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Article

Circulating Metabolites as Biomarkers of Disease and Pharmacoresistance in Patients with Mesial Temporal Lobe Epilepsy

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Abstract: A major challenge in the clinical management of patients with mesial temporal lobe epilepsy (MTLE) is identifying those who do not respond to antiseizure medication (ASM), allowing for the timely pursuit of alternative treatments, such as epilepsy surgery. Here, we investigated changes in plasma metabolites as biomarkers of pharmacoresistance in patients with MTLE. Furthermore, we used the metabolomics data to gain insights into the mechanisms underlying MTLE and response to ASM. We performed an untargeted metabolomic method using magnetic resonance spectroscopy and multi- and univariate statistical analyses to compare data obtained from plasma samples of 28 patients with MTLE compared to 28 controls. The patients were further divided according to response to ASM: 20 patients were refractory to treatment, and eight were responsive to ASM. We only included patients using carbamazepine in combination with clobazam. We compared the group of patients with controls and found that the profiles of glucose ($p = 0.01$), saturated lipids ($p = 0.0002$), isoleucine ($p = 0.0001$), β-hydroxybutyrate ($p = 0.0003$), and proline ($p = 0.02$) were different in patients compared to controls ($p < 0.05$). In addition, lipoproteins ($p = 0.05$), lactate ($p = 0.05$), glucose ($p = 0.05$), unsaturated lipids ($p = 0.05$), isoleucine ($p = 0.05$), and proline ($p = 0.05$), could discriminate between the two groups of patients classified according to response to ASM. The identified metabolites were linked to different biological pathways related to cell energy metabolism, and pathways linked to inflammatory processes and the modulation of neurotransmitter release and activity in MTLE. In contrast, we found that pyruvate metabolism may be linked to resistance to ASM. In conclusion, in addition to insights into the mechanisms underlying MTLE and the response to treatment with ASM, our results suggest that plasma metabolites may be used as biomarkers of disease and response to ASM in patients with MTLE. These findings warrant further studies exploring the clinical use of metabolites to assist in decision-making when treating patients with MTLE.

Keywords: metabolomics; antiseizure medication; ^{1}H Nuclear Magnetic Resonance; focal epilepsy; response to treatment

1. Introduction

Epilepsy is a chronic neurological disorder characterized by persistent and long-lasting hyperactivity of groups of neurons, increasing one's propensity to develop epileptic seizures [1,2]. In mesial temporal lobe epilepsy (MTLE), the epileptogenic focus is localized in the medial structures of the temporal lobe, mainly in the hippocampus [3–5]. MTLE is the most common type of focal epilepsy in adults. About 67%–89% of patients with MTLE with hippocampal sclerosis do not respond to currently available antiseizure

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medications (ASMs) [6,7]. In addition to being exposed to the seizures' intrinsic deleterious effects, pharmacoresistant patients experience the severe side effects of the ASM, often prescribed as polytherapy and in high doses [8]. Therefore, a significant challenge in the clinical management of patients with MTLE is identifying those who do not respond to ASM, allowing the pursuit of other types of treatment, such as epilepsy surgery [9,10]. Indeed, recent reports have highlighted the importance of identifying biomarkers for response to treatment in epilepsies, including MTLE [11]. Several hypotheses explain the mechanism of ASM resistance. The current consensus is that this is a multifactorial condition where gene-gene and gene-environment interactions play important roles [11].

Metabolomics based on Nuclear Magnetic Resonance (^{1}H-NMR) is used for complex sample analysis, principally because of the high reproducibility of NMR data, little or no sample preparation, and sample integrity preservation, which is particularly relevant when evaluating rare clinical samples [12, 13]. In addition, NMR-based metabolomics may lead to the identification of changes in biological samples linked to morphological and biochemical alterations associated with disease, thus assisting in early and precise diagnosis. Furthermore, identifying metabolites associated with specific phenotypes can contribute to a better understanding of disease mechanisms [14].

This study investigated the feasibility of using plasma metabolites as disease biomarkers and pharmacoresistance in patients with MTLE. In addition, we used the metabolomics data generated to gain insights into the mechanisms underlying MTLE and response to ASM.

2. Results

2.1. Characteristics of Study Population

We ascertained 28 patients with a mean age of 54 years (ranging from 26 to 70), eight patients were responsive to treatment with ASM, and 20 were classified as refractory to treatment with ASM. The main clinical characteristics of the patients studied are reported in Table 1. We only included patients currently using carbamazepine combined with clobazam. However, all patients considered here as pharmacoresistant have failed several other ASM regimens. None of the patients presented generalized seizures 24 hours before blood collection. Most patients presented signs of hippocampal sclerosis on magnetic resonance image-decreased hippocampus volume in T1 images with increased signal in T2/FLAIR [3]. Age at onset of epilepsy varied from 1 to 30 years old. We also studied samples from 28 controls with a mean age of 49 years. These were adults between the ages of 29 and 63 years.

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Table 1. Main characteristics of the 28 patients with MTLE included in the study. Most patients presented signs of hippocampal sclerosis on magnetic resonance imaging – decreased hippocampus volume in T1 images with increased signal in T2/FLAIR images. **Notes and abbreviations:** MTLE: mesial temporal lobe epilepsy; ID: patient identification; sex: male/female (M/F); age: the age at investigation. LHS: left hippocampal sclerosis; RHA: right hippocampal sclerosis; bilateral: bilateral hippocampal sclerosis.

ID	Sex	Age (years)	Age at onset of epilepsy	Hippocampal abnormalities	Group	Response to treatment with antiseizure medication
1	F	63	15	LHS	MTLE	Refractory
2	M	60	26	Bilateral	MTLE	Refractory
3	F	57	1	LHS	MTLE	Refractory
4	M	59	15	LHS	MTLE	Refractory
5	F	70	17	LHS	MTLE	Refractory
6	M	58	14	RHS	MTLE	Refractory
7	F	50	2	LHS	MTLE	Refractory
8	M	50	19	LHS	MTLE	Refractory
9	F	37	7	LHS	MTLE	Refractory
10	M	26	5	LHS	MTLE	Refractory
11	M	54	10	LHS	MTLE	Refractory
12	F	60	16	RHS	MTLE	Refractory
13	F	26	7	RHS	MTLE	Refractory
14	F	62	23	Bilateral	MTLE	Refractory
15	M	60	20	RHS	MTLE	Refractory
16	M	62	14	LHS	MTLE	Refractory
17	F	61	2	RHS	MTLE	Refractory
18	M	46	1	LHS	MTLE	Refractory
19	F	54	8	LHS	MTLE	Refractory
20	F	51	30	None	MTLE	Refractory
21	F	43	7	LHS	MTLE	Responsive
22	M	45	8	RHS	MTLE	Responsive
23	F	65	3	LHS	MTLE	Responsive
24	M	55	31	RHS	MTLE	Responsive
25	F	58	17	RHS	MTLE	Responsive
26	M	47	19	LHS	MTLE	Responsive
27	F	56	20	LHS	MTLE	Responsive
28	F	70	18	LHS	MTLE	Responsive

2.2. Metabolomic Analysis

Overall, we identified 27 metabolites in the samples studied (Appendix A, Table A1). Additionally, we found different groups of biomolecules, such as lipids, in the plasma of patients and controls. These biomolecules corresponded to different saturations' fatty acids; amino acids such as alanine, isoleucine, leucine, valine, and glutamine; and aromatic amino acids such as tyrosine, histidine, phenylalanine, lactate, and glucose (Figure 1). See Appendix A, Table A1, and Figure A1 for the chemical shift assignments.

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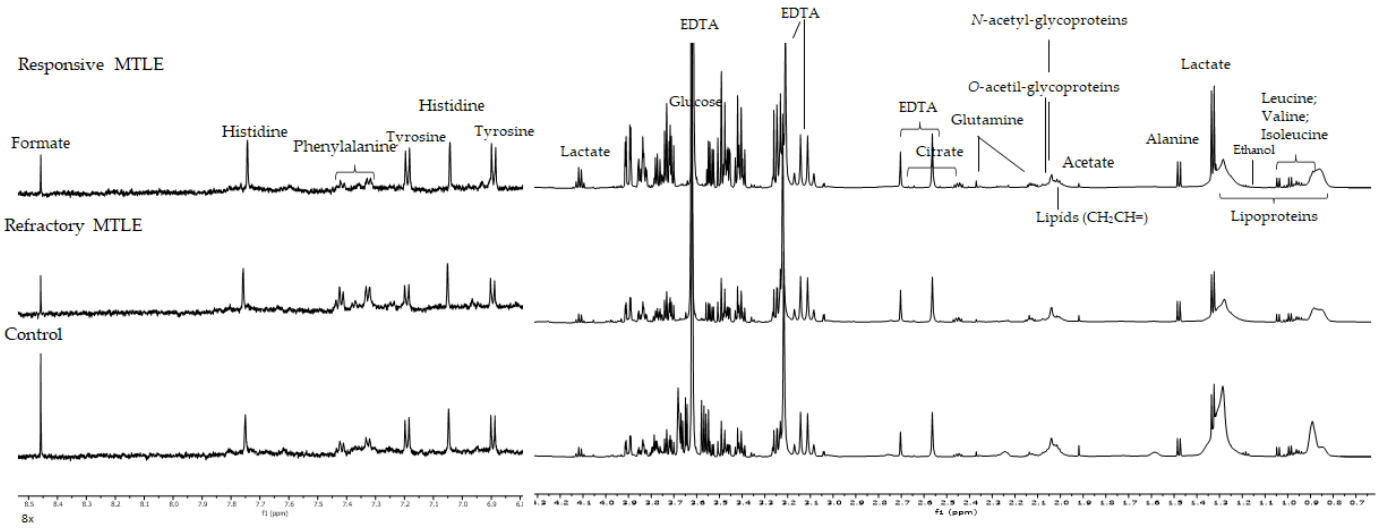

Figure 1. The representative ^{1}H-NMR spectra of plasma samples control, responsive, and refractory MTLE subjects. Spectral regions from 0.5 to 9.0 ppm, acquired using CPMG (cpmgpr1d) pulse sequence, are shown. The regions from 7.0 to 8.5 ppm were zoomed-in (8 x) for better visualization. The regions of D_2O and EDTA were removed (//). The following metabolites were identified: Lipoproteins; Leucine, Valine; Isoleucine; Lactate; Alanine; Acetate; *N*-acetyl-glycoproteins; *O*-acetyl-glycoproteins; Glutamine; Glucose; Tyrosine; Histidine; Phenylalanine; Formate.

The largest variation in the acquired NMR data directions was visualized using the PLS-DA method. The PLS-DA scores plot (Figure 2A) clusters data for the patients and controls and the first principal component (1), explaining 39.1% of the data variation. The PLS-DA model goodness of fit (R^2) value was 0.75, with a prediction (Q^2) of 0.35. The metabolites that discriminate between the groups were identified based on the variables' importance in projection score (VIP score) from the PLS-DA analysis. The first 15 VIPs were combined with univariate analysis such as t-test and fold change (FC) (Table 2). As a result, we identified five significantly altered metabolites when comparing patients with controls: glucose, saturated lipids, isoleucine, β-hydroxybutyrate, and proline (Table 2 and Figure 2C).

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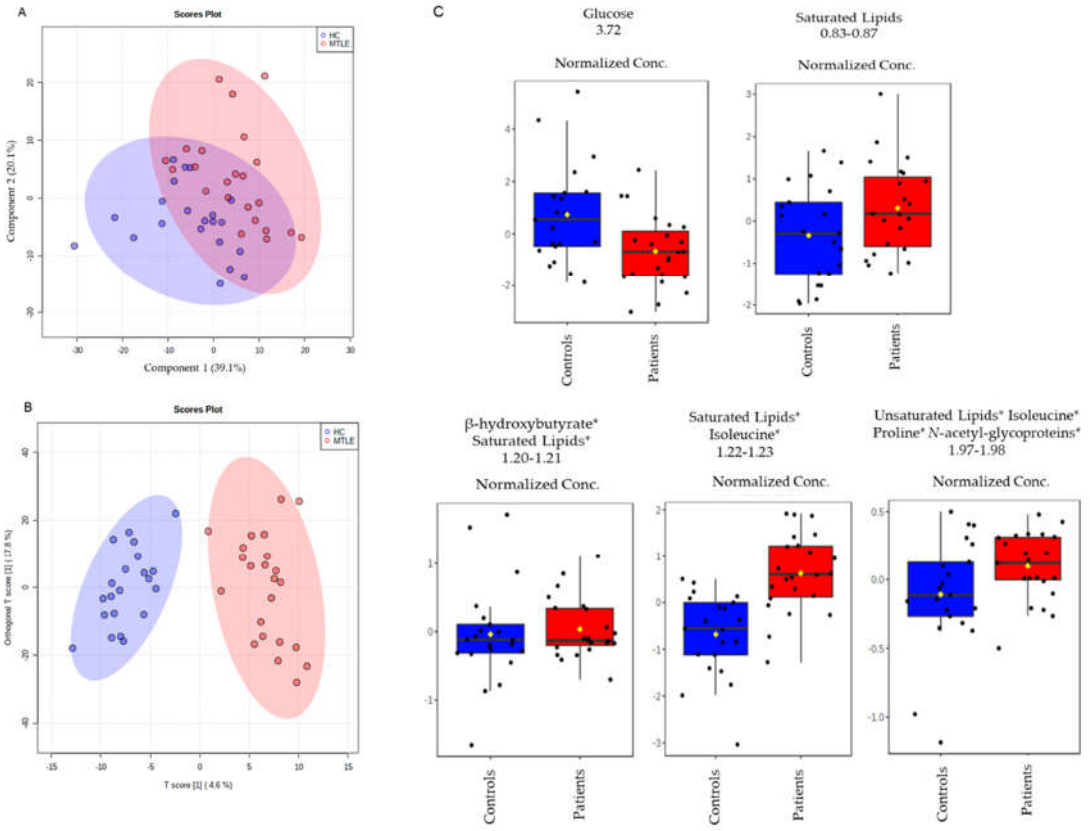

Figure 2. Multivariate analysis of ^{1}H-NMR (CPMG) plasma spectra. (A) PLS-DA analysis with accuracy 73%, R2 0.75, and Q2 0.35. (B) O-PLS-DA analysis. The information about response to treatment with ASM in patients with MTLE was not implemented into the models. (C) Box plots representing the variations of the relative concentrations (measured as peak intensities) of metabolites whose VIP scores > 2 according to PLS-DA results. The black dots represent the metabolite levels in all samples, and the yellow diamond represents the average value. Patients with MTLE (red) and controls (blue).

Table 2. Table showing the metabolites identified in different concentrations and their respective chemical shifts elucidated by the highest VIP values (VIP score). The p-values, -log10(p), calculated from the t-test, FC (Fold change), and log2fold change (logFC) are also shown. **Notes and abbreviations**: multip = multiplicity, where s (singlet), d (doublet), t (triplet), dd (doublet of doublets), m (multiplet), l (broad); assign. = assignment of these signals; * = overlaid signals.

MTLE x Control - VIP score						
Metabolites	**Chemical shift (multip; atrib)**	**Vip Score**	**P value**	***-log10(p)**	**FC**	***log2(FC)**
Glucose	3.68 - 3.78 (m, CH)	5.12	1.00E-02	1.99	0.76	-0.38
Lipids	0.83 - 0.87 (m, CH_3)	5.02	1.67E-04	3.78	1.29	0.37
Isoleucine*; Lipids	1.21 - 1.25 (m, CH_2)n	3.41	1.49E-04	3.82	1.20	0.26
β-hydroxybutyrate* Lipids	1.20 - 1.24 (m, CH_2)n	3.15	2.34E-04	3.63	1.20	0.26
Proline, Isoleucine, and lipids	1.96 - 1.98 (m, CH_2CH=)	2.16	1.92E-02	1.71	1.17	0.23

Furthermore, the PLS-DA model (Figure 3A) scores plot – developed by comparing the patients with refractory and responsive MTLE – displays the clustering for the two groups and the first principal component (1), which explains 44.6% of the data variation. The PLS-DA model goodness of fit (R^2) value was 0.13, with a poorness of prediction (Q^2) of -0.19. Also, a constructed model alone was not enough to consider the model prediction. However, it was possible to identify some candidate variables according to their PLS regression coefficients, and these were classified based on the variables' relative importance in projection or VIP. These metabolites were lipoproteins, lactate, glucose, proline,

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isoleucine, and unsaturated lipids (Table 3 and Figure 3C). Nonetheless, these metabolites did not present significant differences by t-test when comparing patients with responsive and refractory MTLE:

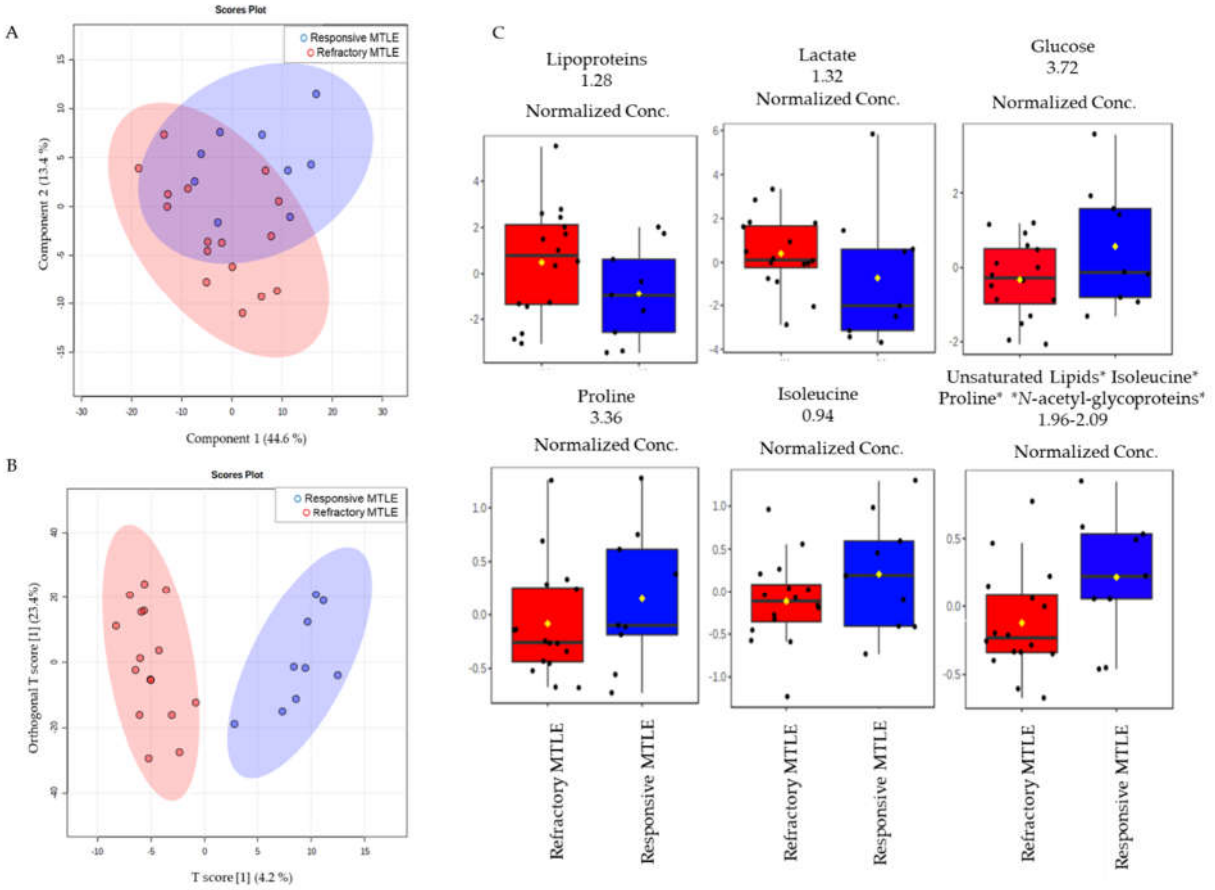

Figure 3. Multivariate analysis of ^{1}H-NMR (CPMG) plasma spectra of responsive and refractory MTLE patients. (A) PLS-DA analysis with accuracy 44.6%, R2 0.13, and Q2 -0.19. (B) O-PLS-DA analysis. The information about response to treatment with ASM in patients with MTLE was not implemented into the models. (C) Box plots representing the variations of the relative concentrations (measured as peak intensities) of metabolites whose VIP scores > 2 according to PLS-DA results. The black dots represent the metabolite levels in all samples, and the yellow diamond represents the average value. Responsive MTLE (blue) and refractory MTLE (red).

Interestingly, only β-hydroxybutyrate has discriminated patients from controls but not between the two groups of patients. By contrast, lipoproteins and lactate discriminate between the two groups of patients, refractory and responsive MTLE, but not between the patients and controls. Furthermore, we observed that most of the metabolites identified as significantly different in patients compared to controls were increased in patients, except for glucose, which was reduced in patients (Table 2 and Figure 2C). In addition, it seems that all metabolites found in the responsive group were increased, and lipoproteins and lactate were increased in the refractory group (Table 3 and Figure 3C).

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Table 3. List of metabolites identified in different concentrations of refractory and responsive MTLE and their respective chemical shifts elucidated by the highest VIP values (VIP score). The p-values, -log10(p), calculated from the t-test, FC (Fold change), and log2fold change (logFC) are also shown. **Notes and abbreviations**: multip = multiplicity, s (singlet), d (doublet), t (triplet), dd (doublet of doublet), m (multiplet), l (broad); assign. = assignment of these signals; * = overlaid signals.

Refractory MTLE x Responsive MTLE – VIP Scores						
Metabolites	**Chemical shift (multip.; assign.)**	**VIP score**	**p-value**	**-log10(p)**	**FC**	**log2(FC)**
Lipoproteins	1.28 (m, CH)	6.66	0.05	1	1.209	-0.274
Lactate	1.33 (d, CH_3)	5.41	0.05	1	1.159	0.212
Glucose	3.41 (m, CH_2)	4.81	0.05	1	0.752	-0.412
Exclusively unsaturated lipid	2.06 (l, CH_2-CH=)	1.71	0.05	1	0.857	-0.222
Isoleucine	0.94 (t, CH_3)	1.57	0.05	1	0.886	-0.173
Proline	3.36 (m, CH)	1.13	0.05	1	0.658	-0.603

Furthermore, we performed pathway enrichment analysis with the differentially abundant metabolites. We first compared patients with controls and identified the following as main altered pathways: lactose degradation, glucose-alanine cycle, lactose synthesis, transfer of acetyl groups into mitochondria, glycolysis, gluconeogenesis, fatty acid biosynthesis, galactose metabolism, sphingolipid metabolism, arginine, and proline metabolism, Warburg effect, valine, leucine, and isoleucine degradation pathway as main altered pathways in patients. Subsequently, we compared the two groups of patients classified according to response to ASM: refractory and responsive MTLE. We found the same enriched pathways as reported above and an additional one, the pyruvate metabolism, which was not enriched when comparing patients and controls (Figures 4A and B).

We also analyzed the diseases to which the differently abundant metabolites have been previously linked. They are diabetes mellitus (non-insulin-dependent), persistent hyperinsulinemic hypoglycemia, pyruvate carboxylase deficiency, respiratory chain deficiencies, long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency – for a complete overview of the diseases found to be linked to the differentially abundant metabolites, refer to appendix A, Figure A2.

Finally, we built networks correlating metabolites with their putative-related genes using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. We found 18 genes associated with the metabolites capable of discriminating patients from controls (Figure 4C) and 21 associated with metabolites able to differentiate between the two groups of patients, refractory and responsive MTLE (Figure 4D). Then, this set of genes was used to search the human phenotype ontology gene setlist and the Gene Set Enrichment Analysis (GSEA) database. We found genes related to elevated hepatic transaminase, energetic metabolism, amino acids metabolism, inflammatory processes.

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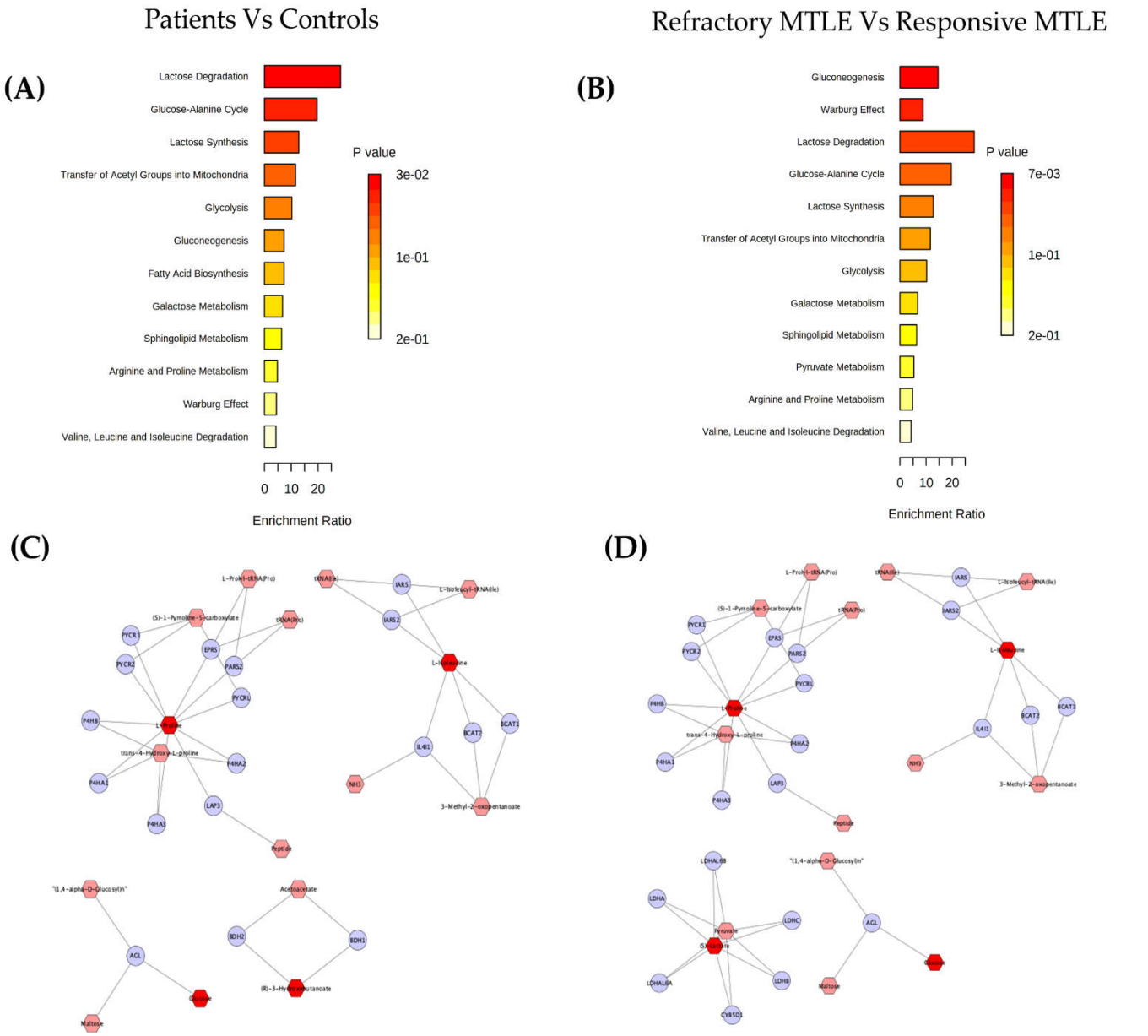

Figure 4. Metabolic pathways identified by the Metabolite Set Enrichment Analysis (MSEA) of the discriminant metabolites identified when comparing **A**. Patients and controls and **B.** Refractory MTLE with responsive MTLE. **C.** Network Compound-Genes built with the discriminant metabolites comparing patients and controls. **D**. Network Compound-Genes built with the discriminant metabolites from comparing refractory MTLE and responsive MTLE. Blue circles – annotated genes; Pink Hexagon – metabolite's ligands; Red Hexagon – discriminant metabolites present in the KEGG database.

3. Discussion

We used ^{1}HNMR-based metabolomics to investigate plasma samples of patients with MTLE and controls. Also, we compared patients with different responses to ASM, i.e., those refractory and responsive to ASM. We searched for metabolic traits that could discriminate between the groups using a hypothesis-free design. Furthermore, we aimed to gain insights into the mechanisms underlying MTLE and response to treatment with ASM with the metabolomics data obtained. Because different epilepsy syndromes present different mechanisms, which may influence the biological processes leading to pharmacoresistance [11], we performed our study exclusively in patients with a single, well-defined epilepsy syndrome, MTLE. Furthermore, the use of ASM may lead to different

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metabolomics signatures [19,43]; thus, we selected patients using the same therapeutic regimen, carbamazepine in combination with clobazam, minimizing the source of bias. Also, the occurrence of seizures in proximity to the time of sample collection may temporarily affect the metabolic profile in plasma; hence, we only collected samples from patients who were seizure-free 24 h before sample collection.

We found five altered metabolites comparing patients and controls: glucose, saturated lipids, 3-hydroxybutyrate, isoleucine, and proline. These biomolecules are related to metabolic processes involved mainly in energetic and amino acid metabolisms, especially those related to the cell's anaplerotic reactions. Noteworthy is the presence of lipids as one of the discriminating elements between patients and controls. Changes in the energetic metabolism of neurons and glial cells have been frequently reported in neurological disorders. They are related to the preferential and intense usage of glucose by the nervous tissue. In addition, neurotransmitters, such as glutamate, acetylcholine, and gamma-aminobutyric acid, are dependent on energy metabolism [15]. The main molecule responsible for transferring and storing energy, the adenosine 5'-triphosphate (ATP), is produced through the glucose catabolism in the glycolysis, tricarboxylic acid (TCA) cycle, oxidative phosphorylation, and several other pathways related to the energetic metabolism, which were also present in the enriched pathways analysis performed in this study (Figure 4). Moreover, it is well known that energy metabolism is indispensable for neurotransmitter production and release and the activity of ion channels. These are the essential players in the known mechanisms leading to epilepsy [16,17]. Therefore, an energetic failure due to abnormalities in glucose concentration may lead to increased seizure susceptibility [18]. Indeed, previous ^{1}H-NMR studies revealed abnormal glucose concentrations in adult patients under different combinations of ASM [19] and in drug-free pediatric patients [20], presenting focal and generalized seizure types. Unfortunately, these studies presented important limitations since the diagnosis of epilepsy syndrome was not informed for both studies [19,20], and one of the studies included patients using different types of ASM [19]. Furthermore, none of the studies discussed above [19, 20] informed if the patients presented seizures 24h before sample collection, which may have introduced significant heterogeneity and bias toward identifying altered metabolites due to the ASM used and/or/or after a major seizure.

In this study, we identified lower glucose levels in patients compared to controls and increased levels of lactate in patients with pharmacoresistant MTLE compared to the patients with responsive MTLE. Additionally, in our enrichment analysis, we found exclusively in comparing refractory and responsive MTLE the pyruvate metabolism pathway, which may also highlight the relevance of the energetic metabolism in the drug resistance phenomenon [21]. Pyruvate metabolism has a pivotal role in glucose catabolism, maintaining the concentrations of TCA cycle intermediates, supporting several mitochondrial functions, and even maintaining cell viability by decreasing cell growth and increasing cell death [22].

Also, other energy sources become necessary in an energy failure scenario since the decrease in ATP availability requires the recruitment of other biomolecules for energy production. In this context, lipidic support is crucial to cell maintenance [23]. In our study, some lipids corresponding to saturated fatty acids have been found elevated in patients and can be related to altered energetic metabolism since this group of molecules, abundantly found in lipoproteins, such as VLDL, HDL, and LDL can be constantly displaced to many different tissues to supply the energy input [24]. We also identified altered levels of unsaturated fatty acids when comparing the two groups of patients, with decreasing levels in refractory MTLE. These findings could suggest that the patients, especially the responsive ones, had a higher intake of food rich in unsaturated fatty acids (such as seeds and nuts) [24]. Interestingly, several studies have demonstrated that this class of lipids, mainly the polyunsaturated fatty acids (PUFAs), can contribute to an improvement in seizure control, possibly by binding to peroxisome proliferator-activated receptor (PPAR)α and PPARγ receptors [24] and by exhibiting anti-inflammatory properties [25–27].

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Other important energy sources for neural cells are gluconeogenesis, glycogenolysis, and beta-oxidation. The ketone bodies can also perform this role, being synthesized depending on the metabolic disposition of the cell in prolonged periods of energetical deficit [28]. These molecules – acetoacetate, acetone, and β-hydroxybutyrate – contribute mainly to the brain tissue energy input, as they can cross the blood-brain barrier, supplying about 60% of the central nervous system (CNS) [29]. Our study found increased levels of β-hydroxybutyrate in patients, even though signals of saturated lipids overlay the chemical shift identified for this molecule. This finding also indicates a state of energetic failure in the cells of patients with MTLE.

In addition to the well-known therapeutic strategy of the ketogenic diet in epilepsy, diets rich in BCAA have been shown to have positive effects in seizure control in an animal model [30] and combined with the ketogenic diet in children with epilepsy [31]. However, a recent study failed to demonstrate that chronic ingestion of BCAA is effective in the long-term reduction of seizures in an animal model of MTLE [32]. It has been proposed that an imbalance in the concentrations of amino acids such as valine, leucine, and isoleucine may lead to increased glutamate levels in the nervous tissue and, consequently, to increased hyperexcitability and excitotoxicity, resulting in neuroinflammation, which is known to occur in epilepsy [31,33,34]. Interestingly, we found increased levels of isoleucine in patients compared to controls and reduced levels of this same amino acid in patients with refractory MTLE compared to responsive MTLE, indicating a complex relationship between isoleucine concentration in MTLE and response to ASM treatment.

Furthermore, we found increased proline levels in patients compared to controls and reduced levels of this same amino acid in patients with refractory MTLE compared to responsive MTLE. This nonpolar amino acid has been linked to seizures and cognitive dysfunction [38], as well as to *PYCR2* gene expression linked to microcephaly and hypomethylation [39], along with the *PARS2 gene* related to infantile-onset encephalopathy [40]. Furthermore, isoleucine is a metabolite linked to genes involved in neurological disorders, such as dementia and seizures: *BCAT1*, *BCAT2*, and *IARS2* (26980008 and 30098844).

Interestingly, we found that the metabolic profile of patients with MTLE was also linked to non-insulin-dependent diabetes mellitus (Mody). This association has been reported previously, and it may be disease-induced or linked to the chronic use of ASM [51-53]. Indeed, a disruption of glucose metabolism in epilepsy leading to the unusual use of glucose as a source of energy [18–54,55] [24–26,32] and pro-inflammatory processes occurring in epilepsy [55–57] may be risk factors for the development of diabetes in patients with MTLE.

Overall, our study found that the levels of metabolites such as glucose, saturated lipids, β-hydroxybutyrate, isoleucine, and proline, can distinguish patients with MTLE from controls. Most importantly, glucose levels, lipoproteins, isoleucine, proline, unsaturated lipids, and lactate are different in patients with pharmacoresistant MTLE than those with responsive MTLE. Also, the computational integration of the data obtained in the metabolomic analysis suggests that pathways related to energetic metabolism, excitatory neurotransmission, and inflammatory processes are abnormally regulated in patients with MTLE. Notably, recent studies using tissue from animal models of MTLE reported similar altered biological pathways and processes [55–57]. Finally, we found that isoleucine and proline, amino acids present in abnormal levels in patients, may be involved in the increased susceptibility to seizures.

3.1. Strengths and limitations

Strengths of this study include a well-characterized and homogeneous cohort since only patients with confirmed MTLE using the same type of ASM were included in the study. All patients were followed prospectively by the same group of neurologists in an epilepsy clinic at a university hospital. Also, we only included patients without generalized seizures in the last 24 hours of blood collection. The diagnostic criteria used to

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identify patients with MTLE and characterize the response to treatment with ASM followed the recommendations by the International League Against Epilepsy [18,58]. Furthermore, we used an agnostic approach to identify metabolites in the patients' plasma and controls through a robust, highly reproducible analytical technique. To our knowledge, this is the first study to comply with all the elements listed above. Finally, because we recruited such a homogenous group of patients and controls, our study limited the number of included individuals.

4. Materials and Methods

4.1. Study Population

We studied 28 patients with MTLE at the University of Campinas (UNICAMP) hospital's outpatient epilepsy clinic. All patients had confirmed MTLE diagnoses according to International League Against Epilepsy (ILAE) criteria [58] and were investigated using a standard protocol including serial interictal electroencephalogram high-resolution 3 Tesla magnetic resonance imaging [59,60]. We only included adults between the ages of 18 and 65. Patients were classified into two groups according to the patients' responses to the treatment with ASM as refractory or responsive following ILAE recommendations [18]. In addition, we only included patients using carbamazepine (CBZ) with clobazam (CLB), the therapy most frequently used by our epilepsy clinic patients. None presented generalized seizures 24 hours before blood collection. Furthermore, we excluded patients using drugs for secondary clinical conditions that could have interacted with the ASM. We also studied samples from 28 control individuals. These were adults between 18 and 65 who were not in treatment for any neurological or chronic clinical conditions. We also excluded individuals under therapy with medications that could alter the function of key metabolism enzymes, such as cytochrome P450 isoforms.

4.2. Blood Collection and Plasma Preparation

Peripheral blood (4 mL) was collected in standard EDTA tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ, USA). Blood samples were kept on ice for up to two hours until the plasma was separated and centrifuged at 2500 rpm for 10 minutes at 4 °C. Then, the obtained plasma was aliquoted and stored at -80 °C until further analysis.

4.3.1. H-NMR Spectroscopy Analyses

Samples were thawed in an ice bath (4 °C), diluted 1:1 (v/v) in deuterium oxide (D_2O, 99.9% with 0.03% of trimethylsilyl propanoic acid, TSP, from Sigma Aldrich), and transferred into 5 mm NMR tubes. A Bruker AVANCE III 600 spectrometer (Bruker Biospin, Karlsruhe, Germany), equipped with a TBI (Triple Resonance Broadband Inverse) probe, was used. All ^{1}H-NMR spectra were acquired at 25 °C, for example, Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence (cpmgpr1d), with 128 scans were explored further. The two-dimensional (2D) total correlation spectroscopy (TOCSY) experiments were recorded with mlevgpphw5 pulse sequence and 300 scans to randomly selected samples.

4.4. Data Analysis: NMR Data Processing

The obtained spectra were processed and standardized using MestreNova software (MestrelabResearch S.L.). First, the spectra were referenced using TSP (0.0 ppm), then aligned, and their phase was corrected. Next, peaks were normalized by the total area, and the matrix was prepared using spectral bins (0.005 ppm). After normalization with a constant sum (100) of the entire spectrum intensity, which reduced the differences in concentration between the plasma samples, water, and EDTA spectral regions were removed.

Such a data-matrix was processed using MetaboAnalyst 5.0 platform (Xia, McGill University) [61]. Data filtering, normalization by the sum, and Pareto scaling (mean-centered and divided by the square root of the standard deviation of each variable) were used in data preprocessing before the statistical tests, and 56 samples against 1620 variables (bins) for ^{1}H-NMR CPMG were analyzed.

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Multivariate Principal Component Analysis (PCA), Partial Least Squares-Discriminant Analysis (PLS-DA), orthogonal (o) PLS-DA, Leave-one-out cross-validation (LOOCV), Variable importance in projection (VIP) scores were explored. The first fifteen VIPs were correlated with univariate analyses, such as Fold-Change (FC) and the t-test. Multiple comparison correction was performed through the false discovery rate (FDR) at a level of 0.05, but it did not result in any significant metabolite (p.adjust). Therefore, we considered statistically significant results for which p-values were < 0.1. The chemical compounds were identified with the support of the Human Metabolome Database (HMDB) (http://www.hmdb.ca/) [62], and Biological Magnetic Resonance Data Bank (BMRB) (http://www.bmrb.wisc.edu/) [63]. Metabolic and disease pathways analysis were performed using the Metaboanalyst function Metabolic Set Enrichment Analysis (MSEA) with an Over Representation Analysis (ORA) algorithm. We also built a network of compound-gene relations using the MetScape app from Cytoscape [64] to identify enriched pathways and visualize changes in metabolite data. All the above enrichment analyses were generated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) [65] and the Small Molecule Pathway Database (SMPDB) [66].

5. Conclusions

Overall, plasma levels of glucose, saturated lipids, β-hydroxybutyrate, isoleucine, and proline can distinguish patients with MTLE from controls. Most interestingly, glucose levels, isoleucine, proline, lipoproteins, unsaturated lipids, and lactate are different in patients with pharmacoresistant MTLE compared to responsive MTLE. Also, the computational integration of the data obtained in the metabolomic analysis suggests that pathways related to energetic metabolism, excitatory neurotransmission, and inflammatory processes are abnormally regulated in patients with MTLE. The identified metabolites were linked to biological pathways related to cell energy metabolism, inflammatory processes, modulation of neurotransmitter release and activity, which can potentially contribute to the mechanisms underlying MTLE. In contrast, pyruvate metabolism may be contributing to resistance to ASM. Thus, in addition to insights into the mechanisms underlying MTLE and response to treatment with ASM, our results show suggestive evidence that plasma metabolites may be used as biomarkers of response to ASM in patients with MTLE. These findings warrant further studies exploring the clinical use of metabolites to assist in medical decisions in treating patients with MTLE.

Author Contributions: AG, AMC, and IC designed the research; AG, AMC, AD, DB, MA, CY, FC, and IC selected the cases, collected and provided samples, revised and provided clinical data of patients; AG, AMC, AD, DCR, MQE, and LGM performed the NMR experiments; AG and MQE performed the chemometrics analysis; LT provided reagents and supervised NMR metabolomic experiments; IC provided reagents and supervised the study; AG, AMC, MQE, LT, and IC wrote the paper. All authors read and approved the final manuscript.

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Appendix A

Table A1. ^{1}H-NMR chemical shifts assignments of the metabolites found in the plasma of patients and controls. Metabolites considered to be variables of importance in projection (VIPs) by the PLS-DA model are shown in red for the comparison between patients and

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controls and underlined for the comparison between patients with refractory MTLE and responsive MTLE.

Sugars		
1.	Glucose	3.41 (H4′), 3.54 (H2′), 3.72 (H3′), 3.84 (H5′), 5.24 (H1′) 3.25 (H2′), 3.41 (H4′), 3.47 (H5′), 3.72 (H6′), 3.90 (H6′), 4.65 (H1′)
Amino acids		
2	Isoleucine	0.94 (δ-CH_3), 1.01 (γ-CH_2), 1.42 (γ-CH_2), 3.67 (α-CH)
3	Leucine	0.96 (δ-CH_3), 1.71 (β-CH_2)
4	Valine	0.99 (γ-CH_2), 1.04 (γ-CH_3), 2.25-2.31 (β-CH), 3.60 (α-CH)
5	Alanine	1.48 (β-CH_3), 3.78 (α-CH)
6	Glutamate	2.04 (β-CH_2), 2.36 (γ-CH_2), 3.71 (α-CH)
7	Glutamine	2.13 (β-CH_2), 2.45 (γ-CH_2), 3.78 (α-CH)
8	Arginine	1.72 (γ-CH_2), 1.89 (β-CH_2), 3.23 (δ-CH_2), 3.73 (α-CH)
9	Proline	3.36 (δ′δ′-CH_2), 3.41 (δ-CH_2), 4.14 (α-CH)
10	Histidine	7.06 (H4), 7.77 (H2)
11	Tyrosine	6.89 (H3, H5), 7.19 (H2, H6)
12	Phenylalanine	3.24 (β′β′-CH), 7.32,7.36 (H2, H6), 7.42 (H3, 5H)
Lipids		
13	Low-density lipoproteins (LDL)	0.90 (CH_3), 1.30 (CH_2)n
14	Very-low-density lipoproteins (VLDL)	0.76-0.93 (CH_3), 1.24-1.37 (CH_2)n
15	High-density lipoproteins (HDL)	0.80-0.85 (CH_3), 1.21-1.23 (CH_2)n
16	Fatty acid chain	1.53-1.65 (-CH_2CH_2CO)
17	Unsaturated lipids and *N*-acetyl glycoproteins	1.96-2.09 (-CH_2-CH=)
18	Lipids HC=CH	5.29-5.43 (CH)
Organic acids		
19	Lactate	1.33 (CH_3), 4.11 (CH)
20	Acetic acid	1.92 (CH_2)
21	Acetone	2.24 (CH_2)
22	Acetoacetic acid	2.28 (CH_2)
23	Citrate	2.54 (CH_2), 2.68 (CH_2)
24	Formate	8.47 (CH)

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Other compounds		
25	Ethanol	1.20 (CH_3); 3.67 (CH_2)
26	Creatinine	3.94 (CH_2), 3.04 (CH_2)
27	Creatine	4.06 (CH_2), 3.05 (CH_2)

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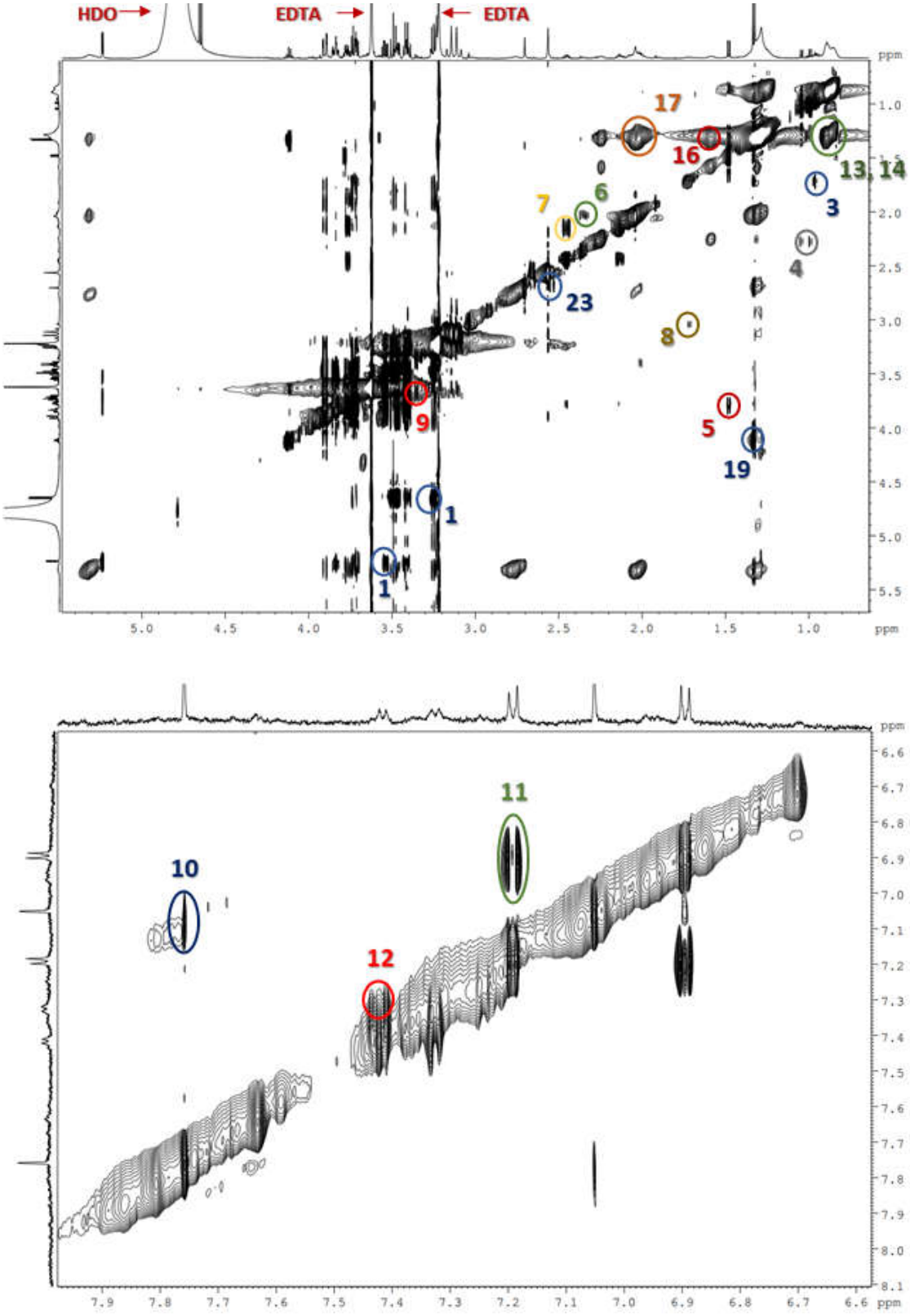

Figure A1. 2D TOCSY ^{1}H-^{1}H NMR obtained for one plasma sample. Spectral region 0.50 to 5.50 ppm is shown on the upper panel, and region from 6.6 to 7.9 ppm is given on the bottom panel. Metabolites identified: 1. Glucose; 3. Leucine; 4. Valine; 5. Alanine; 6. Glutamate; 7. Glutamine; 8. Arginine; 9. Proline; 10. Histidine; 11. Tyrosine; 12. Phenylalanine;

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13. LDL: Low-Density Lipoproteins; 14, VLDL: Very Low-Density Lipoproteins; 16. Fatty Acid Chain; 17. Unsaturated lipids and *N*-acetyl-glycoproteins; 20. Lactate; 23. Citrate. (Numbers refer to ones shown in Table A1)

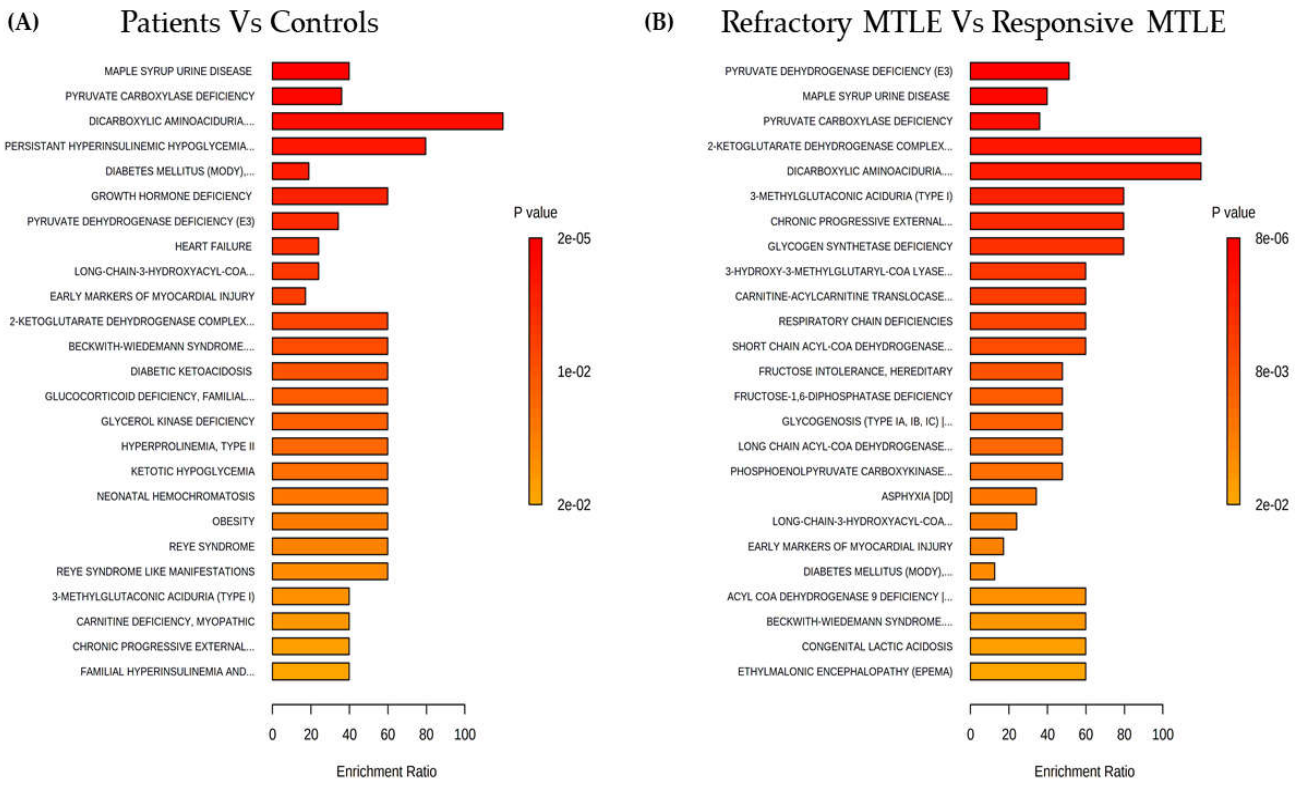

Figure A2. List of diseases identified by the Metabolite Set Enrichment Analysis (MSEA) using the metabolites found to discriminate **A**. Patients and Controls and **B.** Refractory MTLE and Responsive MTLE.