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PhD Thesis

“Plasma phospholipid dysregulation in patients with cystathionine β- synthase deficiency”

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Abstract

Patients with cystathionine β-synthase deficiency (CBSD) exhibit extremely high circulating levels of homocysteine (Hcy) and clinical signs and symptoms whose pathophysiological understanding is poorly elucidated. The aberrant Hcy metabolism could lead to the redox imbalance and oxidative stress resulting in elevated lipid peroxidation. Although phospholipid metabolism is key for a variety of cell functions, no information is available about lipidomic profile in CBSD patients. We have characterized plasma lipidome in CBSD patients and related lipid abnormalities with reactions underlying enhanced homocysteine levels.

Using an untargeted lipidomic approach, changes in phospholipid metabolism were determined by comparing the plasma of 11 CBSD patients with that of 11 healthy subjects (CTRL). CBSD patients had a higher medium and long-chain polyunsaturated fatty acids (PUFA) content in phosphatidylethanolamine (PE) and lysophosphatidylethanolamine (LPE) species ($p=0.02$), and depletion of phosphatidylcholine (PC; $p=0.02$) and of lysophosphatidylcholine (LPC; $p=0.003$) species containing docosahexaenoic acid (DHA), suggesting impaired phosphatidylethanolamine-N-methyltransferase (PEMT) activity. PEMT converts PE into PC using methyl group by S-adenosylmethionine (SAM) thus converted in S-adenosylhomocysteine (SAH). Whole blood SAM and SAH concentrations by liquid chromatography tandem mass spectrometry were 1.4-fold ($p=0.015$) and 5.3-fold ($p=0.003$) higher in CBSD patients than in CTRL. A positive correlation between SAM/SAH and PC/PE ratios ($r=0.520$; $p=0.019$) was found.

Our findings reveals e novel biochemical alteration in CBSD patients consisting in depletion of PC and LPC species containing DHA and accumulation of PUFA in PE and LPE. Variations in plasma SAM and SAH concentration are associated with such phospholipid dysregulation. Given the key role of DHA in thrombosis prevention, reduction of PC species containing DHA in CBSD patients provides a new direction to understand the poor cardiovascular outcome of patients with CBSD.

1. Introduction

1.1. Cystathionine β-synthase deficiency (CBSD): definition and overview

Cystathionine β-synthase deficiency (CBSD, EC 4.2.1.22), also known as homocystinuria (OMIM 236200), is a rare (prevalence 1:1,800-1:900,000) inherited metabolic disease with an autosomal recessive transmission that involves methionine (Met) metabolism. [1,2] The responsible gene is cystathionine β-synthetase (CBS, 21q22.3) with more than 150 mutations identified in different ethnicities. [1] Under physiological conditions, CBS transforms homocysteine (Hcy) into cystathionine in the transsulfuration pathway of the Met cycle requiring pyridoxal 5-phosphate as a cofactor. The other two factors involved in Met cycle are vitamin B12 and folic acid. When CBS function is impaired, an insufficient amount of Hcy is converted to cystathionine resulting in an accumulation of Hcy. [2] The biochemical picture of CBSD patients shows severe accumulation of Hcy in plasma (up to 200 μM) and urine (homocystinuria), decreased synthesis of cystathionine and cysteine and usually an increased Met.[2] CBS deficiency is a multisystem disease and the major clinical manifestations are ectopia lentis, mental retardation, occasional hepatic steatosis, connective tissue disturbances, including skeletal abnormalities (marfanoid habitus), osteoporosis and enhanced tendency to venous and arterial thrombosis. [1,2,3] Overall, venous and arterial thrombotic complications are the leading cause of death in such patients. [1,2] Clinical diagnosis of CBS deficiency is confirmed by measuring plasma levels of total homocysteine (tHcy) and amino acids in plasma, through enzyme analysis of CBS activity and screening for CBS mutations. [3] In infants, the goal is to prevent the development of symptoms and ensure normal physical and intellectual development. When the diagnosis is made later in life and when some symptoms are already present, the goal of therapy is to prevent further complications (such as thromboembolic events, stroke, heart attacks) and prevent worsening of symptoms. [4] The therapeutic treatment is

determined by the age at which homocystinuria is diagnosed and the severity of the disease. To achieve these goals, the various treatment modalities aim to reduce plasma levels of Hcy. Three types of treatment are currently available depending on the patient's responsiveness to pyridoxine (Vit. B6). [3,4] For patients who respond to Vit B6 (cofactor of CBS), treatment includes vitamin B6 at pharmacological doses, in combination with folic acid and vitamin B12 supplementation. In patients unresponsive to Vit B6, the recommended treatment is a diet low in methionine and high in cysteine, in combination with supplementation of pyridoxine, folic acid and vitamin B12. [4] Treatment with betaine anhydrous, a methyl donor, reduces Hcy levels in body fluids by remethylation of Hcy to Met and could help to prevent further complications, particularly thrombotic events. [5]

1.2. Homocysteine metabolism

Hcy is a non-structural amino acid formed following the loss of the methyl group by methionine, an essential amino acid introduced with the diet. [1] Hcy is found at the intersection of two important pathways of methionine metabolism, the remethylation pathway and the transsulfuration pathway. [6] (Fig.1)

Met is converted to Hcy, via S-adenosylmethionine (SAM), and S-adenosylhomocysteine (SAH), by the release of a methyl group that will be used in methylation reactions (e.g., via phosphatidylethanolamine N-methyltransferase, PEMT). [7]

- In the *remethylation pathway*, Hcy is converted back to Met through the action of methionine synthetase, which uses vitamin B12 (cobalamin) as a cofactor and 5-methyltetrahydrofolate (5-MTHF) as a donor of methyl groups. There is another pathway of remethylation, which uses betaine as a donor of methyl groups. The methionine that forms in this pathway is activated by adenosine triphosphate (ATP) to SAM. SAH, which is formed from the transmethylation of SAM, is then converted to Hcy with the release of

adenosine. The 5,10-methylenetetrahydrofolate reductase (MTHFR) enzyme, which uses nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor, is a key enzyme of this pathway because it converts 5,10-methylenetetrahydrofolate to 5-MTHF, a donor of the methyl group in the methionine remethylation reaction. [7,8]

- In the *transsulfuration pathway*, excess homocysteine is conjugated with serine (Ser) and converted to cystathionine, in an irreversible reaction catalyzed by the enzyme cystathionine β-synthetase, which requires pyridoxal-5'-phosphate (PLP), the active form of the vitamin B6, as a cofactor. Cystathionine is then hydrolyzed to cysteine by another PLP enzyme (PLP), γ-cystationase, thus forming cysteine (Cys) and α-ketobutyrate. Excess cysteine undergoes a reaction of oxidation to form taurine and inorganic sulphates, or it is eliminated in the urine. [7,8]

SAM and SAH acts as a fine regulator of these two metabolic pathways, being both an allosteric inhibitor of MTHFR and an activator of CBS. It is clear how, an optimal functioning of the enzymes involved in the two metabolic pathways, together with an adequate supply of vitamin cofactors with the diet (vitamins B6, B12 and folates), is essential for the maintenance of the plasma levels of homocysteine. [9] Moreover, the intracellular content of SAM is the likely gauge for the availability of Met, either excess or insufficiency, while intracellular SAH content is the likely measure of Hcy excess. SAM is also the main donor of methyl groups for most of the methylation reactions occurring inside the cell (e.g. those catalyzed by phosphatidylethanolamine N-methyltransferase, PEMT), while SAH is a potent inhibitor of several SAM-dependent methyltransferases. The SAM / SAH ratio can therefore be defined as an indicator of the methylation potential of a cell. Therefore, an increase in SAH due to high concentrations of Hcy alters the SAM / SAH ratio. Consequently a decrease in the SAM / SAH ratio may be indicative of a reduced cellular methylation capacity. In addition, some studies claim that SAH is a more sensitive indicator of cardiovascular risk than homocysteine.[9]

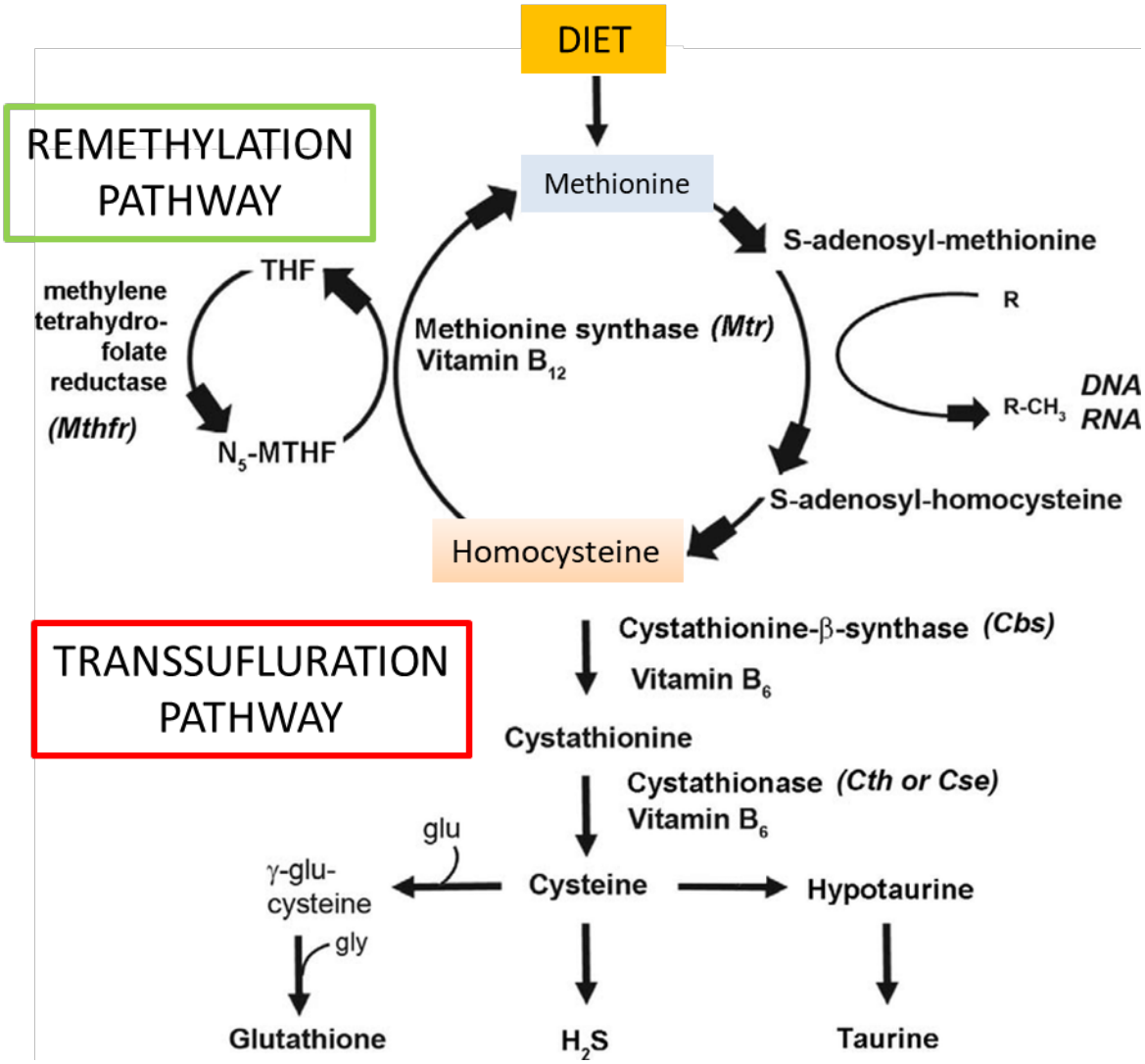


Figure 1: Schematic representation of Hcy metabolism showing remethylation and transsulfuration pathways.

1.3. Toxic effects of Homocysteine

Several hypotheses have been developed on the toxicity of Hcy, but none is able to clearly explain these effects. As reported in literature, Hcy exerts its cytotoxic action through three mechanisms: *homocysteinylation, oxidative stress and excitoxicity*.

- *Homocysteinylation*

Homocysteinylation is the binding of Hcy to proteins and constitutes a post-translational modification. The binding leads to an alteration of the three-dimensional conformation of the protein, as well as to a change in its functions and is proportional to the concentration of Hcy. (Fig. 2) [10]

It is called S-homocysteinylation when the bond occurs between two thiolic groups (-SH), one of the Hcy, the other present on a Cys residue in the amino acid sequence of a protein. This covalent bond radically changes the redox status of the protein. [11]

N-homocysteinylation occurs between the amino group of homocysteine and the ε-amino group of a lysine residue present in the protein. This type of covalent bond causes a profound change in the structure and function of the protein. [11]

Hcy can undergo an intramolecular cyclization reaction that leads to the formation of homocysteine thiolactone (Hcy-TL) which exerts its toxicity through N-homocysteinylation. In vivo studies have shown that Hcy-TL is able to binds and modify hemoglobin, immunoglobulins, low-density lipoprotein (LDL), high-density lipoprotein (HDL), transferrin, antitrypsin and fibrinogen. [12]

N-homocysteinylation, leading to the formation of a free thiol group, produces a perturbation of the redox potential of proteins increasing oxidative stress. N-homocysteinylation, also has a cytotoxic effect due to the stress of the endoplasmic reticulum, increased protein turnover, caused by an increased catabolism of misfolded structures, and deactivation of some enzymes. [13]

Several studies have shown that proteins that have undergone the N-homocysteinylation process act as neoantigens, capable of triggering an inflammatory response, an essential component for the processes of atherogenesis and atherothrombosis. Furthermore, these neoantigens, when present on the surface of endothelial cells, activate the macrophages that cause endothelial damage. Furthermore, the damage cannot be repaired as HcY-TL inhibits lysyl oxidase. [14]

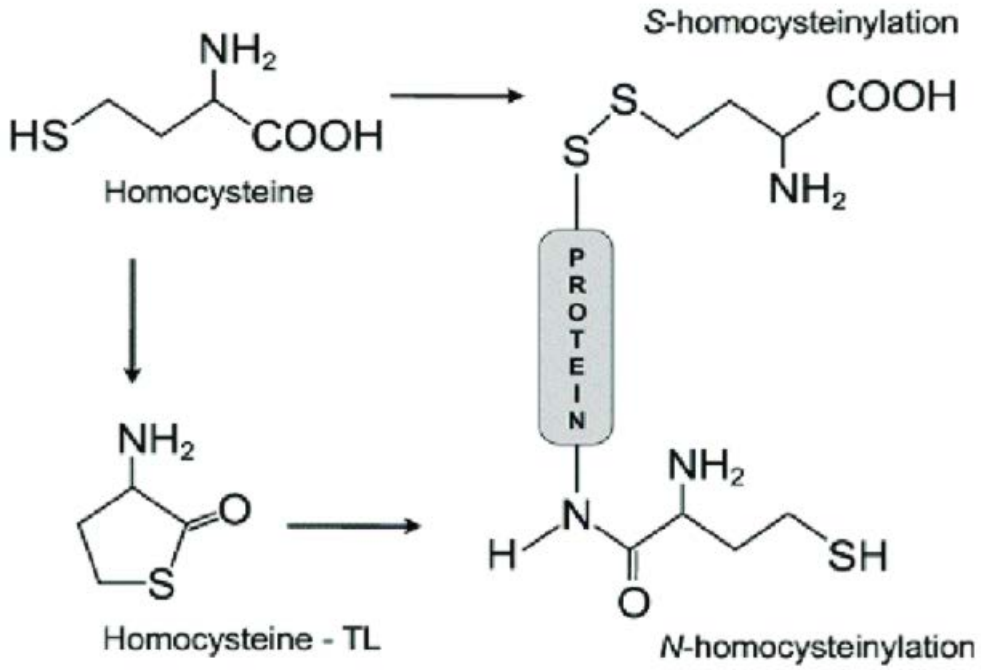


Figure 2 The structure of homocysteine, homocysteine-thiolactone (homocysteine-TL; Hcy-TL) and S/N-homocysteinylation of proteins.

- *Oxidative stress*

High levels of homocysteine have been correlated with an increase in oxidative stress, that is, with an increase in the production of oxygen free radicals (ROS) and a reduction in antioxidant species. Homocysteine, containing a reactive sulfhydryl group (-SH), can go through oxidation processes. This processes leads to the formation of several ROS species, such as superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2). [15] These oxygen-derived molecules are responsible for the

subsequent oxidation of lipids present on the surface of endothelial cells and inside plasma lipoproteins which can lead to endothelial dysfunction with consequent platelet activation and thrombus formation. [16, 17] Thus, this findings suggest that oxidative stress induced by high level of Hcy may explain the pathogenesis of atherothrombosis in patients with disorder of Hcy metabolism. In fact, some studies have shown that, in patients with CBS deficiency, there is a link between high levels of Hcy and increased peroxidation of arachidonic acid, which leads to the formation of F2-isoprostane 8-iso-PGF2a. Since this compound modulates platelet activity in humans, a correlation between an increase in F2-isoprostane 8-iso-PGF2a and persistent platelet activation has also been described. [17] However, it seems that other mechanisms can also be attributed to the oxidative stress induced by homocysteine such as reduced production and inactivation of nitric oxid (NO), inhibition of glutathione peroxidases , activation of NADPH oxidases and disruption of extracellular superoxide dismutase (SOD) from endothelial surfaces. [18]

- *Excitoxicity*

Hcy is particularly toxic to brain tissue as this, unlike other tissues, lacks two of the main metabolic pathways for the elimination of Hcy; that is the betaine-mediated conversion pathway which converts Hcy to Met and the transsulfuration pathway which converts Hcy to Cys. It should also be noted that homocysteine in brain tissue is able to act as an agonist of ionotropic and metabotropic receptors for glutamate, as well as N-methyl-d-aspartate receptor (NMDA), causing an increase in calcium ions at the cytoplasmic level with a consequent increase in free radicals, activation of caspases and apoptosis. [19]

In addition, homocysteine is also capable of acting on some enzymes, like metalloproteinases (MMPs), causing a destruction of the blood-brain barrier (BBE). [20, 21]

1.4. Homocysteine and lipid dysregulation

Hyperhomocysteinemia (HHcy) as well as dyslipidemia are considered risk factors for cardiovascular diseases (CVD). [22] Studies in the literature show that there is an association between HHcy, dyslipidemia and atherosclerosis. [23] Homocysteine exerts its atherogenic activity through various mechanisms including the oxidation of LDL and the reduction of high-density lipoprotein cholesterol (HDL-C). [24] Several studies have associated HHcy with an altered metabolism of HDL-C, in fact it is believed that Hcy is able to down regulate the protein synthesis of ApoA-I and lecithin-cholesterol acyltransferase (LCAT), which play a key role in HDL production. Furthermore, Hcy promotes the clearance of HDL. [24, 25] There are other hypotheses that explain how Hcy is capable of impacting lipid metabolism. The importance of the balance between S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and choline has recently been demonstrated. Perturbations in Hcy metabolism cause a reduction in SAM or an increase in SAH, this alteration is able to influence the synthesis of phospholipids (PC and PE). [24]

1.5 Lipidomics

The term "lipidome" refers to the totality of lipids present in a cell. [26] Lipidomics, together with genomics, transcriptomics, proteomics and metabolomics, are part of the "omics" sciences. [27] It represents an innovative approach to understanding lipid biology. Lipidomics is not limited to the simple characterization of all the lipids present in a cell type. The structural diversity of the building blocks of lipids and the multiple ways in which they can combine, generates a vast number of possible molecular lipids in a given biological system. Through a global approach, it aims to understand the effect of changes in all lipids on a biological system, whether it is in a physiological or pathological state, in relation to lipid-mediated cell signaling and lipid metabolism. [28] The analytical techniques that allow the complete characterization of lipids at the molecular level derive

from metabolomics. Mass spectrometry, usually preceded by separation by liquid chromatography or gas chromatography, is the main analytical technique for lipidomics. [29, 30] This technique allows the study of lipids starting from very small quantities of samples, for example from plasma, cells or tissue biopsies. [31] Two experimental approaches can be used for lipidomic analysis: one *targeted*, used for the study of a specific lipid class; and an *untargeted*, broad-spectrum, aimed at the analysis of entire lipid extracts. The typical work flow of an untargeted lipidomics (Fig.3) experiment begins with the definition of an experimental design followed by sample collection and preparation, data acquisition via instrumental analysis, data analysis, data processing, statistical analysis and interpretation of the results.

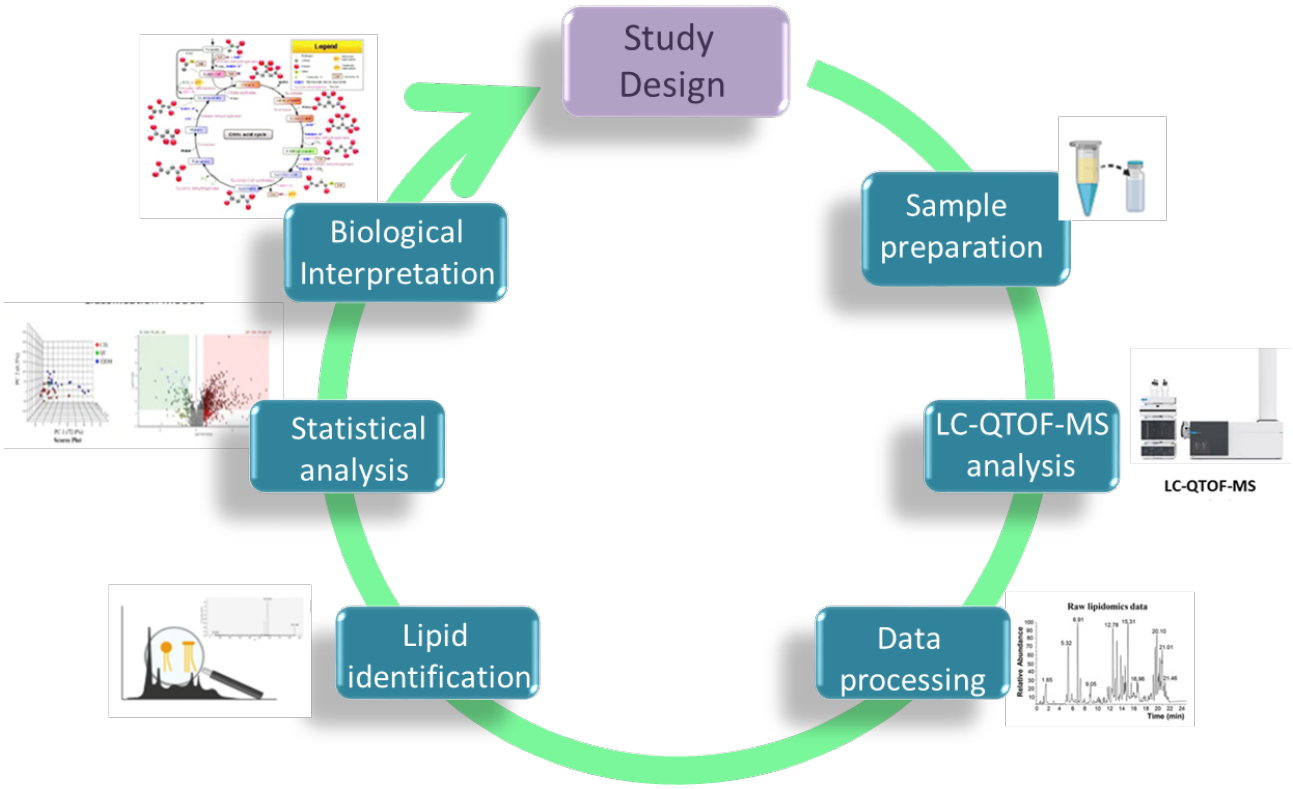


Figure 3 A schematic representation of the steps involved in a lipidomic workflow.

2. Materials and Methods

2.1 Study population

After informed consent, 11 patients with mutations in the gene encoding cystathionine β-synthase (CBS), in the absence of other polymorphisms impacting Hcy metabolism (Hcy ≥ 15 μM [32]), receiving long-lasting treatments with folate and pyridoxine or betaine, were recruited.

Exclusion criteria were: age < 18 years, history of vascular events, end-stage renal disease (estimated glomerular filtration rate < 30 ml/min/m^2), current or previous (2 years) malignant disease. In parallel, 11 non-CBSD, age and sex-matched subjects, without a history of diseases known to alter Hcy or lipid profile, nor receiving drugs affecting Hcy or lipid metabolism, were included as controls (CTRL) in the present study.

The study protocol (CE reference code: 6598/18) was approved by the Ethics Committee of the Federico II University Hospital and was in accordance with the Helsinki Declaration.

2.2 Homocysteine measurement

Fasting total Hcy (tHcy) concentrations (i.e., the sum of the disulphides [≈30%], protein-bound species [≈70%], and the free form [1%]) [35], were determined by HemosIL Homocysteine immunoassay (HemosIL™ Instrumentation Laboratory Company e Lexington, MA, USA).

2.3 Lipidomic analysis

2.3.1 Lipid extraction

Plasma aliquots (200 μl) were extracted two times with water:methanol:chloroform 1:1:2 (v:v:v) using a slightly modified Bligh and Dyer method [33]. Extracts were brought to dryness, reconstituted in 500 μl of methanol: chloroform 9:1 (v:v) and analyzed by Ultra-High-Performance Liquid Chromatography-Electrospray Ionization-Quadrupole-Time of Flight-Mass Spectrometry (UHPLC-ESI-Q-TOF-MS).

2.3.2 Ultra-High-Performance Liquid Chromatography-Electrospray Ionization- Quadrupole-Time of Flight- Mass Spectrometry (UHPLC-ESI-Q-TOF-MS) analysis

Lipids were injected in an Agilent 1290 Infinity UHPLC connected with an Agilent 6550 iFunnel Q-TOF mass spectrometer equipped with Dual-Jet electrospray ionization source (ESI, Agilent Technologies, Santa Clara, CA, USA).

A ZORBAX Eclipse Plus C18 Rapid Resolution HD column (2.1×150 mm, 1.8 µm; Agilent Technologies) was eluted at 0.3 ml/min. Mobile phase A was acetonitrile: water 50:50 (10 mM ammonium formate, 0.1% formic acid) and mobile phase B was acetonitrile: water: isopropanol 10:2:88 (10 mM ammonium formate, 0.1% formic acid).

The linear gradient, starting at 65%A:35%B, reached 95%B in 20 min and 100%B in 5 min. Full scan analysis over mass/charge (*m/z*) range 50- 1200 was performed injecting 0.2 µl in positive and 2 µl in negative ion mode. Data were acquired using the MassHunter software (version B.07.00; Agilent Technologies).

2.3.3. Lipid annotation

Lipids were annotated according to their *m/z* ratios and denoted by head group, total fatty acyl carbon atoms and unsaturation content. Unsaturation Index (UI) was calculated as: $UI_y = [\Sigma (\%\ area\ lipid_x \times number\ of\ double\ bonds\ lipid_x)]/100$, where $lipid_x$ represents each single molecular species belonging to the $_y$ lipid class. The Average Chain Length (ACL) of each lipid class was calculated using the formula: $ACL_y = [\Sigma (\%\ area\ lipid_x \times total\ number\ of\ acyl\ chains\ -\ carbon\ atoms\ of\ lipid_x)]/100$.

2.4 SAH and SAM measurements

Whole blood SAH and SAM concentrations were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) method by Birsa et al. with some modifications [34]. Briefly, an aliquot of whole blood (50 µl) was separated using a LunaNH2 100 Å column (3.0 × 150 mm, 3 µm; Phenomenex, Bologna, Italy) at 30 °C under isocratic conditions with 5% acetonitrile and 95% ammonium formate 10 mM, pH 3.4. Mass spectrometric analysis was performed using a 5500 QTrap linear ion trap quadrupole mass spectrometer (AB Sciex, Milan, Italy) outfitted with ESI source operating in positive mode. For single reaction monitoring (SRM) the transitions *m/z* 399.0 → *m/z* 298.0 + 250.0 + 161.9, *m/z* 136.2 (SAM), *m/z* 402.2 → *m/z* 301.3 + 250.2 + 136.2 (SAM-d3), *m/z* 385.1 → *m/z* 250.1 + m/z 135.9 (SAH), *m/z* 389.1 → 254.1 + 138.0 + 136.1 + 92.0 (SAH-d4) were employed. The operating MS conditions were: gas 1, nitrogen 20 psi; gas 2, nitrogen 30 psi; ion spray voltage, 4000 V; ion source temperature, 400 °C; curtain gas, 20 psi; collision gas, medium. Data acquisition and analysis were performed with MultiQuant™ software (AB Sciex).

2.5 Detection of liver fibrosis and steatosis

Liver fibrosis in CBSD patients was non-invasively assessed by Transient elastography (TE) with the FibroScan (®) M probe (FibroScan; Echosens, Paris, France) by measuring liver stiffness [36]. In detail, TE is equipped with a probe consisting of an ultrasonic transducer mounted on the axis of a vibrator. The mild-amplitude low-frequency vibration transmitted causes an elastic shear wave that propagates through the tissue. To follow the shear wave propagation and to measure its velocity, which is directly related to tissue stiffness, pulse-echo ultrasonic acquisitions are carried out in parallel. Cut-off TE values for liver disease are: F0/F1- absent to mild fibrosis: <7.3 Kilopascal (kPa); F2- moderate fibrosis: 7.3-12.5 kPa; F3- severe fibrosis: 12.6-17.6 kPa; F4-cirrhosis: >17.6 kPa. Such

values are derived from a large population study that included patients with different types of chronic liver disease [37]. TE data also help calculate the Controlled Attenuation Parameter (CAP), an accurate non-invasive tool to detect different degrees of liver steatosis [38]. The CAP score is measured in decibels per meter (dB/m), and optimal cut-off values for the detection of steatosis grade (S0-S3) are: CAP < 215 dB/ m (≤11% liver with steatosis): S0; CAP between 215 and 252 dB/m (11-33% liver with steatosis): S1; CAP between 253 and 296 dB/m (33 and 66% liver with steatosis): S2; and CAP > 296 dB/m (>66 liver with steatosis): S3 [39].

2.6 Raw data processing and statistical analyses

Raw data were normalized by the Loess normalization using the Normalyzer tool [40], after discarding lipids with more than 3 missing values and performing the missing value imputation; the imputation step was carried out by the 'randomForest' R package [41]. Differential analyses were performed by the 'limma' package [42], adjusting for the 'Smoking' effect [43,44]. The Benjamini-Hochberg procedure was used to control for the false discovery rate (FDR). A lipid was deemed significant if the FDR adjusted *p*-value was <0.05 and the $|\log_2(\text{Fold Change})| > 1.5$. The cohort characteristics were presented as mean ± standard deviation, and Student's *t*-tests were employed for comparisons. Pearson correlations and multivariate regressions were used to evaluate associations between continuous features.

3.Results

3.1 Study population

Eleven patients (4 males, 7 females, age 35-68 years) with homozygous or compound heterozygous mutations in the gene encoding CBS (CBS; 613381) on chromosome 21q22, belonging to 7 unrelated families, were recruited (***Table 1***). Genetic data concerning these individuals have been reported in detail elsewhere [17]. Leukocyte DNA analysis of the CBS locus showed that 3 individuals were compound heterozygotes for the mutations C262T and T833C. For all the data reported in the present study, no differences were found between the 3 subjects with the C262T and T833C mutations and the other 8 patients. All patients had a history of severe HHcy with homocystinuria, ectopia lentis, osteoporosis, and different degrees of mental retardation. All had been receiving daily doses of folic acid (400 μg/day). All but one patient was responsive to pyridoxine and had been on pyridoxine treatment (500 mg/day) from the time of diagnosis. The pyridoxine-unresponsive subject was on 8 g/day betaine. All CBSD subjects were compliant to a restricted dietary intake of the Hcy precursor Met. None of the CTRL and CBSD patients showed ST depression or Q waves on the ECG, and their clinical records were negative for angina pectoris, myocardial infarction, venous thromboembolism, and renal dysfunction. All had normal peripheral pulses and were negative for bruits over the carotid vessels. Duplex scanner analysis confirmed the absence of haemodynamically significant peripheral artery stenosis. A family history of coronary artery disease (CAD) was present in 10 out of 11 CBSD patients and in 3 out of 11 CTRL subjects. Folic acid, pyridoxine, aspirin, and statins were the medications in use in the CBSD patients but not in CTRL (***Table 1***). LDL-cholesterol was lower in CBSD patients than in CTRL, but total and HDL-cholesterol concentrations were comparable. Finally, 7 of the CBSD patients and 1 CTRL

subject were on treatment for hypertension, while 3 of the CTRL and none of the CBSD patients were current smokers.

Table 1 *Baseline Characteristics for CBSD patients and control subjects*

Variables	**CBSD (n=11)**	**CTR (n=11)**	***p* value**
Demographic characteristics			
Age (years)	49.84 ± 10.53	48.84 ± 11.17	0.83
Male gender, no. (%)	4 (36.4)	4 (36.4)	1
Comorbidities			
Homocysteine (µMol)	65.70 ± 46.65	7.63 ± 2.87	**<0.001**
Family history of CAD, no. (%)	11 (100)	3 (27.3)	**<0.001**
Diabetes mellitus, no. (%)	1 (9.1)	1 (9.1)	1
Active smoking, no. (%)	0 (0)	3 (27.3)	0.06
Hypertension, no. (%)	7 (63.6)	1 (9.1)	**0.004**
Obesity, no. (%)	2 (18.2)	1 (9.1)	0.500
Medications in use			
Folic acid, no. (%)	11 (100)	0	**<0.001**
Pyridoxine, no. (%)	10 (90.9)	0	<0.001
Aspirin, no. (%)	4 (36.4)	0	**0.038**
Statins, no. (%)	5 (45.4)	0	0.15
Anti-hypertensive drugs, no. (%)	7 (63.6)	1 (9.1)	0.004
Biochemistry			
Total cholesterol (mg/dL)	169.45 ± 22.26	192.02 ± 23.85	0.33
LDL cholesterol (mg/dL)	87.18 ± 17.36	117.51 ± 24.86	**0.003**
HDL cholesterol (mg/dL)	65.91 ± 14.61	54.45 ± 12.59	0.63
Triglycerides (mg/dL)	71.4 ± 24.47	100.45 ± 52.85	0.129

Values are expressed as means ± SD or as n (% of total). Continuous variables were compared by *T-test* while categorical ones by Fischer exact test.
[a]All but 1 CBSD patients were responsive to pyridoxine and had been on pyridoxine treatment (600-900 mg/day) from the time of diagnosis. The pyridoxine-unresponsive subject had been on 8 g/day betaine.

3.2. Lipid detection in plasma

The lipidomics analysis in positive ion-mode revealed 672 features, 507 being putatively assigned to lipid molecular species, including adducts. In negative ion mode, 880 features were detected and 510 were putatively assigned to lipid molecular species and adducts. According to MSI, identified metabolites were annotated at level 2 that is based on *m/z* recorded in high

resolution mode, MS/MS experiments in positive and negative ion modes and on matching to data available in public databases. All other features were annotated at level 4 as unknown (or unidentified compounds). In the dataset recorded in positive ion mode, the phospholipid phosphatidylcholine (PC; *n* = 112), its lysoforms (LPC; *n* = 38), and the neutral lipids diacylglycerols (DAG; *n* = 14), sphingomyelins (SM; *n* = 35), cholesteryl esters (CE; *n* = 12), and triacylglycerols (TAG; *n* = 241) species were identified, quantified and annotated according to MS/MS fragmentation pattern (***Fig.*4A**). In the negative ion mode analysis, PC (*n* = 117), phosphatidylethanolamine (PE; *n* = 43) and phosphatidylinositol (PI; *n* = 19), their lyso-forms (LPE; *n* = 10 and LPC; *n* = 28), SM (*n* = 45), ceramides (CER; *n* = 239) and their glycosylated species (hexosyl and lactosyl ceramides) were detected (***Fig.* 4B**). Few PE and PI were detected in positive ion mode. A better characterization of these lipid classes was achieved in negative ion mode. As expected [45], we did not detect any form of other phospholipids, such as phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylserine (PS), and cardiolipins. Clustering analysis, performed by multi-dimensional scaling (MDS) showed that, except for smokers, CBSD and CTRL groups were well separated both in positive (***Fig.* 5A**) and in negative (***Fig.* 5B**) ion mode.

Figure 4 Full scan spectra of major plasma lipid classes detected and quantified in (A) positive and (B) negative ion mode. *Acronyms:* Lyso-phospholipids (lyso-PL); Phospholipids (PL); Sphingomyelin (SM); Tricylglycerol (TAG); Cholesterol esters (CE); Diacylglycerol (TAG); Ceramides (CER).

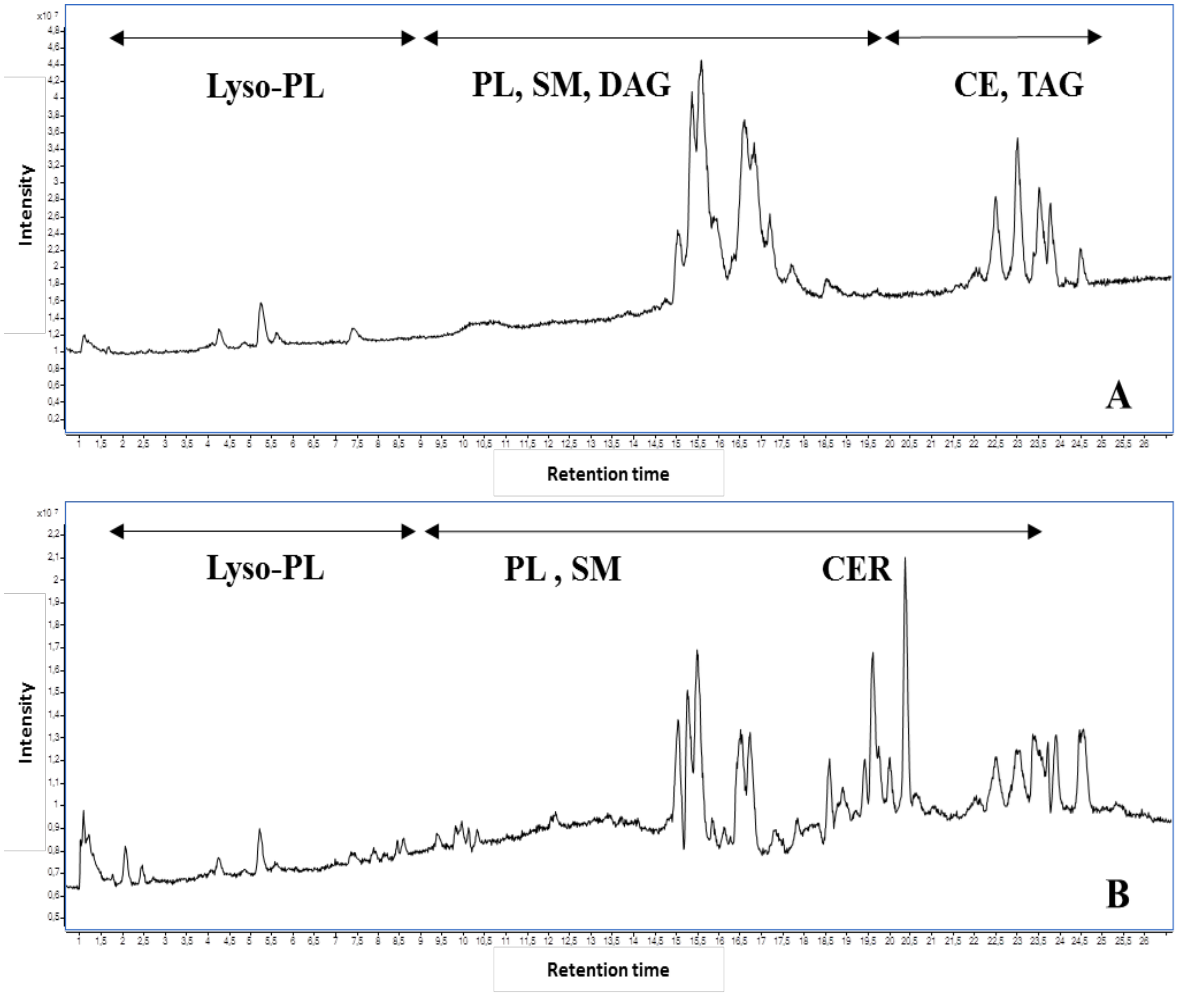
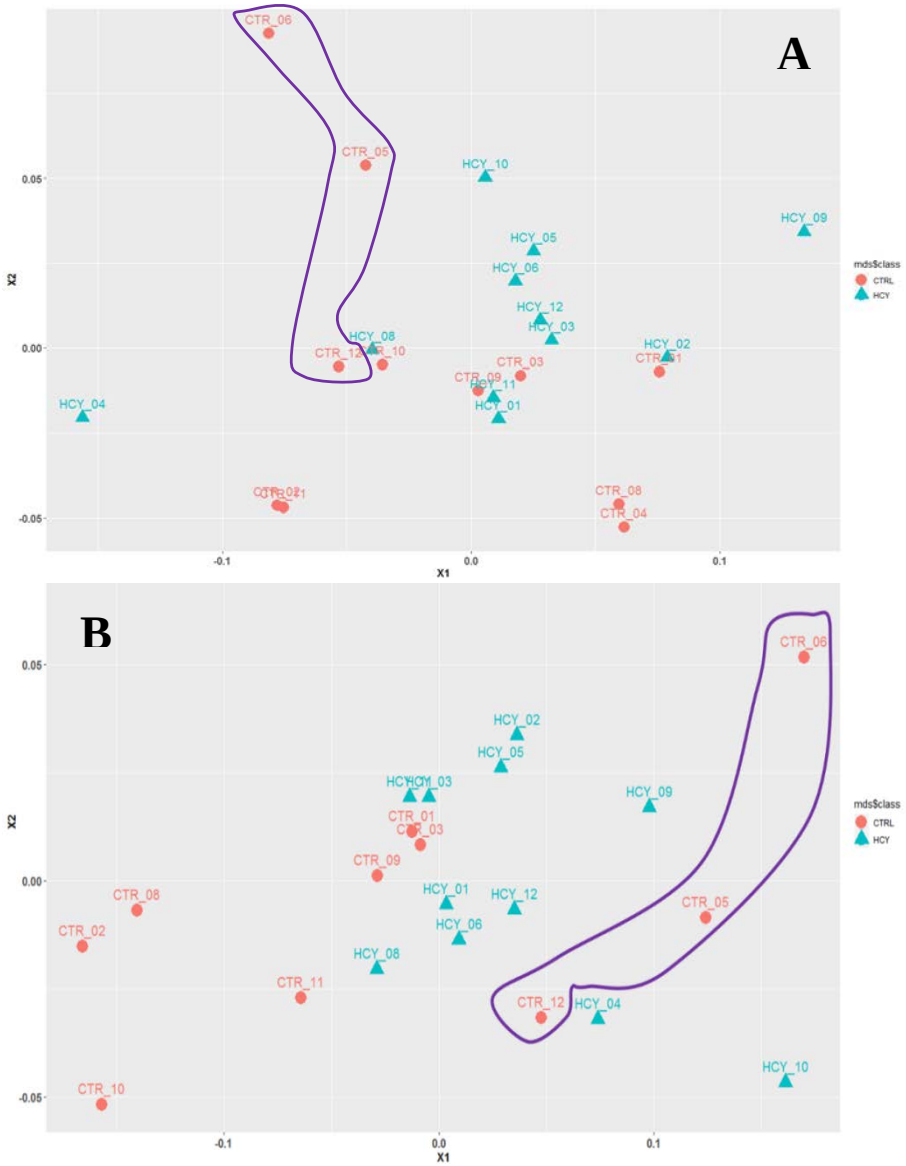


Figure 5 Multi-Dimensional Scaling (MDS) plots of data recorded in positive (A) and negative ion mode (B). Red dots represent CTRL subjects; blue dots represent CBSD patients. We delimited current smokers by the purple boundary.



3.3. Lipid species identified: structural differences between CBSD patients and CTRL

Results obtained by splitting the data with respect to lipid species, the Average Chain Length (ACL) of each lipid class (calculated using the formula: $ACL_y = [\Sigma$ (% area $lipid_x \times$ total number of acyl chains - carbon atoms of $lipid_x)]/100$), and the Unsaturation Index (UI) -calculated using the formula: $UI_y = [\Sigma$ (% area $lipid_x \times$ number of double bonds $lipid_x)]/100$, where $lipid_x$ represents each single molecular species belonging to the $_y$ lipid class UI-, are shown in *Table 2*. No statistically significant difference between CTRL and CBSD patients was found as to ACL of the lipid species analyzed. Consistent with the possibility that the fatty acid elongation pathway was not altered by high circulating Hcy levels, the average number of carbon atoms forming fatty acyl chains within a specific lipid class did not differ within the two groups. In contrast, significant differences were documented in the UI of LPC and TAG (adjusted for FDR, $p < 0.05$ for both). The only fatty acid chain present in LPC in CBSD patients contained, on average, a lower number of double bonds compared to that of CTRL (0.7 *vs*. 0.8 respectively). In contrast, TAG detected in plasma of CBSD patients were more unsaturated than those detected in CTRL (3.2 double bonds/molecule compared to 2.8, respectively, adjusted for FDR, P-value < 0.05). A significant difference (1.1 in CTRL vs. 0.4 in CBSD patients; adjusted for FDR, *P*-value < < 0.05) was also observed when the oxidized/non-oxidized ratio of CE species was evaluated.

Table 2. Average chain length and unsaturation indices for each lipid class in CTRL and CBSD patients. Lipids were denoted by head group, total fatty acyl carbon atoms and unsaturation content (e.g., PC 34:1).

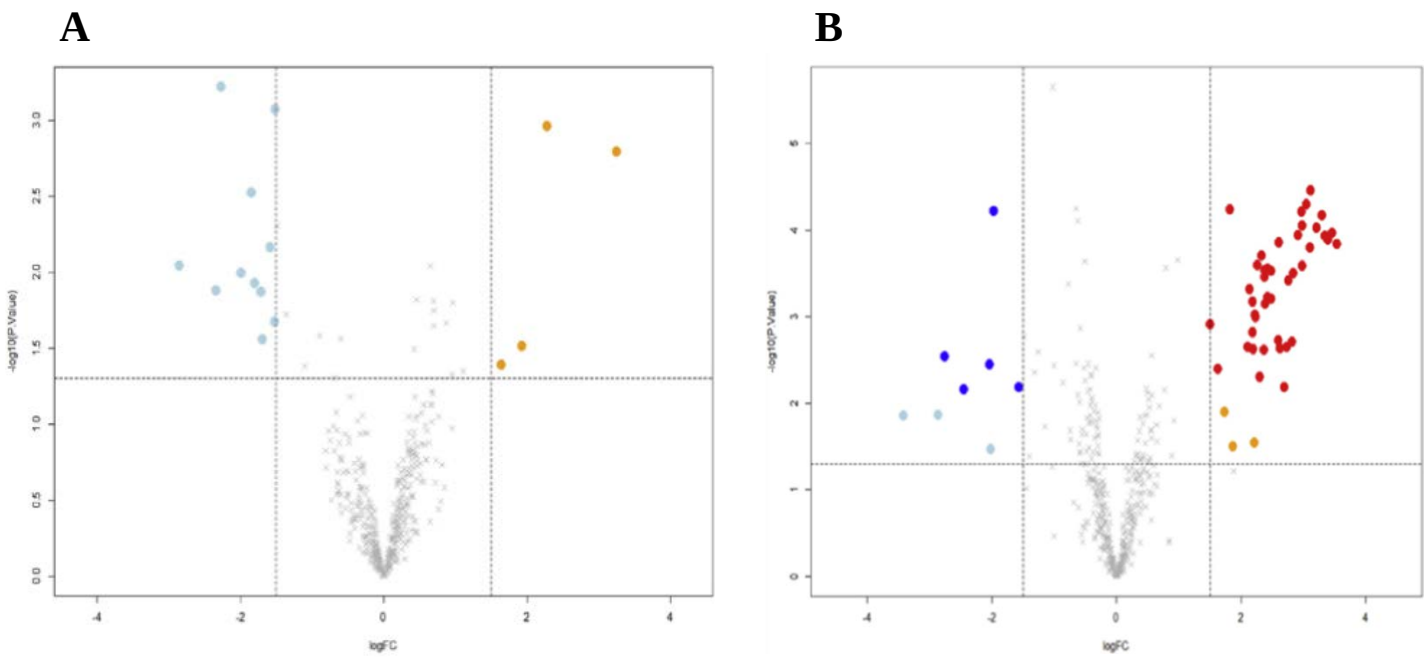
		PC	**LPC**	**PE**	**LPE**	**PI**	**CER**	**SM**	**DAG**	**TAG**	**CE**
Average Chain Length **(ACL)**	**CTRL**	35.5	17.2	37.5	19.1	37.2	40.0	37.3	35.8	51.8	18.4
	CBSD Patients	35.6	17.1	37.5	18.9	37.9	39.9	37.9	36.0	52.2	18.5
Unsaturation Index **(UI)**	**CTRL**	2.6	0.8*****	4.5	2.8	3.3	1.3	1.4	3.1	2.8*****	2.3
	CBSD Patients	2.7	0.7*****	4.5	2.5	3.4	1.3	1.4	3.3	3.2*****	2.4

3.4. Accumulation of PE and depletion of PC in plasma from CBSD patients

Differential analyses revealed that plasma lipid composition was heterogeneous in CBSD patients and CTRL. Light blue and orange dots in ***Fig. 6*** represent lipids differentially expressed in CBSD and CTRL. Light blue dots refer to lipids with $\log_2$ fold change < 0.05; orange dots to those with $\log_2$ fold change >1.5 and a pp value < 0.05, and blue dots and red dots represent lipid classes that were differentially expressed at an adjusted *P*-value < 0.05. At variance with the positive ion mode (***Fig. 6A***), 71 lipids were found to be differentially expressed in negative mode (adjusted p-value < 0.05), 42 of them having a Log Fold Change [$\log_2$(FC)] > 1.5 and 5 a $\log_2$(1FC) < 1.5 (***Fig. 6B***). Accumulation of diacyl-, ether-, and vinyl-ether-PE and LPE was documented, being 29 out of 44 PEs and 5 out of the 10 LPEs identified at higher concentrations in CBSD plasma samples than in CTRL (adjusted $P < 0.02$). These differentially expressed PEs and LPE species represented 93 and 74% of the total PE, respectively. PEs contained saturated fatty acids or the ether/vinyl ether group at *sn*-1 position of glycerol backbone and polyunsaturated fatty

acids (PUFA) at *sn*-2. Compared to CTRL, PE from CBSD patients had a significantly (adjusted $p < 0.02$) higher content of medium/long chain PUFA, such as linoleic acid, arachidonic acid, eicosapentaenoic acid, docosapentaenoic acid, and docosahexaenoic acid (DHA). On the other hand, significantly lower concentrations of LPC (adjusted $p = 0.0033$) and PC ($p = 0.0248$) species containing DHA were found in CBSD patients. Due to statin treatment, LDL cholesterol was significantly lower in CBSD patients than in CTRL (see ***Table 1***). However, no statistically significant difference in lipid classes was observed between CBSD patient groups stratified by ongoing statin treatment, in both positive and negative ion mode datasets (data not shown).

Figure 6 Volcano plots showing results of differential analysis of lipids identified in (A) positive and (B) negative ion mode analysis.



3.5. PC/PE ratio

The PC/PE ratio calculated on raw plasma PC and PE data was 2.2-fold lower in CBSD patients than in CTRL (63.5 ± 25.5 in CBSD *vs*. 141.9 ± 98.5 in CTRL, $p = 0.029$). After adjusting for smoking habits, a significant association between CBSD status and the PE/PC

ratio was found (multivariate linear analysis, β = -0.749; p > 0.001). No significant difference in PC/PE ratio was found between CBSD males and females and between pre- and postmenopausal CBSD females.

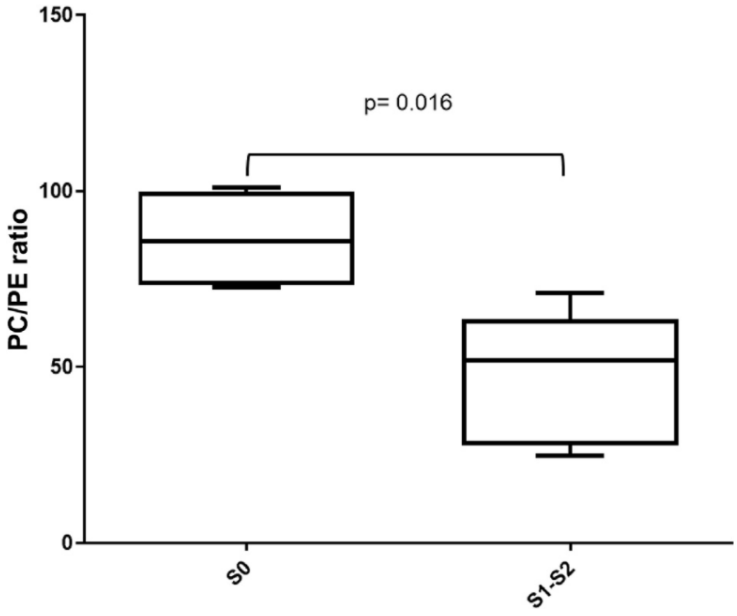
3.6. SAM and SAH measurements

Mean SAM concentrations were 1.4-fold higher in CBSD patients (8.12 ± 1.97 µM) than in CTRL (5.99 ± 0.72 µM; P = 0.015). Likewise, mean SAH concentrations were 5.3- fold higher in CBSD patients than in CTRL (0.20 ± 0.29 µM *vs*. 0.038 ± 0.014 µM, respectively; P = 0.003). SAM/SAH ratio was significantly lower in CBSD than in CTRL (88.42 ± 48.7 *vs*. 202.8 ± 121.2, respectively; p = 0.003). A significant positive correlation was found between SAM/ SAH ratio and PC/PE ratio (r = 0.520; p = 0.019). In addition, after adjusting for smoking habits, CBSD status was significantly associated with the SAM/SAH ratio (multivariate linear analysis, β = -0.568; p = 0.009). No significant difference in SAM/SAH ratio was found between CBSD males and females and between pre- and post-menopausal females.

3.7. Association between liver steatosis and PC/PE ratio

Controlled Attenuation Parameter (CAP), an accurate noninvasive tool to detect different degrees of liver steatosis, showed the absence of liver steatosis (S0) in 5 and its presence in 6 out of 11 CBSD patients (four S1 and two S2). CBSD patients with liver steatosis had a significantly lower PC/PE ratio than those without steatosis (48.26 ± 18.7 *vs*. 86.28 ± 14.4, respectively; p = 0.016; ***Fig. 7***). Accordingly, after correcting for age and gender, PE/PC ratio was associated with liver steatosis in a multivariate linear analysis (β = -0.770; p = 0.009). TE values < 7.3 kPa were found in all CBSD patients, which ruled out the presence of liver fibrosis (not shown).

Figure 7 PC/PE ratio stratified according to the absence (S0) or the presence (S1-S2) of steatosis (at ultrasonographic evaluation).



4. Discussion

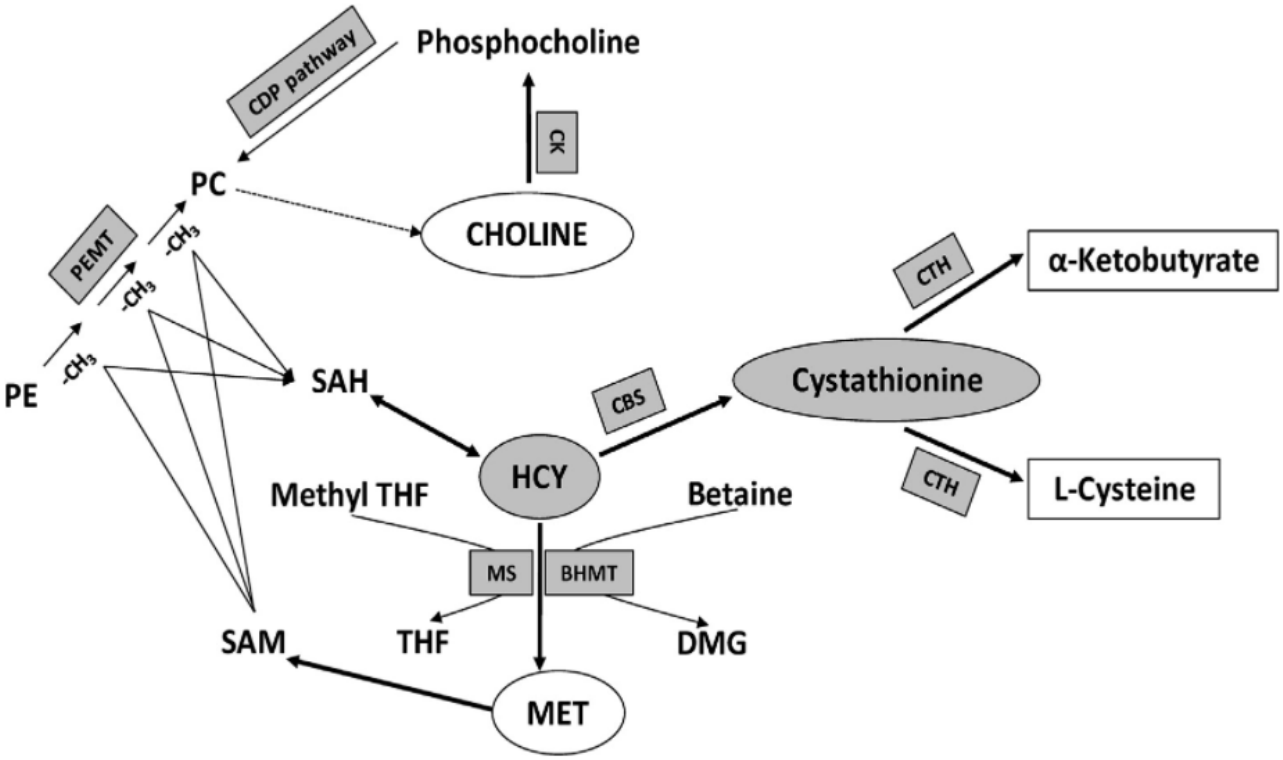
This study first shows a differential plasma lipidomic profile in patients with CBSD compared with age- and sex-matched controls. By showing medium/long chain PUFA enrichment in PE, depletion of DHA in PC, and lower than normal LPC concentrations, our untargeted lipidomic approach reveals significant abnormalities in PE and PC composition in CBSD patients. The depletion of PC species containing DHA may play a role in the subtle balance between AA, DHA/EPA and platelet activation [46] and in the tendency to thrombosis and poor cardiovascular outcome of patients with HHcy and homocystinuria. In addition to being important constituents of lipoproteins circulating in the bloodstream, PC and PE play a key role in the cell membrane structure [47]. The PC/PE balance is important for proper integrity and function of the membrane, a reduced PC/PE ratio resulting in leakage of molecules and enzymes to the outer side in hepatic steatosis [48]. In the liver, PE is converted into PC mainly via the cytidine-diphosphate-choline: 1,2-diacylglycerol

choline phosphotransferase (CDP-choline) biosynthetic route (accounting for ≈70% of total PC enriched in medium-chain saturated fatty acids) [49,50]. Alternatively, PE→PC conversion takes place through the PEMT pathway (accounting for ≈30% of total PC, the latter being particularly rich in PUFA, such as AA, EPA and DHA). PEMT converts PE into PC *via* three sequential methylations of ethanolamine (***Fig. 8***) that employ SAM as the methyl donor [51,52]. Once de-methylated, SAM is converted into SAH and subsequently to adenosine and Hcy by SAH hydrolase [53-55]. Once the intracellular concentration of Hcy increases (*e.g.*, in CBSD), the equilibrium of the reaction catalysed by SAH hydrolase shifts towards SAH synthesis starting from Hcy and adenosine, and SAH potently inhibits further transmethylation reactions (*i.e.*, the conversion of PE into PC) [47,56]. Binding of SAM activates CBS [57], a critical event to maintain the balanced methylation and the redox potential of the CBS enzyme [47]. The presence of high plasma levels of PE (both diacyland ether forms) and depletion of PC species containing DHA in CBSD patients, allowed us to speculate that dysfunctions in the key plasma source of methyl groups SAM and in the major transmethylation inhibitor SAH (and/or an impairment in PEMT activity) play a key role in such abnormal PC/PE ratio. Indeed, SAM and SAH concentrations were on average 1.4- and 5.3-fold higher, respectively, and SAM/SAH ratio was positively associated with the abnormal PC/PE ratio, in CBSD patients. In the present setting, SAM and SAH concentrations are in keeping with previous findings in severe and moderate HHcy [58,59]. Together, these findings argue for changes in the SAM/SAH ratio as being associated with the phospholipid dysregulation in CBSD patients and related to Hcy levels on SAM/SAH levels regardless of the underlying genetic defect. SAH accumulation inhibits PEMT, and SAH-mediated impairment of PEMT is linked to hepatic steatosis [60-62]. The concept that hepatic steatosis is common in CBSD patients [63] has been challenged [64]. In the present setting, CAP evaluation documents liver steatosis in 6 out of the 11 CBSD patients, the latter patients having a significantly lower PC/PE ratio than those

without steatosis. In addition, we found a significant association between liver steatosis and PE/PC ratio. The association between diminished PC concentrations and hepatic steatosis has been also documented in dogs [65] and in rats [66]. Lack of fibrosis in our cohort hampered the possibility to perform needle biopsy and to quantitatively and functionally evaluate hepatic PEMT in CBSD patients [67]. However, in a transgenic model (HO mice) that expresses very low levels of CBS and high plasma concentrations of Hcy and SAH, a post-translational repression of PEMT that inversely correlates with the scale of liver steatosis (but not fibrosis) is present, together with upregulation and down-regulation of phospholipid species and SAM/ SAH ratios in keeping with the ones reported in the present report on patients with CBSD [68]. Hepatic steatosis is associated with enhanced cardiovascular risk, and often co-exists with major risk factors of atherosclerosis, *e.g.* diabetes mellitus, obesity, hyperlipidemia [69]. The information that liver steatosis is associated with a significantly lower PC/PE ratio in CBSD patients (***Fig. 7***) is new and deserves further evaluation. Compared to CTRL, plasma from CBSD patients had a higher medium and long-chain PUFA content in PE and LPE species, and depletion of PC and LPC species containing DHA. Hepatic lipid composition is key for PUFA delivery to plasma and peripheral tissues [70]. In the PUFA scenario, DHA is the most common omega-3 fatty acid in human diet and acts on the platelet membrane to reduce platelet aggregation and thromboxane formation. Indeed, it competes with arachidonic acid for the biosynthesis of prostaglandins and thromboxane, playing key roles in cellular hemostasis, platelets activation and endothelium integrity [71,72]. The relevance of the present findings as to the propensity of CBSD patients to venous and arterial thrombosis is matter of our present investigation. Likewise, it remains to be determined whether major lipid changes in this setting might be reversed by changing the PUFA ratio in plasma [73] as suggested by a recent report in the area [74]. A major limitation of the present study is the relatively small sample size. However, based on birth incidence detected by new-born screening, rate of

consanguinity, and founder effect and/or estimates from clinically ascertained patients, the mean prevalence of CBSD worldwide is 1:335,000 (ranges 1:1800- 1:900,000) [75], thus CBSD can be considered a rare disease. To reduce the risk of false positive results and focus on the top differences in this cohort, patients were matched for age and sex with CTRL, and only those differences in lipids classes that potentially discriminate at most CBSD patients from CTRL we considered statistically significant. The present lipidomic approach first provides evidence of a novel biochemical abnormality in CBSD patients consisting in depletion of PC and LPC species containing DHA and accumulation of PUFA in PE and LPE species. Changes in plasma SAM and SAH concentrations are associated with such phospholipid dysregulation, arguing for an impaired PEMT-mediated PE→PC conversion in CBSD patients. Our results provide the rationale for testing new directions to elucidate mechanism(s) underlying key clinical features and unsolved questions in homocystinuria and suggest innovative strategies to counteract the tendency to thrombosis and the poor cardiovascular outcome in this clinical setting. With respect to the latter, the role of hepatic steatosis [76] deserves to be deeply investigated in CBSD patients.

Figure 8 The homocysteine pathway and the biosynthesis of PC and PE in the human body. BHMT: betaine homocysteine methyl transferase; CBS: cystathionine β synthase; CTH: cystathionine γ-lyase; DMG: dimethylglycine; Hcy: homocysteine; L-Cys: L-cysteine; Methyl THF: Methyl tetrahydrofolate; MET: methionine; MS: methionine synthase; PC: phosphatidylcholine; PE: phosphatidylethanolamine SAH: S-adenosylhomocysteine; SAM: Sadenosylmethionine; THF: tetrahydrofolate.



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