H¹ NMR-BASED METABOLOMICS APPLIED TO THE SEARCH FOR DRUG RESISTANCE BIOMARKERS IN PATIENTS WITH MESIAL TEMPORAL LOBE EPILEPSY


Abstract
Mesial Temporal Lobe Epilepsy (MTLE) stands out among the different epilepsy types due to its prevalence and the high rate of resistance to treatment with antiseizure drugs (ASDs). Due to the challenge of predicting which patients will respond to drug treatment, alternative therapies, such as epilepsy surgery, can take many years to be indicated. Thus, it is necessary to search for more effective methods, such as biomarkers capable of predicting resistance to ASDs. One approach to identifying biomarkers for a disease is to focus on metabolites from a biological matrix, called metabolomics. Metabolomics has many advantages over other molecular techniques, such as closer proximity of the metabolites to the phenotypic profile, high robustness, and specificity in detecting these analytes. It can be used as a powerful tool in the identification of biomarkers for ASDs in MTLE patients. Thus, we aim to analyze metabolites in blood plasma from drug-resistant (n=19) and responsive (n=10) patients with MTLE, using H¹ NMR and chemometric analysis. Spectral differences were identified when comparing the two groups, such as in the signals corresponding to the hydrogens of L-arginine, citrate, creatine, L-glutamine, and L-tyrosine. These findings may indicate an increase in inflammatory events, energy metabolism failure, and increased neuronal hyperexcitability in patients with drug-resistant MTLE.

Keywords:
Mesial Temporal Lobe Epilepsy; Metabolomics; Proton Nuclear Magnetic Resonance Spectroscopy.

Introduction
Mesial Temporal Lobe Epilepsy (MTLE) is the most frequent type of epilepsy in adults. It is characterized by recurrent simple or complex partial seizures, at times accompanied by secondary generalization. Also, about 30% of patients with MTLE do not respond to the prescribed antiseizure drugs (ASDs). It is common in these patients to find the association with a specific neuropathological lesion named mesial temporal sclerosis (MTS). For these patients with MTS and ASD resistance, surgical intervention may be an alternative procedure for treatment. Epilepsy surgery has a good chance of promoting a significant improvement in seizure frequency and severity. However, surgical intervention is not always performed early enough, resulting in a long period of exposure to the side effects of the ASDs and the occurrence of uncontrolled seizures. In this sense, an investigation of the metabolic profile can offer better tools to identify which patients are resistant to ASD therapy and help identify metabolic factors associated with the resistance to ASD therapy, thus accelerating and optimizing the indication of epilepsy surgery. Metabolomics usually employs several analytical techniques to provide data on the metabolic profile through various physical-chemical principles. One of the most widely used methods is the proton nuclear magnetic resonance spectroscopy (H¹-NMR). The use of H¹-NMR in the context of metabolomics is supported by its high reproducibility, without the need for complex sample preparation, and associated with the chemometric analysis; it can provide information about specific metabolites which could be related to a particular profile of drug resistance to ASDs in patients with MTLE. Suppose we find a distinct metabolic profile associated with ASD resistance. In that case, this could be used as a biomarker to assist in the early indication of epilepsy surgery in patients with MTLE. Thus, this study aims to compare the metabolic profile of patients with MTLE who presented different responses to pharmacological treatment of MTLE.
Material and Methods
Twenty-nine patients with MTLE were divided into two groups according to their response to pharmacological treatment, patients with drug-resistant MTLE (n=19) and patients with responsive MTLE (n=10). All patients were prospectively followed at the epilepsy clinic of the UNICAMP University Hospital by the same group of neurologists (MKA, CLY, and FC) and according to a semi-structured protocol, which includes clinical assessment as well as state-of-the-art neuroimaging evaluation. The classification of patients in the two groups of response to pharmacological treatment was performed by the treating physician according to previously published criteria by the International League Against Epilepsy \(^7\) and has been used regularly by our group in previous works \(^8,9\). Also, to avoid possible confounding factors related to the type of ASD the patients were taken, we only included individuals using clobazam combined with carbamazepine, the most common ASD regimen given to patients in our hospital.

The plasmatic metabolic profile of both groups was analyzed by liquid-state NMR (600-MHz BurkerAscend™ Spectrometer) data acquisition combined with chemometric analysis. The obtained spectra with the CPMG pulse sequence from these groups were processed using MestreNova and then analyzed using Metaboanalyst. Subsequently, the specific metabolites were identified with HMDB and BMRB database.

Results and Discussion
The statistical models were constructed using different regions of the H\(^{1}\)-NMR spectrum edited with the T\(_2\) filter (CPGM) to assess general differences in the metabolites present in the plasma of the investigated groups. Patients in the drug-resistant group (n=19) had a mean age of 52.35 ± 12.03 years and a female percentage of 50%. Patients in the responsive group (n=10) had a mean age of 53.9 ± 8.5 years and a female percentage of 60%. There were no significant differences between the patients' ages and sex distribution in the two groups (U and chi-square tests).

Data acquired were analyzed in the regions: a) 0-9 ppm, with 2429 variables, for a global analysis of the metabolites; b) from 6.5-9 ppm, with 630 variables, for an analysis of aromatic compounds; c) 0-4.5 ppm, with 993 variables, for an analysis of aliphatic compounds. The choice of this division was based on a more specific analysis for the identification of aromatic amino acids, since they have a fundamental role in the biosynthesis of neurotransmitters, and, therefore, an important role in the pathophysiology of epilepsies \(^10,11\).

Using a multivariate analysis, the PCA did not show a clear separation between the two groups in three different spectra divisions, which indicates the homogeneity of metabolic profiles. However, using supervised tests such as PLS-DA and OPLS-DA, observing a separation between the groups was possible. Despite allowing a better separation, a visual inspection of the cluster profile was insufficient to provide reliability and statistical relevance \(^12\), as shown in Figure 1.
Figure 1. Figure showing the graphs of (1) PLS-DA, (2) OPLS-DA, and (3) PCA tests of the three spectral divisions, constructed from $\text{H}^1$-NMR spectra with the CPMG pulse sequence. The areas circumscribed to the samples of both groups represent a 95% confidence interval. The red samples represent the drug-resistant patients, and those depicted in green represent the responsive patients.

We subsequently performed a 5-fold cross-validation (Q²) approach in order to assess the predictability of the PLS-DA model: a) 0-9 ppm, with Q² = -0.193; b) 6.5-9 ppm, with Q² = -0.121; c) 0-4.5 ppm, with Q² = -0.221. The low values found for Q² may show a low sample N or an overfitting process of the method\textsuperscript{13,14}. With this statistical model, chemical shifts with higher VIP scores were evaluated - using component 1 of the PLS-DA test -, using the sum of all the VIPs evidenced by the 3 proposed spectral divisions, to highlight which metabolites are more representative and differential between the two groups, at a p-value ≤ 0.01. These were: δ1.702 (p = 0.01) and δ1.706 (p = 0.002), corresponding to L-arginine hydrogen atoms; δ2.654 (p = 0.09) to citrate hydrogens; δ3.914 (p = 0.08) to creatine hydrogens; δ2.130 (p = 0.03) to L-glutamine hydrogens and δ6.861 (p = 0.05) to L-tyrosine hydrogens.

Some of these metabolites, such as L-arginine, citrate, and creatine, were found in greater quantities in drug-resistant patients, which may characterize a more significant presence of inflammatory events and mechanisms of failure in energy metabolism\textsuperscript{15,16,17,18}. On the other hand, L-glutamine and L-tyrosine were found to be decreased in drug-resistant patients, which may characterize increased neuronal hyperexcitability in these individuals\textsuperscript{19,20,21}, Figure 2.
Conclusions
We found significant differences in the plasma metabolites of patients with MTLE according to response to ASDs. There were five metabolites present in different abundances in the two groups of patients, L-arginine, citrate, creatine, L-glutamine, and L-tyrosine. Together, these molecules could be considered potential biomarkers with discriminatory capacity for the two groups analyzed. These metabolites were related to several biological pathways, such as energy metabolism and the biosynthesis and modulation of neurotransmitter activity. In addition, we found evidence pointing to an energy failure leading to susceptibility to seizures in drug-resistant patients.

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References


