Propionic Acidemia, Methylmalonic Acidemia, and cblC Defect: Comparison of Untargeted Metabolomic Profiles

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Article

Propionic Acidemia, Methylmalonic Acidemia, and cblC Defect: Comparison of Untargeted Metabolomic Profiles

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Abstract: Methylmalonic acidemia (MMA), propionic acidemia (PA), and cobalamin C deficiency (cblC) share a defect in propionic acid metabolism. In addition, cblC is also involved in the process of homocysteine remethylation. These three diseases produce various phenotypes and complex downstream metabolic effects. In this study we used an untargeted metabolomics approach to investigate the biochemical differences and the possible connections with the pathophysiology of each disease. Untargeted urine metabolomic profiles of 21 patients (7 MMA, 7 PA, 7 cblC) were identified through statistical analysis ($p<0.05$; $log2FC > |1|$) and then used for the annotation. Annotated features were associated with different metabolic pathways potentially involved in the diseases development. Comparative statistic showed markedly different metabolomic profiles between MMA, PA and cblC, highlighting characteristic species for each disease. The most affected pathways were related to the metabolism of organic acids (MMA, PA, cblC), amino acids (MMA, PA, cblC), glycine and its conjugates (PA), transsulfuration pathway (cblC), oxidative processes (cblC) and neurosteroid hormones (cblC). The untargeted metabolomics study highlighted the presence of significant differences between the three diseases, pointing to the most relevant contrast of cblC profile compared to MMA and PA. Some new biomarkers are proposed for PA, while novel data regarding the alterations of steroid hormone profiles and biomarkers of oxidative stress were obtained for cblC disease. The elevation of neurosteroids in cblC may indicate a potential connection with the development of ocular and neuronal deterioration.

Keywords: methylmalonic acidemia; propionic acidemia; cblC deficiency; untargeted metabolomics

1. Introduction

Propionic academia (PA) and methylmalonic academia (MMA) belong to the category of "classic" organic acidemias, a group of inherited diseases that result from deficient activity of enzymes involved in the metabolism of propionate, whose precursors are the amino acids valine, isoleucine, threonine, methionine, along with odd chain fatty acids, cholesterol side chains, and propionate produced by the gut flora [1]. PA is caused by mutations in PCCA and PCCB genes, coding for the two subunits of propionyl-CoA carboxylase, while MMA is caused by mutations in MUT gene coding for the synthesis of methylmalonyl-CoA mutase [1]. A third disease, Cobalamin C defect (cblC), due to mutations in MMACHC gene, which encodes for a protein involved in intracellular vitamin B12 metabolism, impairs the synthesis of adenosylcobalamin and methylcobalamin, the cofactors of methylmalonyl-CoA mutase and of methionine synthase [2]. These three diseases, sharing a deficient propionate metabolism, produce various phenotypes and complex downstream metabolic effects. Clinically, PA and MMA can present with recurrent attacks of metabolic decompensation, mainly characterized by acidosis, hyperammonemia, ketosis and elevated blood lactate. Acute neurological symptoms are common and can range from mild encephalopathy to metabolic strokes involving the basal ganglia [1]. Chronic complications of both diseases include failure to thrive, developmental delay, movement disorder, deafness, and optic

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atrophy [1]. PA can additionally present with cardiomyopathy and QT prolongation while MMA is also associated with a chronic kidney disease leading to progressive renal failure [1]. Patients with cblC present a major involvement of central nervous system (CNS) with neurocognitive impairment and develop a multisystem disease with progressive visual dysfunction, atypical hemolytic-uremic syndrome, and pulmonary hypertension [3].

Biochemically, PA, MMA and cblC accumulate in body fluids "primary" metabolites, which derive from the disruptions of metabolic pathways. These include propionyl-carnitine, 3-hydroxypropionic acid, and 2-methylcitric acid, all deriving from the un-metabolized propionyl-CoA [4]. As a consequence of the deficient activity of methylmalonyl-CoA mutase, MMA and cblC share the "primary" accumulation of methylmalonic acid, while the defect of methionine synthase in cblC cause the accumulation of homocysteine and the reduced synthesis of methionine [2]. Changes in in the levels of biomarkers, such as 2-methylcitric acid, propionylcarnitine and methylmalonic acid have demonstrated clinical relevance in patients with PA, MMA [4, 5]. Moreover, the elevated homocysteine in cblC causes a diffuse macroangiopathic damage, which is responsible for many of the clinical manifestation [3]. Abnormal circulating levels of ammonia, lactic acid, glycine, glutamine, FGF21, which represent the "secondary" impact of the underlying genetic defect on metabolic and cellular pathways, are detectable in PA and MMA and only very rarely in cblC [3-5].

These abnormalities, detectable in patient's body fluids through targeted metabolomics analyses, represent the biochemical hallmarks which characterize the three disorders and have been validated for diagnosis and to evaluate the response to therapeutic interventions, including liver or liver and kidney transplantation in MMA [2, 4, 6, 7]. However, there is still a need to determine whether the discovery of other metabolites can provide novel insight to understand the mechanisms that underlie the pathophysiology and aberrant processes of diseases in relation to the clinical manifestations. To this purpose, thanks to rapid developments in bio-informatics and analytical technologies, it has been possible to expand metabolomics from targeted to untargeted analyses. The evolution of analytical platforms based on high-resolution mass spectrometry (HR-UHPLC-MS/MS), has allowed the simultaneous measurement of the broadest range of metabolites without a priori knowledge of the metabolome, in contrast with targeted metabolomics, in which metabolites are analyzed on the bases of a priori information [8]. The power of the untargeted metabolomics therefore lies in its potential to broaden our understanding of disease biochemistry, identifying new biomarkers, and providing more precise disease categorization in relation to treatment modalities and responses.

Considering the relatively recent entry of untargeted metabolomics in the area of inborn errors of metabolism [9], only a few studies have so far addressed MMA and PA, showing the impairment of serine, thiol and propionate metabolism [10, 11], dysregulation of the tricarboxylic acid cycle [12] and changes of some known biomarkers in relation to patient's status [13]. As for cblC, untargeted metabolomic was applied as a screening tool with the aim of selecting diagnostic biomarkers and confirm methylmalonic and 2-methylcitric acids, propionylcarnitine, C4DC-carnitine, homocysteine and methionine as the most reliable in discriminating cblC from other metabolic diseases [14, 15].

In this work we studied and compared the untargeted urinary metabolomics profiles of MMA, PA and cblC to determine expanded diseases-specific biomarker signatures, aiming at providing novel insights on the biological and pathophysiological differences between the three diseases.

2. Experimental Design

2.1. Patients

Urine samples were obtained from 21 patients attending the Division of Metabolic Diseases at the Bambino Gesù Children's Hospital in Rome. All patients had confirmed biochemical and molecular diagnosis of MMA, PA or cblC. Each disease group was composed of 7 patients of both sex, the age was matched among groups spanning the following ranges in years: MMA 0.3-22; PA 0.4-20.4; cblC 0.4-17.1. All patients within the same disease group were treated with standard therapy, which included natural protein restriction, supplemented with amino acid mixture free of offending

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precursors (MMA, PA), oral carnitine (MMA, PA, cblC), parenteral hydroxocobalamin (cblC) and oral betaine (cblC) therapy. Clinically, all patients presented with a severe phenotype, characterized by early onset of the disease and by a multiorgan involvement. Two out of 3 patients identified by newborn screening were already symptomatic. At the time of urine collection for untageted metabolomics analysis, no one of the 21 patients was subjected to organ transplantation or dialysis. During disease course, patients PA 1, PA 3, PA 5, PA 6, MMA 2, MMA 5 were treated with liver transplantation, and patients MMA 1, MMA 4 and MMA 7 with combined liver and kidney transplantation, that further confirms the severity of disease phenotype. Table 1 describes more in detail the clinical phenotypes of each patients.

Table 1. Clinical data and symptomatic of the patients.

Patient	Onset	Cardiac involvement	Pancreatitis	Renal disease	Visual dysfunction	Epilepsy	Abnormal MRI	Developmental delay/ intellectual disablity	Deafness	Age at urine sampling (years)
PA 1	Neonatal	-	-	-	-	-	+	+	+	2.1
PA 2	7 months	+	-	-	-	-	+	+	-	9.9
PA 3	3 months	+	-	-	-	-	-	-	-	20.4
PA 4	Neonatal	+	-	-	-	-	-	+/-	+	15.2
PA 5	Neonatal (NBS symptomatic)	-	-	-	-	-	-	+/-	-	0.4
PA 6	Neonatal (NBS symptomatic)	-	-	-	-	-	+	+	-	0.8
PA 7	Neonatal	+	-	-	-	-	-	-	-	7.0
MMA 1	Neonatal	-	-	++	-	-	+/-	-	-	0.6
MMA 2	Neonatal	-	+	+/-	-	-	+/-	+	+	1.2
MMA 3	Neonatal	-	-	-	-	-	++	+	NA	10.6
MMA 4	17 months	-	-	++	+	-	-	-	-	20.6
MMA 5	NBS (asymptomatic)	-	-	-	-	-	-	-	-	0.3
MMA 6	Neonatal (NBS symptomatic)	-	-	-	-	-	-	-	NA	3.7
MMA 7	6 months	-	+	++	+	-	++	-	-	22.2
cblC 1	Neonatal (NBS symptomatic)	+	-	-	+	-	-	+/-	-	0.4
cblC 2	Neonatal	-	-	+	+	+	+	+	-	3.7
cblC 3	2 months	+	-	+	+	+	++	++	-	2.8
cblC 4	Neonatal	-	-	-	+	+	+	+	-	3.3

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cblC 5	5 months	-	-	-	+	++	+	++	-	13.7
cblC 6	Neonatal	-	-	-/+	+	-	+	+/-	-	13.2
cblC 7	3 months	++	-	+	-	+	+/-	+/-	-	17.1

++ severe; + present; +/- mild; - absent; NA – non avaliable; *long-term follow-up, urine samples for metabolomics analysis were collected before transplantation.

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The research was not a clinical trial and the procedure was in accordance with the Declaration of Helsinki of the World Medical Association. The study has been approved by the Ethical Committee of the Bambino Gesù Children's Hospital (n. 2119_OPBG_2020) and the informed consent was obtained from all patients/caregivers.

3. Procedure

3.1. Sample Preparation

Urine samples were collected during scheduled follow-up visits and immediately frozen at -80 0C prior to analysis. Sample preparation consisted in mixing 200 µL of urine with 200 µL of organic solvent (acetonitrile and methanol 3:1) for HILIC analysis or with 200 µL of water for reverse phase UHPLC experiment. After 20 sec vortex and 10 min centrifugation at 13.000 rpm the supernatants were transferred into UHPLC vails for injection. A pooled sample was prepared by mixing equal aliquots of all urine samples. This pool underwent the same preparation steps and was used for data dependent MS2 acquisitions and as Quality controls (QC).

3.2. UHPLC and HRMS

Ultra-high-performance chromatographic separation was executed on Ultimate 3000 (Thermo Fisher Scientific, Waltham, MA, USA) system by reverse phase and HILIC conditions. Reverse phase experiment was carried on Luna Omega C18 column (100 x 2.1 mm, 1.6 µm PS, Phenomenex, Torrance, CA, USA) with mobile phases A composed of water with 0.1 % formic acid and B composed of acetonitrile with 0.1 % formic acid. HILIC chromatographic separation was run on Accucore-150-Amide column (100 x 2.1 mm, 2.6 µm, Thermo Fisher Scientific, Waltham, MA, USA) with mobile phase A (95 % acetonitrile, 0.1 % acetic acid, 10 mM ammonium acetate) and B (50 % acetonitrile, 0.1 % acetic acid, 10 mM ammonium acetate). Chromatographic gradients and instrument sets are available in Tables 1,2 in Supplement materials. All samples were injected three times as technical replicates in random order. QC samples for peak area and RT correction were analyzed repetitively during the whole batch after

The UHPLC system was coupled with a mass spectrometer Q Exactive (Thermo Fisher Scientific, Waltham, MA USA) scanning in full MS and dd-MS2 modes. Full scan MS experiment was set to 70-1050 m/z range with 140,000 resolution, 3x106 AGC target and maximum IT 100 ms. dd-MS2 mode, used only for pool sample acquisition, was set at 17,500 resolution, 2x104 AGC target, maximum IT 35 ms, TopN 12, isolation window 1.5 m/z and dynamic exclusion 2.5 sec. The features have been fragmented with three different normalized collision energy set to 20, 35 and 50 V. Source ionization parameters were: spray voltage ± 3.5 kV; capillary temperature 380 °C; sheath gas 60; auxiliary gas 20; S-Lens level 50. The data were acquired in both positive and negative modes independently. Calibration was performed before each analysis using calibration mixes (Piercenet, Thermo Fisher Scientific, Rockford, IL USA) to minimize the ppm error of the intact mass.

3.3. Metabolomic Data Analysis

The raw data were processed on Compound Discoverer 3.3 (Thermo Fisher Scientific, Waltham, MA USA) software for deconvolution, compound identification and statistical analysis. To account for variations in sample dilution factors, the acquired data were normalized by Constant Sum algorithm integrated in Compound Discoverer 3.3. The time-dependent batch effects were corrected with the use of QC samples, implemented to build the linear regression model describing the variability of chromatographic peak area of each m/z at different acquisition time. After alignment and grouping of chromatographic peaks with the same molecular weight and retention time, the background features were removed from result table. Subsequently, the features were statistically analyzed to identify characteristic compound discriminating each disease. Principal component analysis (PCA) and volcano plots were applied to highlight the differences between groups. Data annotation was made searching matching compounds through home-made library (90 metabolites), ChemSpider, Metabolika and mzCloud databases. All proposed metabolite structures were verified

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by the FISH score, which is assigned by comparing experimental and in silico fragmentation patterns. PCA and box-and-whisker plots were generated by Compound Discoverer 3.3, heatmaps and pathway enrichment analysis were made with MetaboAnalyst 6.0 web platform. Pearson correlation coefficients (r) were calculated by Excel Microsoft 2016.

4. Results

After spectra deconvolution, alignment, QC and background corrections, the data from HILIC, in both polarity modes, and from reverse phase (C18), only in negative mode, were considered for subsequent elaborations. The data from reverse phase C18 positive mode were discarded because did not fulfill the Compound Discoverer 3.3 quality criteria.

Untargeted metabolomic data showed the net differences between the three diseases. The PCA plots obtained from the different chromatographic conditions demonstrated coherent picture pointing to the most characteristic metabolomic profile of cblC group that resulted in the highest separation from other diseases (PC1 > 24.6 %), while the profiles of MMA and PA were more similar between them (PC2 > 9.5 %), Figure 1.

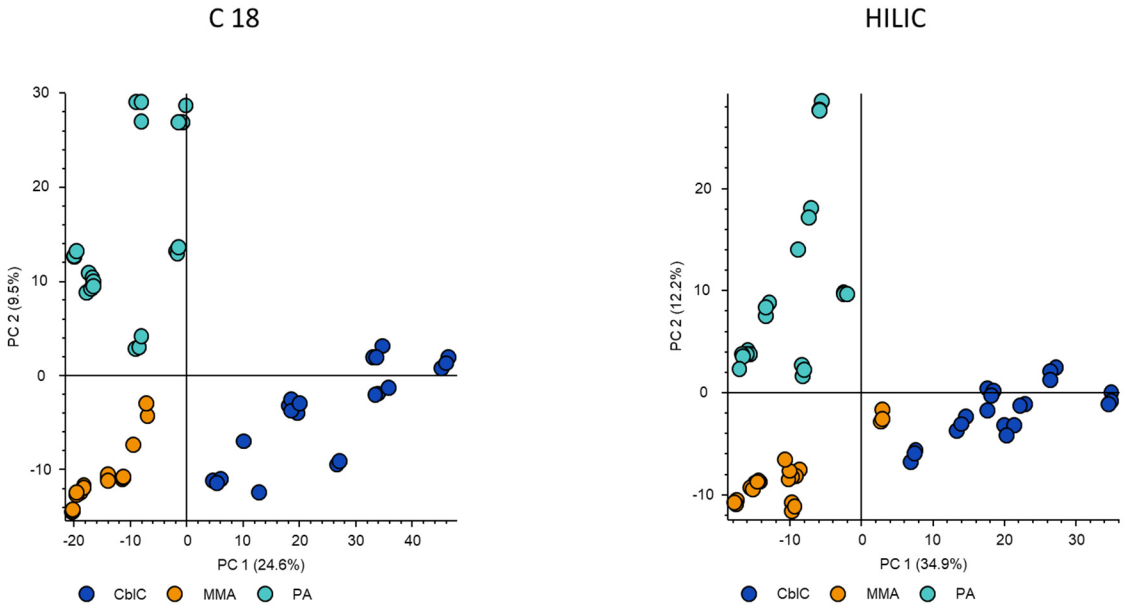

Figure 1. PCA obtained from untargeted metabolomic data acquired by C18 and HILIC columns in negative ionization mode.

4.1. Significantly Different Metabolites

Volcano plots, set to $p < 0.05$, $\log_2FC > |1|$, revealed 530 characteristic features for MMA, 450 for PA and 1700 for cblC. Only a part of characteristic features was annotated. Additionally, we selected a group of features with molecular formula CxHyNO, likely belonging to the same chemical class, that were significantly increased in PA. The most relevant findings are summarized in Table 2, while the whole list of putative annotations is given in the Supplementary Table 3.

Table 2. Significantly different metabolites in cblC, MMA and PA and their relative fold-change values.

Metabolite	cblC/MMA	cblC/PA	PA/MMA	KEGG Pathway
Organic acids and ketones				
Citric and isocitric acids	3.3**	2.5**	ns	• Tricarboxylic acid cycle (TCA)

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				• Glyoxylate and dicarboxylate metabolsim • Alanine, aspartate and glutamate metabolism
2-butanone	0.5*	0.13***	3.9***	
Fumaric acid	0.3*	0.2***	ns	• TCA • Arginine biosynthesis • Pyruvate metabolism • Alanine, aspartate and glutamate metabolism
Malic acid	0.2*	0.08***	ns	• TCA • Glyoxylate and dicarboxylate metabolism • Pyruvate metabolism
Methylmalonic acid	0.06***	19***	0.003***	• Valine, leucine and isoleucine degradation • Propanoate metabolism • Pyrimidine metabolism
MW 74.036 (propionic acid isobar)	0.05***	12***	0.004***	
2-methylcitric acid	ns	0.2***	4.3***	• Propanoate metabolism
2-methyl-3-hydroxy-valeric acid	ns	0.3***	5.4***	
3-oxo-valeric acid	ns	0.21***	3.4***	
2-methyl-3-oxo-valeric acid	ns	0.09**	8.3**	
3-pentanone	ns	0.06***	10***	
Glycine and carnitine conjugates				
C4DC-carnitine	2.1*	348***	0.006***	
Propionylcarnitine	0.06***	0.05***	ns	
Propionylglycine	ns	0.01***	60***	
Propionylcarinitine glycine conjugate	ns	0.05**	23***	
Tiglylglycine	ns	0.07***	21***	
Butyrylglycine	ns	0.2**	11***	
Amino acids and peptides				
Threonine	83***	36***	ns	• Glycine, serine and threonine metabolism • Valine, leucine and isoleucine biosynthesis
Butyl-α-aspartyl-allothreoninate	28***	20***	ns	
Isoleucine	24**	50***	ns	• Valine, leucine and isoleucine biosynthesis • Valine, leucine and isoleucine degradation

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Dimethylglycine	21***	36***	ns	• Glycine, serine and threonine metabolism
Isoleucylalanine	15***	3.4**	ns	
Aspartylphenylalanine	9.6*	23.4**	ns	
Glutamylisoleucine	9.4***	19*	ns	
Prolylproline	8.0**	4.4*	ns	
Valylvaline	7.7**	3.6*	ns	
Glycylglycyl-alanyl-2-methylalanine	6.7*	11**	ns	
Isoleucylvaline	5.0**	4.9**	ns	
Lysine	4.1**	ns	ns	• Biotin metabolism
Citrulline	3.9**	2.3*	ns	• Arginine biosynthesis
Valine	3.8***	2.4***	1.6*	• Valine, leucine and isoleucine biosynthesis • Valine, leucine and isoleucine degradation • Pantothenate and CoA biosynthesis
Methionine	ns	0.07**	16**	• Cysteine and methionine metabolism • 2-oxocarboxylic acid
Glycine	ns	0.04***	33***	• Glycine, serine and threonine metabolism • Glyoxylate and dicarboxylate metabolism • Glutathione metabolism • Lipoic acid metabolism • Porphyrine metabolism
Transsulfuration pathway metabolites				
Cystathionine	78***	20***	3.8**	• Glycine, serine and threonine metabolism • Cysteine and methionine metabolism
Homocysteine	37***	39***	ns	• Cysteine and methionine metabolism
Thiosulfuric acid	17*	18**	ns	• Sulfur metabolism
Cysteine	5.7**	3.5*	ns	• Glycine, serine and threonine metabolism • Pantothenate and CoA biosynthesis • Glutathione metabolism • Thiamine metabolism
2-hydroxybutyric acid	5.0***	3.2*	ns	• Propanate metabolism

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Sulfuric acid	4.2***	2.5**	ns	• Sulfur metabolism
Biomarkers of oxidative damage				
Thioproline	96***	45***	2.1*	
α-tocopheronic acid	14**	5.9*	ns	
α-TLHQ glucuronide	8.0**	4.7*	ns	
α-TLHQ sulfate	6.8**	3.3*	ns	
α-tocopheronic acid sulfate	5.9*	4.9*	ns	
CxHyNO features				
C4 H9 N O	ns	0.02***	48***	
C6 H9 N O	ns	0.05***	21***	
C6 H11 N O	ns	0.06***	30***	
C6 H13 N O	ns	0.04***	44***	
C7 H11 N O	ns	0.01***	72***	
C7 H13 N O	ns	0.01***	142***	

* – p-value < 0.05; ** – p-value < 0.01; *** – p-value < 0.001; ns – non-significant.

The significantly increased metabolites in each disease were associated with known KEGG metabolic pathways. Figure 2 demonstrates the main pathways involved in three metabolic diseases.

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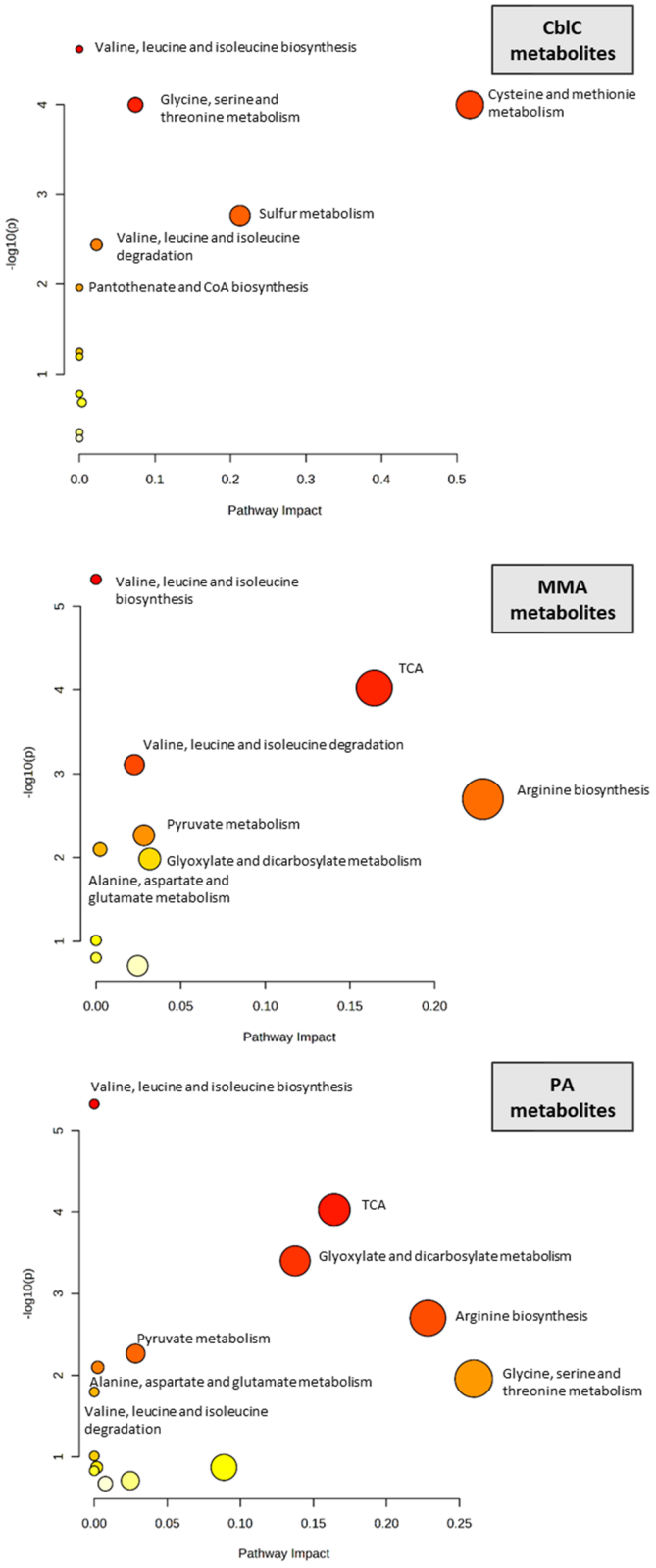

Figure 2. Pathway enrichment analysis. Node size (pathway impact) corresponds to the relative number and position of matched metabolites in the selected pathway, colours varying from yellow to red mean the different levels of significance. The named pathways include only those with $p<0.05$.

All diseases shared the involvement of valine, leucine and isoleucine biosynthesis and degradation pathways. CblC and PA had a common involvement of glycine, serine and threonine

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metabolism related-pathway, while PA and MMA shared pathways related to Krebs cycle (TCA), pyruvate metabolism, glyoxylate and dicarboxylate metabolism, arginine biosynthesis, along with alanine, aspartate and glutamate metabolism. The main pathways involved in cblC and not in MMA and PA include cysteine and methionine metabolism, sulfur metabolism, pantothenate and CoA biosynthesis.

4.1.1. Organic Acids and Ketones

The untargeted metabolic study confirmed the presence of known primary disease related biomarkers, revealing a significant increase of methylmalonic acid in MMA and in cblC compared to PA, while methylcitric acid was significantly increased in PA compared to MMA and cblC (Table 2, Figure 3). The most discriminant organic acids and ketones in PA were 3-pentanone, 2-methyl-3-oxo-valeric acid, 2-methyl-3-hydroxy-valeric acid, 2-butanone and 3-oxo-valeric acid (Table 2, Figure 3). Moreover, 3-hydroxypropionic acid was also increased in PA but, due to the low quality of chromatographic peak integration caused by the presence of coeluting isomers, it was not considered for quantitative estimation.

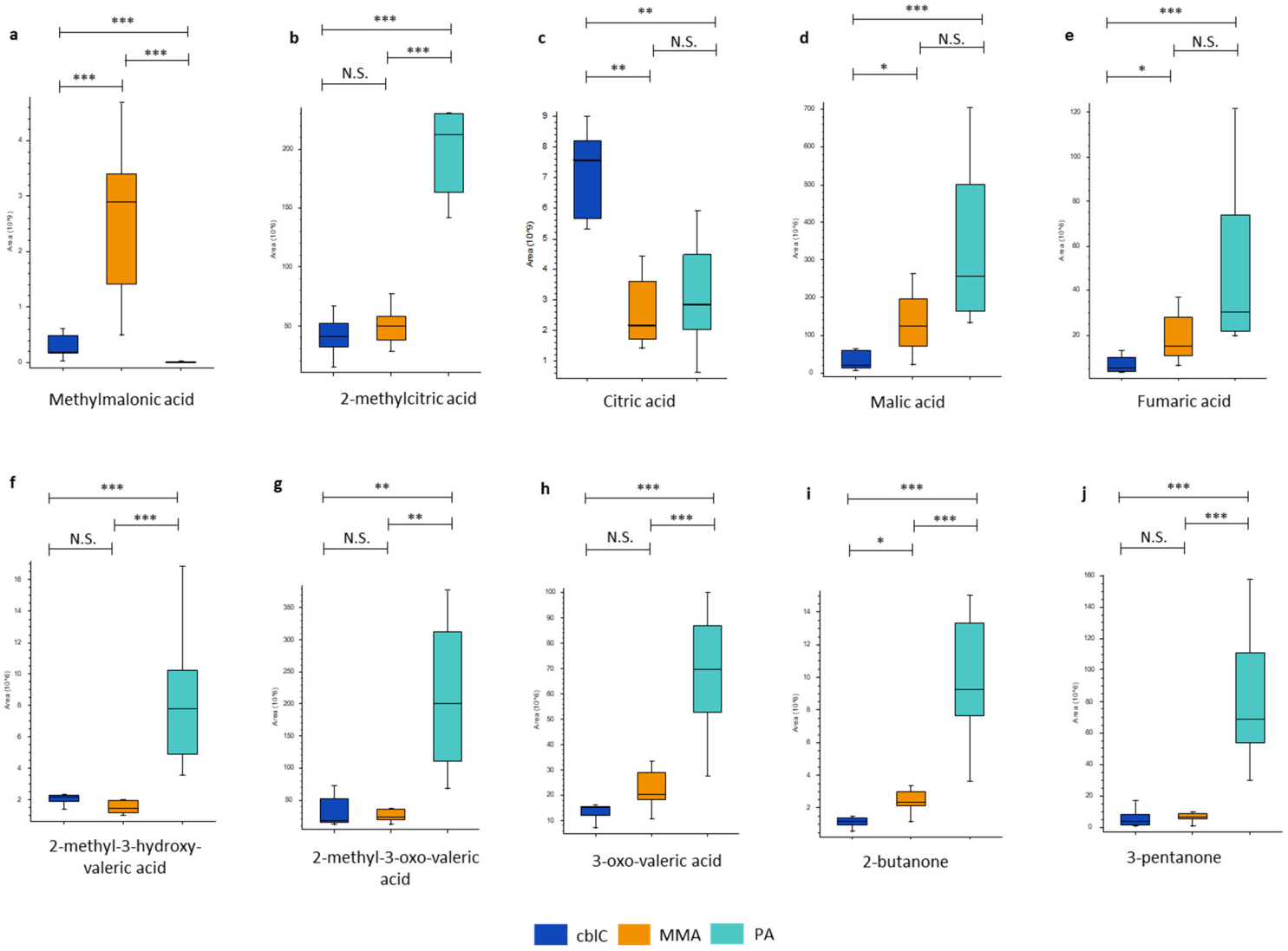

Figure 3. Organic acids and ketones significantly changed between three acidemias. * – p-value < 0.05; ** – p-value < 0.01; *** – p-value < 0.001; N.S. – non-significant.

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The profiles of organic acids related to Krebs cycle showed significantly reduced levels of citric and isocitric acids (not separated chromatographically) along with increased malic and fumaric acids in MMA and PA compared to cblC, Figure 3. Other organic acids related to TCA cycle did not show differences between the three disease groups.

A feature with m/z and fragmentation pattern putatively corresponding to propionic acid, was highly increased in MMA and in cblC group respect to PA (Table 2, Supplementary Figure 1a). However, its levels were strongly correlated across all samples ($r > 0.9$) with methylmalonic acids (Supplement Figure 1b), allowing to consider this compound as propionic acid derived from in-source fragmentation of methylmalonic acid.

4.1.2. Amino Acids and Peptides

Several free amino acids and small peptides demonstrated significantly different levels between the three disease groups (Table 2, Figure 4).

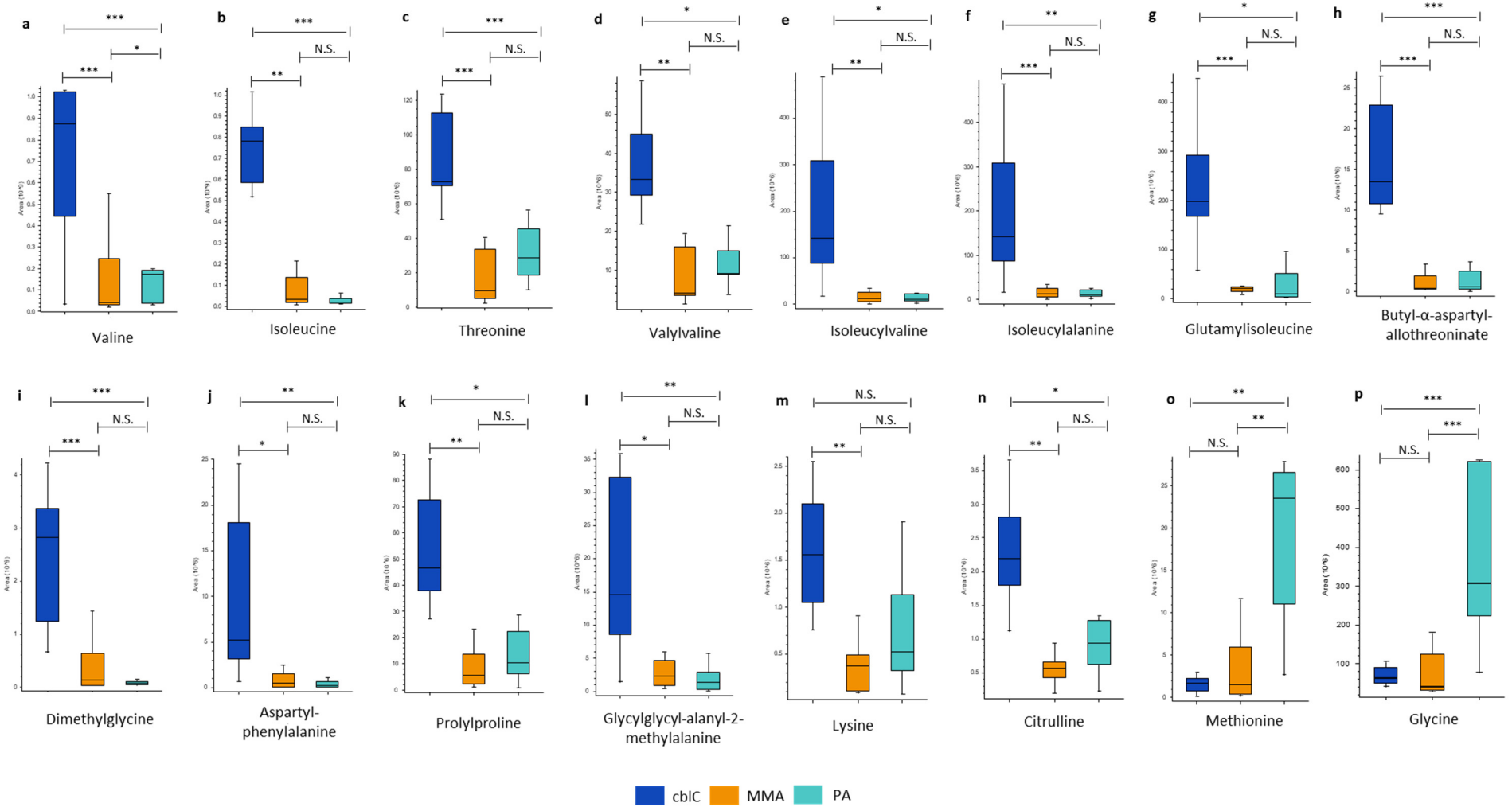

Figure 4. Significantly changed amino acids and small peptides. * – p-value < 0.05; ** – p-value < 0.01; *** – p-value < 0.001; N.S. – non-significantCoherently with dietary restrictions, MMA and PA showed reduced levels of valine, isoleucine and threonine, and of di- and tri-peptides composed of branched-chain amino acids and/or threonine (i.e. isoleucylalanine, glutamylisoleucine, valylvaline, isoleucylvaline, butyl-alpha-aspartyl-allothreoninate). In addition, other amino acids and peptides (i.e. dimethylglycine, aspartylphenylalanine, prolylproline, glycylglycyl-alanyl-2-methylalanine, lysine and citrulline) were relatively higher in cblC compared to MMA and PA.

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Methionine was significantly reduced in cblC, and also in MMA, compared to PA (Figure 4o). Glycine level in PA was increased up to 30- and 20-fold compared to MMA and cblC, respectively (Figure 4p). The levels of glycine and methionine have no significant differences between MMA and cblC.

4.1.3. Glycine and Carnitine Conjugates

Three glycine esters, propionylglycine, butyrylglycine and tiglylglycine were markedly increased in PA compared to MMA and cblC (Table 1, Figure 5).

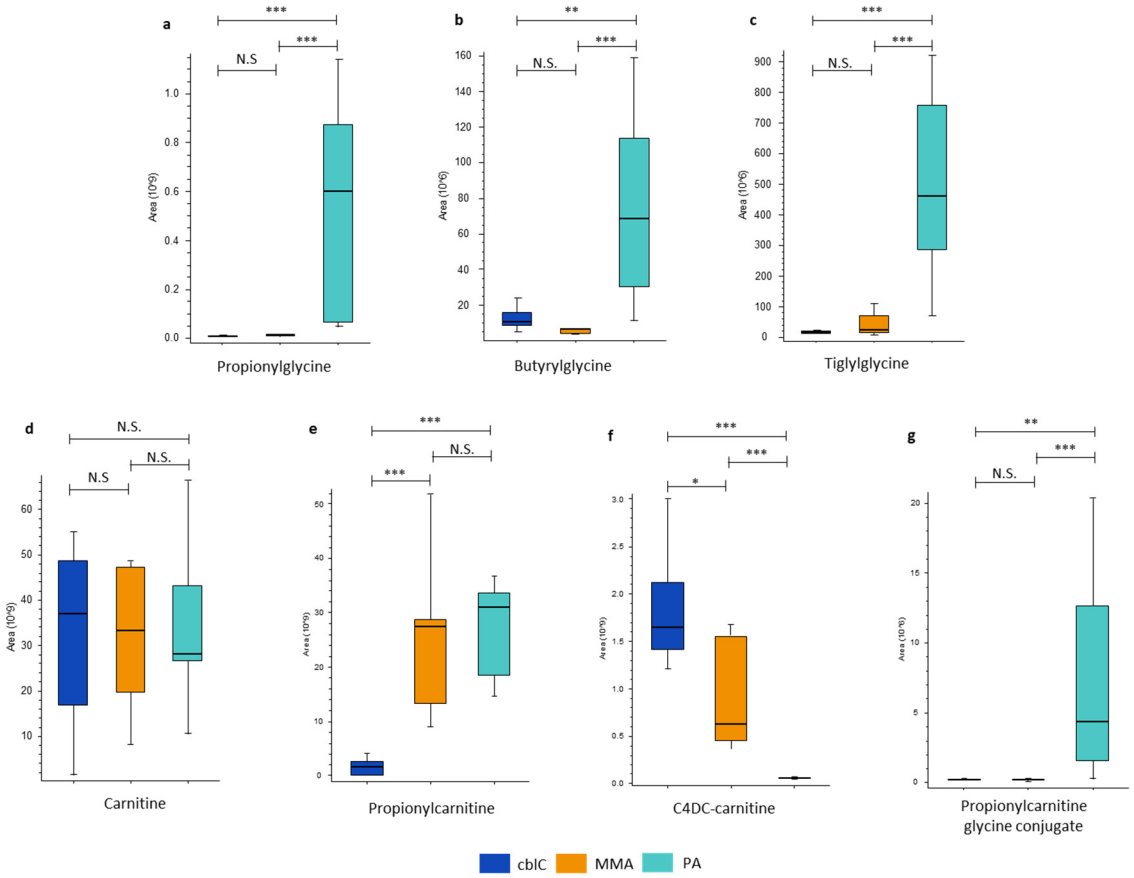

Figure 5. Glycine and carnitine conjugates. * – p-value < 0.05; ** – p-value < 0.01; *** – p-value < 0.001; N.S. – non-significant.

In particular, propionylglycine was increased up to 100-fold and 60-fold respect to cblC and MMA. Propionylglycine ($r > 0.9$) and butyrylglycine ($r > 0.7$) were positively correlated with glycine, and between them ($r > 0.7$) The levels of other glycine conjugates like N-acetylglycine, glutamylglycine, isovalerylglycine, isocaprolylglycine, hexanoylglycine and hippuric acid had no differences between the three disease groups.

Different from glycine and its esters, free carnitine had similar levels in cblC, PA and MMA, while propionylcarnitine was equally increased in PA and MMA compared to cblC (Table 1, Figure 5e). C4DC-carnitine levels in cblC exceeded those in MMA and PA (Table 1, Figure 5f). Interestingly, a metabolite corresponding to propionylcarnitine conjugated to glycine was selectively elevated only in PA and not in MMA nor cblC (Table 1, Figure 5g). The proposed putative structure of this double carnitine/glycine conjugate produced the in-silico fragmentation pattern corresponding to experimental MS/MS data at 93.7 % (Supplementary Figure 2a,b).

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4.1.4. Transsulfuration Pathway Metabolites

Several metabolites belonging to transsulfuration pathway, including homocysteine, cystathionine, cysteine, sulfuric and tyhiosulfuric acids were selectively increased in cblC (Table 1, Figure 6).

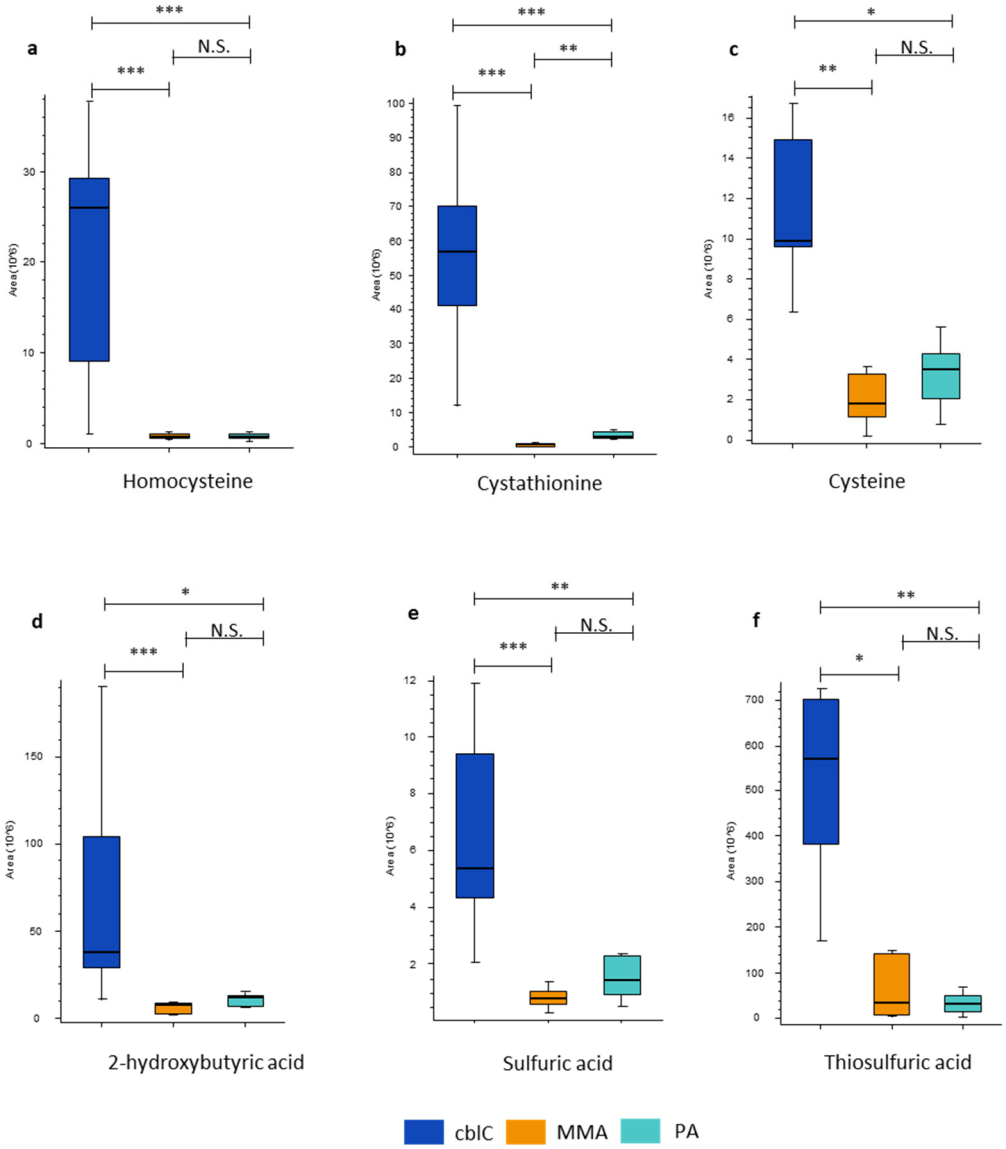

Figure 6. Transsulfuration pathway metabolites increased in cblC. * – p-value < 0.05; ** – p-value < 0.01; *** – p-value < 0.001; N.S. – non-significant.

Among these metabolites, cystathionine and homocysteine demonstrated the most relevant and significant increase, reaching 78-fold and 39-fold changes compared to the levels in MMA and PA. Moreover, 2-hydroxybutyric acid, putatively derived from alpha-ketobutyric acid, was also elevated in cblC.

4.1.5. Biomarkers of Oxidative Damage

Urine obtained from cblC patients showed a major increase of compounds related to oxidative stress, Figure 7.

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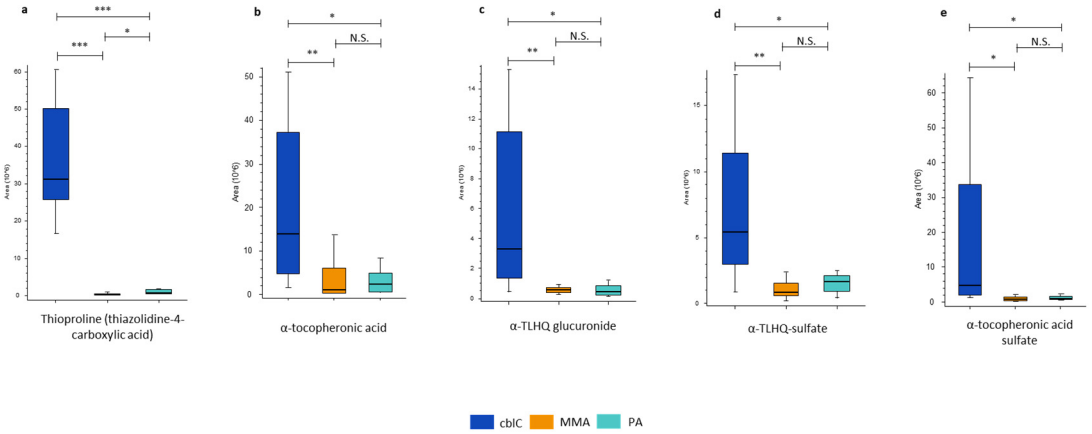

Figure 7. Oxidative stress biomarkers characteristic of cblC disease. * – p-value < 0.05; ** – p-value < 0.01; *** – p-value < 0.001; N.S. – non-significant.

These included thioproline, a sulfur-containing non-proteinogenic amino acid, formed by the condensation between cysteine and formaldehyde, increased up to 96-fold and 45-fold compared to MMA and PA, respectively (Table 1, Figure 7a). Other metabolites significantly elevated in cblC group, derive from oxidation of vitamin E by peroxyl radicals. These included α-tocopheronic acid, α-tocopheronolactone hydroquinone (α-TLHQ) glucuronide, α-TLHQ sulfate and α-tocopheronic acid sulfate (Table 1, Figure 7b-e).

4.1.6. Steroid Hormones

CblC group was characterized by the increased level of steroid compounds with androstane or pregnane core. The putative annotations corresponding to derivatives of dehydroepiandrosterone (DHEA), pregnanolone, and others are given in Table 3.

Table 3. Steroid hormones increased in cblC group.

IUPAC or common names of putatively annotated structures	ChemSpider ID	Hierarchical class	Ratio (CblC)/(MMA)	Ratio (CblC)/(PA)
3β,8xi,9xi,14xi,17β -Androst-5-ene-3,17-diyl bis hydrogen sulfate (androstendiol disulfate)	21403154	androstane	114.5	32.9
Androsterone sulfate	140383	androstane	97.4	37.1
16α- hydroxydehydroepiandrosterone-3-sulfate (OH-DHEAS)	24850136	androstane	53.4	15.9
4-androstene-3β,17β -diol disulfate (androstendiol disulfate)	58163615	androstane	34.3	20.0
Dehydroepiandrosterone sulfate (DHEAS)	12074	androstane	24.7	15.4
16,17-Dihydroxyandrost-5-en-3-yl hydrogen sulfate	133127	androstane	20.1	29.4
9-Hydroxyandrosta-1,4-diene-3,17-dione	211114	androstane	8.2	3.8
3β -(Phenylacetoxy)pregna-5-ene-20-one	58539760	pregnane	40.4	33.7
3,20-Dioxopregn-4-en-17-yl 4-methylbenzoate	9180030	pregnane	34.9	28.5

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3α,5α-20-Oxopregnan-3-yl beta-D-glucopyranosiduronic acid (3α-allopregnanolone 3β-D-glucuronide)	68025990	pregnane	19.2	7.5

Six out of 10 compounds were in sulfate form and one was a glucuronic acid conjugate. The urinary levels of these hormones in cblC group largely exceeded those in MMA and in PA, Figure 8.

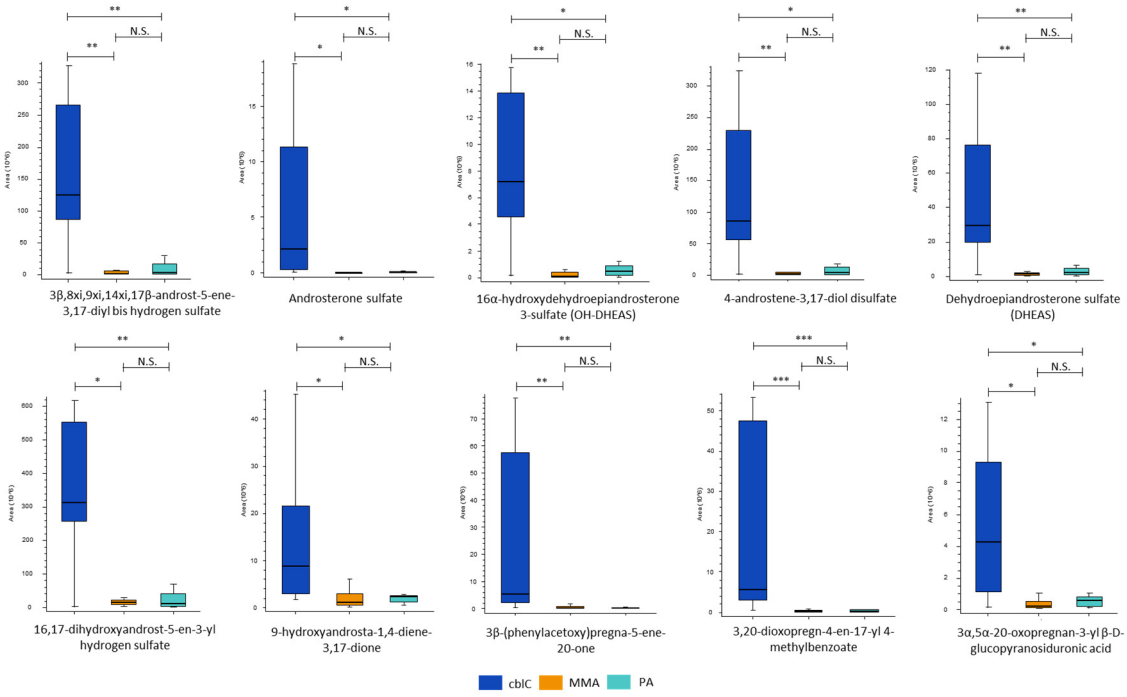

Figure 8. The levels of steroid class hormones with putative annotations significantly increased in cblC. * – p-value < 0.05; ** – p-value < 0.01; *** – p-value < 0.001; N.S. – non-significant.

4.1.7. Non-Annotated Metabolites CxHyNO in PA

A group of six non-annotated metabolites, having CxHyNO elemental composition with carbon atoms length C4-C7, were significantly increased in the group of PA. C7H13NO was the most increased one, up to 142-fold change respect to MMA and cblC. Other features were C7H11NO, C6H13NO, C6H11NO, C6H9NO and C4H9NO (Table 1, Figure 9).

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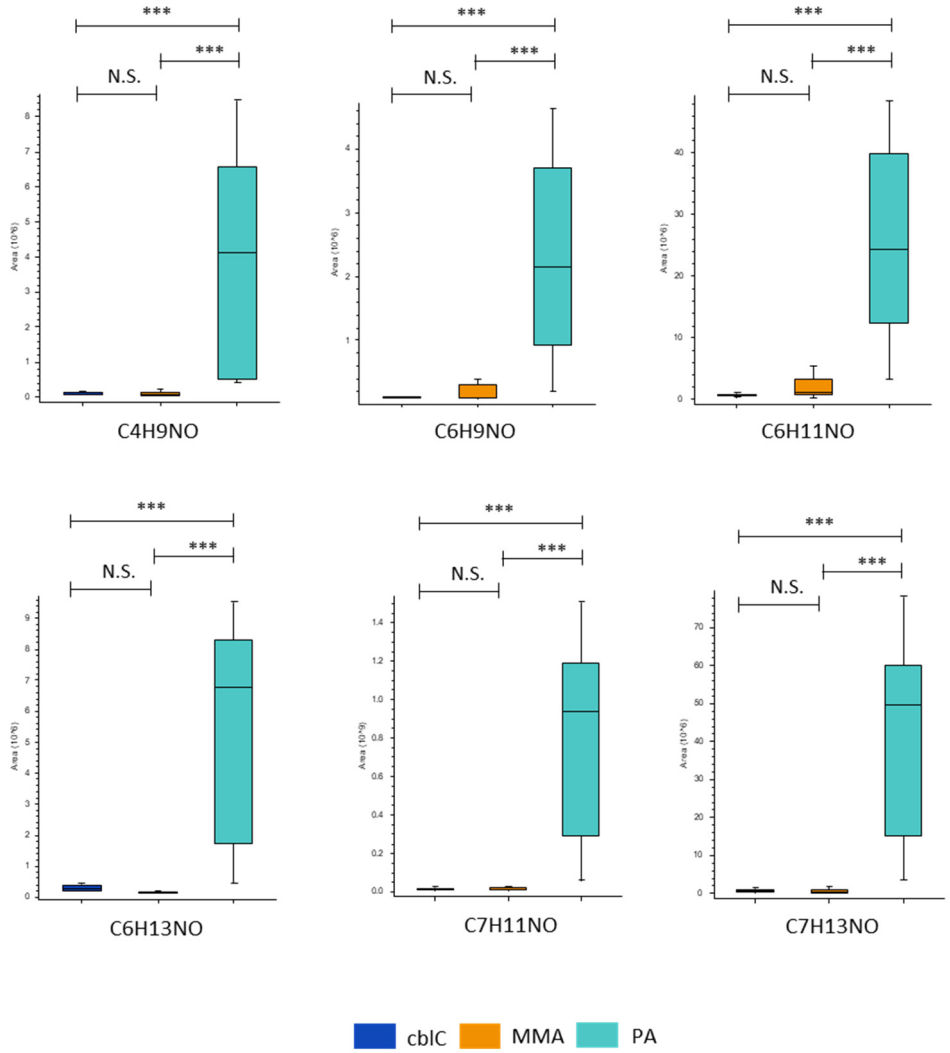

Figure 9. The CxHyNO features significantly increased in PA. * – p-value < 0.05; ** – p-value < 0.01; *** – p-value < 0.001; N.S. – non-significant.

4.1.8. The Most Dysregulated Non-Annotated Features

The 25 most up- and down-regulated non-annotated features for each disease ($p < 0.001$) are listed in Supplementary tables 5-7. Heatmaps with hierarchical clustering of these features are shown in Figure 10.

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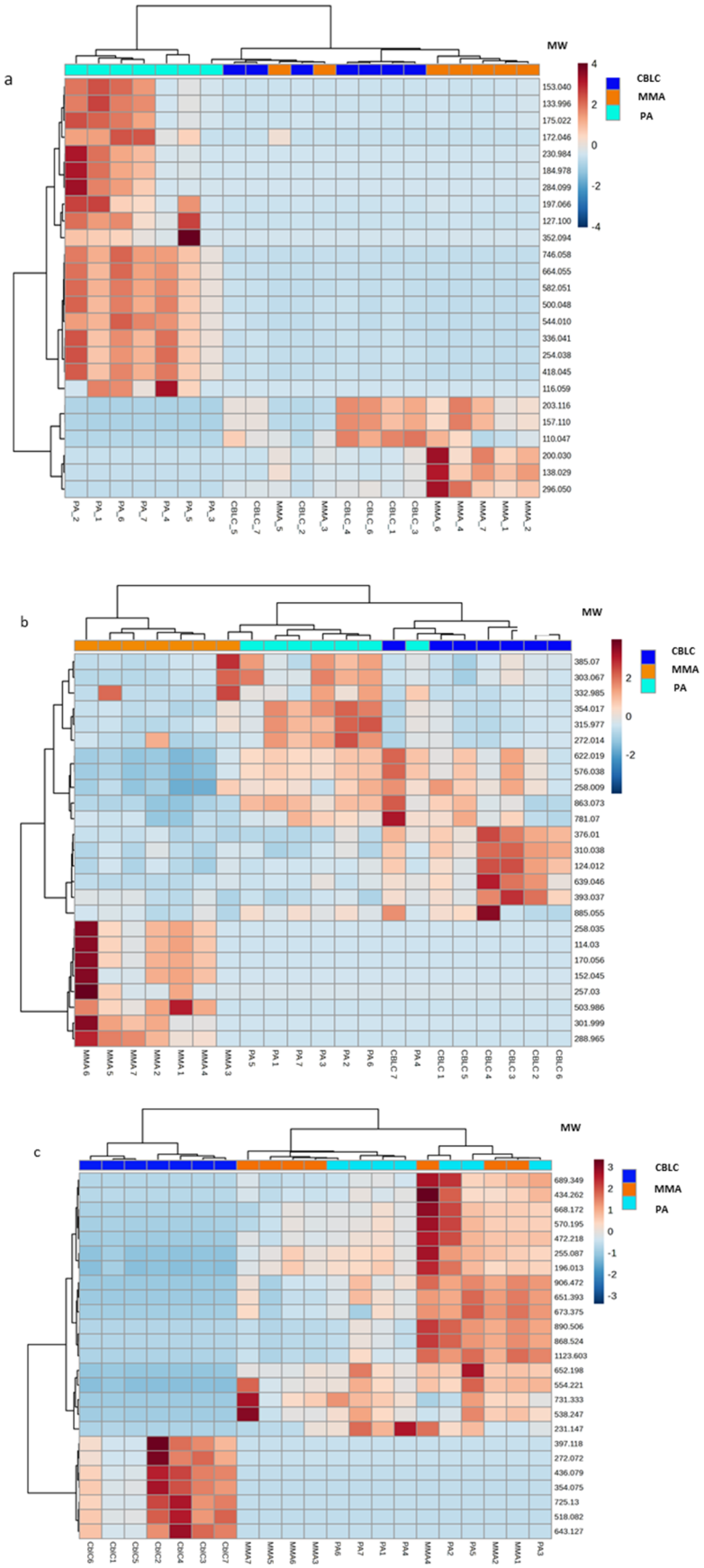

Figure 10. (**a**) The heatmap based on the 25 most up-/down-regulated features in PA ($p < 0.001$); (**b**) the heatmap based on the 25 most up-/down-regulated features in MMA ($p < 0.001$); (**c**) the heatmap based on the 25 most up-/down-regulated features in cblC ($p < 0.001$).

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PA group was characterized by elevated levels of 19 features with molecular weights 116-746 Da, 8 of them coherently increased across all PA samples. Six features with lower molecular weights (110-296 Da) were decreased in all in PA samples respect to cblC and MMA group (Figure 10a). MMA group was characterized by lower levels of 17 species with molecular weights 124-885 Da. A single feature, with molecular weight 288.965 Da, was constantly increased in all MMA samples (Figure 10b). In cblC group 18 out of 25 features were significantly decreased compared to MMA and PA. These features generally had high molecular weights (up to 1123 Da) and demonstrated constantly decreased levels across all cblC samples (Figure 10c).

5. Discussion

In this study we performed a comparison of untargeted metabolic profiles in the urine of three organic acidemias, MMA, PA and cblC, sharing the involvement of propionate pathway. Our results highlighted a clear distinction between the three diseases, showing up the relative differences in the levels of known biomarkers and described new characteristic metabolites allowing a more profound knowledge and understanding of (biochemical) phenotypic disease characteristics and pathophysiology.

Pathway enrichment analysis indicated the involvement of branched chain amino acid metabolism in all diseases, pointing at major perturbations affecting the Krebs cycle in PA and MMA. The specificities of cblC, contributing to the highest separation from other diseases, were related not only to sulfur metabolism, but also to other novel pathways.

The relative quantification in untargeted metabolomics applied to urine requires normalization of data. To this purpose, different normalization strategies were developed to reduce the measurement errors [16-18], but no consensus has been so far reached on the most suitable approach for urine standardization [17, 19, 20]. The normalization to creatinine is the most used way in targeted metabolomic analyses [21, 22], however MMA and PA may affect creatine metabolism, leading to a reduced creatinine formation [23-26]. For this reason, for our study we applied the Constant Sum algorithm as post-acquisition normalization.

By targeted metabolomics analyses, the main shared biomarkers of PA, MMA and cblC are 3-hydroxypropionic and 2-methylcitric acids [2-5]. Coherently, the results of our untargeted metabolomic study in urine showed a major increase of 2-methylcitric acid in PA compared to MMA and cblC. As for methylmalonic acid, shared by MMA and cblC [27], it was predominantly increased in MMA. Unexpectedly, and similar to a previous report [13], we identified a metabolite with molecular weight and fragmentation pattern corresponding to propionic acid, highly increased in MMA and cblC but not in PA. However, the levels of this compound and methylmalonic acid were strongly correlated across the samples and a more accurate evaluation allowed to demonstrate that the presumed "propionic acid" was an artifact, derived from the in-source fragmentation of methylmalonic acid molecule due to the loss of a CO_2 [28, 29].

As for other biomarkers, a selective increase of 2-methyl-3-oxo-valeric acid, 2-methyl-3-hydroxy-valeric acid, 3-oxo-valeric acid, 3-pentanone and 2-butanone was detected in PA. Oxo-valeric acid-related metabolites derive from the self-condensation of propionyl-CoA, 3-pentanone may result from the decarboxylation of 2-methyl-3-oxo-valeric acid [30], while 2-butanone is a product of methylacetoacetate decarboxylation in the catabolic pathway of isoleucine [31]. From a clinical perspective, the presence of alpha- and beta-ketoaciduria may explains the frequent observation in PA patients of positive ketones in urine as detected by commercial strips (testing for acetoacetate through a nitroprusside reaction), which does not correspond the presence of ketonemia (elevated beta-hydroxybutyrate levels in blood (personal observation of Carlo Dionisi-Vici).

Focusing on TCA metabolites, the citrate levels in MMA and PA were significantly reduced compared to cblC, likely reflecting the major accumulation of propionyl-CoA that competes with acetyl-CoA as substrate for citrate synthase, generating 2-methylcitrate after condensation with oxaloacetate [32]. The accumulation of 2-methylcitrate and of others "toxic" compounds derived from methylmalonyl-CoA and propionyl-CoA metabolism, may impact on TCA cycle and on urea cycle, leading to a shortage of TCA intermediates, to a mitochondrial dysfunction and to hyperammonemia

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[32, 33]. In accordance, increase of malate, fumarate and lower citrulline levels were found in PA and MMA samples. Perturbations of these metabolites, which are known to be related with mitochondrial dysfunction [34-37], may therefore reflect and explain a greater mitochondrial involvement in PA and MMA compared to cblC.

MMA and PA are both characterized by an abnormal glycine metabolism [38]. Our study, while confirming this pertubation, showed that the levels of glycine in PA were about 30 times higher than those seen in MMA. This difference highlights that different (patho)mechanisms may lead to increased glycine in the two diseases. In MMA, a widespread posttranslational modification, methylmalonylation, inhibits the glycine cleavage pathway [38]. In PA, as shown by ^{13}C-flux experiments in PCCA-null cells, an increased de novo synthesis of glycine occurs from serine [10]. Remarkably, we found that the increased glycine levels in PA significantly correlated with the increase of its esters, propionylglycine and butyrylglycine, further confirming that PA affects glycine metabolism to a most extent than it occurs in MMA. Taken together, all these findings seem to indicate that the increased glycine synthesis in PA may be considered as a "defense" mechanism, aimed to buffer the toxic accumulation of propionyl-CoA, through the action of glycine-N-acylase, as seen in isovaleric acidemia [39-41]. Consistently, we identified a novel glycine conjugate of propionyl-carnitine that was significantly elevated in PA.

The analysis of carnitine derivatives, reflecting the buffering action of carnitine-acetyltransferase on mitochondrial excess of propionyl-CoA, showed less pronounced differences than those seen for glycine esters. Propionylcarnitine was equally increased in PA and MMA in comparison to cblC, while C4DC-carnitine, combining methylmalonyl- and succinyl-carnitines [42], was higher in cblC than in MMA.

The study showed significant differences in the excretion of amino acids and small peptides in cblC in comparison to PA and MMA. The relative elevation in cblC of the majority of these compounds is likely the consequence of the dietary differences between the three diseases. All cblC patients were on a free diet, while PA and MMA patients were treated with a protein restricted diet to limit the intake of propionic acid precursors [6]. Accordingly, the levels of isoleucine, valine, threonine and of related small peptides were significantly reduced in PA and MMA. On the other hand, a significant reduction of methionine was detectd in cblC, reflecting the deficient activity of methionine synthase as a consequence of the reduced availability of its cofactor methylcobalamin [2]. The high increase of dimethylglycine in cblC samples is consistent with the activation of the folate-indipendent homocysteine remethylation pathway through the action of betaine therapy [2].

As expected, increased levels of metabolites linked to transsulfuration pathway were exclusively detected in cblC group, as also reported by previous researches [43, 44]. The elevated levels of thiosulfate and sulfate ions indicate that, among the pathways of homocysteine and cysteine degradation, the most active would be the one related to H_2S production, which is further oxidized to sulfates [45, 46].

A common characteristic of MMA, PA and cblC is an elevated oxidative stress. Previous studies demonstrated the presence of reactive oxygen species and oxidative damage in these diseases [47-51]. In addition, cblC lymphocytes were characterized by an imbalance between reduced/oxidized forms of glutathione [52]. The comparison of metabolomic profiles between the three diseases allowed to distinguish some specific pathways of oxidative stress that were characteristic only for cblC, rather than for MMA and PA. We identified oxidized forms of vitamin E metabolites, such as α-TLHQ, α-tocopheronic acid and their conjugates. These molecules have been associated with multiple diseases characterized by lipid peroxidation and were defined as the urinary biomarkers of oxidative stress [53-55]. Another specific indicator of oxidative stress damage uniquely detected in cblC patients was thioproline, which is related to formaldehyde metabolism. Thioproline is generated in oxidant-exposed cells and exerts a protective role through sacrificial oxidation and nitrile ion trapping [56]. Being a product of formaldehyde and cysteine conjugation, thioproline may be therefore considered as a scavenger of free endogenous formaldehyde released during demethylation, protein oxidation, and from the metabolism of methanol, methylamine and adrenaline [56]. The excess of formaldehyde in cblC may also be related to the oxidative breakdown

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of tetrahydrofolate not employed for the folate-dependent methylation of homocysteine [57, 58]. Beside the conjugation with cysteine, other ways for formaldehyde buffering exist in mammals, such as the enzymatic conversion through glutathione, and the adduction with histidine to form spinacine [59]. However, in our study thioproline was the unique formaldehyde adduct increased in cblC, while the levels of spinacine did not differ between the three diseases. Furthemore, we did not find features corresponding to glutathione-related products, such as S-hydroxymethyl-glutathione or S-formyl-glutathione [59]. Therefore, the elevated levels of thioproline in cblC may be explained by a preferential buffering of formaldehyde through its condensation with cysteine. The potential role of formaldehyde and thioproline in the pathophysiology of cblC disease is worthy of further elucidations. Interestingly, both formaldehyde and thioproline accumulation have been associated with neurodegeneration [59, 60].

Untargeted metabolomic study revealed the sharp increase of steroid hormones in the urine of cblC patients. The majority of these compounds belongs to the class of "neurosteroids", which can also have androgenic or estrogenic activity. Neurosteroids, synthetized in brain or in peripheral nervous system, can be transformed in sulfate or glucuronide conjugates and then excreted in urine. The role of neurosteroids is very complex and lies in the ability to act as neuro-hormones, neuromodulators, neurotransmitters and as neurotrophic factors. They regulate arousal, sleep, learning, social and sexual behaviors and are implicated in several (patho)physiological conditions, such as pain, stress, anxiety, depression, but also in autism, neurodegenerative diseases (multiple sclerosis, Alzheimer's disease, Parkinson's disease), in Niemann-Pick type C disease, and in traumatic brain injury [61-70]. The presence of increased levels of neurosteroids in cblC patients may therefore indicate the potential connection with the peculiar neurological manifestations in this disease [2].

Furthermore, involvement of steroid hormones in cblC may also regard the characteristic disease-associated visual deterioration [2]. Specific receptors have been found on the visual system, including lacrimal and meibomian glands, conjunctiva, cornea, lens, retina, and choroid [71]. Numerous studies on ocular disease have revealed the net connection between steroid hormones and retinal, corneal and optic nerve disorders such as cataract, dry eye disease, glaucoma, age-related macular degeneration, retinitis pigmentosa and central serous chorioretinopathy [71-75]. It has also been reported that both estrogens and androgens affect retinal thickness, and their elevated levels were found in patients with chronic central serous chorioretinopathy [72, 73, 76].

Our study identified a potentially novel class of PA biomarkers characterized by the molecular formula CxHyNO with carbon lengths C4-C7. The similar elemental composition and close elution times may indicate the presence of the same functional group(s) derived from coherent metabolic pathways. These metabolites may belong to the class of amides (from decarboxylation of acylglycines [77]), aminoaldehydes (from oxidative degradation of polyamines [78]), nitroso-compounds, and/or acyl-pyrrolidine/pyrrolines.

It is well known that the annotation of features detected by untargeted metabolomics represents the biggest challenge. In this study, we selected the 25 most significantly increased/decreased non-annotated features for each disease and reported their accurate molecular weights to allow their confirmation and characterization in future metabolomic researches on MMA, PA or cblC.

The most relevant results of our study, demonstrating the different and common aspects in metabolomic profiles of cblC, MMA and PA acidemias, are summarized in Figure 11.

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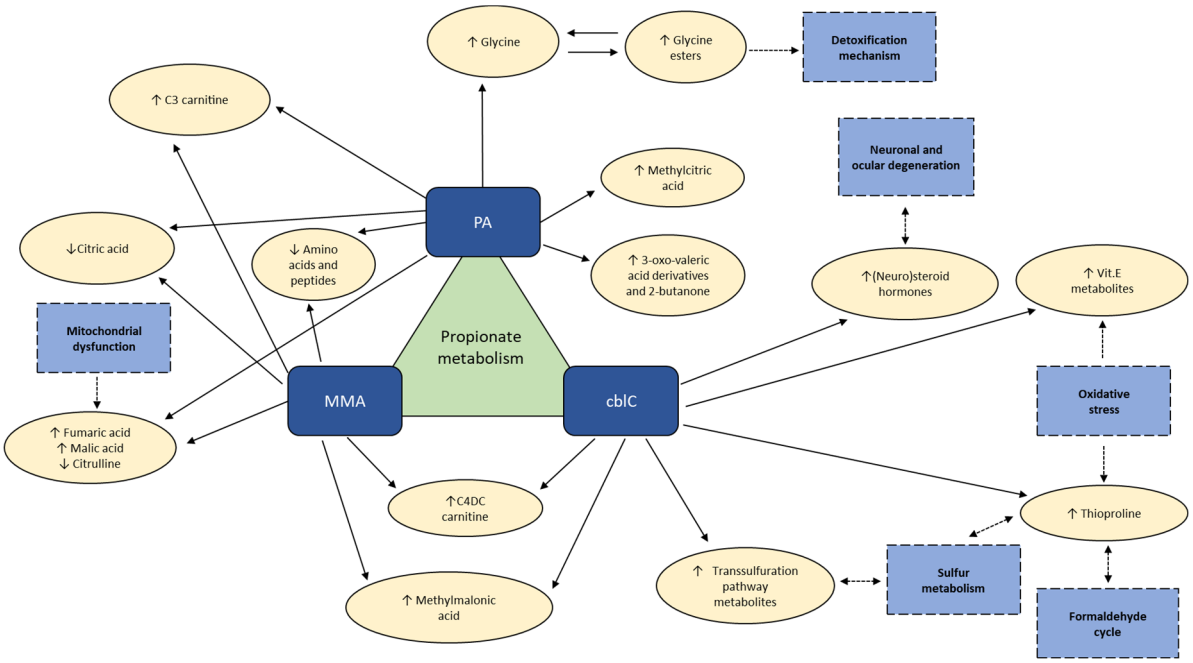

Figure 11. The most relevant findings from relative comparison of metabolomic profiles in PA, MMA and cblC. ↑ (↓) – metabolites significantly increased (decreased) respect to other two acidemias. Dashed boxes – physiological processes involving significantly changed metabolites.

6. Conclusion

Our study allowed the discovery of new disease-related biomarkers, expanding knowledge on novel potential pathophysiological mechanisms, demonstrating the importance of exploring through untargeted metabolomics the biochemical profiles of metabolic disease.

Author Contributions: Anna Sidorina: Conceptualization; Methodology; Investigation; Acquisition of data, Data curation and interpretation, Writing - original draft; Giulio Catesini: Conceptualization; Methodology; Acquisition of data, Data interpretation, Writing - review & editing; Elisa Sacchetti: Visualization; Investigation; Cristiano Rizzo: Conceptualization, Supervision, Writing - review & editing; Carlo Dionisi-Vici: Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethical Committee of the Bambino Gesù Children's Hospital n. 2119_OPBG_2020.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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