^{1}H-NMR spectroscopy in serum suggests altered concentrations of several metabolites in active migraineurs

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Abstract

Objective – Migraine is a prevalent neurovascular disorder that is diagnosed based on consensus criteria due to the lack of reliable biomarkers. We here used a single-point measurement of serum samples to identify low-molecular-weight metabolites associated with migraine, which may help unraveling pathophysiological mechanisms involved in the disease.

Methods – From 289 migraine patients and 1,360 controls without severe headache from the Erasmus Rucphen Family (ERF) study fasting serum samples were available for proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. A total of 100 signals representing at least 49 metabolites were detected. Using elastic net regression analysis we identified a subset of metabolites associated with lifetime or active migraine and cross-validated these analyses two-fold. Odds ratio, p-values and explained variance of the scores were calculated using logistic regression analysis.

Results – The subset of peaks that was identified for lifetime migraine status was not significantly associated with the outcome after age and sex correction. However, for active migraine we identified a set of 20 peaks, containing amino acids, lipids, pyruvate, dimethylglycine, 1,5-anhydrosorbitol and glucose, which significantly ($p = 6.2*10^{-3}$) associated with active migraine status after correction for age and sex.

Interpretation – Our results show that serum concentrations of several low-molecular weight metabolites measured by ^{1}H-NMR spectroscopy differ between migraine patients and controls, especially in active migraineurs. Future studies with similar NMR data containing all associated peaks could validate our findings. The associated metabolites provide important information that might help elucidate the pathophysiological processes underlying this disorder.

Key Words: ^{1}H-NMR spectroscopy ■ Migraine ■ Biomarker

Introduction

MIGRAINE IS A HIGHLY PREVALENT episodic neurovascular disorder that is characterized by recurrent throbbing, unilateral headache of moderate to severe intensity, which is aggravated by physical exercise and lasts 4-72 hours.[1] Attacks are accompanied by nausea, vomiting, photophobia, and/or phonophobia. Due to the lack of reliable biomarkers, migraine diagnoses is still based on history taking and interviews according to the consensus criteria of the International Classification of Headache Disorders (ICHD-2) from the International Headache Society.[2]

Various studies have attempted to find reliable clinical, genetic, radiological and CSF biomarkers to diagnose migraine patients.[3] Studies that focused on identifying biochemical biomarkers in blood until now focused on only a limited amount of molecules and these studies have been hypothesis-driven, that is they focused on e.g. neuroexcitatory amino acids,[4,5] inflammatory markers,[6-10] vasoactive neuropeptides,[11] and cardiovascular risk factors.[12,13]

In order to search for migraine biomarkers in serum in a hypothesis-free manner, we performed proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, which is widely used to acquire metabolic profiles from large groups of patients because of the robustness and relatively low costs of this method.[14] With ^{1}H-NMR spectroscopy it is possible to quantify up to several hundreds of low-molecular weight metabolites in a single measurement in serum or plasma. We analyzed metabolite profiles in serum samples of migraine patients and controls from the Erasmus Rucphen Family population, a large Dutch population-based family study from the Southwest of the Netherlands in which we previously had identified migraine cases.[15] By comparing metabolic profiles of migraine patients and control individuals we set out to identify potential (sets of) biomarkers that may also provide valuable insights into pathophysiological mechanisms underlying migraine.

Material and Methods

Study population

The study flow is depicted in Figure 1. Subjects were participants of the Erasmus Rucphen Family (ERF) study. This is a population-based family study from a genetically isolated community in the Southwest of the Netherlands. The ERF study population includes 3,465 living descendants of 22 couples that had at least six children baptized in the community church between 1850 and 1900. Participants were not selected based on disease of interest. The extensive genealogy and pedigree of the population has been published previously.[16] The Medical Ethical Committee of the Erasmus Medical Center approved of the study and all participants provided written informed consent.

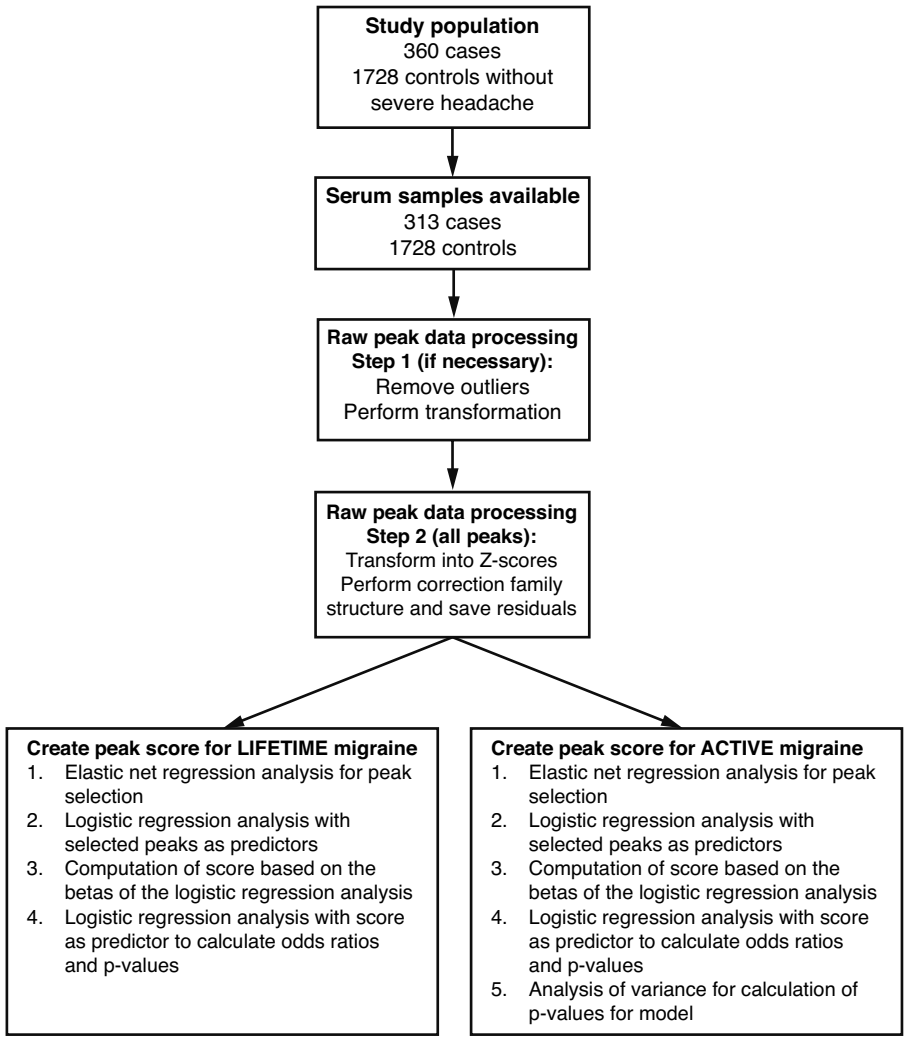

Figure 1 - Study flow.

Migraine diagnoses

Participants were selected between 2005 and 2007 using a validated three-stage screening procedure[17] assessing lifetime occurrence of migraine based on the second edition of the International Classification of Headache Disorders (ICHD-2).[2] Details on the migraine case-finding procedure have been published.[15] In short, stage one consisted of a five-item screening questionnaire on headache and aura symptoms. If the screening criteria were met, participants entered the second stage and completed a detailed questionnaire on headache and aura symptoms. The final stage consisted of telephone interviews with

participants meeting the screening criteria specified by step one. In addition, two groups of subjects were contacted by telephone directly: (i) screen-positives subjects who did not return the extended questionnaire and (ii) subjects providing no or incomplete data on the screening questionnaire. A study physician, who consulted a headache specialist in case of ambiguous symptoms, made final diagnoses. A total of 360 lifetime migraineurs and 1,728 controls were identified. Fasting serum samples were available from 313 of these migraine patients and 1,512 controls without severe headache, which were used for ^{1}H-NMR spectroscopy profiling.

^{1}H-NMR spectroscopy metabolite profiling: data processing and quality control

Details on the acquisition of NMR spectra can be found in the Supplementary Materials and Methods section. Good-quality NMR spectra were obtained from 289 migraine patients and 1,360 controls. Post-acquisition data processing and quality control (QC) on the set of ^{1}H-NMR spectra were carried out in Matlab® (R2009a, The Mathworks Inc., Natick, MA, USA). For QC, a set of spectroscopic parameters (i.e. shim values and intensity of the water signal) was examined and spectra were visually inspected. Spectra that failed the QC were not included for further analysis. Subsequently, the spectra that passed the QC were scaled with respect to the sensitivity of the experiment, which is inversely proportional to the pulse length. After subtracting a constant value as a simple baseline correction, the spectra were calibrated to the anomeric resonance of α-D-glucose (δ = 5.23 ppm).[18] Since there are small deviations of the peak position in the different ^{1}H-NMR spectra, alignment was performed using the correlation optimized warping algorithm by Tomasi et al.[19] This was performed actively for the Carr–Purcell–Meiboom–Gill (CPMG) spectra, after which the same warping was applied to the J-resolved spectra (JRES) projection. The peaks in the JRES projection were automatically deconvoluted by fitting the spectra with mixed Gauss-Lorentz lineshapes using the Simplex method. As the fitting algorithm incidentally converges to a local minimum, values further from the median than three times the interquartile range were discarded. Using partial least square regression, the remaining peak intensities were used to build a linear model that predicts the intensities directly from the unwarped spectrum, yielding also reasonable values for the cases where the deconvolution or warping algorithms failed.

Using the abovementioned procedure, 100 metabolite peaks were detected in the JRES projection and quantified in the ^{1}H-NMR spectra. In total, we had available spectra of 289 migraine patients and 1,360 controls for further analysis. For 82 peaks, metabolites could be assigned using information from the Human Metabolome Database (HMDB)[20] and the Pearson correlation coefficients between the peaks intensities. These 82 peaks represented 49 different metabolites (i.e. after subtracting the peaks representing EDTA,

Table 1 - Overview of peaks identified in the 2D J-resolved spectrum and performed transformations.

Peak number	Chemical shift (ppm)	Assignment (Jan 2012)	Trans-formation	Peak number	Chemical shift (ppm)	Assignment (Jan 2012)	Trans-formation
1	0,85113	unknown	none	51	2,93035	asparagine	none
2	0,87072	lipids CH3	LN	52	3,02340	lysine	none
3	0,89006	cholesterol	LN	53	3,03376	creatine/creatinine	none
4	0,92847	isoleucine	none	54	3,04941	ornithine	none
5	0,94597	leucine	none	55	3,14304	unknown	SD
6	0,95118	unknown	none	56	3,20231	choline	none
7	0,95702	leucine	none	57	3,21429	phosphorylcholine	none
8	0,97200	unknown	none	58	3,24004	glucose	none
9	0,98082	valine	none	59	3,26710	1,5-anhydrosorbitol	none
10	0,98612	unknown	none	60	3,33236	proline	LN
11	0,99919	isoleucine	none	61	3,34504	1,5-anhydrosorbitol	none
12	1,03145	valine	none	62	3,35396	unknown	none
13	1,06258	unknown	none	63	3,38052	unknown	none
14	1,11139	ketoisovalerate	none	64	3,40064	glucose	none
15	1,13461	unknown	SD	65	3,48648	glucose	none
16	1,16301	isopropyl alcohol	SD	66	3,53111	glucose	none
17	1,17437	ethanol	SD	67	3,55110	glycine	none
18	1,19057	3-hydroxybutyrate	SD + LN	68	3,55914	glycerol	none
19	1,21117	unknown	none	69	3,58832	1,5-anhydrosorbitol	none
20	1,26482	lipids CH2	LN	70	3,59782	valine	none
21	1,31981	lactate	none	71	3,62232	myoinositol	SD
22	1,38997	unknown	none	72	3,65021	ethanol	SD + LN
23	1,40660	unknown	none	73	3,65855	isoleucine	none
24	1,42426	unknown	none	74	3,71204	glucose	none
25	1,47045	alanine	none	75	3,72103	glucose	none
26	1,70571	unknown	none	76	3,74475	unknown	none
27	1,90859	acetate	SD	77	3,75932	glucose	none
28	1,99964	lipids	none	78	3,77643	alanine	none
29	2,03401	N-acetyl glycoproteins	none	79	3,80094	glucose	none
30	2,06653	O-acetyl glycoproteins	none	80	3,81746	unknown	none
31	2,10168	glutamine/glutamate	none	81	3,82382	glucose	SD
32	2,11814	glutamine/glutamate	none	82	3,83140	unknown	none
33	2,13291	acetylcarnitine	none	83	3,83856	glucose	SD
34	2,22215	lipids (CH2CO)	LN	84	3,87709	1,5-anhydrosorbitol	none
35	2,26037	valine	none	85	3,89397	glucose	none
36	2,27276	acetoacetate	SD	86	3,92001	creatine	none
37	2,30052	3-hydroxybutyrate	SD	87	3,93298	unknown	none
38	2,34857	glutamate	SD	88	3,95567	serine	none
39	2,36196	pyruvate	none	89	3,97538	phenylalanine/histidine	none
40	2,39235	3-hydroxybutyrate	SD	90	4,04386	creatinine	none
41	2,42815	glutamine	none	91	4,10334	lactate	LN
42	2,44561	glutamine	none	92	4,12106	proline	none
43	2,46282	glutamine	none	93	4,23715	threonine	none
44	2,52733	citrate	SD	94	4,50117	unknown	SD
45	2,59803	unknown	none	95	5,17855	mannose	none
46	2,63742	methionine	none	96	5,22921	glucose	SD
47	2,66908	citrate	SD	97	5,29802	lipids (CH=CH)	LN
48	2,70842	dimethylamine	none	98	6,89014	tyrosine	none
49	2,89562	unknown	none	99	7,18628	tyrosine	none
50	2,91618	dimethylglycine	SD	100	8,44976	formate	none

LN: log transformation performed; SD: outliers > 4 standard deviations from the mean removed.

see Table 1 for peak assignment). The other 18 peaks could not be annotated yet and were discarded from further analysis.

^{1}H-NMR metabolite profiling: acquisition of NMR spectra

Stored fasting serum EDTA samples were thawed at 4°C and were mixed by inverting the containers ten times. Samples (300 µL) were mixed with 300 µL 75 mM disodium phosphate buffer in H_2O/D_2O (80/20) with a pH of 7.4 containing 6.15 mM NaN_3 and 4.64 mM sodium 3-[trimethylsilyl] d4-propionate (TSP) using a Gilson 215 liquid handler in combination with a Bruker SampleTrack system (Bruker, Karlsruhe, Germany). Samples were transferred into 5mm SampleJet NMR tubes (Bruker, Karlsruhe, Germany) in 96 tube racks using a modified Gilson 215 tube filling station (Gilson, Middleton, WI, US) and kept at 6°C on a SampleJet sample changer (Bruker, Karlsruhe, Germany) while queued for acquisition.

All proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy experiments were acquired on a 600 MHz Bruker Avance II spectrometer (Bruker, Karlsruhe, Germany) equipped with a 5 mm TCI cryogenic probe head with Z-gradient system and automatic tuning and matching. All experiments were recorded at 310K. Temperature calibration was done prior to each batch of measurements using the method of Findeisen et al.[21] The duration of the $\pi/2$ pulses were 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.[22]

For water suppression pre-saturation of the water resonance with an effective field of $\gamma B_1 =$ 25 Hz was applied during the relaxation delay.[18] J-resolved spectra (JRES)[23] 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 x 12,288 data points was collected covering a sweep width of 78 x 10,000 Hz. A sine-shaped window function was applied and the data was zero-filled to 256 x 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*(128-k) points and symmetrised about the central horizontal lines in order to compensate for the skewness of the multiplets in the F1 dimension. For T2-filtered ^{1}H-NMR spectra a standard 1D CPMG pulse sequence,[24,25] was used with a relaxation delay of 4 s. A pulse train of 130 refocusing pulses with individual spin echo delays of 0.6 ms were applied resulting in a total T2 filtering delay of 78 ms. 73,728 data points covering a spectral width of 12,019 Hz were collected using 16 scans. The FID was zero-filled to 131,072 complex data points and an exponential window function was applied with a line broadening factor of 1.0 Hz prior to Fourier transformation. The spectra were automatically phase and baseline corrected.

Quality control, scaling and calibration of the NMR spectra

Further data processing was performed in Matlab® (R2009a, The Mathworks Inc., Natick, MA, USA). The spectra and associated data were converted into Matlab files using in-house code These files can be quickly loaded into Matlab for further processing. First, the spectra were combined into one file while removing superfluous information. For CPMG this included dropping the imaginary part of the spectrum, while for the JRES spectra the sum projection along the indirect dimension was taken. Quality control (QC) on the set of ^{1}H-NMR spectra was carried out by examining a set of spectroscopic parameters such as shim values and intensity of the water signal, and subsequently visually inspecting the spectra. The spectra that failed the QC were not included for further analysis. The spectra were then scaled with respect to the sensitivity of the receiver coil. This sensitivity is inversely proportional to the pulse length, which is dependent on the tuning of the RF coil. After subtracting a constant value as a simple baseline correction, the spectra were calibrated with respect to the anomeric resonance of α-D-glucose (δ = 5.23 ppm).[20] Since there are small deviations of the peak position in the different ^{1}H-NMR spectra, alignment was performed using the correlation optimized warping algorithm by Tomasi et al.[19] This was performed actively for the CPMG spectra, after which the same warping was applied to the JRES projection. The peaks in the JRES projection were automatically deconvoluted by fitting the spectra with mixed Gauss-Lorentz lineshapes using the Simplex method. As the fitting algorithm incidentally gets stuck in local minima, values further from the median than 3 times the interquartile range are discarded. Using PLS regression, the remaining peak intensities were used to build a linear model that predicts the intensities directly from the unwarped spectrum, yielding also values for the cases where the deconvolution values were discarded and eliminating the problem of faulty warping.

The peaks were assigned using information from the Human Metabolome Database[26] and the Pearson correlation coefficients between the peaks intensities.

^{1}H-NMR spectroscopy metabolite profiling: statistical analyses

Demographic characteristics of cases and controls were compared using a Student t test for continuous variables and χ^2 statistics for dichotomous variables. Raw ^{1}H-NMR peak data were processed in four steps. First, we removed outliers from the data by filtering out all data points more than four standard deviations (SD) away from the mean. This was necessary for 19 of the 100 peaks. Second, the distribution was checked for normality, and data were transformed if necessary. We performed log-transformations on nine peaks (of which two were also processed in the first step). For the remaining 74 peaks additional processing was not necessary. Analyses were performed using SPSS software version 20.0 (SPSS-IBM, NY). Third, we adjusted the peak data for family structure by using the

Table 2 - Demographic characteristics of the study population.

Variable	Cases (N=289)	Controls (N=1,360)	p-value
Age (years)	46.5 ±12.1	48.7 ±14.5	0.013*
Female sex (%)	76.1	49.5	<0.000*
Education (%)			0.185
high	3.8	6.6	
medium	63.1	62.8	
low	33.1	30.6	

*Difference significant between migraine cases and controls ($p < 0.05$).

information from the kinship matrix in a linear regression analysis in GenABEL.[27] Fourth, the residuals from this linear regression model were transformed into Z-scores and used for further analyses.

After processing of the raw data we aimed to identify subsets of predictive peaks for (1) life-time diagnosis and (2) a diagnosis of active migraine (defined as having migraines in the last 12 months). We used a special form of regression analysis, called elastic net regression analysis, to overcome the problems encountered in conventional logistic regression analyses in studies like ours where the number of predictors (i.e. the ^{1}H-NMR peak data, age and sex) is much larger than the number of observations on the outcome measure (i.e. migraine status). We used the glmnet package for elastic net regression analysis[28] with alpha set to 0.5 and 50-fold cross-validation using R software version 2.14.2 (www.r-project.org). In this first cross-validation step we validated the selection of the peaks by performing our regression analysis on 50 randomly chosen samples of our study population. We performed an additional cross-validation step to assess the fit of the selected model on different subpopulations of our sample.

After identification of the set of associated peaks we entered these variables in a logistic regression model to determine the weights for each peak for this population. Subsequently, we used the betas from this regression analysis to transform the set of associated peaks into a weighted score per individual participant. This score was used in a second logistic regression analysis to calculate odds ratios, p-values and the proportion of explained variance. To correct for confounding by differences in age and gender distribution we also calculated adjusted odds ratios and p-values by performing a logistic regression analysis with these confounders as covariates.

To validate the findings from the previous analysis we performed analysis of variance (ANOVA) in which we compared the performance of the identified scores for migraine with the performance of a model containing only information on age and sex. The resulting p-value can be interpreted as the likelihood that all peaks identified by the elastic net analysis are only associated with migraine status by chance.

Table 3 - ^{1}H-NMR peaks associated with lifetime prevalence of migraine

Chemical shift (ppm)	Metabolite	Beta
0,928474601	Isoleucine	-,045
0,999191706	Isoleucine	-,206
1,406597219	Unknown	,177
2,637416682	Methionine	-,100
3,588320905	1,5-anhydrosorbitol	-,163

Table 4 - ^{1}H-NMR peaks associated with active migraine

Chemical shift (ppm)	Metabolite	Beta
0,890058516	Cholesterol	-,0101
0,928474601	Isoleucine	-,084
0,957021758	Leucine	-,0036
0,999191706	Isoleucine	,001
1,264820045	Lipds CH2	-,029
1,406597219	Unknown	,161
2,222145112	Lipids (CH*2CH=CH)	-,018
2,361963346	Pyruvate	,224
2,637416682	Methionine	-,084
2,916183974	Dimethylglycine	-,196
3,353959417	Unknown	-,159
3,588320905	1,5-anhydrosorbitol	-,114
3,59781855	Valine	-.023
3,721027815	Glucose	-,0318
3,95566792	Serine	,152
4,043855495	Creatinine	-,0098
4,121059468	Proline	-,122
4,50117004	Unknown	-,111
5,229206003	Glucose	-,239

Results

Demographic characteristics

We compared several demographic characteristics in our migraine patients compared to the control group (see Table 2). Migraine patients tended to be younger and, as can be expected, they were more often female than the controls. There was no significant difference in educational level between cases and controls. Of the 289 migraineurs in our study, 150 patients (58%) reported at least one migraine attack in the 12 months preceding the interview and were assigned to the group of "active migraineurs".

^{1}H-NMR score for life-time diagnosis of migraine

Using the complete group of 289 migraineurs we identified a subset of five peaks that were associated with migraine status using the elastic net regression analysis (see Table 3). One of these peaks could not be annotated yet, so it is unclear which metabolite it represents. The other four peaks represent isoleucine (2 peaks), methionine and 1,5-anhydrosorbitol. This score containing these five metabolites explained 3.6% of the variance in migraine status and was significantly associated with migraine status in the uncorrected analysis (OR = 2.72 (1.94 – 3.81), $p = 6.8*10^{-9}$). As it is known that NMR spectra are highly dependent on age and sex we corrected the score for these confounders. Unfortunately, after correction the association with migraine was no longer significant (OR = 1.40 (0.95 – 2.06), p = 0.088).

^{1}H-NMR score for diagnosis of active migraine

When including only the 150 active migraineurs, we identified a subset of 20 associated peaks using elastic net regression analysis (see Table 4). Notably, all five peaks that were part of the subset associated with lifetime migraine were also part of the 20 peaks that associated with active migraine status. Three of these 20 peaks have not been annotated yet. The remaining 17 peaks represent 14 metabolites: leucine, isoleucine (2 peaks), methionine, proline, serine, valine, dimethylglycine, glucose (two peaks), 1,5-anhydrosorbitol, cholesterol, lipids CH2, lipids CH2CO, creatinine and pyruvate. This score explained 8.7% of the variance and was significantly associated with active migraine status in the uncorrected analysis (OR = 2.72 (2.09 – 3.53), $p = 9.5*10^{-14}$). After correcting the score for age and sex it remained strongly associated (OR = 1.74 (1.27 – 2.40), $p = 6.2*10^{-3}$). The outcome of our ANOVA analysis was also significant ($p = 1.5*10^{-12}$), adding to the evidence for involvement of these metabolites in active migraine.

Table 5 - Associated serum metabolites previously studied in migraine patients and controls.

Metabolite	Concentration compared to controls	Number of patients	Number of controls	Reference
Creatinine	No difference	5,087	22,539	Kurth, Ridker & Buring 2008
Glucose (fasting)	No difference	111	463	Schwaiger et al. 2008
HDL-cholesterol	No difference	620	5135	Scher et al. 2005
	No difference	111	463	Schwaiger et al. 2008
	No difference	165	1285	Benseñor et al. 2011
	Decreased	5,087	22,539	Kurth et al. 2008
	Decreased	3,412	28,115	Winsvold et al. 2011
Total cholesterol	Increased	620	5,135	Scher et al. 2005
	Increased	5,087	22,539	Kurth et al. 2008
	No difference	111	463	Schwaiger et al. 2008
	No difference	165	1,285	Benseñor et al. 2011
	Decreased	3,412	28,115	Winsvold et al. 2011
Pyruvate	Increased	14	12	Okada et al. 1998
Leucine	No difference	31	9	Ferrari et al. 1990
Isoleucine	No difference	31	9	Ferrari et al. 1990
Methionine	No difference	31	9	Ferrari et al. 1990
Proline	No difference	31	9	Ferrari et al. 1990
Serine	No difference	31	9	Ferrari et al. 1990
Valine	No difference	31	9	Ferrari et al. 1990

Discussion

The aim of this study was to search for (a set of) serum metabolites associated with migraine status. Using ^{1}H-NMR spectroscopy metabolic profiling we were able to identify a subset of metabolites significantly associated with active migraine, but not with lifetime migraine in the large population-based Erasmus Ruchen Family (ERF) sample. Our study has several strengths compared with previous studies on plasma concentrations of metabolites in migraine patients. Our sample consists of a large number of well-characterized migraine patients and controls that are not affected by severe headaches. Participants were selected based on their ancestry and came from a relatively homogeneous population. Using ^{1}H-NMR spectroscopy, which is considered a highly robust method [14], we were able to test a large number of metabolites for their association with migraine in a hypothesis-free manner.

In our 150 migraine patients with active migraine in the year preceding the interview and the 1,360 controls we found an association with 22 peaks representing 16 known metabolites. In contrast, in the combined analysis of the same controls with our 150

patients with active and 139 patients with inactive migraine (i.e. lifetime migraine status) the subset of peaks associated with lifetime migraine was no longer significant after correcting for age and sex. The five peaks that showed some association with lifetime migraine status were also present among the 22 peaks that were associated with active migraine. The fact that we only saw significant association with active migraine may be due to the fact that that group is more homogeneous compared to the group with lifetime migraine, which allows smaller concentration differences to be detected. Alternatively, one could envisage that some metabolites undergo persistent concentration changes and are therefore picked up in our lifetime migraine analysis, while concentrations of other metabolites alter only temporarily, explaining why these metabolites are only significantly associated with active migraine status.

The 22 peaks associated with active migraine represent 19 annotated peaks and three unknown metabolites, which is a common problem in this type of studies, as the human metabolome has not been annotated completely.[14] The 19 annotated peaks represent leucine, isoleucine, methionine, proline, serine, valine, dimethylglycine, glucose, 1,5-anhydrosorbitol, cholesterol, lipids CH2, lipids CH2CO, creatinine, myoinositol, acetate and pyruvate. Of these metabolites, 1,5-anhydrosorbitol (inert metabolite from the diet that competes with glucose for reabsorption in the kidneys), myoinositol (involved in cerebral calcium homeostasis) and dimethylglycine (produced upon metabolizing choline into glycine) have not been studied in the serum of migraine patients before (www.hmdb.com). The other metabolites have been studied to some extent in migraine (for overview, see Table 5). Comparing the previous studies with our study is difficult, because they all used conventional platforms to assess metabolite concentrations. For the cardiovascular disease markers (total cholesterol, HDL-cholesterol, creatinine), results of these studies are not unequivocal. In our study, all lipid levels were decreased in migraine patients, which is in line with the findings from another European population-based study,[29] but is different from findings in other studies.[12,30-32] It should be noted, though, that comparison of these results is hampered because [1]H-NMR spectroscopy subdivides the lipids in different subclasses than conventional analyses.

Pyruvate levels in our migraine patients were higher than in controls. A small study with only 14 migraine patients also found increased plasma concentrations of pyruvate and suggested a role for abnormal mitochondrial metabolism in the pathophysiology of migraine.[33] Interestingly, lactate and pyruvate concentrations are also raised in patients with mitochondrial myopathy, encephalopathy, lactic acidosis with stroke-like episodes (MELAS) in which migraine frequently occurs as one of the presenting symptoms.[34]
Lastly, we found an association of migraine status with several amino acids. We found decreased concentrations of leucine, isoleucine, methionine and proline and increased concentrations of serine and valine in our migraineurs compared to controls. Some studies

from the early nineties addressed the role of neuroexcitatory amino acids in migraine patients and measured interictal serum levels using conventional assays.[4,35-37] Most of these studies focused mainly on plasma levels of glutamate and glutamine, but all amino acids associated with migraine in the ERF population were also measured in the study by Ferrari et al.[4] No differences were found between cases and controls in that study. This may be due to the small sample size of this study (including only 31 migraine patients) and the technical advances in ^{1}H-NMR spectroscopy that made it possible to detect much smaller differences in concentration than with the older met, but future studies using state of the art detection methods are necessary to seek confirmation of the association of migraine with these various amino acids.

Over the years, the important role of glutamate in monogenic and complex forms of migraine has been established firmly in several genetic and functional studies.[38] Interictal plasma glutamate levels also have been studied in several studies. Glutamate levels were shown to be increased in three of the four studies, either in the migraine without aura patient group[35] or in all migraine patients.[4,37] In contrast, a small study in 34 children with migraine found decreased plasma glutamate concentrations compared to controls.[36] Since all studies had relatively small sample sizes, it remains unclear to what extent plasma glutamate concentrations are altered in migraineurs. In our study, glutamine/glutamate and glutamate peaks were not associated with migraine status. It could be that there is indeed no difference between migraine patients and controls. An alternative explanation would be that our group with active migraine is too heterogeneous to pick up differences that could occur shortly after a migraine attack and do not persist for a long time.

Several limitations of our study have to be acknowledged as well. First, the set of metabolites that we studied covered only a small part of the human metabolome using our single metabolic profiling method.[14] Future complementary studies using different platforms may identify additional metabolites associated with migraine status. The second limitation is that our group of active migraine patients is defined by migraine attacks occurring in the last twelve months prior to the interview. Transient changes occurring shortly after a migraine attack in plasma concentrations may therefore be detectable in this study. The third limitation of our study is that we used the same population for discovery of the associated peaks as well as for assessing the magnitude of the association. Ideally, we would have been able to perform a replication study to validate our findings, but no ^{1}H-NMR spectroscopy study is currently available that measured at least the vast majority of the associated metabolites. We took specific precautions in our statistical analysis to minimize the lack of replication problem by using two cross-validations and performing the ANOVA analyses. In addition, the ANOVA analysis indicated that it is highly unlikely that none of these peaks has any association with active migraine. This shows that our set of associated peaks is an interesting starting point for further studies.

In conclusion, using [1]H-NMR spectroscopy, we were able to measure a large set of metabolites and perform a hypothesis-free metabolic-profiling study in migraine. We identified a subset of 22 metabolites that is associated with active migraine in our ERF population. For most of these metabolites, the relationship with migraine has not been studied before. Further studies are needed to validate our findings and to elucidate the possible pathophysiological basis of the association between migraine and each of these metabolites.

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