Metabolomic changes in CSF of migraine patients measured with $^1$H-NMR spectroscopy†

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Background. Migraine is a common episodic brain disorder. Treatment options and diagnosis are hampered by an incomplete understanding of disease pathophysiology and the lack of objective diagnostic markers. The aim of this study was to identify biochemical differences characteristic for different subtypes of migraine in cerebrospinal fluid (CSF) of migraine patients using an exploratory $^1$H-NMR-based metabolomics approach.

Methods: CSF was obtained, in between migraine attacks, via lumbar puncture from patients with hemiplegic migraine, migraine with aura, migraine without aura, and healthy controls. Metabolite concentrations were measured by quantitative $^1$H-NMR spectroscopy. Multivariate data analysis was used to find the optimal set of predictors, generalized linear models (GLM) were used to ascertain the differential significance of individual metabolites. Results: In CSF samples from 18 patients with hemiplegic migraine, 38 with migraine with aura, 27 migraine without aura, and 43 healthy controls, nineteen metabolites were identified and quantified. Hemiplegic migraine patients could be discriminated from healthy controls using supervised multivariate modelling with 2-hydroxybutyrate and 2-hydroxyisovalerate as the most discriminant metabolites. Univariate GLM analysis showed 2-hydroxybutyrate to be lower in hemiplegic migraine compared with healthy controls; no significant differences were observed for other metabolites. It was not possible to discriminate migraine with and without aura from healthy controls based on their metabolic profile. Conclusions: Using an exploratory $^1$H-NMR metabolomics analysis we identified metabolites that were able to discriminate hemiplegic migraine patients from healthy controls. The lower levels of 2-hydroxybutyrate found in patients with hemiplegic migraine could indicate a dysregulation of the brain’s energy metabolism. An experimental confirmation in vitro or in animal models will be required to confirm or discard this hypothesis. Migraine with and migraine without aura patients did not reveal a metabolic profile different from healthy controls.

Introduction

Migraine is a common brain disorder that is typically characterized by recurrent, disabling attacks of severe unilateral headaches that are accompanied by photo- and phonophobia, nausea and vomiting. In one-third of patients attacks are preceded by transient neurological symptoms, called aura. Patients with and without aura form the two main migraine subtypes. When left untreated attacks can last from several hours to several days and are very disabling for patients. About 15% of the general population suffers from migraine; women three times more often than men. Hemiplegic migraine is a rare subtype of migraine with aura that is characterized by a transient hemiplegia (one-sided motor weakness) during the aura phase of a migraine attack and has been proposed as a good model to study the common forms of migraine. Thus far, three hemiplegic migraine genes have been identified (CACNA1A, ATP1A2 and SCN1A) that all encode subunits of ion transporters that seem to play a role in (glutamatergic) neurotransmission.
Repeated migraine attacks are highly disabling for the patient and pose a great burden to society, especially medication to prevent migraine attack from occurring are not adequate in many patients, therefore more effective and well-tolerated preventative agents are urgently needed. However, our incomplete understanding of migraine pathophysiology hampers the development of such new treatment strategies. Although various biochemical mechanisms have been proposed, biochemical knowledge has not let to a panel of biomarkers that could aid migraine diagnosis or predict treatment outcome. For practical reasons, urine and blood (plasma or and serum) are the most frequently used body fluids in biomarker discovery projects. However, for migraine, as it is a brain disorder, peripheral body fluids may not be ideal because biochemical changes occurring in the diseased brain may not be detected or become too diluted. Therefore, given its close proximity to the central nervous system (CNS), cerebrospinal fluid (CSF) obtained via lumbar puncture is a more preferred body fluid for monitoring the physiology of the CNS. The CSF compartment lies between the endothelial blood–brain barrier and the epithelial blood–cerebrospinal fluid barrier; the brain parenchyma is only separated from CSF by the glia limitans through which small metabolites can readily pass. Thus, analysis of the biochemical composition of CSF offers a more direct insight into biochemical mechanisms in migraine. Here, we apply an exploratory 1H-NMR-based metabolomics approach to identify biochemical differences characteristic for different subtypes of migraine, i.e., migraine with aura, migraine without aura, and hemiplegic migraine.

Methods

Subjects

Patients with migraine with aura, migraine without aura or hemiplegic migraine fulfilling the International Classification of Headache Disorders third edition (beta-version) were selected. Healthy volunteers were free of (a) specific headaches. All CSF samples of migraine patients were taken in between menstrual cycle, i.e., free of an attack for at least three days prior to lumbar puncture. For patients and healthy controls exclusion criteria were: (i) body mass index (BMI) > 28; (ii) any oncological history; (iii) severe psychiatric disorders; (iv) epileptic disorders; (v) contra-indication for a lumbar puncture (including signs and symptoms of increased intracranial pressure, local infection of the skin, and a coagulopathy including use of anti-coagulant drug or platelet-inhibitors); (vi) use of chronic medication (other than oral contraceptives, and migraine prophylactic drugs) in the four weeks preceding the lumbar puncture; (vii) use of acute migraine-specific or headache drug in the three days preceding the lumbar puncture; and (viii) use of acute migraine drugs on more than 6 days per month. Additional exclusion criteria for healthy volunteers were: (i) suffering from periodic pain attacks or a brain disorder; (ii) personal or family history in first-degree relatives of migraine or trigeminal autonomic cephalalgia; and (iii) more than one tension-type headache episode in the last 3 months. The study was approved by the Medical Ethical Committee of the Leiden University Medical Centre and all subjects provided written informed consent prior to study.

CSF sampling and sample handling

CSF sampling via lumbar puncture was performed in the morning (before 12.00 a.m.). All subjects were subjected to overnight fasting from at least 8 hours before the lumbar puncture and were only allowed water prior to the lumbar puncture. CSF samples were taken via lumbar puncture between the L3/L4 or L4/L5 interspace with a Medi Plast 0.9(20G) × 90 mm traumatic needle. For routine CSF diagnostics 3 mL CSF was sampled in a 12 mL polystyrene tube (Cat. No. 160 172, Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands). For NMR analysis a total of 4.8 mL of CSF was sampled directly in a 15 mL polypropylene falcon tube (tube P1a; Cat. No. 188 271, Greiner Bio-One) and centrifuged at 4 °C for 5 minutes (2000 rpm, 747 g) directly after sampling. Following centrifugation, the supernatant was transferred to a new 15 mL polypropylene falcon tube, and divided in 0.5 mL aliquots into eight 1.0 mL Nunc cryotubes (Cat. No. 3666656, Sigma-Aldrich, Chemie B.V., Zwijndrecht, the Netherlands) and placed on dry ice. All samples were handled exactly the same after sampling and all sample handling steps were carried out on ice. All sample aliquots were placed on dry ice within 30 minutes from sampling and were transferred to −80 °C for storage within 60 minutes from sampling. All samples remained at −80 °C until sample preparation, no additional freeze-thaw cycles were allowed.

CSF sample pre-treatment

To 1.5 mL standard eppendorf tubes (placed on ice), 25 μL of pH 7.0 phosphate buffer (50 mM) in D2O containing 4 mM of sodium 3-trimethylsilyl[2,2,3,3]tetraduteropropionate (TSP) and 2.0 mM NaN3 was added to 225 μL of CSF. Following thorough mixing by repeated inversion, 190 μL of the sample was transferred into 3 mm NMR tubes (Part No. Z112272, Bruker Biospin GmbH, Rheinstetten, Germany). Samples were kept in a cooled rack at 6 °C pending measurement.

1H-NMR data acquisition

1H-NMR data were obtained using a Bruker 600 MHz AVANCE II spectrometer (Bruker BioSpin, Karlsruhe, Germany) equipped with a 5 mm TCI cryo-probe and a z-gradient system; a Bruker SampleJet sample changer system was used for sample transfer; samples were kept at 6 °C while queued for acquisition. One-dimensional (1D) 1H-NMR spectra were recorded at 300.0 K using the first increment of a nuclear overhauser effect spectroscopy (NOESY) pulse sequence with presaturation (B1 = 50 Hz) during a relaxation delay of 4 s and a mixing time of 10 ms for efficient water suppression. Duration of 90 degree pulses was automatically calibrated for each individual sample using a homonuclear gated nutation experiment on the locked and shimmed samples after automatic tuning and matching of the probe head. Sixteen scans of 200704 points covering 18 028 Hz were recorded and zero-filled to 262 144 complex points prior to Fourier transformation, an exponential window function was
applied with a line-broadening factor of 1.0 Hz. The spectra were manually phased and baseline corrected and automatically referenced to the internal standard (TSP = 0.0 ppm). Phase offset artefacts of the residual water resonance were manually corrected using a polynomial of degree 5 least square fit filtering of the free induction decay (FID).

J-Resolved spectra (JRES) were recorded with a relaxation delay of 2 s and a total of one scan for each increment in the indirect dimension. A data matrix of 40 × 12 288 data points was collected covering a sweep width of 78 × 10 000 Hz. A sine-shaped window function was applied and the data was zero-filled to 256 × 16 384 complex data points prior to Fourier transformation. The resulting data matrix was tilted along the rows by shifting each row \((k)\) by \(0.4992 \times (128 – k)\) points and symmetrised about the central horizontal lines in order to compensate for the skewness of the multiplets in the F1 dimension.

**Data processing**

The NMR spectra of all samples were visually inspected for technical failure of the measurement. A dataset consisting of 74 peak integrals was created from 2D-JRES spectra using in-house deconvolution routines. A peak-picking routine was performed on the 2D-JRES skyline projections of a subset of spectra. Peaks which were present in at least 50% of the subset were grouped and fitted by a simplex-fitting procedure, which was used to build a linear model to estimates the peak surface of the skyline projection from their raw projections. This linear model was used to estimate the peak surface of all spectra. This estimate was used to construct the dataset.

Subsequently, outliers were detected by principal component analysis (PCA) of the entire dataset composed of 74 peak integrals, standardized by scaling to mean zero and unit variance. In contrast to the multivariate analysis, the entire dataset was used to prevent bias towards the identified metabolites. Specific cases with a large distance-to-model centre, and/or cases with large orthogonal distance to the model plane were treated as potential outliers. Spectra of potential outliers were again visually inspected before classifying cases as true outliers. To make sure that differences in profiles were not related to disease status, class-distribution of all outliers was checked for overrepresentation of diagnostic groups. Further analysis was performed on a dataset with outliers removed.

To identify different metabolites, peaks of the JRES NMR spectrum were compared to in-house databases of sample spectra, comprising all metabolites described by D. S. Wishart et al. except 3-hydroxyisobutyrate. Public spectra of 3-hydroxyisobutyrate were used for its identification. To verify the assignment, hierarchical cluster analysis (as presented by “R” package “Hmisc”) was performed to visualize the correlation structure of the dataset, which was compared to the known peak correlation of the candidate metabolite. In total, 20 different metabolites could be identified (Table S1, ESI†). Of these 20 metabolites, 16 were used in the final analysis. Ethanol was excluded due to its use as a skin disinfectant during CSF collection, thereby able to diffuse freely. Previous observations suggested ethanol contamination is likely to take place unless great caution is observed and non-ethanol skin disinfectants are used. Pyruvate and acetate were excluded due to an apparent batch effect, likely due to pyruvate instability (Fig. S1, ESI†). Acetone was excluded due to likely contamination. Spread in acetone concentration exhibited an batch effect, correlated with the order in which the samples were collected, stored, prepared and measured (Fig. S2, ESI†), and therefore has likely an external origin. Prior to further analysis, the data was normalized using cubic-spline normalization. This type of normalization scales individual spectra to fit the average distribution of peak intensities, instead of equalizing total spectral intensity.

**Multivariate statistical analysis**

Statistical analysis for annotated metabolites was performed using “R” (cran.r-project.org), release 2.15.2 for Windows. PCA analysis (as implemented in the “R”-package “rcov”) was used to detect trends, and grouping among cases, based on similarities of the multi-metabolite spectrum. Plots of cases in PCA-space, with cases labelled according to a chosen covariate, allowed to visually inspect trends of possible covariates. Prior to PCA-analysis, data was standardized by scaling to mean zero and unit variance, and log-transformed.

To detect differences between migraine and control samples, penalized logistic regression was used, as implemented in the elastic net algorithm (“R”-package “GLMNET”) (Fig. S3, ESI†). This penalized form of linear regression allows for an unique solution when the number of variables is larger than the number of cases. The elastic net penalty term is able to deal with highly correlated variables, and does both regression coefficient shrinkage and variable selection.

Prior to analysis, equally sized groups were selected based on age criteria, with total number of cases being twice the sample size of the smallest group. Groups were selected using the pairmatch algorithm (“R”-package “optmatch”) by first matching males and females based on age in the smallest diagnostic group, followed by matching based on age and gender with the contrasting diagnostic group. The model was optimized by minimizing the binomial deviance, keeping mixing parameter \(z = 0.5\) constant. To obtain model regression coefficients, model performance statistics, and their respective confidence intervals, 500 time-repeated cross-validation was performed. In each single run, a complete 10-fold cross-validation was performed. 10 fold cross-validation consisted of randomly splitting the matched dataset into 10 equal parts, where one part constituted the test sets, and the remaining cases constituted the training set. Test sets were alternated 10 times so every case was included in a test set once. Training data sets were scaled to zero mean and unit variance. Test datasets were scaled using mean and standard deviation from training dataset. From every run, root mean squared prediction error (RMSEP), receiver operator characteristic (ROC) and its area under the curve (AUC), and regression coefficients were extracted. In addition, the uniqueness of the RMSEP to the original class assignment was tested using permutation routines. In short, the class vector was permuted at random, and used to construct and
validate a model. By repeating this process 2000 times, the percentage of models with equal or lower RMSEP compared to the original model were calculated.

Univariate statistical analysis

Medians of different migraine groups were compared by Mann–Whitney test, implemented in the “R” stats package. In total three comparisons were made: hemiplegic migraine versus control, migraine without aura versus control, and migraine with aura versus control. To minimize the false discovery rate (FDR), acquired p-values were compensated using Benjamini and Hochberg FDR. In addition, the same comparison was performed by linear model, correcting for both age and gender. Contrast functions were used to obtain p-values.

Generalized linear models (GLM) (implemented in the “R”-package “rms”) were employed to study the partial effects of migraine diagnoses on metabolite levels. Full models were constructed for the predictors; age, gender, and diagnosis, allowing 3-way variable interaction. Reduced models were obtained using backward variable selection, by Akaike’s Information Criterion. Hypothesis-testing was performed using general contrast functions. When age × diagnosis interactions were present, an additional GLM model was build using age-tertiles to allow for better study of age interaction effect. Metabolite levels were log-transformed if judged necessary from residual analysis.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Baseline characteristics of subjects (N = 126) used in statistical data analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of subjects</strong></td>
<td>Healthy controls (CO)</td>
</tr>
<tr>
<td>Females (%)</td>
<td>22 (51.2%)</td>
</tr>
<tr>
<td>Years of age (±SD)</td>
<td>34.2 (±3.7)</td>
</tr>
<tr>
<td>BMI (±SD)</td>
<td>23.9 (±3.0)</td>
</tr>
<tr>
<td>Overnight fasting</td>
<td></td>
</tr>
<tr>
<td>Fasting time in hours (±SD)</td>
<td>11.9 (±2.6)</td>
</tr>
<tr>
<td><strong>Migraine characteristics</strong></td>
<td></td>
</tr>
<tr>
<td>Migraine frequency (attacks per month)</td>
<td>—</td>
</tr>
<tr>
<td>Headache days (days per month)</td>
<td>—</td>
</tr>
<tr>
<td>Migraine &lt; 3 days after LP</td>
<td>—</td>
</tr>
<tr>
<td>Yes (%)</td>
<td>—</td>
</tr>
<tr>
<td>Unknown (%)</td>
<td>—</td>
</tr>
<tr>
<td><strong>Medication use</strong></td>
<td></td>
</tr>
<tr>
<td>Prophylactic medication (%)</td>
<td>—</td>
</tr>
<tr>
<td>β-Blocker (%)</td>
<td>—</td>
</tr>
<tr>
<td>Antiepileptic drug (%)</td>
<td>—</td>
</tr>
<tr>
<td>Serotonine-antagonist (%)</td>
<td>—</td>
</tr>
<tr>
<td>Angiotensin II receptor antagonist (%)</td>
<td>—</td>
</tr>
<tr>
<td>Attack medication</td>
<td>—</td>
</tr>
<tr>
<td>Triptan (%)</td>
<td>—</td>
</tr>
<tr>
<td><strong>Sampling characteristics</strong></td>
<td></td>
</tr>
<tr>
<td>Opening pressure in mmH₂O (±SD)</td>
<td>17.1 (±3.6)</td>
</tr>
<tr>
<td>Sampling time in seconds (±SD)</td>
<td>105.5 (±63.0)</td>
</tr>
<tr>
<td><strong>CSF characteristics</strong></td>
<td></td>
</tr>
<tr>
<td>Protein concentration in g L⁻¹ (±SD)</td>
<td>0.33 (±0.11)</td>
</tr>
<tr>
<td>Glucose in mmol L⁻¹ (±SD)</td>
<td>3.0 (±0.25)</td>
</tr>
</tbody>
</table>

BMI = body mass index; CSF = cerebrospinal fluid; LP = lumbar puncture. Age, BMI, migraine frequency, headache days, migraine duration values are averages with standard deviation in brackets. For the variables that differed significantly between groups one-way ANOVA for continues data, chi-square for proportions, significant p-values (< 0.05) are depicted in bold.

Results

Patients and controls

We obtained CSF and clinical data from 91 patients with migraine (19 had hemiplegic migraine, 39 had migraine with aura, and 33 had migraine without aura) and 45 healthy controls (Table 1). CSF was collected in adherence to a strict sampling protocol and was obtained for research purposes only. There was no statistically significant difference in gender distribution between the groups (p = 0.313). The mean age of the controls was statistically significant lower compared with the migraine patients (p = 0.009). The mean attack frequency was 2.5 (±2.8) attacks per month resulting in an average of 4.9 (±2.8) headache days per months in migraine patients. All migraine patients were sampled in between migraine attacks and were attack-free for at least three days before lumbar puncture; seven patients experienced a migraine attack in the three days following CSF sampling. There were no statistically significant differences in routine CSF measurements (erythrocytes, leukocytes, total protein, and glucose levels) between the groups.

Data pre-processing and outlier detection

After visual inspection of the 136 2D-JRES NMR, four spectra had to be removed due to insufficient water suppression and one spectrum because of a highly elevated and fluctuating spectral baseline (Fig. S4, ESI†). In the 2D-JRES skyline projections, some
peaks were obscured by spectral crowding, or hard to quantify due to low signal-to-noise. The peaks of which the integrated intensity could be reliably determined in at least 50% of all spectra were integrated and used in further analysis. This procedure resulted in a data table, consisting of peak position and integrated intensity of 74 features. For the purpose of visualisation, 1D-NOESY spectra were obtained, of which representative examples are included in the ESI,† Fig. S5.

Subsequently, this dataset was investigated for the presence of outliers using PCA (Fig. S6, ESI†). Outlier detection was carried out including all 74 features. A total of five cases (three subjects with migraine, one with hemiplegic migraine, and one healthy control) showed highly different feature profiles. Visual inspection of the NMR spectra showed increased lipid profiles or the presence of additional compounds, as possible explanations of these altered profiles. These cases were classified as outliers and left out of further analysis.

**Multivariate statistical analysis**

PCA analysis was performed to detect the effects of main covariates, such as age, gender, and disease status in the data (Fig. 1A and B). This unsupervised method allows detection of trends and groupings, based only on similarities over the integrated intensity of 74 features. No clear effect of migraine status was observed with PCA analysis, for any of the principle components (Fig. 1B). A slight grouping of hemiplegic migraine cases was observed in the 4th principle component. Of particular importance for further analysis was the presence of age effects in the first two principle components that explain 45% of the total variance (Fig. 1A). Because of the high prevalence of migraine prophylactic and/or triptan use, PCA analysis was also utilized to detect any effects of different types of prophylactics and triptan. Being performed on the entire dataset, PCA analysis would allow for both the detection of residual substances, as well as detection of extended alterations of the entire metabolome. No major effects of migraine prophylactic and/or triptan use were observed. Only a slight effect of the subgroup of the migraine patients using antiepileptic drug as migraine prophylactic treatment was detected in the 6th principle component, accounting for 4.1% of all variance (Fig. S7, ESI†). However, important loadings constituting the 6th PC do not contain any known metabolites or peaks used in the final analysis, and its effects on the final analysis are therefore minimal.

A targeted approach was used to determine if there were any differences between the migraine groups, and the matched control group, based on general trends in the set of 16 identified and quantified metabolites. Only identified metabolites were included in order to maximize the interpretability of the model. A pair wise comparison of control groups with equally-sized, age-matched migraine groups was employed, both consisting of equal numbers of males and females. To reduce model stability issues resulting from collinearity problems, a penalized form of logistic regression was used: the elastic net. The elastic net routine employs logistic regression, but regularizes the size of the regression coefficients by including a penalty term. The elastic net does automatic variable selection by allowing some regression coefficients to shrink to zero, while treating highly correlated variables as a group by assigning similar coefficients, thereby yielding a sparse and less noisy model.29 The predictive ability of the model was evaluated by repeated 10-fold cross-validation. In addition, random permutation routines were used to assess the amount of random findings.

The logistic regression model of hemiplegic migraine cases versus healthy controls was able to partially separate the two diagnostic groups in the modelled data (Fig. S8, ESI†). To test the predictive capabilities of the model repeated 10 fold cross-validation was used. Cross-validation results were visualized and quantified by ROC analysis. This type of analysis visualizes the model its ability to correctly predict the test-cases their true class, and the degree of overlap and separation between the predictions of the two diagnostic groups. ROC analysis showed test-cases could be predicted with a moderate degree of accuracy, with an AUC average of 0.65 (Fig. 2B). In addition, an ANOVA approach was used to model the cross-validated predictions. In comparison to

![Fig. 1](image-url)  
**Fig. 1**  
PCA analysis of 1H-NMR migraine data. PCA analysis, showing that there is no clear separation of groups. (A) PCA plot showing an effect of age in the first component. (B) PCA plot showing that there is no clear separation between groups in the first two components. HM: hemiplegic migraine, MO: migraine without aura, MA: migraine with aura.
variation around the mean, the average cross-validated predictions were marginally significant, at $p = 0.08$ (Table S2, ESI†). To analyse which variables were important for prediction, both the variable inclusion in the model by variable selection, and regression coefficients were observed. Both 2-hydroxybutyrate and 2-hydroxyisovalerate were included in all cross-validated models with relatively high regression coefficients, indicating their relative importance in HM prediction. Fructose obtained relatively high regression coefficients, but was not included in every single model. Other metabolites picked by this routine were only included in a subset of models, and had comparatively low average coefficients (Fig. 2A). The model outperformed over 95% of all randomly generated models, suggesting a low degree of additional structures in the dataset (Fig. 2C). No predictive model could be generated that separated either migraine without aura or migraine with aura from the control group.

Univariate statistical analysis

The findings with the multivariate method raised questions about the behaviour of individual metabolites, and the influence of the covariates age and gender on the metabolite levels, perhaps interacting with the migraine status. Mann–Whitney tests were used to study the behaviour of individual metabolites (Fig. 3). The levels of only three metabolites showed a significant difference ($p < 0.05$) when comparing hemiplegic migraine and control samples: 2-hydroxybutyrate, fructose, and 2-hydroxyisovalerate. Since these results could also come from the age and gender imbalance present in the dataset, GLMs were built for all individual metabolites. Thereby, the effect of age and gender, as well as migraine status, could be studied. Migraine status showed to be a significant factor for three metabolites (Fig. 4). 2-Hydroxybutyrate showed to be significantly lower for hemiplegic migraine (23 μM ± 9.2) compared with controls (29 μM ± 12, $p = 0.003$) with no significant effect of the covariates age and gender. 2-Hydroxyisovalerate levels were influenced by both migraine status and gender, and were significantly lower for hemiplegic migraine (5 μM ± 1.4) compared with controls (7 μM ± 2.1, $p = 0.003$).

Choline showed to be significantly lower in both migraine with aura and migraine without aura, in an age-dependent manner.

Discussion

The aim of this study was to search for biochemical changes in CSF composition to learn more about potential biochemical differences between the different subtypes of migraine. To this end, we applied an exploratory $^1$H-NMR-based metabolomics approach to study the interictal (i.e., in migraine-free periods) biochemical composition of CSF of migraine patients compared with healthy controls. $^1$H-NMR is commonly accepted as a robust
and standardized platform in metabolomics with the potential to be used for routine diagnostics. H-NMR analysis of CSF has been used in neurological disorders, such as complex regional pain syndrome, multiple sclerosis, Alzheimer’s disease, motor neuron disease, and schizophrenia. These studies seemed to imply that untargeted approaches, which make use of the spectral bins or peak integrals (i.e., features), are not optimal. We therefore used a semi-targeted approach aiming to annotate as many metabolites as possible. The 2D-JRES-NMR method used in this study allowed us to identify a large set of metabolites in CSF, by reducing the problem of overlapping metabolite peaks. Reducing the complexity and dimension of the data, this approach simplifies the biological interpretation. However, by working only with a set of annotated metabolites, we might have missed some potentially relevant information from yet unidentified spectral features.

To date only a few metabolomics studies of migraine have been published (for reviews see ref. 38–40). Consequently, our knowledge of migraine-related biochemical changes is limited. Cortical spreading depression-induced brain metabolic changes have been captured in plasma of a transgenic migraine mouse model of familial hemiplegic migraine using CE-MS and multivariate data analysis. As part of routine CSF measurements glucose levels have been frequently reported to be normal in migraine patients. A recent meta-analysis on biochemical compounds measured in migraine found that glutamate, calcitonin-gene related peptide and nerve growth factor concentrations are consistently higher and β-endorphin concentrations are lower in CSF from migraine patients compared with non-headache controls. These compounds could not be analyzed using the platform of the present study. Rothrock et al. have reported higher r-glutamine levels in patients with episodic and chronic (i.e., patients that have headache/migraine more than 15 days per month) migraine compared with healthy controls. However, the healthy controls in that study were significantly younger than the migraine patients and no correction for age effect was applied. Our data show no significant differences in r-glutamine levels between age- and gender-matched migraine patients and controls.

The present metabolomics study describes the largest sample set of migraine patients and healthy controls so far (126 subjects). Yet, considering the number of subgroups included, group sample sizes are still relatively small (depending on the analyses between 18 and 43 subjects per group). Special care was taken regarding the slight imbalance in both age and, to a lesser extent, gender between groups, by correcting for age and gender effects in univariate statistical analysis, and constructing age- and gender-balanced groups for multivariate analysis. Unfortunately, the sample sizes did not allow for the use of an independent validation set, we therefore used repeated 10-fold cross-validation to evaluate the predictive capabilities of the multivariate models. Differences in CSF composition observed between controls and migraine patients were modest. Most notable is the finding of lower concentrations of 2-hydroxybutyrate and 2-hydroxyisovalerate in CSF of patients with hemiplegic migraine. These were also the two most important metabolites in the multivariate model for hemiplegic migraine versus healthy controls. Both metabolites were also confirmed by univariate Mann–Whitney tests and by linear models that allowed for age and gender compensation.

2-Hydroxybutyrate is one of the compounds that is consistently reported as a constituent of CSF. Moreover, it was shown “borderline elevated” in CSF of patients with lactic acidosis and consistently elevated in CSF of patients with propionic acidemia, a metabolic disorder in which a defective form of the enzyme propionyl-coenzyme A (CoA) carboxylase results in the accumulation of propionic acid. Increased levels of urinary 2-hydroxybutyrate are found in early stage type II diabetes. Notably, in our study the levels of 2-hydroxybutyrate are found decreased for hemiplegic migraine compared to control subjects. Exploratory case-control studies give little room for a functional interpretation. By providing a static ‘snapshot of the metabolome’, such a study design makes mapping of individual metabolites to pathways rather speculative.

2-Hydroxybutyrate is not an exception. The bulk of 2-hydroxybutyrate is derived from alpha-ketobutyrate, which can be e.g. a product of threonine catabolism or the lysis of cystathionine. The direction of the enzymatic conversion (alpha-ketobutyrate to 2-hydroxybutyrate or vice versa) is also dependent on NADH2/NAD ratio. Thus, one could speculate that lower levels of 2-hydroxybutyrate are a consequence of the dysregulation of the brain’s energy metabolism. This hypothesis is in part supported by observations of Uncini et al. who used 31P magnetic resonance spectroscopy (13P-MRS) and found higher concentrations of adenosine diphosphate (ADP) in the brains of the patients with familial hemiplegic migraine. It was concluded that the metabolic rate of mitochondria in these patients is close to the maximum rate. A 31P-MRS study of patients with migraine with aura and migraine without aura reported similar findings of increased metabolic activity and a reduced energy reserve. Alternatively, one could explore a link with the...
threonine metabolism: threonine is an essential amino acid and its brain pool is larger than the one in peripheral tissues. An experimental confirmation in vitro or in animal models will be required to confirm or discard these hypotheses.

Building a plausible functional hypothesis around 2-hydroxyisovalerate (2-hydroxy-3-methylbutyric acid, 2-oxyisovalerate), which is also a usual constituent found in CSF,18,42 also is a daunting challenge. The compound is a minor biochemical hub in amino acid metabolism and its source cannot be easily be determined.47 Moreover, it has been show that levels of 2-hydroxyisovalerate in CSF of multiple sclerosis patients are lower than in controls.18 Notably, the compound was included in a panel of biomarkers predicting progression of insulin resistance-related disorders.49

We here present the largest metabolomics study of CSF of migraine patients thus far. Using an exploratory 1H-NMR metabo-lomics analysis we identified metabolites 2-hydroxybutyrate and 2-hydroxyisovalerate that are able to discriminate hemiplegic migraine patients from healthy controls. None of the analysed metabolites was able to separate common migraine with or without aura from controls.

Abbreviations

AUC Area under the curve
BMI Body mass index
CNS Central nervous system
CSF Cerebrospinal fluid
FDR False discovery rate
FID Free induction decay
GLM Generalized linear models
1H-NMR Proton nuclear magnetic resonance
JRES J-Resolved spectra
NOESY Nuclear overhauser effect spectroscopy
PCA Principle component analysis
RMSEP Root mean squared prediction error
ROC Receiver operator characteristic
TSP Sodium 3-trimethylsilyltetradeuteriopropionate

Disclosures

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References

2 B. K. Rasmussen and J. Olesen, Cephalalgia, 1992, 12, 221–228.