglycerols		2.28	0.0076	High
6.82_775.5759n	$C_{42}H_{82}NO_9P$	Glycerophospholipids	Glycerophosphoserines			2.35	0.0025	Low
8.49_829.6823 m/z	$C_{47}H_{90}NO_7P$	Glycerophospholipids	Glycerophosphoethanolamines	1-(1Z-alkenyl),2-acylglycerophosphoethanolamines	1-(1Z-eicosenyl)-2-(13Z,16Z-docosadienoyl)-glycero-3-phosphoethanolamine (PE(P-20:0/22:2))	2.23	0.0049	Low
5.53_717.5549 m/z	$C_{39}H_{74}NO_7P$	Glycerophospholipids	Glycerophosphoethanolamines	1-alkyl,2-acylglycerophospho-ethanolamines		2.11	0.0032	Low
7.74_733.5631n	$C_{40}H_{80}NO_8P$	Glycerophospholipids				2.11	0.0030	High
8.21_809.5962n	$C_{46}H_{84}NO_8P$	Glycerophospholipids				2.32	0.0049	High
8.88_837.6267n	$C_{48}H_{88}NO_8P$	Glycerophospholipids				2.37	0.0020	High
9.14_467.3095 m/z	$C_{28}H_{44}O_4$	Sterol lipids				2.24	0.0026	Low

[a] Mean VIP values of the 5 principal components based on the PLS-DA model.
[b] False Discovery Rate, corrected p-values according to Benjamini-Hochberg test.
[c] Mean ion intensity of the CGL group/mean ion intensity of the EUT group.

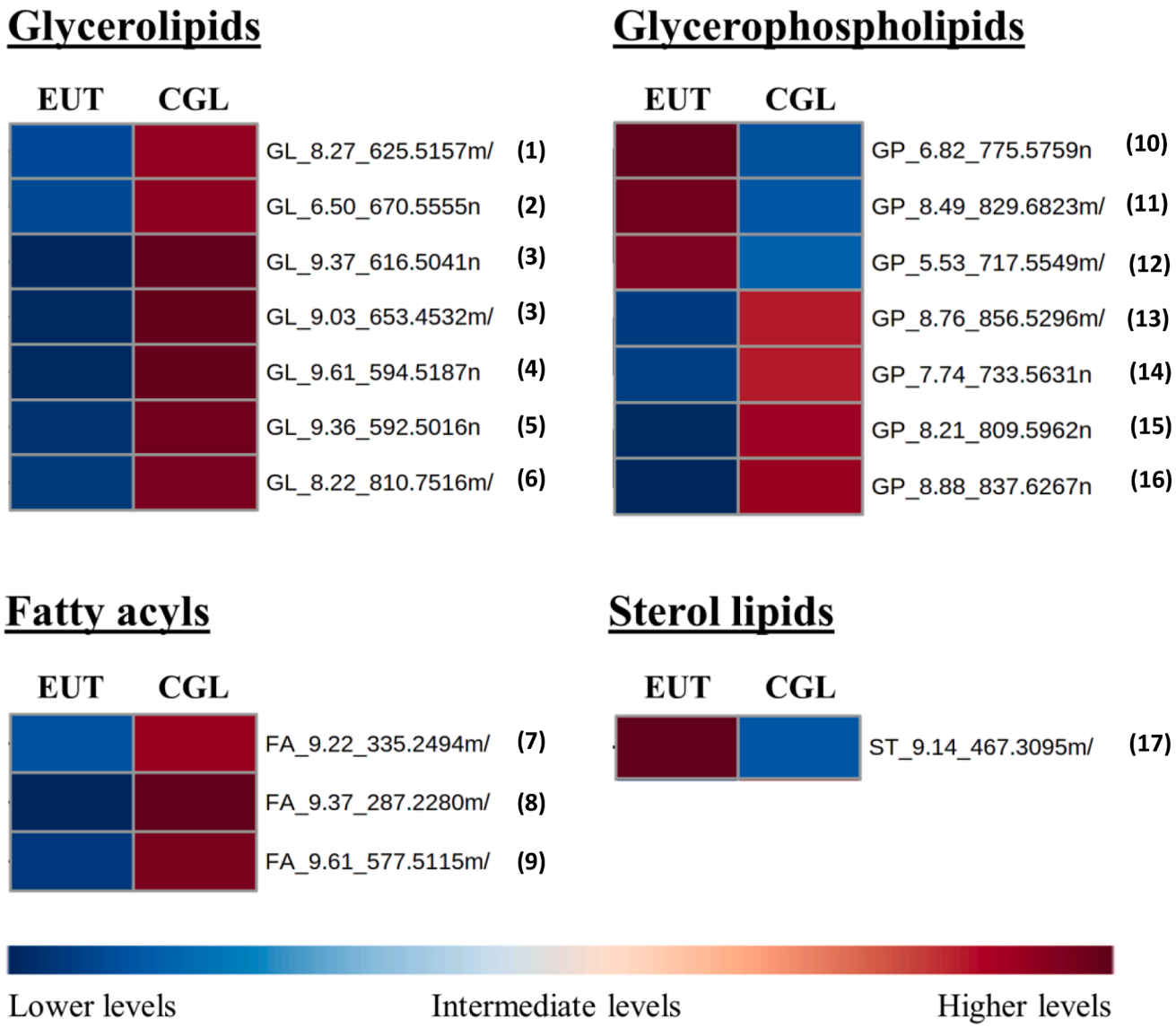

Fig. 3. Mean intensity heatmaps of the 17 compounds identified at lipid category level between EUT and CGL at T0. (1) Diacylglycerol O-36:4; (2) Diacylglycerol 40:5; (3) Diacylglycerol 36:4*; (4) Diacylglycerol 34:1; (5) Diacylglycerol 34:2; (6) Triacylglycerol 40:7; (7) Fatty acid 19:1; (8) Fatty acid 16:1;O2; (9) Fatty alcohol 37:4;O3; (10) Glycerophosphoserine O-36:1; (11) Glycerophosphoethanolamine O-42:3; (12) Glycerophosphoethanolamine O-34:3; (13) Glycerophosphoserine 42:9; (14) Glycerophosphocholine 32:0; (15) Glycerophosphocholine 38:4; (16) Glycerophosphocholine 40:4; (17) Sterol 28:3;O4. *Same MS-annotated lipid identified in two features.

[23].

GLs are generated by esterification of glycerol with one, two or three FAs, generating monoacylglycerols (MAG), diacylglycerols (DAG) and triacylglycerols (TAG), respectively. We found elevated levels of several DAG species in CGL patients, both at T0 and T90, as compared to EUT. DAGs act as second messengers and mediate the activation of protein kinase C (PKC) [24]. Increased levels of DAGs and their accumulation in hepatocytes has been linked to non-alcoholic fatty liver disease (NAFLD), insulin resistance and T2D, via PKC-induced impairment of insulin signaling [25,26].

The only glycerolipid found at decreased levels (8.63_781.5983 m/z) in our CGL patients was monogalactosyl diacylglicerol (MGDG), a compound associated with myelination [27]. Manifestations associated with demyelinating syndrome have been described in a 22-years old CGL sufferer [28]. Paranodal myelin swellings have been observed in sural nerve biopsies of patients with Dunnigan familial partial lipodystrophy [29].

Due to its rarity, only very few CGL studies have investigated the underlying mechanisms explaining hypertriglyceridemia in CGL. One putative pathway suggests that the acylation of 2-monoacylglycerol by monoacylglycerol acyltransferases (MGAT) are the source of DAG for TAG synthesis in the liver [3,30]. The expression of MGAT1 has been reported to be increased in the liver of AGPAT2-null mice, a CGL rodent model, suggesting that this alternative pathway is activated. Moreover, the *de novo* lipogenesis was also elevated in that model [30].

Glycerophospholipids (GP), main components of cell membranes, are formed by a glycerol backbone to which usually a saturated and an unsaturated fatty acid are attached at *sn-1* and *sn-2* positions, respectively, with a phosphate head group attached to the *sn-3* position. The GP lipid category comprises glycerophosphocholines (PC), glycerophosphoethanolamines (PE), glycerophosphoserines (PS), cardiolipins (CL), glycerophosphates (PA), and glycerophosphoglycerols (PG), among others. Functionally, GP can be the precursors of DAG, eicosanoids, phosphatidylinositol phosphates and lysophospholipids, which are lipid subclasses with second messenger activity [31,32].

Changes in PC/PE ratio across the cell membrane are associated with

Fig. 4. Score plots of PLS-DA model for serum lipidomic data on negative and positive ionization modes at T90. PLS-DA: Discriminant analysis by partial least squares.

deranged membrane integrity and permeability [33]. Although we did not find changes in PC in CGL patients, we did find decreased levels of two PE species, suggesting an elevated PC/PE ratio. Reduced PE levels have been identified in the liver of rats with diabetic ketoacidosis [34]. Although some investigations have reported an association between decreased PC/PE ratio and metabolic disorders [35–37], other investigations have shown elevated PC/PE ratio in analogous circumstances [38,39]. Either case appears to suggest that both increased and decreased PC/PE ratio can result in deleterious conditions. Moreover, as PE is naturally much richer in both omega 6 and 3 PUFAs than PC in plasma and erythrocytes of healthy subjects [40], the decreased PE levels found in CGL further corroborate the hypothesis of deranged membrane integrity and permeability in CGL.

Importantly, elevated PC/PE ratio has been implicated in the impairment of calcium homeostasis at the endoplasmic reticulum (ER), contributing to ER stress in the liver [38]. Additionally, it has been shown that a modest 20% – 30% depletion of PE in the mitochondria, an organelle where PE is highly abundant, induced mitochondrial fragmentation, impaired oxidative phosphorylation, decreased ATP production and reduced cell growth [41]. As we did find significantly decreased levels of two PE species in CGL, such finding may imply a condition of elevated ER stress and oxidative stress, processes previously associated with CGL [42–44].

We found in CGL altered levels of two molecular species of PS at T0, one at lower and the other at higher level. At T90, the latter remained increased, and another one was also elevated. PS are synthetized in the ER and participate in the activation of both PKC and Akt signaling as they are translocated from the cytosol to the plasma membrane [45,46]. Moreover, when PS translocate to the outer membrane leaflet, they trigger an apoptotic signal during tissue damage [47]. The roles of PKC and Akt signaling, and of apoptosis, in the development and progression of nonalcoholic steatohepatitis and NAFLD have been demonstrated [48, 49,50]. PS serum levels have been identified as biomarkers of hepatic inflammation and fibrosis in patients suffering with chronic hepatitis B [51]. Our results showing an imbalance of PS species may indicate a role for this lipid subclass in the hepatic impairment seen in CGL patients.

CL are synthesized in the inner mitochondrial membrane (IMM), with phosphatidic acid as precursor. CL interactions with IMM proteins influence apoptosis, fusion and fission processes, and oxidative phosphorylation. Disturbances in CL content, composition or oxidation level are associated with mitochondrial dysfunction and elevated oxidative stress [52]. Depletion of the most abundant CL molecular species in myocardium of diabetic mice has been demonstrated [53]. Decreased CL content has also been observed in Barth syndrome, a rare disease that courses with hypertrophic cardiomyopathy [54]. Here we found decreased levels of one CL molecular species in CGL at T90. Our finding is compatible with the presence of hypertrophic cardiomyopathy in CGL patients [55,56].

We found decreased levels of one glycosphingolipid (Glc) and one sphingomyelin (SM) species in CGL patients. Sphingolipids (SL) are cell membrane components with important signaling roles. In the SM-ceramide-Glc pathway, ceramides (Cer) are both precursors for Glc and SM synthesis, and products of their metabolism [57]. Disturbed levels of Cer and SM have been identified in metabolic diseases [58]. Moreover, disturbances in the SM-Cer-Glc pathway, due to either increase or decrease of one or more intermediaries, has been associated with insulin resistance and fatty liver disease [57]. In liver cirrhosis patients, SM and Cer molecular species levels correlated inversely with scores of hepatic dysfunction [59].

Interestingly, the insulin-sensitizing effect of adiponectin has been associated with its ability to decrease Cer levels by stimulating a ceramidase that produces sphingosine from Cer [57,60]. We have reported hypoadiponectinemia in our CGL patients [10]. Considering the signs of imbalances in the SM-Cer-Glc metabolism observed in the present study, an in-depth analysis of how hypoadiponectinemia could be associated with disturbed SL in CGL deserves further investigation in view of novel therapeutic strategies.

A weakness of our study is that our equipment does not have ion mobility spectrometry capacity, therefore preventing us to confirm with absolute assurance the stereochemistry for some of the lipids featuring overlapping spectrometric identities. Nonetheless, it is known that dietary fats are involved in the regulation of food intake, and we have

Table 2
Discriminant ions (PLS-DA, VIP > 2.0) with statistically significant differences (*t*-test, FDR < 0.05) between groups at T90.

Ion	Chemical formula	Lipid category	Main class	Subclass	Systematic name	Mean VIP[a]	FDR[b]	Observed change[c]
Negative ionization mode								
5.40_357.2793 m/z	$C_{20}H_{38}O_5$	Fatty acyls	Fatty acids and conjugates			2.15	0.0016	High
5.58_333.2787 m/z	$C_{22}H_{38}O_2$	Fatty acyls				2.07	0.0005	High
3.29_275.2016 m/z	$C_{18}H_{28}O_2$	Fatty acyls				2.11	0.0020	High
9.67_653.5348 m/z	$C_{42}H_{70}O_5$	Glycerolipids	Diradylglycerols	Diacylglycerols		2.07	0.0018	High
9.67_639.5222 m/z	$C_{41}H_{68}O_5$	Glycerolipids	Diradylglycerols	Diacylglycerols		2.39	0.0006	High
9.67_629.4990 m/z	$C_{40}H_{70}O_5$	Glycerolipids	Diradylglycerols	Diacylglycerols		2.20	0.0005	High
8.63_781.5983 m/z	$C_{45}H_{82}O_{10}$	Glycerolipids	Glycosyldiradylglycerols	Glycosyldiacylglycerols		2.15	0.0021	Low
8.67_1395.8680 m/z	$C_{77}H_{138}O_{17}P_2$	Glycerophospholipids	Glycerophosphoglycero-phosphoglycerols	Cardiolipins		2.08	0.0034	Low
8.76_856.5296 m/z	$C_{48}H_{76}NO_{10}P$	Glycerophospholipids	Glycerophosphoserines	Diacylglycerophosphoserines		2.14	0.0011	High
8.76_848.5571 m/z	$C_{47}H_{80}NO_{10}P$	Glycerophospholipids	Glycerophosphoserines	Diacylglycerophosphoserines		2.19	0.0005	High
5.61_683.4688 m/z	$C_{38}H_{69}O_8P$	Glycerophospholipids	Glycerophosphates	Diacylglycerophosphates		2.14	0.0011	Low
8.84_754.5742 m/z	$C_{43}H_{82}NO_7P$	Glycerophospholipids				2.05	0.0033	Low
9.29_728.5584 m/z	$C_{41}H_{80}NO_7P$	Glycerophospholipids				2.25	0.0016	Low
6.07_640.4763 m/z	$C_{36}H_{67}NO_8$	Sphingolipids	Neutral glycosphingolipids	Simple Glc series	N-(hexadecanoyl)-1-β-glucosyl-4E,6E-tetradecasphingadienine (GlcCer(d14:2(4E,6E)/16:0))	2.16	0.0007	Low
Positive ionization mode								
9.61_577.5115 m/z	$C_{37}H_{68}O_4$	Fatty acyls	Fatty alcohols	–	(3S,4R,9R,11S)-29-(4-hydroxyphenyl)-3-methoxy-4-methylnonacosane-9,11-diol (phenolic phthiocerol)	2.43	0.0001	High
9.37_287.2280 m/z	$C_{16}H_{30}O_4$	Fatty acyls	Fatty acids and conjugates	Dicarboxylic acids		2.27	0.0019	High
9.22_335.2494 m/z	$C_{19}H_{36}O_2$	Fatty acyls				2.15	0.0003	High
9.78_630.5262n	$C_{40}H_{70}O_5$	Glycerolipids	Diradylglycerols	Diacylglycerols		2.42	0.0015	High
Ion	**Chemical formula**	**Lipid category**	**Main class**	**Subclass**	**Systematic name**	**Mean VIP[a]**	**FDR[b]**	**Fold-change[c]**
9.61_594.5187n	$C_{37}H_{70}O_5$	Glycerolipids	Diradylglycerols	Diacylglycerols		2.64	0.00005	High
9.36_592.5016n	$C_{37}H_{68}O_5$	Glycerolipids	Diradylglycerols	Diacylglycerols		2.19	0.0001	High
9.37_616.5041n	$C_{39}H_{68}O_5$	Glycerolipids	Diradylglycerols	Diacylglycerols		2.24	0.0003	High
6.50_670.5555n	$C_{43}H_{74}O_5$	Glycerolipids	Diradylglycerols	Diacylglycerols		2.18	0.0011	High
8.60_651.5358 m/z	$C_{43}H_{70}O_4$	Glycerolipids	Diradylglycerols	1-acyl,2-alkylglycerols		2.30	0.0008	High
8.49_829.6823 m/z	$C_{47}H_{90}NO_7P$	Glycerophospholipids	Glycerophospho-ethanolamines	1-(1Z-alkenyl), 2-acylglycero-phosphoethanolamines	1-(1Z-eicosenyl)-2-(13Z,16Z-docosadienoyl)-glycero-3-phosphoethanolamine (PE(P-20:0/22:2(13Z,16Z))	2.14	0.0005	Low
6.60_763.5485 m/z	$C_{41}H_{79}O_{10}P$	Glycerophospholipids	Glycerophosphoglycerols	Diacylglycero- phosphoglycerols		2.30	0.0028	Low
2.56_480.3045 m/z	$C_{23}H_{46}NO_7P$	Glycerophospholipids				2.17	0.0056	Low
8.88_837.6267n	$C_{48}H_{88}NO_8P$	Glycerophospholipids				2.33	0.0024	High
8.21_809.5962n	$C_{46}H_{84}NO_8P$	Glycerophospholipids				2.23	0.0037	High
6.35_726.5690n	$C_{41}H_{79}N_2O_6P$	Sphingolipids	Phosphosphingolipids	Ceramide phosphocholines (sphingomyelins)	N-(9Z-octadecenoyl)-4E,14Z-sphingadienine-1-phosphocholine (SM(d18:2/18:1))	2.19	0.0003	Low

[a] Mean VIP values of the 5 principal components based on the PLS-DA model.
[b] False Discovery Rate, corrected p-values according to Benjamini-Hochberg test.
[c] Mean ion intensity of the CGL group/mean ion intensity of the EUT group.

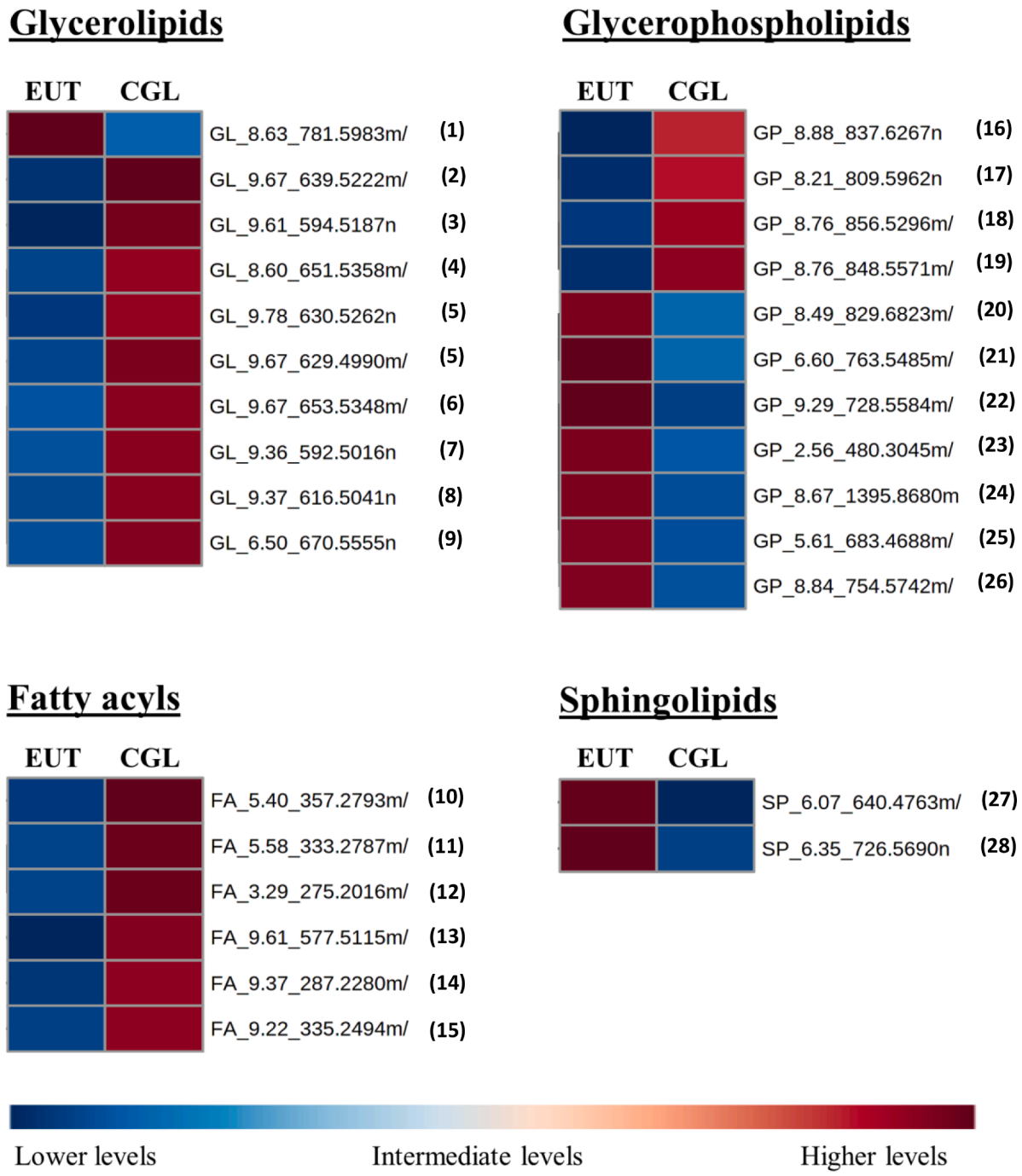

Fig. 5. Mean intensity heatmaps of the 29 compounds identified at lipid category level between EUT and CGL at T90. (1) Glycosyldiradylglycerol 36:2; (2) Diacylglycerol 38:6; (3) Diacylglycerol 34:1; (4) Diacylglycerol O-40:8; (5) Diacylglycerol 37:4*; (6) Diacylglycerol 39:6; (7) Diacylglycerol 34:2; (8) Diacylglycerol 36:4; (9) Diacylglycerol 40:5; (10) Fatty acid 20:1;O3; (11) Fatty acid 22:3; (12) Fatty acid 18:4; (13) Fatty alcohol 37:4;O3; (14) Fatty acid 16:1;O2; (15) Fatty acid 19:1; (16) Glycerophosphocholine 40:4; (17) Glycerophosphocholine 38:4; (18) Glycerophosphoserine 42:9; (19) Glycerophosphoserine 41:6; (20) Glycerophosphoethanolamine O-42:3; (21) Glycerophosphoglycerol 35:1; (22) Glycerophosphocholine O-33:2; (23) Glycerophosphocholine 15:1; (24) Cardiolipin 68:6; (25) Glycerophosphate 35:3; (26) Glycerophosphocholine O-35:3; (27) Neutral glycosphingolipid d30:2; (28) Phosphosphingolipid 36:3;O2. *Same MS-annotated lipid identified in two features.

previously reported that our CGL patients showed shorter satiation and satiety times compared to controls [10]. The technical limitation herein presented does not affect our overall conclusion that a fat-containing breakfast meal did not change the lipid profile in CGL as intensely as it did for control participants. CGL patients showed resistance to lipid changes which were expected after a meal, and our results may have implications for clinical practice.

Our study is the first to investigate how a 12-hour fasting followed with an *ad libitum* fat-containing breakfast meal could impact the lipidomic profile of CGL patients in comparison to matched eutrophic controls. We have identified significant differences in the lipidomic profile between CGL and EUT, with molecular species of fatty acyls, glycerolipids, glycerophospholipids and sphingolipids significantly affected in CGL. Our findings indicate that, besides the intrinsically deleterious impact of dyslipidemia, impaired lipid droplet formation and ectopic fat deposition, the metabolic profile of complex lipids is

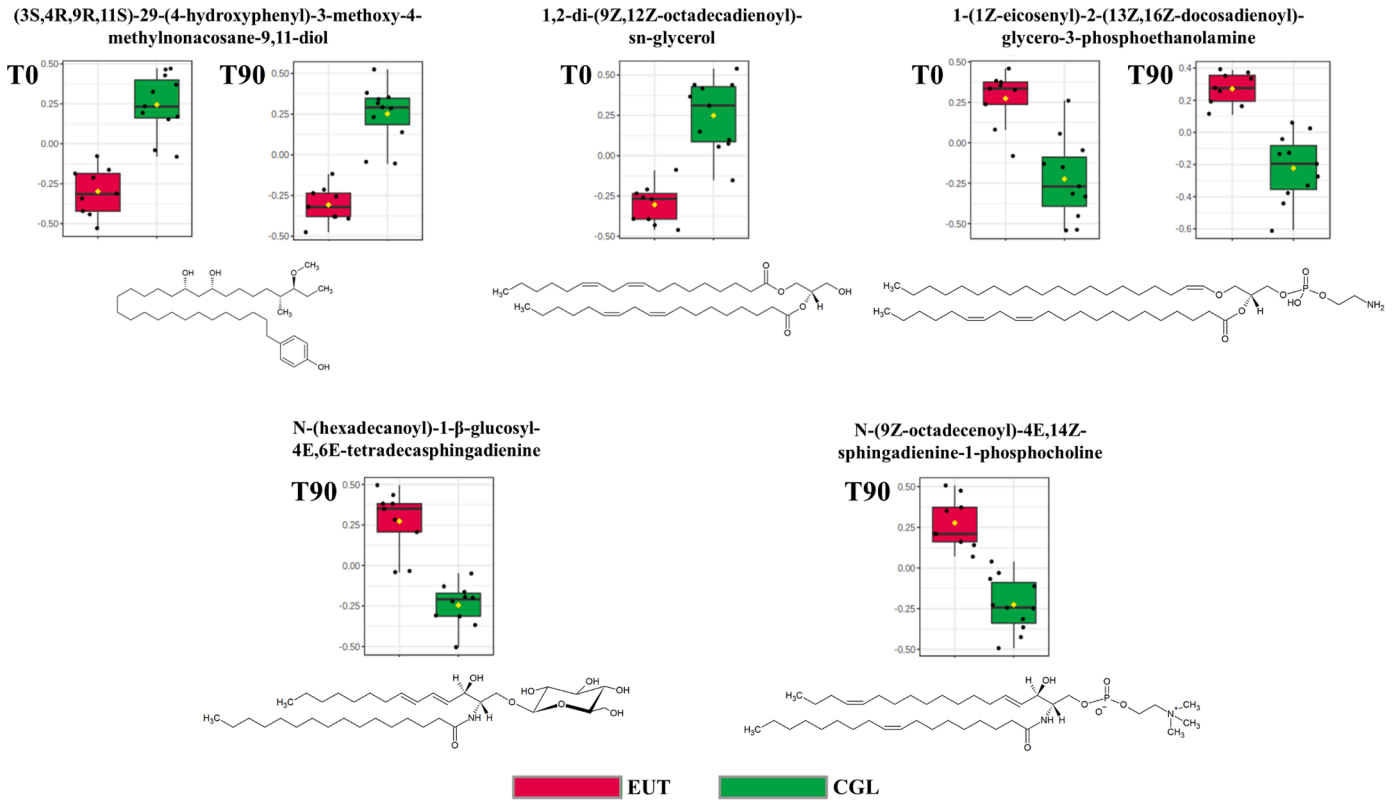

Fig. 6. Chemical structure and relative intensities of the 5 compounds identified at the level of systematic name.

severely affected in CGL patients, but not severely impacted by a very short-term dietary intervention. Our study highlights the importance of a deeper comprehension of the role of specific lipid categories in the metabolic derangements present in CGL. Novel therapeutic strategies to be investigated could include longer term dietary intervention combined with adiponectin and leptin analogues or receptor agonism.

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CRediT authorship contribution statement

Camilla O.D. Araújo: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. **Amanda P. Pedroso:** Formal analysis, Writing – original draft. **Valter T. Boldarine:** Data curation, Investigation, Methodology. **Anna Maria A. P. Fernandes:** Data curation, Investigation, Methodology. **José J.M. Perez:** Data curation, Investigation, Methodology. **Renan M. Montenegro:** Methodology, Project administration. **Ana Paula D.R. Montenegro:** Methodology, Project administration. **Annelise B. de Carvalho:** Methodology, Project administration. **Virgínia O. Fernandes:** Methodology, Project administration. **Lila M. Oyama:** Formal analysis. **Patrícia O. Carvalho:** Data curation, Investigation, Methodology. **Carla S.C. Maia:** Conceptualization, Data curation. **Allain A. Bueno:** Formal analysis, Validation, Visualization, Writing – original draft, Writing – review & editing. **Eliane B. Ribeiro:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Mônica M. Telles:** Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.plefa.2023.102584.

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