Title

Lipidome profile of Cystic Fibrosis Related Diabetes, Type 1 and Type 2 Diabetes Mellitus: potential links to inflammation and glucose and lipid metabolism.

Running title: Lipidome profile in Cystic Fibrosis and Diabetes Mellitus

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Abstract

Cystic Fibrosis (CF) is a genetic disease that primarily affects the pancreas and lungs. CF dyslipidaemia is characterized by decreased circulating lipids and increased ectopic lipid deposition in liver, pancreas, and lungs. Pancreatic exocrine insufficiency precedes the onset of CF related diabetes (CFRD). We hypothesized that different mechanisms contribute to CFRD development and progression, including features of Type 1 and Type 2 diabetes mellitus (T1DM and T2DM). Thus, we compared their plasma inflammatory, metabolic/hormonal, and lipidomic profiles, using Luminex assays and untargeted mass spectrometry analyses. Then, we compared the lipidomic profiles of lung biopsies and plasma extracellular vesicles (EVs) of CFRD and patients with other lung diseases (LD). Inflammatory cytokines (IL6 and IL1β) and chemokines (IL8 and MCP-1) were increased in the plasma of CFRD as compared with T1DM, whereas only cytokines increased when comparing with T2DM. Low insulin and C-peptide characterized CFRD and T1DM. Phosphatidylcholine, phosphatidylethanolamine and storage lipids were reduced and free fatty acids (FA) were increased in CFRD plasma compared with T1DM and T2DM.

When comparing CFRD with LD, systemic inflammation was increased to a similar extent. Increased levels of sphingolipids, glycerolipids, acylcarnitines were found in lung biopsies of CFRD as compared to LD. Increased triacylglycerols in lung biopsies positively correlated with lung inflammatory infiltrates (CD68 positive cells) of CFRD patients. In conclusion, CFRD is characterized by altered lipid metabolism, insulin deficiency and insulin resistance, partially overlapping with both T1DM and T2DM. CFRD also involves ectopic lung lipids accumulation correlating with increased *in situ* inflammation

New and noteworthy

CFRD is characterized by altered lipid metabolism, insulin deficiency, and insulin resistance, which are distinctive features that partially overlap with both T1DM and T2DM. Systemic inflammation with elevated free FA and reduced plasma lipids is also present in CFRD. Lipids are increased in lung biopsies, whose lipidomic profiles are similar to those of blood-derived EVs. CFRD develops ectopic lipid accumulation in the lungs, correlating with heightened local inflammation and reduced plasma lipid transport.

Keywords 5: Cystic fibrosis related diabetes, Lipidomic analysis, Diabetes Mellitus, Extracellular vesicles, Lung transplantation

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Introduction.

Cystic fibrosis (CF) is a genetically inherited disease caused by several mutations in the CF transmembrane conductance regulator (CFTR), an ABC transporter for carbonate and chloride ions [1, 2]. CFTR is expressed in the apical membrane of epithelial cells, and its mutations cause a multi-organ disease that primarily affects the pancreas and lungs. CFTR pharmacological modulators dramatically increased life expectancy, although lung transplantation remains the inevitable last therapeutic option, due to the disease-related recurrent and severe infections and progressive inflammatory damage leading to terminal respiratory insufficiency[3-5]. CF patients are often characterized by malnutrition, low body fat (fat-free mass FFM), low BMI, but increased visceral adipose tissue[6, 7], which are associated with worsening pulmonary function[8-13] and complications [14, 15]. Interestingly, dyslipidemia has been reported in CF, with reduced plasma lipoprotein levels [16-25], but increased lipid concentrations in bronchoalveolar lavage (BAL) fluid[26] and other organs [16, 24, 27-30]. Elevated cholesterol levels have been observed in the lung, trachea,[31] and surfactant [32]. Increased levels of inflammatory lipids, such as ceramides and lysophosphatidylcholines, in the BAL and sputum are associated with severe pulmonary inflammation and worse disease scores[33].. Reduced levels of circulating lipids have been linked to exocrine pancreatic insufficiency (PI) and also to beta cell dysfunction, [9], which is responsible for severe insulinopenia, similar to Type 1 Diabetes Mellitus (T1DM) and final stages of Type 2 Diabetes Mellitus (T2DM) [34-36]. Prediabetes with insulin resistance and ectopic fat accumulation are also quite common in CF patients, progressing to CF-related diabetes (CFRD), which occurs in approximately 20% of adolescents and 70-80% of long-standing CF, contributing to increased mortality[37-41]. Systemic inflammation of CFRD, is strongly associated with insulin resistance and beta cell failure, similarly to what occurs in obesity and T2DM[23, 42-48].[49, 50]. Interestingly, CFRD patients rarely develop ketoacidosis despite severe insulin deficiency. They do, however, show increased hepatic gluconeogenesis, lipogenesis[24] and steatosis[51] [51].CFRD is also associated with a decline in FFM due to enhanced proteolysis, which is a consequence of reduced insulin levels, while fat mass is relatively preserved[24, 52, 53]. Interestingly, overweight CF patients are at higher risk for developing insulin resistance and exhibit features of T2DM [43, 54]. The CFTR genotypes are differentially correlated with CFRD, and several other loci, in addition to CFTR, may act as genetic modifiers [55]. Adipokines are primarily produced by adipose tissue, but are also expressed in other organs, including lungs. Leptin, ghrelin, resistin and adiponectin are altered in CF plasma and in diabetes [56, 57]. These hormones finely regulate inflammatory processes and energy metabolism, and thus may contribute to the progression of CF [58, 59]. Inflammatory and anti-inflammatory adipokines are associated with alterations in disease severity and comorbidities in both T1DM and T2DM [60].[61-63] [64]. While still controversial,[65] a few studies have reported elevated ghrelin and decreased leptin levels in CF patients [57]. The hypothesis of this study is that in CFRD, dyslipidemia secondary to exocrine pancreatic insufficiency chronic inflammation, ectopic fat deposition and altered glucose metabolism, due to a combination of insulin resistance and insulinopenia, all concur to CFRD pathogenesis.

Therefore, in the first part of the manuscript, we compared CFRD hormone and lipid profiles with T1DM and T2DM patients, respectively affected by insulinopenia, and systemic inflammation, insulin resistance, and also with LD patients, with inflammation and fibrotic deterioration of the lungs, as an additional control. To the best of our knowledge, this is the first time that a study has dissected the metabolic and inflammatory-related components of CFRD as compared with other types of diabetes mellitus.

In the second part of the study, we focused on evaluating whether lipid metabolism alterations within the lungs correlated with circulating lipids and with increased lung inflammation, comparing lipidomics of biopsies from CF and LD with plasma and plasma-derived EVs. The data presented here support the concept that CFRD displays multiple lipidomic alterations that correlate with lung inflammation and which could also contribute to deranged glucose metabolism.

Results

Inflammatory cytokines are elevated in CF and LD.

CF is a chronic inflammatory disease, very frequently associated with prediabetes/diabetes mellitus and dyslipidemia. We selected 9 CF patients undergoing lung transplant (Table 1). As an additional control group, we also selected non-CF lung disease patients, who were lung transplanted because of bronchiectasis/idiopathic pulmonary fibrosis (lung disease, LD), and healthy subjects (H), type I and type II diabetes patients (T1DM and T2DM) (Table 2). We also compared the plasma inflammatory profile of CFRD with all other groups. LD and CFRD exhibit a very severe inflammatory status. In particular, IL1 β, IL8, IL6, and MCP1were all significantly increased in CF as compared to H, T1DM, and T2DM (Figure 1).

Insulin production and adipokines are altered in CF.

CFRD patients were affected by diabetes mellitus and received exogenous insulin treatment. We evaluated fasting insulin and C peptide in all patients' groups and observed a significant reduction (0,4 and 0,5 -fold decrease) of C peptide and insulin in CFRD as compared with H. C-peptide was even lower in T1DM as compared to CFRD, while C-peptide and insulin were highest in patients with T2DM, as expected (Figure 2). Next, we evaluated a panel of adipokines and lipid metabolism-related plasma proteins. Adiponectin did not change significantly, while there was a significant decrease of leptin and adipsin in CFRD as compared to H and T2DM. Ghrelin level was higher in CFRD as compared to LD and diabetes, whereas adipsin was lower in CFRD as compared to all other groups. A similar trend, although not significant, was observed when comparing CFRD and T1DM. Moreover, an increase in the fatty acids transporters FABP-2 and –4 and a concomitant decrease in LPL characterized CFRD as compared to H, T1DM, and T2DM. (Figure 3). CFRD shows reduced c-peptide and insulin secretion, similar to T1DM. Moreover, they also show reductions of circulating adipokines and altered lipid transport and metabolizing proteins as compared with healthy individuals, T1DM, and T2DM. (Figure 4).

CF plasma lipid profile differs from LD, T1DM , T2DM and H.

We then compared plasma lipid profiles of CFRD, LD, T1DM, T2DM, and H. By untargeted lipidomic analysis, we found a distinct profile of CFRD as compared to the other groups (Figure 5 A and B). By evaluating the abundance of lipid classes (medium value among species belonging to each class), there was a general trend towards reduction of circulating sphingolipids, structural and remodeling lipids, and storage-related lipids in CFRD as compared to H (Figure 5C). The reduction of lipids observed in CFRD compared to H was notable, particularly in representative species like sphingomyelin (SM), phosphatidylcholine (PC), its deacylated form (LysoPC), and cholesterol esters (CE). Similarly, such lipids tended to be lower in CFRD as compared with T1DM, T2DM, and LD, although not significantly. Conversely, we observed a significant reduction of monoglycosylated ceramides (Hex Cer) in CF compared to H, DM, and LD. Interestingly, CFRD, but also LD, exhibited a higher concentration of plasma fatty acids (FA) as compared to H (and DM), with a 2,4 (2,0) and 2,7 (2,4) -fold, respectively. Only CFRD showed an increase of acylated glycine (NAGly), which was 4,4 fold higher than H, and tended to be higher as compared to T1DM, T2DM, and LD. Finally, acylated carnitines (CAR) were significantly reduced in CFRD as compared to H (0,7-fold reduction) and tended to be reduced as compared to all other groups (Figure. 5C). The significant differences in a) inflammation; b) lipid transport and oxidation; c) metabolism regulation; d) lipid species of CFRD patients as compared to T1DM, T2DM and H (upper panel) and CFRD and LD (lower panel) are summarized in Figure 6.

In summary, CFRD differs from H, T1DM, and T2DM for higher inflammation, lower plasma lipids, together with an increase in free fatty acids and their transporters. CFRD and LD share a similar inflammatory status and no change in the levels of free fatty acids. Finally, CFRD also displays major reductions in plasma lipid species as compared with LD.

Lipid profiles of CF lung biopsies and plasma vesicles differ from plasma.

Next, we evaluated the lipid profiles of lung biopsies in CFRD and LD patients undergoing lung transplantation. Since different body districts may communicate by releasing extracellular vesicles (EVs), we obtained EVs from patients' plasma and evaluated lipid profiles in plasma-derived EVs. Plasma-derived EVs were isolated and characterized for their morphology, size, and markers (Supplementary Figure 1). Lipidomic profiles obtained by untargeted LC-MS analysis of EVs, lung biopsies and plasma are shown in Figure 6. EVs derived from CFRD patients exhibited lipidomic profile patterns which were similar to the lung biopsies, but differed from the plasma profile in the same patients. Likewise, EVs and lung biopsies lipidomic compositions were similar in LD patients, differing from their own plasma lipid content. Notably, CFRD and LD have somehow opposite lipid profiles: low CFRD plasma lipids (blue dots, i.e., CAR) *versus* high LD plasma lipids (red dots, i.e., CAR), a significant number of species at high levels in EVs and biopsies from CFRD patients (red dots, i.e., CER) compared to lower levels of the same species in EVs and biopsies from LD patients (blue dots, i.e., CER) (Figure 7). By evaluating the abundance of the lipid classes in lung biopsies (Figure 8A) and EVs (Figure 8B), we generally observed increased lipids in CFRD versus LD. Significant changes included CFRD ceramides, which increased 8-fold in EVs and 3-fold in biopsies, lactosyl ceramides (Lac~~-~~Cer~~ER~~), which increased 6-fold in EVs and 9-fold in biopsies, and sphingomyelins increased significantly in EVs (5-fold) and the biopsies (2-fold). Among significant changes in the glycerophospholipids, PC increased 6-fold in EVs and 3-fold in biopsies, and ether phosphatidylcholines (Ether PC) increased 4.3-fold in EVs and 2,5 in biopsies. Among storage lipids, TG and CE increased in biopsies only (2-fold and 5-fold, respectively), showing a trend in the EVs. CFRD and LD plasma exhibit a significant increase in free FA as compared to H, and this is paralleled by the FA concentration in lung tissue, which did not differ between the CFRD and LD patients' groups. Among acylated amino acids, acyl glycine is increased in CFRD EVs (3-fold), in agreement with what is observed in CFRD plasma versus LD plasma. Finally, CAR, which is reduced in CFRD plasma as compared to LD, is increased in biopsies (2-fold), and it is significantly increased in EVs (6-fold). A side-by-side comparison of each lipid class in biopsies and EVs is shown in Supplementary Figure 2. A VIP score analysis identified ether-linked PC species among the top 10 species that significantly differed in plasma, EVs, and biopsies in CFRD *versus* LD patients. CER species were among the top 10 differentially regulated species in biopsies and EVs (Supplementary Figure 3). In order to investigate a possible relation between lipid metabolism alterations and pulmonary inflammation, we evaluated the presence of monocytes/macrophages (i.e., CD68-positive cells) in LD and CFRD patients' lung sections, either in alveolar areas or in the consolidated and fibrotic areas (bronchiectasis). Next, we correlated lipid abundances with the inflammatory infiltrate in each patient. We observed a significant increase of macrophages (CD68 positive cells) within the consolidated areas of CFRD as compared with LD ($p<0,05$), whereas no major differences were detected in the alveolar areas. (Figure 9 A and B.)

We evaluated the potential association of specific lipids and inflammatory infiltrate in the biopsies of CFRD and LD. A significant positive correlation between TG and CD68-positive cells was observed within lung biopsies ($p<0.02$). Trends of positive correlations were observed between sphingolipids (LacCer; SM) and the inflammation in plasma-derived EVs and the biopsies' infiltrate. Similarly, a trend of positive correlation was also found between CAR in EVs and lung inflammatory infiltrate. Also, a trend to negative correlation was found between the plasma anti-inflammatory N-acetyl ethanolamine (NAE) levels and lung CD68 positive cells (Figure 9C). The parallel changes in lipids and inflammatory cells, involve lipid classes which increase in CFRD as compared to LD, either in biopsies (TG, Figure 8A) or in plasma derived EVs (LacCer, SM, LPC and CAR, Figure 8B), suggest that a more severe inflammatory state of CFRD is associated with a different lipid profile in the lungs and in circulating EVs.

Data supplements can be accessed here: 10.6084/m9.figshare.29924060

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Discussion

CF patients have pancreatic exocrine insufficiency since birth, together with recurrent lung infections and almost invariably develop early prediabetes and a unique form of diabetes mellitus, during the natural history of the disease [4, 66]. This form of diabetes mellitus shares marked insulin deficiency with T1DM and insulin resistance with T2DM, in the context of a severe chronic inflammatory state and chronic pancreatitis, which also characterizes CFRD [60, 67, 68]. Diabetes mellitus worsens CF patients' prognosis [13, 68]. Given the role of lipid metabolism in inflammation-related diseases, we compared the lipid profiles of CFRD with those of T1DM, T2DM, other chronic lung diseases, and healthy controls, aiming to identify changes related to CFRD. Despite the similarities with T1DM (also characterized by low C-peptide and insulin plasma levels), we found that CFRD exhibited significant changes in hormones that regulate lipid metabolism, differing in this regard from T1DM, T2DM, and H. In CFRD, reduced levels of leptin and adipsin, along with increased ghrelin as compared to H and both T1DM and T2DM, suggest an unbalance between lipid intake and oxidation for energy production. The increase in pro-inflammatory cytokines could also be linked to altered lipid metabolism in these patients. CFRD had higher plasma fatty acid transporters (FABP-2 and -4) and a reduced plasma lipoprotein lipase (LPL), the phospholipase responsible for facilitating peripheral fatty acid tissue uptake. Indeed, CFRD showed a marked increase in free fatty acids and a significant rise in N-acyl-glycine levels as compared to T1DM, T2DM, and H. These findings may be explained by altered body lipid metabolism, with decreased circulating lipids as previously reported data by Topcu et al.[69]. Storage lipids such as triacylglycerols and their derivatives, as well as cholesterol esters and structural lipids (including glycerophospholipids and sphingolipids) are generally reduced in CFRD plasma. Interestingly, reduced plasma phospholipid concentrations are associated with bacterial infections and inflammation and have been considered accurate biomarkers for community-acquired pneumonia [70]. On the other hand, there are increased fatty acids in plasma, both in their free form and bound to transporters (such as fatty acid-binding proteins, FABPs) or to other metabolites (including N-acyl glycine). N-acyl glycine is synthetized upon activation of Glycine Acyl Transferase (GLYAT), an enzyme previously shown to be important in glucose and energy metabolism[71-73]. GLYAT facilitates the conjugation of glycine with acyl-CoA substrates, playing a role in the availability of free CoA within the mitochondria[74, 75]. As expected, T2DM exhibited a trend of increased TG as compared to H, whereas PC, Ether Pc, and LPC are significantly increased in T1DM as compared to CFRD. We observed significant increases in the sphingolipid species (HexCer and SM) in T1DM and a tendency to increased levels, although not significant, in T2DM as compared to CFRD, consistent with previous reports in T2DM [76].

Next, we evaluated lipid content in lung explanted from patients, either affected by CFRD or other pulmonary diseases (LD). Most of the significant changes in lipid content of lung biopsies were increases in lipid species in CFRD as compared to LD. Lipid mediators are involved in regulating inflammation, infection, cell death, and organ demise, triggering apoptosis, [77], and pulmonary fibrosis [78]. The hypothesis of enhanced lipogenesis in CF was suggested by Mailhot and coworkers, who demonstrated that CFTR silencing in the intestinal cells led to increased lipogenesis and delta-7 desaturase activity through elevated expression of SREBP-1c. These findings directly challenge the idea that CFTR dysfunction causes only intestinal malabsorption and suggest the possibility of altered lipid metabolism [79]. We observed significantly higher levels of sphingolipids such as ceramide, along with its derivatives LacCer and SM, of the most representative classes of glycerophospholipids (PC and PE), and of cholesterol esters in lung biopsies from CFRD as compared with LD. Cer is a pro-inflammatory lipid that promotes lung inflammation and infection [31, 80-82]. Pharmacological inhibition of the synthesis [83, 84 85] or inhibition of the release of ceramide from SM, may reduce organ damage [86]. The overall lipid increase in CFRD biopsies may be due by increased *de novo* synthesis, leading to the accumulation of SM and phosphoglycerol lipids. Furthermore, the increase in LacCer, a precursor for glycosphingolipids anabolism, implies the building up of intermediate lipid species implicated in inflammation and lipotoxicity [87, 88]. Ether glycero-phospho lipids were significantly increased in CF lung biopsies. Ether lipids are known to be involved in oxidative stress defence[89]. A significant increase in CAR and CL was observed in CFRD. These two lipid classes are essential for mitochondrial function, and their increase may reflect a compensatory mechanism aimed at enhancing mitochondrial oxidation [90]. Importantly, tissue-derived vesicles (extracellular vesicles) are important carriers of circulating lipids, conveying specific signals among different body districts. The lipid content of plasma vesicles mirrors somehow the lipid composition observed in CFRD and LD lung biopsies. Increased glycerol-phospholipids, storage lipids (TG, DG, and CE), and sphingolipids (DHCer, precursors of ceramides) were significantly increased in EVs from CFRD, while CAR showed a trend to increase. There was a significant correlation between the TG extracted from lung biopsies and the number of infiltrating macrophages (CD68+) within the lungs, suggesting that inflammation associates with lipid accumulation in the lung. This observation is also somehow reinforced by the tendency to positive correlations between lung inflammation and SM, LacCer, LPC and CAR content in plasma derived EVs. Previous studies have demonstrated that lipotoxicity in the lung can result from disturbances- of the PC lipid class[76, 77]. The deacylated form of PC (LPC), regulates lung inflammation, acting as a “find-me” signal, enhancing the phagocytic effect of neutrophils. Additionally, LPC slows the progression of neutrophil extracellular trap (NET) formation, reducing the harmful effects of neutrophil activation, which is particularly excessive in cystic fibrosis (CF)[91].

A limitation of this study is the relatively small number of CF patients included, as it was conducted at a single center and the recent advances in pharmacological therapies have significantly reduced lung transplants in this patients’ population, since the beginning of our enrollment[3-5]. Potential confounders may be present in this clinical study because there were 5 patient categories. However, we matched the patients as much as possible to reduce the impact of potential confounders. With regards to age, there were some differences only between CFRD and LD or T2DM, as these two diseases (LD and T2DM) occur later in life. BMI was only significantly increased in T2DM patients as compared with the other 4 patients’ -groups. The reason is that T2DM occurs more commonly in overweight/obese patients (BMI > 25 kg/m^2). Insulin therapy was not a confounder between CFRD and T1DM, because all these patients were insulin-treated.

In CFRD, glycemia remains slightly but consistently elevated, which can be attributed to the close adherence to exogenous insulin treatment, even during periods of intense inflammation/infective episodes. Our data show the coexistence between chronic systemic inflammation and lipid metabolism dysregulation, even when diabetes mellitus is well-controlled in CFRD patients. In this study, which directly compares for the first time, CFRD, LD, T1DM, and T2DM, an uncoupling between hyperglycemia, insulin resistance, and systemic inflammation is very apparent. The confounder regarding the severe inflammatory status of LD and CFRD could be the occurrence of more frequent lung bacterial infections in CFRD as compared with LD [42, 50, 91-93]. However, only IL6 was significantly higher in CFRD as compared to LD.

In CFRD, severe systemic inflammation occurs in combination with impaired insulin secretion and diabetes mellitus. In LD, there is severe systemic inflammation, although slightly inferior as compared to CFRD, normal insulin secretion, and glucose levels. In T1DM, insulin secretion is severely impaired with substantially normal plasma cytokines, while T2DM is severely insulin resistant with moderate inflammation. We hypothesize that in CFRD, the interaction between insulin deficiency, systemic inflammation, and insulin resistance produces a unique pathophysiological condition with severely altered lipid metabolism, possibly contributing to lung disease severity. Future studies could investigate the molecular mechanisms underlying altered lipid metabolism in CFRD.

Materials and Methods

1. Study population. Nine adults with CF, with heterozygous mutations (four out of nine with F508del mutation) and affected by CFRD, waiting for lung transplant were enrolled for this study at the IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milano, Thoracic Surgery and Transplant Unit. We also enrolled 9 patients, matched for age and waiting for lung transplant because of diseases other than CF (bronchiectasis, interstitial lung diseases, emphysema, COPD) and named LD in the manuscript. Exclusion criteria included current pregnancy and age < 18. In addition, ten patients with type 1 diabetes mellitus (T1DM) and nine with type 2 diabetes mellitus (T2DM) were enrolled, together with 10 healthy volunteer donors; these patients were matched for gender and age with those with CF and were recruited at the U.O. of Endocrinology of the Ospedale L. Sacco, Milan, Italy (Supplementary Table 1). The study was approved by the Ethical Committee of Milan Area2 (G44I19000850005, June 22nd, 2020, amendment **n. 1 February** 24th 2023). Written informed consent was obtained from all participants.

2. Clinical assessments Patients have been subjected to blood sampling at the time of their routine of clinical care. Biopsies were obtained at the time of lung transplantation (CF and LD), concurrently with bioptic sampling of the explanted lung for clinical pathology evaluation. Biopsies were immediately frozen and stored at -80°C. Participants medical history, medication use (including CFTR genotype, pancreatic insufficiency, anthropometric measures, fasting glucose levels, insulin therapy, and CFTR modulators or pancreatic enzyme supplementation) were recorded.

3. Sample size considerations We compared CFRD with the other groups (H, LD, T1DM and T2DM), using Mann Whitney test, two tails, using the medium value and the standard error of each group. We considered a power of 80% and a significance (alpha error) of 0,05 and an effect size in the population of at least 1,4. Therefore, we considered adequate a sample size of 9 subjects per group. Sample size calculation was obtained by using G*Power, version 3.1.9.7 (https://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-und-arbeitspsychologie/gpower).

4 **Evaluation of plasma cytokines and adipokines.** For plasma collection, blood was collected in 4 ml glass Acid Citrate Dextrose (ACD) tubes (BD Vacutainer, Franklin Lakes, NJ) and centrifuged at 1500 g×10 minutes. Plasma samples were stored at −80°C until further analysis. Total inflammatory cytokines cytokines/chemokines (IL1b, IL8, IL6 and MCP1), Adipokines (Leptin, Adipsin, Resistin, Ghrelin, Chemerin), lipid regulating proteins (FABP-2 and –4, LPL) and hormones (insulin and c peptide) were measured using Luminex human magnetic assay (Labospace S.R.L.).

5 Isolation and characterization of plasma EVs To isolate EVs, plasma was preliminary serial centrifuged (1000, 2000 and 3000 g for 10 min at 4 °C) and finally ultracentrifuged (100,000 g for 75 min at 4 °C). EVs were then resuspended in PBS with protease inhibitors and stored at −20 °C until mass spectrometry evaluation. Freshly isolated EVs were characterized by NTA, TEM and Western Blotting, as previously described [52]. Briefly, NTA was carried out with the Malvern NanoSight NS300 system (Malvern Panalytical ltd., Malvern, UK). For TEM analysis, EVs'pellet was fixed in 2.5% glutaraldehyde for 2 h, post-fixed in 1% osmium tetroxide and finally embedded in epoxy resin after dehydration. For Western Blotting analysis, aliquots of protein extracts (20 µg) were separated by a 10% SDS-PAGE and blotted with anti TSG101, #MA5-32463 (Thermofisher), 1:1000.

6. Untargeted lipidomics. Sera (25 µl) , EVs suspension (25 µg), homogenates of bioptical samples (50 µg) were extracted by methanol/chloroform mixture [83]. The addition of butylated hydroxytoluene (BHT) during sample preparation avoided unspecific oxidation. LC–MS/MS consisted of a Shimadzu UPLC coupled with a Triple TOF 6600 Sciex (Concord, ON, CA) equipped with Turbo Spray IonDrive. All samples were analyzed in duplicate in positive mode with electrospray ionization. Spectra were contemporarily acquired by full-mass scan from *m/z* 200–1500 and top-20 data-dependent acquisition from *m/z* 50–1500. Declustering potential was fixed to 50 eV, and the collision energy was 35 ± 15 eV. The chromatographic separation was reached on a reverse-phase Acquity CSH C18 column 1.7 µm, 2.1 × 100 mm (Waters, Franklin, MA, USA) equipped with a precolumn by a gradient between (A) water/acetonitrile (60:40) and (B) 2-propanol/acetonitrile (90:10), both containing 10-mM ammonium acetate and 0.1% of formic acid[83].

7. Lipidomic Data Processing. The spectra deconvolution, peak alignment and sample normalization were attained using MS-DIAL (ver. 4.0). MS and MS/MS tolerance for peak profile was set to 0.01 and 0.05 Da, respectively. Identification was achieved matching spectra with LipidBlast database or in-house built mass spectral library. Intensities of analytes were normalized by Lowless algorithm and those with a CV% superior to 30% in the QC pool sample were excluded.

8. CD68-immunohistochemistry on lung tissues. A representative lung tissue block was retrieved from the archives of the Division of Pathology for all the subjects enrolled in the study. After morphological revision of the blocks, all CFRD patients (n=9) and 7 LD resulted adequate for immunohistochemical analysis of the monocytic/macrophagic immunological infiltrate. Briefly, a CD68 monoclonal antibody (clone KP1, Dako , Agilent) was used with an automatic immunostainer (Ventana Benchmark ULTRA; Roche Diagnostics) as previously described [94]. Then, slides were scanned and digitalized with Aperio AT2 and the CD68-positive infiltrate was quantified in at least two alveolar and fibrotic/consolidated ROI per case using ImageScope and an implemented nuclear algorithm [95] . The number of CD68-positive cells out of the number of cells present in each area was recorded and averaged per ROI type

9. Statistical analysis. Graphs and statistical analyses were prepared with GraphPad Prism 7.0 (GraphPad Software, Inc, La Jolla, California, USA), R (v4.3.1) and with MetaboAnalyst 4.0. Univariate statistical analysis was performed using one-way ANOVA with Bonferroni post hoc test for comparing lipid concentrations across different groups, whereas Mann-Whitney test was used for comparing CFRD with each of the other groups. For multivariate analysis, data were checked for integrity, filtered by interquartile range, log-transformed and auto-scaled. Partial least squares discriminant analysis (PLS-DA) was performed to increase the group separation and investigate the variables with a high Variance Importance in Projection score (VIP > 1.5). p values < 0.05 were considered statistically significant. Data are shown as mean ± SD. Linear correlation were calculated according to Pearson analysis with R (v4.3.1).

Figures legends

Table 1. Mutation and characterization of CF patients. CFRD: Cystic Fibrosis Related Diabetes; EPI: exocrine pancreatic insufficiency.

Table 2. Characterization of patients' groups. M, male; F, female. CF significant difference from other groups is indicated where it is present, (* p<0,05; ** p<0,005).

Figure 1. Plasma inflammatory cytokines concentration. Data are presented as means ± SE (* p<0.05; ** p<0.01; *** p<0.005); Mann-Whitney test was used for all data. H, healthy subjects; CF, Cystic Fibrosis; LD, Lung Disease; T1DM, T2DM, Type1 or Type2 Diabetes Mellitus patients.

Figure 2. Plasma insulin and c peptide concentration. Data are presented as means ± SE (Mann-Whitney test, * p<0.05; ** p<0.01; *** p<0.005). H, healthy subjects; CF, Cystic Fibrosis; LD, Lung Disease; T1DM, T2DM, Type1 or Type2 Diabetes Mellitus in patients'.us patients

Figure 3. Plasma adipokines and lipid regulating proteins concentration. Data are presented as means ± SE (Mann-Whitney test, * p<0.05; ** p<0.01; *** p<0.005). H, healthy subjects; CF, Cystic Fibrosis; LD, Lung Disease; T1DM, T2DM, Type1 or Type2 Diabetes Mellitus patients

Figure. 4. Heatmap of the plasma proteins in the different groups. The median concentration values are log-transformed and scaled to z-values for visualization. The color-scale differentiates values as high (purple), average (white), and low (green).

Figure 5. A) Discriminant analysis (score plot) of the plasma lipidome as a function of diseases. (B) Heatmap of the principal lipid classes of modulated lipids. The mass intensities are log-transformed and scaled to z-values for visualization. The color-scale differentiates values as high (purple), average (white), and low (green). C) Plasma concentration of specific lipid classes: data are

presented as means ± SE (Mann-Whitney test, * $p<0.05$; ** $p<0.01$; *** $p<0.005$). H, healthy subjects; CF, Cystic Fibrosis; LD, Lung Disease; T1DM, T2DM, Type1 or Type2 Diabetes Mellitus patients

Figure 6. Significant changes in selected markers (reported as p-values) that differ between CFRD and all other groups. Green bars indicate markers upregulated in CFRD, while red bars represent those downregulated in each comparison. Significance levels are specified as follows: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

Figure 7. Heatmap of the principal lipid classes of modulated lipids in plasma, extracellular vesicles (EVs) and lung biopsies. The mass intensities are log-transformed and scaled to z-values for visualization. The color-scale differentiates values as high (red), average (white), and low (blu). CF, Cystic Fibrosis, LD, Lung diseases patients.

Figure 8. A). Lung biopsies and B) EVs concentration of specific lipid classes; data are presented as means ± SEM (Mann Whitney test, * $p<0.05$; ** $p<0.01$; *** $p<0.005$). H, healthy subjects; CF, Cystic Fibrosis; LD, Lung Disease patients.

Figure 9. A, Evaluation of the monocytic/macrophagic infiltrate in lung tissues from CF or control subjects. Alveolar and consolidated (bronchiectasis) ROIs of CF (n=9) and LD (n=7) lungs were selected on hematoxylin and eosin (H&E) stained sections and then CD68-positive cells were evaluated by immunohistochemistry separately in each ROI. CD68-positive cells (arrows) C. Pearson's correlations between different lipid classes in lung biopsies, plasma derived EVs, plasma and CD68 positive cells in lung biopsies of CF and LD patients. R, Pearson's correlation coefficients.

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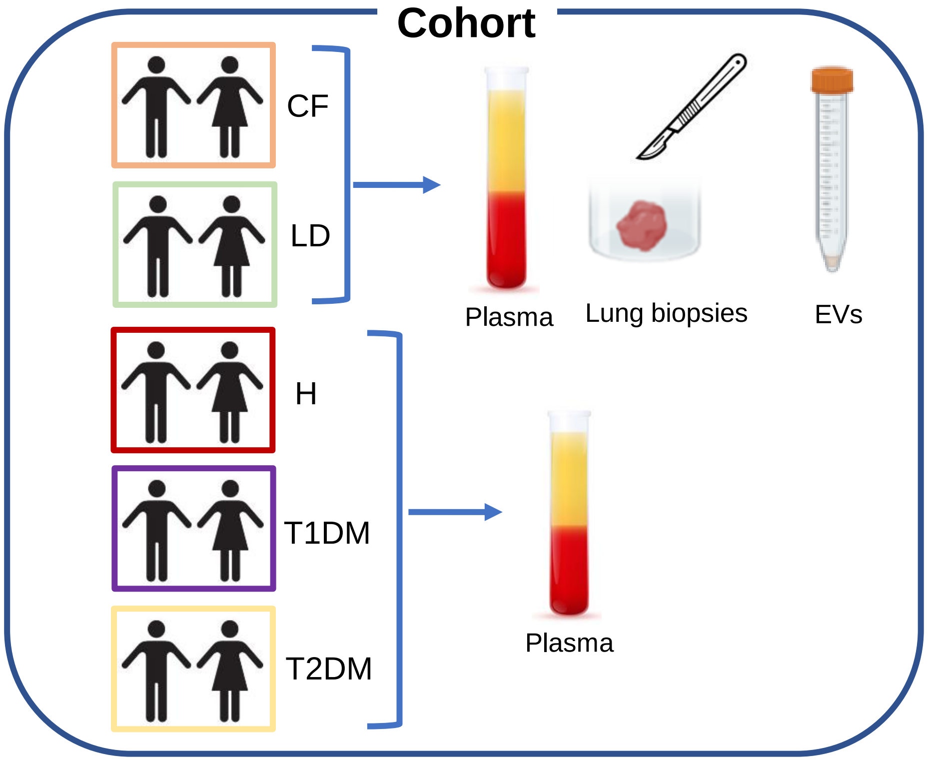
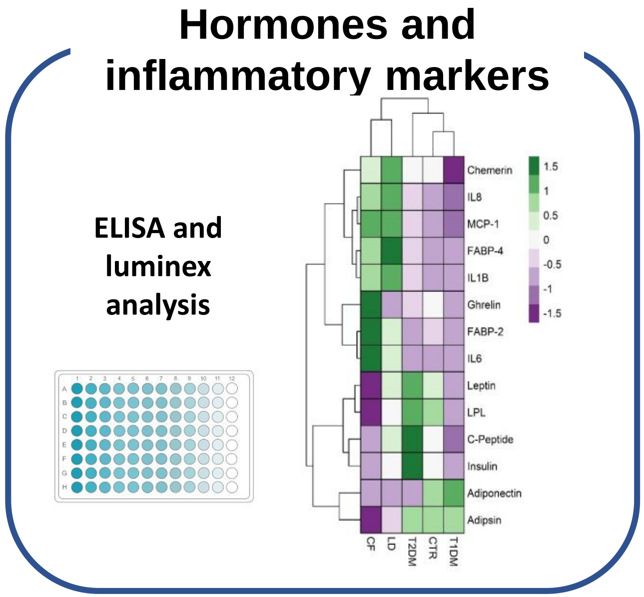
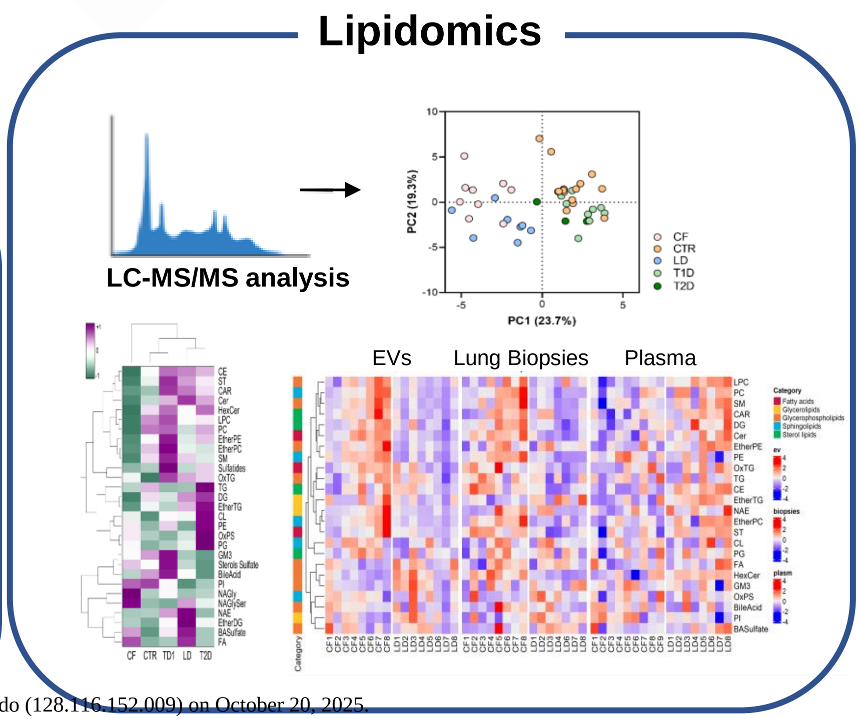
Inflammation and lipid metabolism in Cystic Fibrosis and Diabetes Mellitus patients





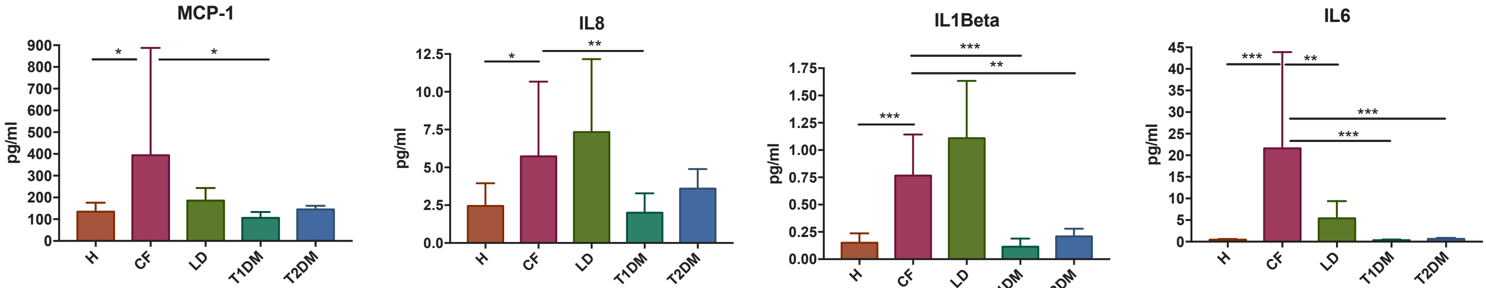


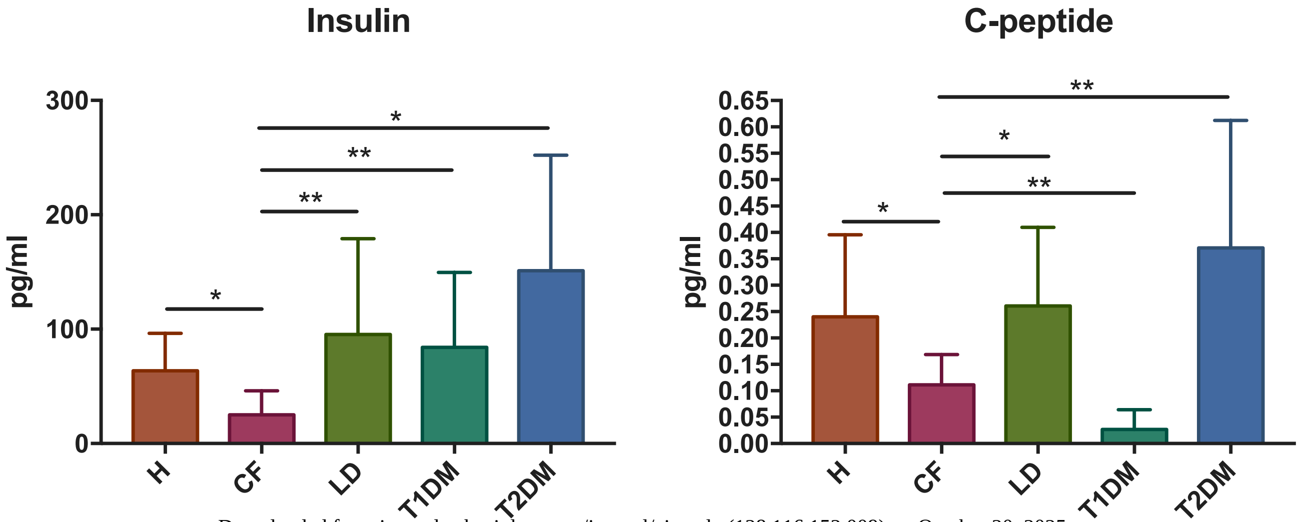
Conclusions

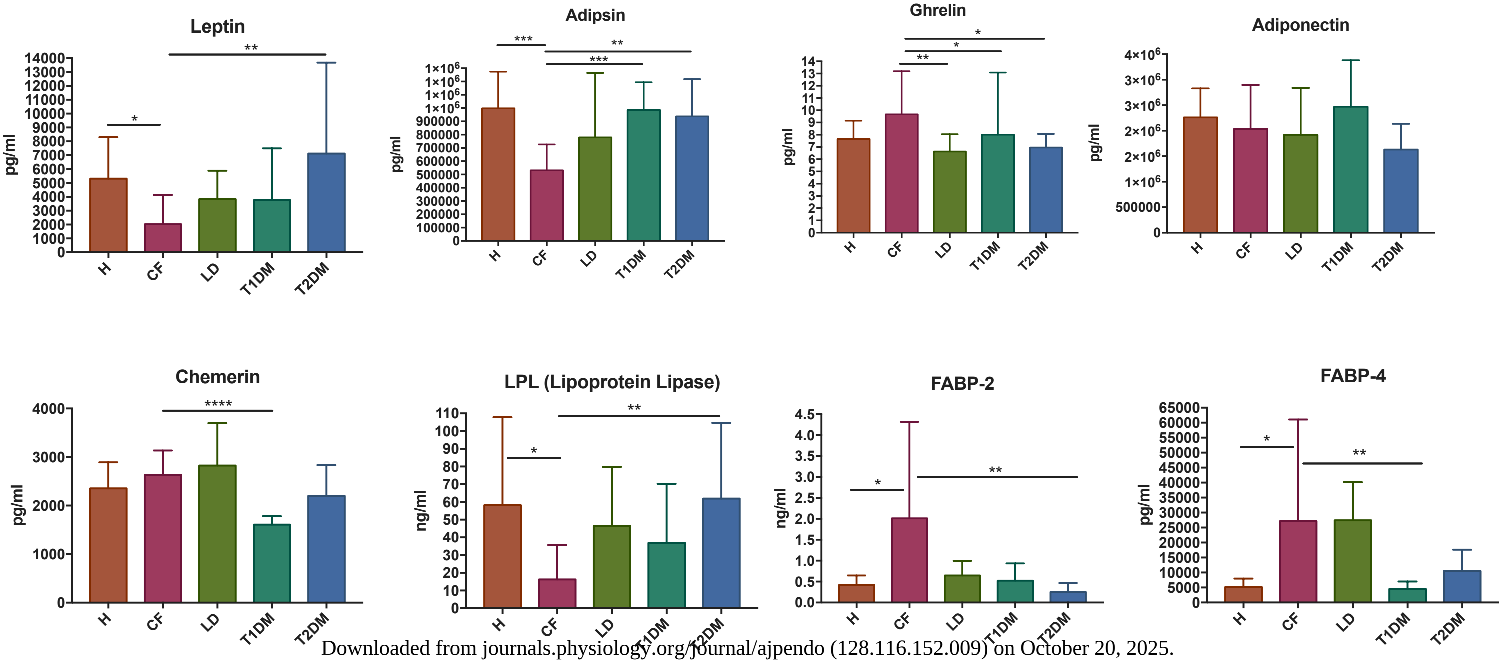
Severe stage of CF is characterized by an altered lipid metabolism that does not overlap neither with type 1 nor type 2 DM. Systemic inflammation associates with elevated FA but reduced total lipids in plasma. The abundance of lipids is increased within the lungs and possibly the peripheral body districts, whose profile can be traced in blood derived EVs. We speculate that CF develops a reduced consume of lipids that correlates with lipids accumulation in peripheral organs, promoting reduced glucose entry, hyperglicemia, inflammation and insulin resistance. The analysis of plasma EVs may identify CF lipids alteration for the staging of the disease and lipid metabolism can be considered a therapeutic target.

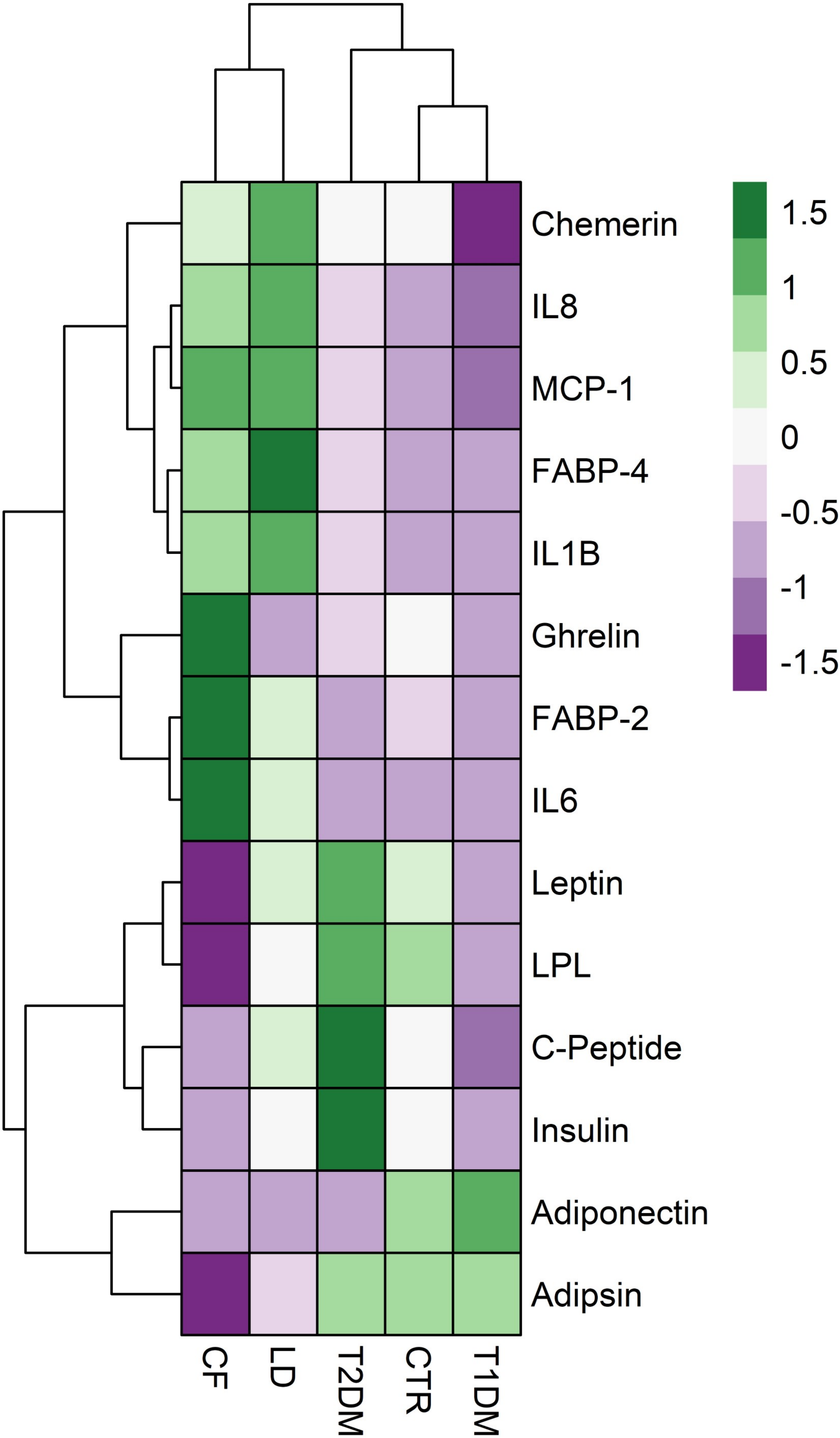
Patient	CFTR mutation	CFRD	EPI	Fasting glucose (mg/dL)
CF 1	2789+5G->A & R1070Q	Yes	Yes	88
CF 2	F508del/E585X	Yes	Yes	98
CF 3	F508del/G542X	Yes	Yes	77
CF 4	N1303K/N1303K	Yes	Yes	165
CF 5	R553X/G85E	Yes	No	105
CF 6	Y563X/Y563X	Yes	Yes	98
CF 7	F508del/R1066H	Yes	No	97
CF 8	F508del/711+5G>A	Yes	Yes	82
CF 9	N1303K/1259insA	Yes	Yes	102

	H	CF	LD	T1DM	T2DM
Age	34 ± 18	34 ± 16	49.5 ± 19 *	35 ± 15	54 ± 12 *
Sex (n;%)	M=4;40% F=6; 60%	M=3; 33,3% F=6; 66,6%	M=7; 87,5% F=1; 12,5%	M=4,40% F=6; 60%	M=5; 55,6% F=4; 44,4%
BMI	21.7 ± 2	20.7 ± 3	23.7 ± 4	21.9 ± 3.7	26,1 ± 4.01*
Glycemia	87,5 ± 5.3	101.25 ± 27	84.5 ± 6	164 ± 43 **	156 ± 56 **

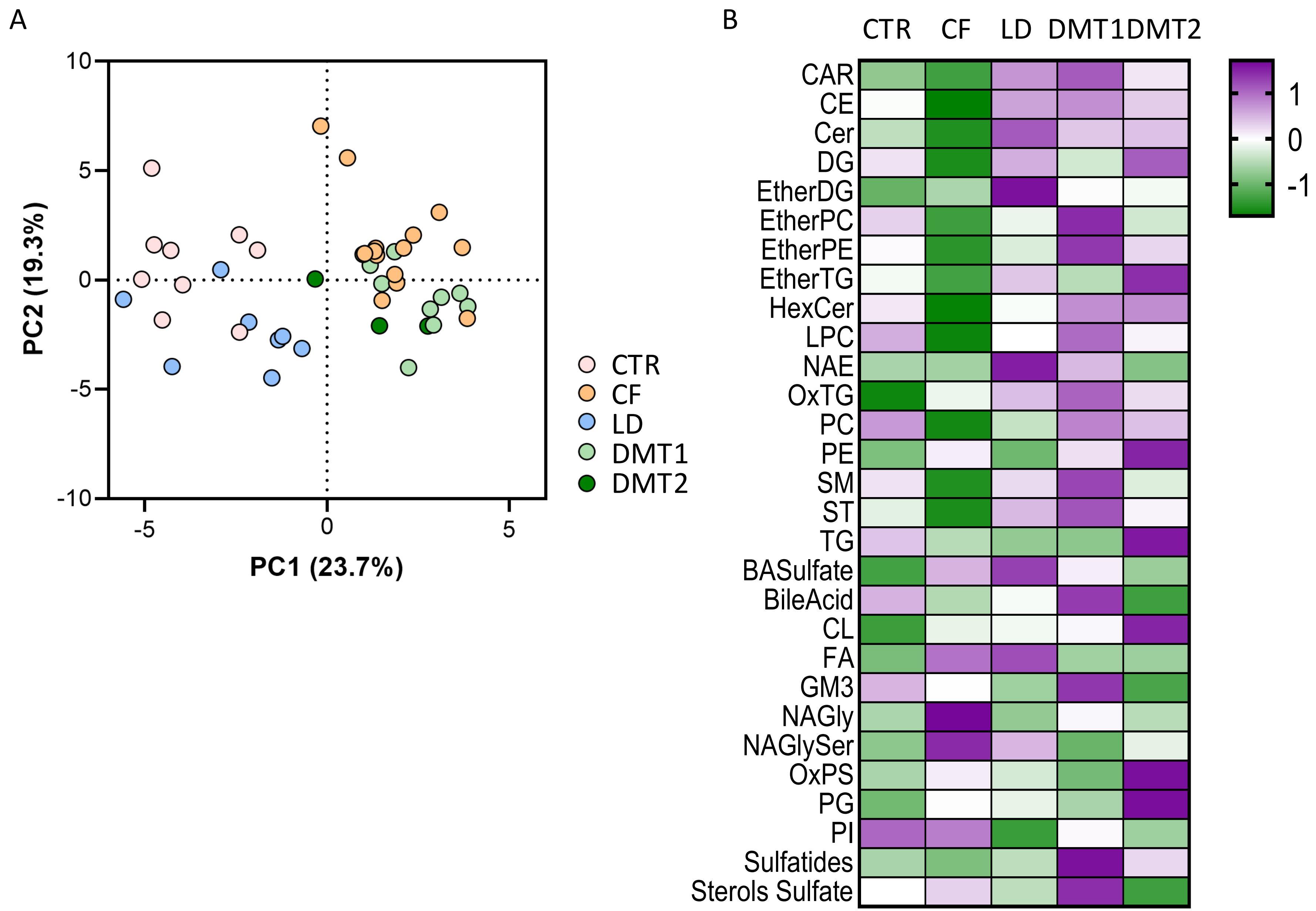

MCP-1
pg/ml
900
800
700
600
500
400
300
200
100
0
*
*
H
CF
LD
T1DM
T2DM
IL8
pg/ml
12.5
10.0
7.5
5.0
2.5
0.0
*
**
H
CF
LD
T1DM
T2DM
IL1Beta
pg/ml
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
***
**
***
H
CF
LD
T1DM
T2DM
IL6
pg/ml
45
40
35
30
25
20
15
10
5
0
***
**
***
***
H
CF
LD
T1DM
T2DM

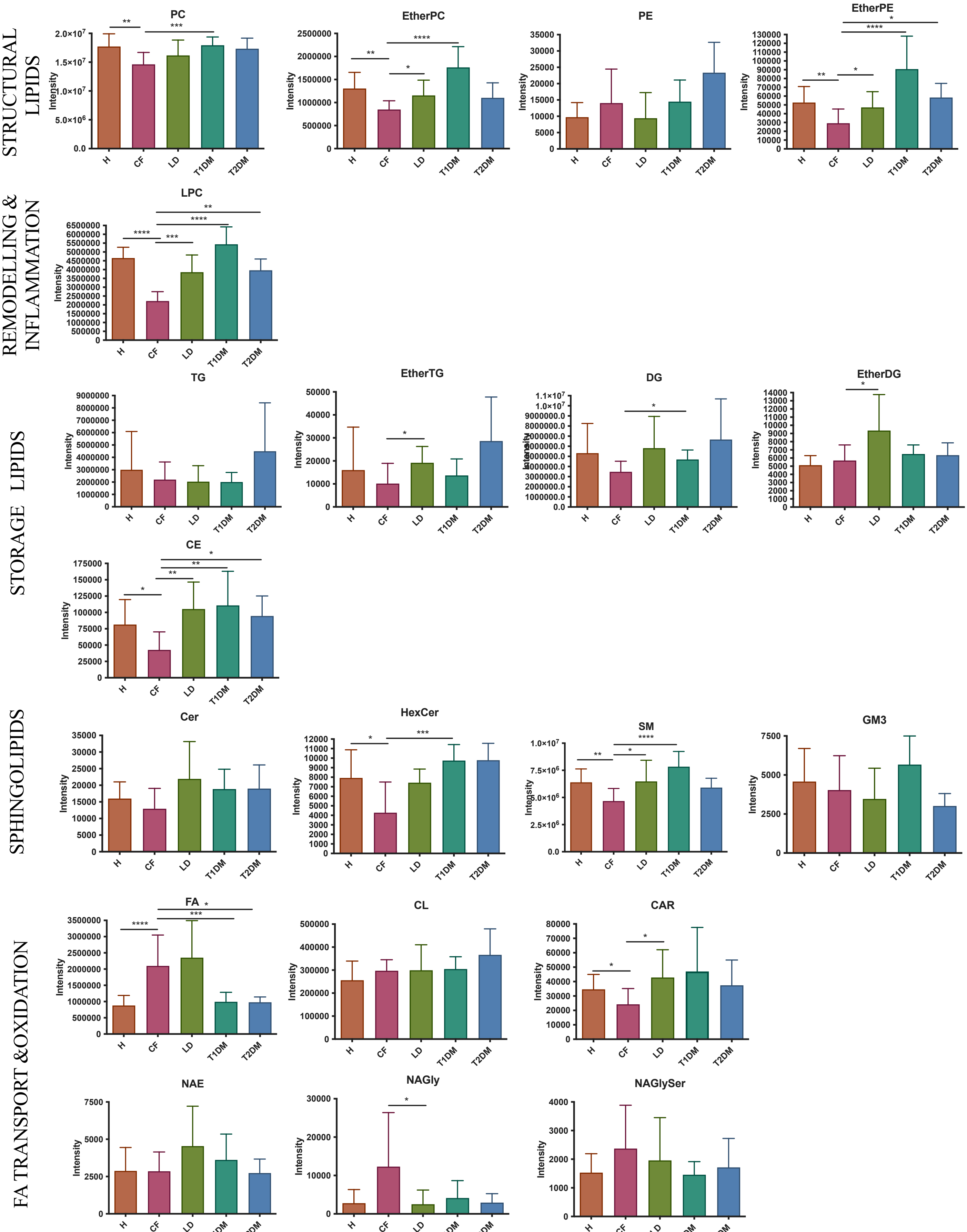

Insulin
C-peptide
pg/ml
300
200
100
0
pg/ml
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
**
**
*
*
**
**
*
H
CF
LD
T1DM
T2DM
H
CF
LD
T1DM
T2DM


Leptin
pg/ml
Adipsin
pg/ml
Ghrelin
pg/ml
Adiponectin
pg/ml
Chemerin
pg/ml
LPL (Lipoprotein Lipase)
ng/ml
FABP-2
ng/ml
FABP-4
pg/ml
H
CF
LD
T1DM
T2DM

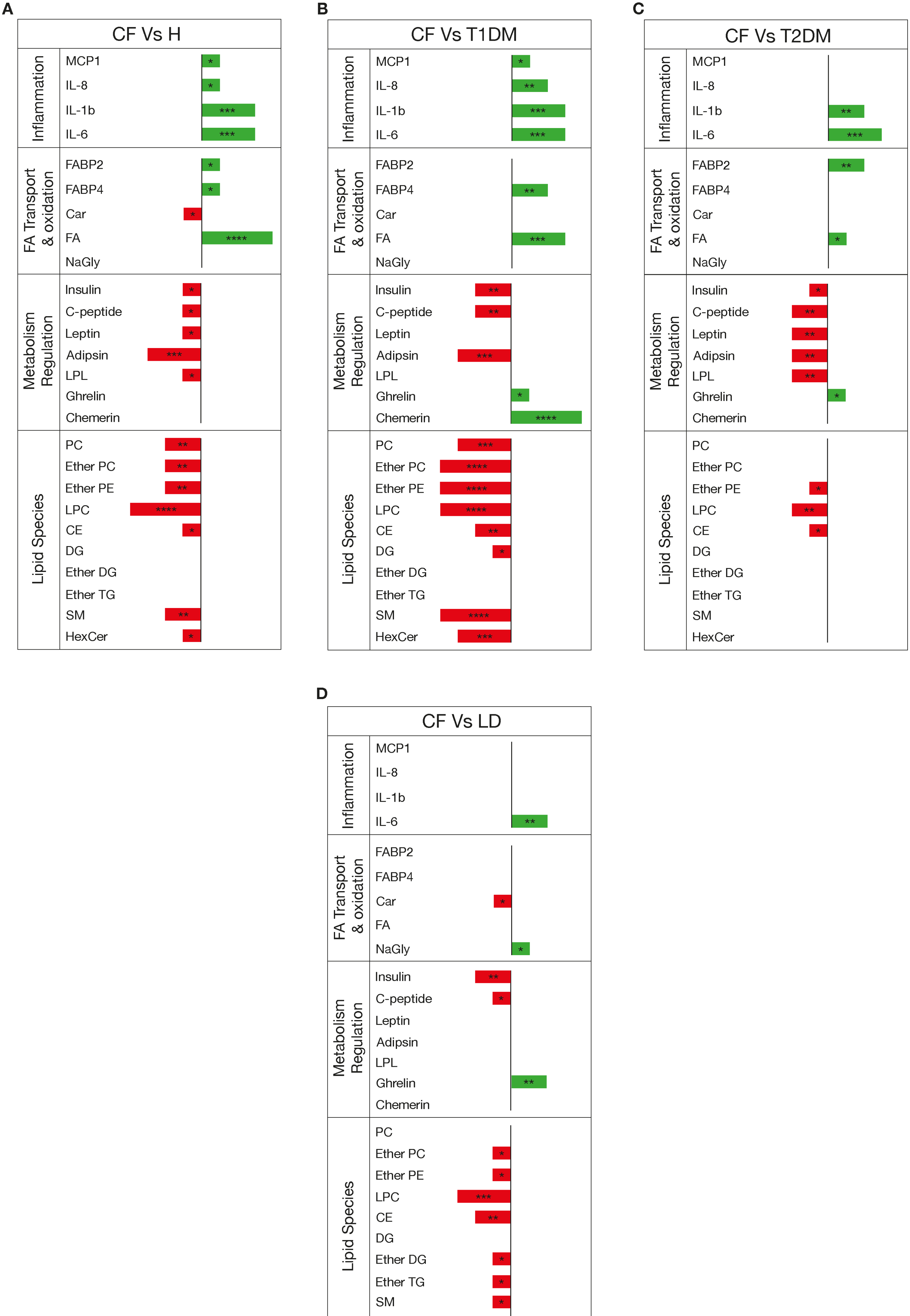


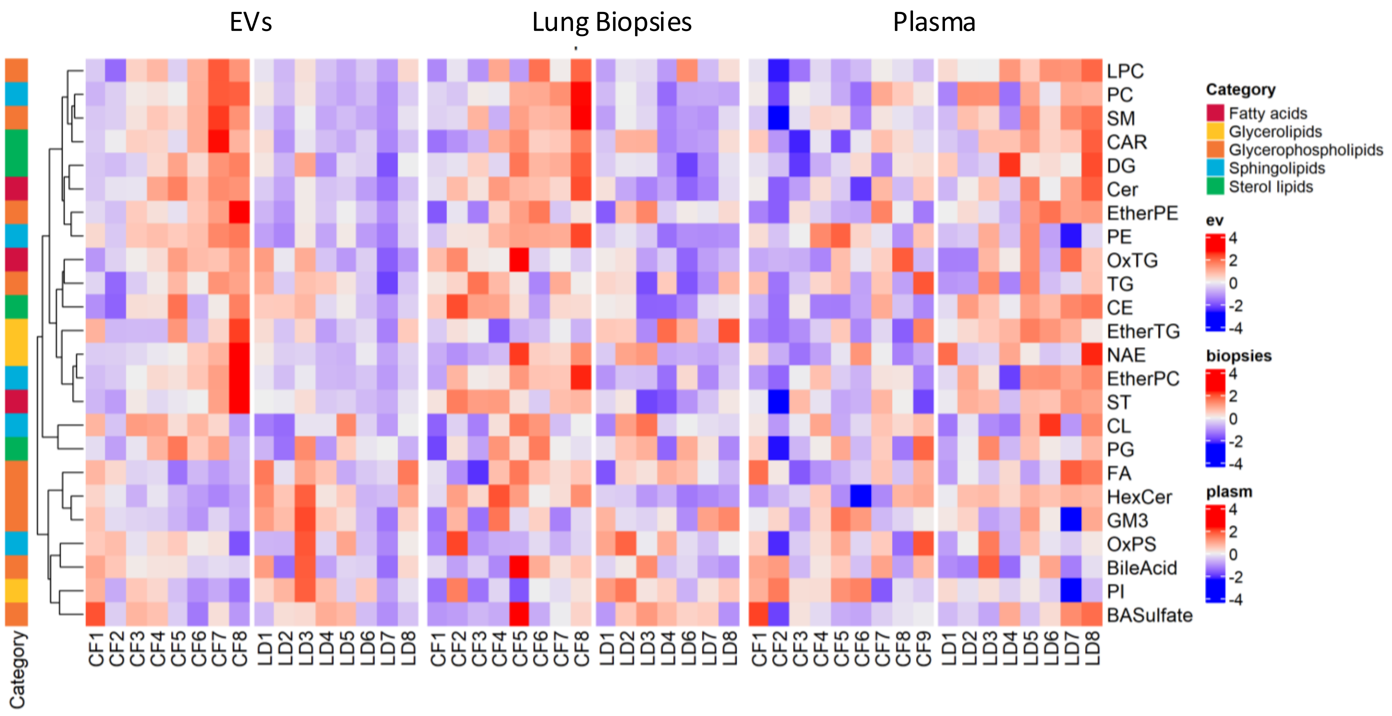
Chemerin
IL8
MCP-1
FABP-4
IL1B
Ghrelin
FABP-2
IL6
Leptin
LPL
C-Peptide
Insulin
Adiponectin
Adipsin
CF
LD
T2DM
CTR
T1DM
1.5
1
0.5
0
-0.5
-1
-1.5


A
PC2 (19.3%)
PC1 (23.7%)
10
5
0
-5
-10
-5
0
5
CTR
CF
LD
DMT1
DMT2
B
CTR
CF
LD
DMT1
DMT2
CAR
CE
Cer
DG
EtherDG
EtherPC
EtherPE
EtherTG
HexCer
LPC
NAE
OxTG
PC
PE
SM
ST
TG
BASulfate
BileAcid
CL
FA
GM3
NAGly
NAGlySer
OxPS
PG
PI
Sulfatides
Sterols Sulfate
1
0
-1

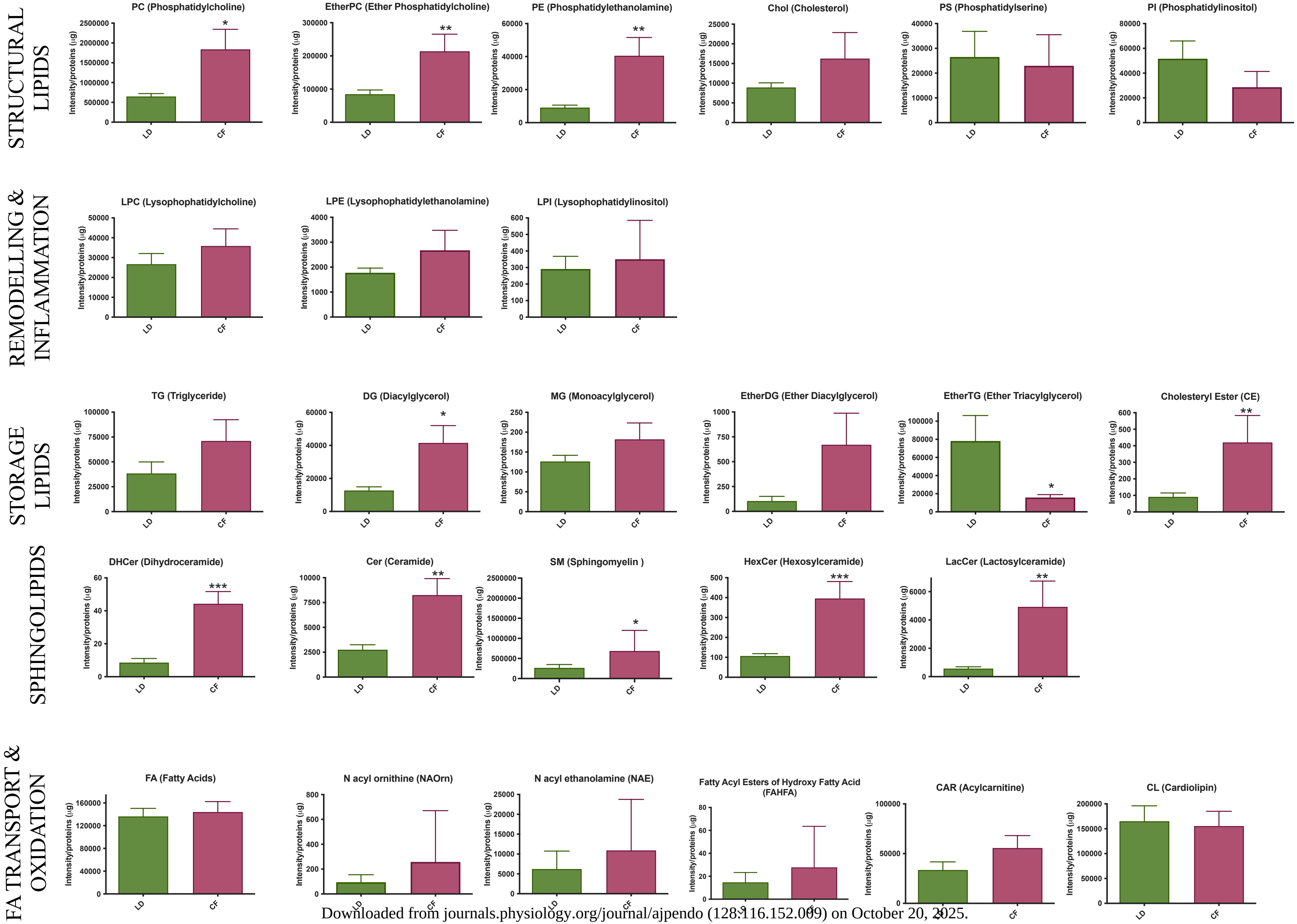


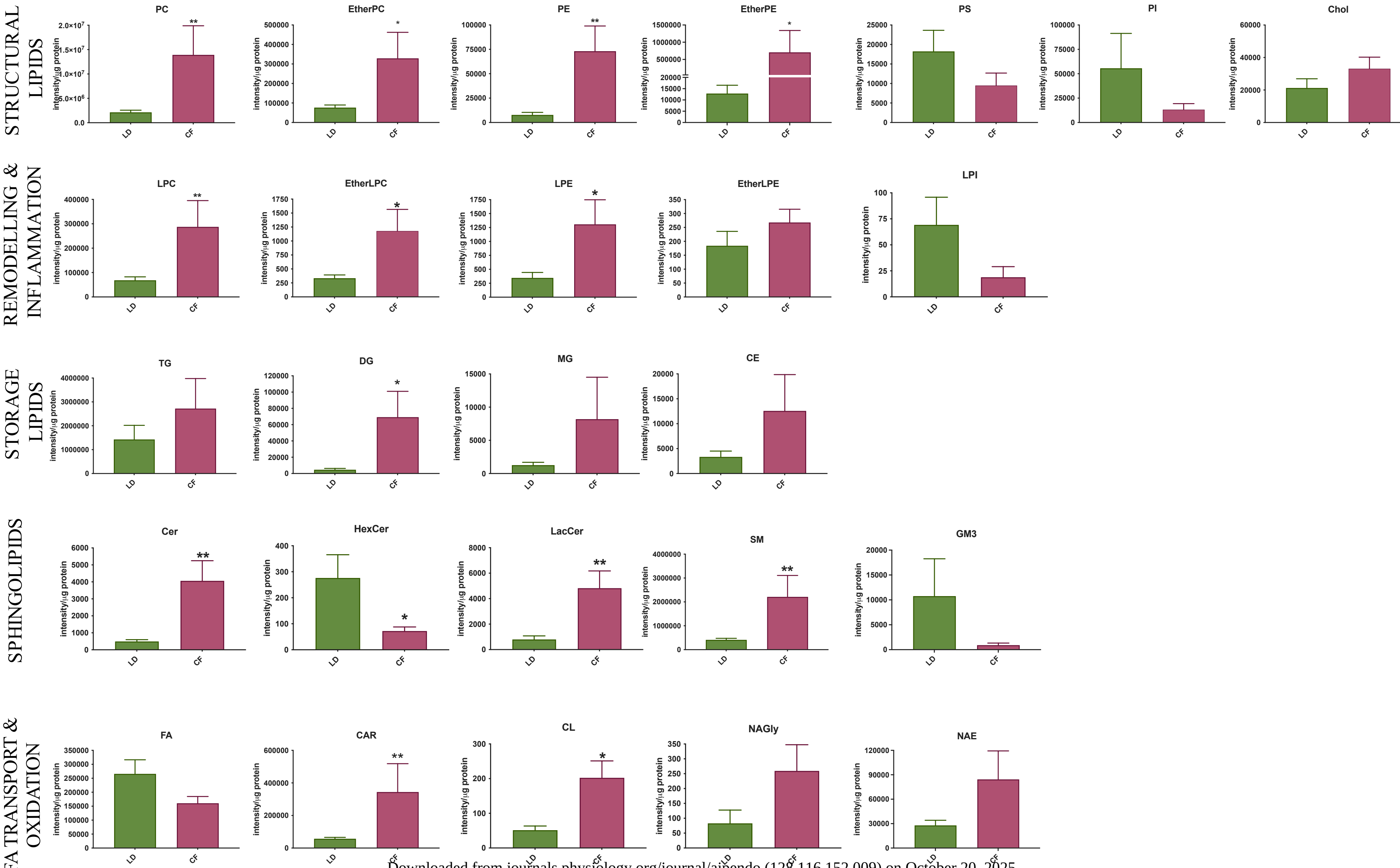
STRUCTURAL LIPIDS
PC
EtherPC
PE
EtherPE
REMODELLING & INFLAMMATION
LPC
STORAGE LIPIDS
TG
EtherTG
DG
EtherDG
CE
SPHINGOLIPIDS
Cer
HexCer
SM
GM3
FA TRANSPORT &OXIDATION
FA
CL
CAR
NAE
NAGly
NAGlySer
Intensity
H
CF
LD
T1DM
T2DM


A
CF Vs H
Inflammation
MCP1
IL-8
IL-1b
IL-6
FA Transport & oxidation
FABP2
FABP4
Car
FA
NaGly
Metabolism Regulation
Insulin
C-peptide
Leptin
Adipsin
LPL
Ghrelin
Chemerin
Lipid Species
PC
Ether PC
Ether PE
LPC
CE
DG
Ether DG
Ether TG
SM
HexCer
B
CF Vs T1DM
C
CF Vs T2DM
D
CF Vs LD



EVs
Lung Biopsies
Plasma
LPC
PC
SM
CAR
DG
Cer
EtherPE
PE
OxTG
TG
CE
EtherTG
NAE
EtherPC
ST
CL
PG
FA
HexCer
GM3
OxPS
BileAcid
PI
BASulfate
CF1 CF2 CF3 CF4 CF5 CF6 CF7 CF8 LD1 LD2 LD3 LD4 LD5 LD6 LD7 LD8
CF1 CF2 CF3 CF4 CF5 CF6 CF7 CF8 LD1 LD2 LD3 LD4 LD5 LD6 LD7 LD8
CF1 CF2 CF3 CF4 CF5 CF6 CF7 CF8 CF9 LD1 LD2 LD3 LD4 LD5 LD6 LD7 LD8
Category
Category
Fatty acids
Glycerolipids
Glycerophospholipids
Sphingolipids
Sterol lipids
ev
4
2
0
-2
-4
biopsies
4
2
0
-2
-4
plasm
4
2
0
-2
-4


STRUCTURAL LIPIDS
PC (Phosphatidylcholine)
EtherPC (Ether Phosphatidylcholine)
PE (Phosphatidylethanolamine)
Chol (Cholesterol)
PS (Phosphatidylserine)
PI (Phosphatidylinositol)
REMODELLING & INFLAMMATION
LPC (Lysophophatidylcholine)
LPE (Lysophophatidylethanolamine)
LPI (Lysophophatidylinositol)
STORAGE LIPIDS
TG (Triglyceride)
DG (Diacylglycerol)
MG (Monoacylglycerol)
EtherDG (Ether Diacylglycerol)
EtherTG (Ether Triacylglycerol)
Cholesteryl Ester (CE)
SPHINGOLIPIDS
DHCer (Dihydroceramide)
Cer (Ceramide)
SM (Sphingomyelin)
HexCer (Hexosylceramide)
LacCer (Lactosylceramide)
FA TRANSPORT & OXIDATION
FA (Fatty Acids)
N acyl ornithine (NAOrn)
N acyl ethanolamine (NAE)
Fatty Acyl Esters of Hydroxy Fatty Acid (FAHFA)
CAR (Acylcarnitine)
CL (Cardiolipin)
Intensity/proteins (µg)
LD
CF


STRUCTURAL LIPIDS
PC
EtherPC
PE
EtherPE
PS
PI
Chol
REMODELLING & INFLAMMATION
LPC
EtherLPC
LPE
EtherLPE
LPI
STORAGE LIPIDS
TG
DG
MG
CE
SPHINGOLIPIDS
Cer
HexCer
LacCer
SM
GM3
FA TRANSPORT & OXIDATION
FA
CAR
CL
NAGly
NAE
intensity/µg protein
LD
CF