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Biochemical profiling of the brain and cerebrospinal fluid in migraine syndromes

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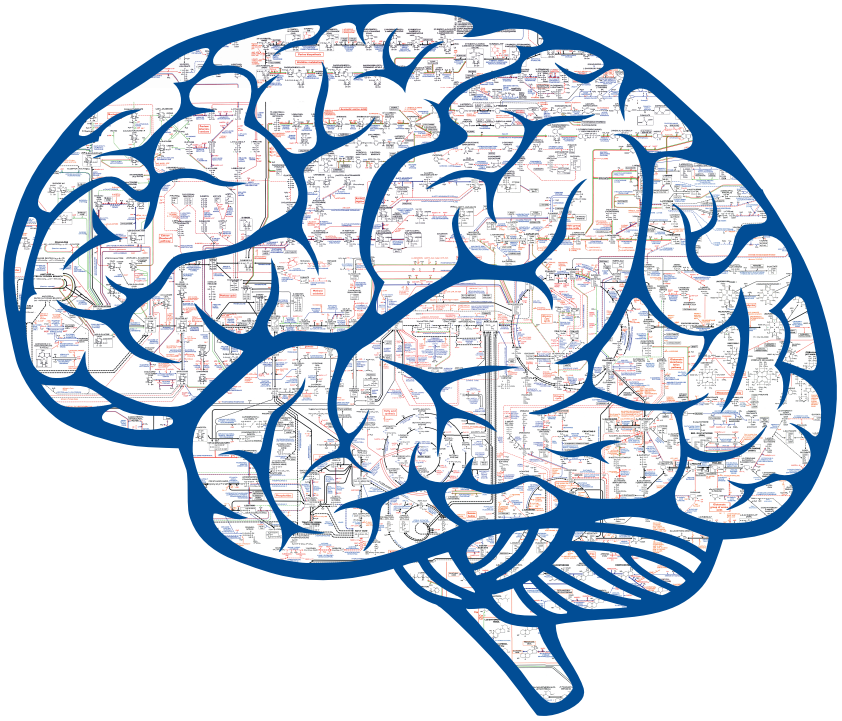
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Biochemical profiling of the brain and cerebrospinal fluid in migraine syndromes



Ronald Zielman

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Colophon

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Biochemical profiling of the brain and cerebrospinal fluid in migraine syndromes

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CHAPTER 1

General introduction

Migraine

Migraine is a common disease which is typically characterized by recurrent, disabling attacks of severe often unilateral headaches that are accompanied by symptoms of photo- and phonophobia, nausea and vomiting.[1] In one-third of patients attacks are preceded by transient neurological symptoms, called migraine aura.[2] When left untreated these attacks can last between several hours up to several days [3] and are very disabling for patients.[4] We lack a satisfying explanation on why and how such events start and end. Identification of the exact biochemical signature that is associated with migraine might help us in a better understanding of the pathophysiological mechanisms that lead to a migraine attack. Our understanding of the biochemical changes that occur around a migraine attack is far from complete and a real challenge because migraine is a very complex disorder, both from a phenotypical and a pathophysiological perspective. There is great variability in type, severity and duration of symptoms between patients. There are, however, commonalities in the clinical phenotype and it is, therefore, possible to distinguish different phases in migraine attacks which may aid biochemical research to identify biochemical signatures of the various phases of a migraine cycle.

Clinical characteristics of migraine

A typical migraine attack cycle consists of up to four phases: 1) the premonitory or prodromal phase, 2) the aura phase, 3) the headache phase, and 4) the postdrome phase.[3,5] Here I will describe the clinical characteristics of the different phases of a migraine attack in more detail.

The premonitory phase

The premonitory or prodromal phase precedes the aura and/or the headache. During this phase patients can experience a wide variety of non-headache symptoms. Premonitory symptoms have not been studied extensively and its exact prevalence rates remain difficult to estimate because of the subjective character of the symptoms. It is supposed that more than 80% of migraine patients experience at least one prodromal symptom.[6,7] The most frequently reported premonitory symptoms are fatigue, weariness, phonophobia, yawning, stiff neck,

gastrointestinal symptoms, mood and cognitive changes, temperature change, smell and taste distortion, and food craving.[6,8,9] The duration of the prodromal phase varies between patients and ranges from a few hours until one to two days before the migraine headache starts.[9] In a diary study, the prodromal symptoms correctly predicted a subsequent headache attack in 72% of patients.[8] Interestingly, the accuracy of migraine attack prediction based on premonitory symptom gradually increased towards a migraine attack, attacks were correctly predicted on 19% of occasions more than 24 hours before the start of the attack, and on 68% of occasions at around 6 hours before the attack.

The aura phase

The aura phase is characterized by transient neurological symptoms. The duration of these symptoms typically ranges from 5 until 60 minutes; in approximately 75% of patients the aura lasts less than 30 min.[10-12] The visual aura is the most prevalent aura symptom (80-90%) and can vary from simple flashes or lights to fortification scotoma or complex hallucinations.[2,13] Other symptoms are sensory paraesthesias mostly with cheiro-oral distribution (30%), aphatic speech disturbances (17%), and motor weakness (10%).[2,12] When several aura symptoms are present, their occurrence often follows a regular sequence: visual, sensory, speech and then motor.[14] The aura phase usually precedes the headache phase, but there may be a short gap between the end of the visual aura and the headache onset,[15] sometimes the headache starts already during the aura phase.[16]

The headache phase

The headache phase is characterized by a moderate to severe unilateral headache which mostly has a pulsating or throbbing character.[5,17,18] The majority of patients consider themselves severely disabled and a significant portion needs to go to bed during an attack.[19] Gastrointestinal disturbances including nausea, vomiting, abdominal cramps, or diarrhea are very common. Nausea occurs in more than 90% of migraineurs; nearly one-third experience nausea during every attack. Vomiting occurs in almost 70% of migraineurs; nearly one-third vomit in the majority of their attacks.[20] Sensory hyperexcitability manifested by photophobia, phonophobia, and osmophobia are frequently experienced.[20] The headache and associated symptoms can last for hours up to several days when no medication is used.[2]

The postdrome phase

After the headache phase, patients recover and might experience postdromal symptoms which can last from several days up till one week.[21] Postdromal symptoms occur in the majority of patients, with tiredness, weakness, cognitive difficulties, and mood changes being the most common; other symptoms include residual head pain, lightheadedness, and gastrointestinal symptoms.[7,22] There is some overlap between premonitory symptoms and postdromal symptoms, raising the possibility that the postdromal symptoms may have been present throughout the attack, but were overshadowed by the headache, nausea, and aura symptoms.[7]

Classification of migraine

Because of the variability in migraine symptoms among patients - even between recurrent attacks in the same patient - defining migraine has been difficult. A major step towards standardizing the diagnosis of migraine was made with the introduction of the International Classification of Headache Disorders (ICHD) as published by the International Headache Society; currently in its third (beta) edition.[23] In this classification the commonalities in symptoms and signs are captured in different diagnostic categories. The introduction of the ICHD reduced variation of diagnosis in population-based surveys and increasing the accuracy of migraine prevalence estimates. Besides epidemiological purposes, the ICHD is also an important tool for patient selection in clinical trials, scientific research and for diagnosis of patients in healthcare. According to the ICHD, migraine can be subdivided in migraine without aura and migraine with aura, distinguished based on the presence of an aura preceding the headache phase.

Migraine with and without aura

Migraine without aura is defined as a recurrent headache disorder manifesting in attacks lasting 4-72 hours. Typical characteristics of the headache are unilateral location, pulsating quality, moderate or severe intensity, aggravation by routine physical activity and association with nausea and/or vomiting and/or photophobia and phonophobia (table 1). Migraine with aura is defined as a recurrent disorder manifesting in attacks of reversible focal neurological symptoms that usually develop gradually over 5-20 minutes and last for less than 60 minutes. Headache with the features of migraine without aura usually follows the aura symptoms. Less

commonly, headache lacks migrainous features or is completely absent (table 1). Many patients who have frequent attacks with aura also have attacks without aura.[23]

Hemiplegic migraine

Migraine with aura including motor weakness is considered a subclass of migraine with aura, called hemiplegic migraine (table 2). It is characterized by a transient hemiplegia that may last from several minutes to hours or even days. If a patient has at least one first- or second-degree relative that also has migraine aura with motor weakness, this patient is classified as having familial hemiplegic migraine (FHM). If a patient has no first-or second-degree relative that has these symptoms,

Table 1: Classification of migraine without aura and migraine with aura (ICHD-III beta criteria)

Migraine without aura	
A.	At least five attacks fulfilling criteria B–D
B.	Headache attacks lasting 4-72 hours (untreated or unsuccessfully treated)
C.	Headache has at least two of the following four characteristics:
	1. unilateral location
	2. pulsating quality
	3. moderate or severe pain intensity
	4. aggravation by or causing avoidance of routine physical activity (e.g. walking or climbing stairs)
D.	During headache at least one of the following:
	1. nausea and/or vomiting
	2. photophobia and phonophobia
E.	Not better accounted for by another ICHD-3 diagnosis.

Migraine with aura	
A.	At least two attacks fulfilling criteria B and C
B.	One or more of the following fully reversible aura symptoms:
	1. visual
	2. sensory
	3. speech and/or language
	4. motor
	5. brainstem
	6. retinal
C.	At least two of the following four characteristics:
	1. at least one aura symptom spreads gradually over 5 minutes, and/or two or more symptoms occur in succession
	2. each individual aura symptom lasts 5-60 minutes
	3. at least one aura symptom is unilateral
	4. the aura is accompanied, or followed within 60minutes, by headache
D.	Not better accounted for by another ICHD-3 diagnosis, and transient ischaemic attack has been excluded.

he or she is classified as sporadic hemiplegic migraine (SHM). Both FHM and SHM patients in many cases also regularly have migraine attacks with or without aura but without the hemiplegia.[24] Therefore, hemiplegic migraine is considered to be part of the migraine spectrum, where migraine without aura is on one end, hemiplegic migraine is on the other end, with migraine with aura in the middle.[25]

Until now three genes that have been identified for monogenic FHM, which allows for a more precise definition of FHM.[26] In FHM1 there are mutations in the *CACNA1A* gene that encodes the pore-forming alpha1 subunit of voltage-gated neuronal Cav2.1 calcium channels. This type of FHM is responsible for over half of the families with FHM.[27] In FHM2 there are mutations in the *ATP1A2* gene that encodes the alpha2 subunit of a Na^+ ,K^+ pump ATPase and is responsible for about a quarter of FHM families.[28] Finally, in FHM3 there are mutations in *SCN1A* that encodes the alpha2 subunit of neuronal sodium channels and so far has been found in only a handful of FHM families.[29] FHM often presents with brainstem symptoms, in addition to the typical aura symptoms.[30] Rarely, during FHM attacks, disturbances of consciousness (sometimes including coma), confusion, fever and CSF pleocytosis occurs, and in some cases FHM attacks can be triggered by (mild) head trauma.[30] In approximately 50% of FHM families, chronic progressive cerebellar ataxia is present.[31]

Table 2: Classification of hemiplegic migraine according to the ICHD-III criteria

Hemiplegic migraine	
A.	At least two attacks fulfilling criteria B and C
B.	Aura consisting of both of the following:
	1. fully reversible motor weakness
	2. fully reversible visual, sensory and/or speech/language symptoms
C.	At least two of the following four characteristics:
	1. at least one aura symptom spreads gradually over 5 minutes, and/or two or more symptoms occur in succession
	2. each individual non-motor aura symptom lasts 5–60 minutes, and motor symptoms last <72 hours
	3. at least one aura symptom is unilateral
	4. the aura is accompanied, or followed within 60 minutes, by headache
D.	Not better accounted for by another ICHD-3 diagnosis, and transient ischaemic attack and stroke have been excluded.

Epidemiology and burden of disease

In western countries, the prevalence of migraine is estimated at approximately 6-8% in men and 16-18% in women; the epidemiologic profile has remained stable during the last decades.[4,19,32-34] The median attack frequency is 1-2 per month and the median attack duration is one day. At least 10% of patients have weekly attacks of 2-3 days each. The burden of migraine is therefore high and it is associated with deteriorated health-related quality of life and lost productivity, and has a big impact on patients and their families.[4,34] Consequently, migraine is rated by WHO among the most disabling chronic disorders,[35] and has been estimated to be the most costly neurological disorder in the EU.[36] The prevalence of hemiplegic migraine is much lower: approximately 0.01%. [24]

Migraine is a multifactorial disorder

The once popular vascular theory of migraine, which suggested that vascular headaches were caused by the dilatation of blood vessels and the aura resulted from vasoconstriction, is no longer considered viable in its original form.[37] The complex phenotype of migraine is mirrored by multiple pathophysiological mechanisms with the proposed mechanisms explaining only a few of the migraine symptoms. Here, I will shortly discuss the current ideas on migraine susceptibility and the underlying pathophysiology for the different phases of the migraine attack.

Genetic susceptibility and modulating factors – setting the baseline

Both genetic and non-genetic factors play a role in the susceptibility for migraine attacks.[38,39] Different mechanisms are involved operating on different time scales: i) *a genetic predisposition* causes lifelong susceptibility to migraine; in part through ion channel dysfunction;[26,40-42] ii) *modulating factors*, such as stress, relaxation, fatigue, and hormonal fluctuations, temporarily increase the susceptibility to migraine attacks;[43-45] and iii) *largely unknown patient-specific attack triggers*, cause the attack threshold to be surpassed.[46-48] While the range of factors that may trigger migraine attacks is very broad, there are communalities. In particular, vulnerability to migraine appears to be associated with altered neuronal excitability.[49,50] For instance, there is increasing evidence for increased glutamatergic excitatory neurotransmission as one of the underlying disease pathways.[51,52] Collectively,

these factors may lead to a brain state that is more susceptible to developing a migraine attack.[53] A clear biochemical correlate or knowledge of a neurobiological cascade of events that could explain how changes in excitability lead to an attack, however, is still lacking and dearly needed.

Prodromal phase and the hypothalamus, dopamine and altered brain excitability

The link between triggering factors and the activation of the migraine pain pathway remains poorly understood. A widely held hypothesis regarding the premonitory phase is that it involves changes in the hypothalamus.[54] A hypothalamic role has been hypothesized because symptoms involving changes in mood, appetite, and energy could be attributed to this brain region.[54] Moreover, positron emission tomography (PET) showed an increased blood flow in hypothalamus during a migraine attack.[55] Also changes in brain activity during premonitory symptoms, evoked by NTG using H2O PET, revealed increases in hypothalamic blood flow point in this direction.[56] Neurophysiological studies that suggested that the migraine brain has a reduced capacity for habituation for repeated sensory stimuli pointed towards cortical or brainstem dysfunction,[57] whether this dysfunction is a neuronal hypo- or hyper-reactivity, still is a matter of debate. Magnetic resonance spectroscopy (MRS) studies suggest a compromised brain energy (mitochondrial) system.[58] A specific hypothesis regarding premonitory symptoms involves neurotransmitter dopamine: exogenously administered dopamine receptor agonists produce some of the symptoms that are also experienced by migraine patients during the premonitory phase, namely yawning, nausea, drowsiness, and light-headedness.[59,60]

Aura phase and cortical spreading depression

The phenomenon of cortical spreading depression (CSD) seems to be the underlying mechanism of the aura symptoms.[39,61,62] This electrophysiological phenomenon starts in the occipital cortex and is characterised by a wave of intense neuronal and glial depolarization that propagates frontal through the cortex, until it hits upon a less sensitive part of the brain.[61] The wave front travels at a speed of 2-5 mm per minute and leaves affected neurons depolarized for several minutes to up to an hour.[61] Animal experiments have indicated that cortical spreading depression can activate the trigeminovascular system, thus suggesting a potential triggering mechanism for the headache in migraine.[63-67] The relevance in humans is still controversial.[63,68,69] Much research has focused on the electrophysiological

basis of the spreading process across the cortex,[70,71] but little is known about the mechanisms causing spreading depression to start. Studies in transgenic mouse models that carry human pathogenic FHM gene mutations that revealed a reduced triggering threshold, an enhanced speed, and subcortical propagation of the spreading depolarization waves,[72,73] provide the most compelling evidence thus far that CSD has relevance to human migraine pathology.

Headache phase and the trigeminovascular system and sensitisation

Activation of the trigeminovascular system (TGVS), which connects brain stem nuclei with dural blood vessels, is thought to be responsible for the headache. The migraine headache is believed to be the consequence of activation of largely unmyelinated fibres originating from the ophthalmic division of the trigeminal ganglion.[74,75] Substance P and calcitonin gene-related peptide (CGRP) are released upon activation and cause vasodilation of the meningeal arteries. CCRP release has been observed in both spontaneous[76-78] and in NTG-triggered attacks.[79] Which mechanisms actually trigger the activation of the TGVS, and thereby cause the migraine headache, is still not clear. One theory assumes firing of first-order peripheral trigeminal neurons in response to nociceptive signals from the meninges,[80] possibly due to cortical events such as a CSD[67], is the primary event. Another theory assumes that the headache starts by a dysfunction of subcortical structures, e.g. diencephalic and brainstem nuclei, that modulate the activation of the TGVS.[81]

There is increasing consensus about the concept that sustained activation of the TGVS leads to peripheral sensitization, where primary afferent nociceptive neurons exhibit increased responsiveness to external stimuli. This is considered the underlying mechanism of the throbbing character of the migraine headache, and may provide an explanation for why this headache aggravates during physical activities that increase intracranial pressure such as coughing and bending over.[80] During the headache phase migraine patients also experience an increased sensitivity to light (photophobia), sound (phonophobia), smell (osmophobia) and touch (allodynia). There is increasing evidence that the underlying mechanism for these symptoms is central sensitization, which is the condition where nociceptive neurons in the dorsal horn of the spinal cord exhibit increased excitability, increased synaptic strength, and enlargement of their receptive fields.[82] For allodynia there is a large body of evidence to show that central sensitization is the underlying mechanism, and it is suggested that the other symptoms of increased sensitivity might be also explained by central sensitization.[83]

Biochemistry of migraine – a biomarker for migraine?

Over the last decades numerous biochemical markers have been measured.[84,85] The majority of these studies were hypothesis-driven and focused on a single laboratory measure, or a few at most. Measured compounds were selected and focussed around one pathophysiological concept regarding migraine. However, most of the reported biochemical findings in literature have never been replicated,[84] and there are many contradictory findings, most likely due to differences in selected patients, moment in the migraine cycle, type of body fluid sampled, and measurement technique. It is therefore difficult to assess the relevance of many of these biochemical findings. Another complicating factor in interpreting the biochemical findings is related to different pathophysiological mechanisms that are related to different phases of the migraine attack. Biochemical compounds that are relevant during the headache phase, may not necessarily also be important during other phases of the migraine attack, or even outside a migraine attack.

There is, however, an increasing need for new biochemical biomarkers. Such biomarkers can help diagnosing patients, can teach us more about the underlying biochemistry of migraine, and may fuel the quest for new preventive medication.[86] A biomarker is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention".[87] The potential roles for biochemical biomarkers for migraine are numerous, they can: (i) provide insight into mechanisms of pathophysiology; (ii) quantify the degree of change (over migraine phases); (iii) distinguishing groups of migraineurs from one another or from controls; (iv) discriminate what may be clinically overlapping disorders; (v) help defining more homogeneous groups of patients for genetic and other migraine research; (vi) predict or monitoring drug response; and (vii) eventually perhaps eventually serve as a widely used diagnostic marker.

Pathophysiological biomarkers leading to migraine treatments

Compounds can be measured as reflections of different pathophysiological mechanisms underlying migraine, this in turn might help the development of migraine specific medication. There are already several excellent examples on how knowledge of biochemical pathophysiology leads to adequate migraine treatment by the development of migraine specific drugs.

As the first signalling molecule to be implicated in migraine pathogenesis, serotonin (5-HT) can be credited for fuelling the early migraine research. In migraineurs lower platelet-free plasma levels of 5-HT and higher levels of 5-HIAA were found between attacks compared with controls.[88] During attacks plasma 5-HT levels were significantly higher and plasma 5-HIAA levels were significantly lower compared with the headache-free period.[88] It was hypothesized that 5-HT metabolism is enhanced in migraineurs during headache-free periods and transiently decreases during attacks and it was postulated that migraine might be a syndrome of chronically low serotonin, with migraine attacks triggered by a sudden raise in 5-HT release.[89] These findings played a significant role in the development of the class of medication called the triptans, which are highly effective in aborting a migraine attack.[90,91] Triptans are agonists of 5-HT1B and 5-HT1D receptors, which are found on bloodvessels and presynaptically on sensory neurons.[90]

Another interesting biochemical compound is calcitonin gene-related peptide (CGRP), which is the most potent endogenous vasodilator. Increased ictal CGRP levels in the external jugular vein of migraine patients have been reported, which normalized after sumatriptan treatment, with simultaneous alleviation of headache in two studies.[78,92] Follow-up studies of antecubital levels have also reported an increase in CGRP levels ictally.[76] Additionally, interictal antecubital levels of CGRP in migraineurs have been reported to be elevated.[93,94] It is tempting to suggest that CGRP may be a biomarker for migraine; however, it is not clear if that is the case as several studies have reported no change in the CGRP levels in migraineurs [95-97]. These clinical observations of increased plasma CGRP levels raised the interest in this compound which eventually led to the development CGRP receptor antagonists, which are also proven to be an effective acute migraine drug.[98,99]

Glutamate is also often implicated in migraine pathophysiology. Neuronal hyperexcitability is the central mechanism in these theories. Glutamate is the major excitatory neurotransmitter in the Central Nervous System and is implicated in several pathophysiological mechanisms related to migraine, including trigeminovascular activation, central sensitization and cortical spreading depression.[100] Between migraine attacks, patients with migraine (more pronounced in migraine with aura in some, but not all, studies) have substantially higher plasma glutamate levels than controls and tension type headache. During migraine attacks, glutamate levels were even further increased.[101,102] Analyses of plasma glutamate have revealed that the uptake and release of glutamate is altered in migraine patients platelets and, moreover, also between migraine patients

with aura and migraine patients without aura.[103] The results suggest a defective cellular reuptake mechanism for glutamate in migraineurs, predisposing the brain of migraineurs to develop spreading depression. Although still in an early phase, glutamate receptor antagonists were developed to alter glutamatergic responses in brain areas involved in migraine, and the first results are promising.[100,104]

Diagnostic biomarkers

The diagnosis of migraine and adequate treatment decisions could be much easier if there would be objective diagnostic biomarkers. Although the introduction of the ICHD has greatly improved the validity of a migraine diagnosis, its differentiation from other headaches, can still only be based on rather time-consuming anamnesis and history taking. None of the suggested 'biomarkers' have sufficient sensitivity or specificity to be practical for diagnosis of an individual patient. The genetic susceptibility SNPs that have been identified in the GWAS studies cannot be applied as diagnostic marker. Even in the monogenetic FHM disorder there are carriers with no clinical phenotype of FHM (non-penetrance) and vice versa patients with the clinical picture of FHM but with no mutation (phenocopies). A fertile area of future research will be to evaluate the usefulness of a battery or combination of these markers for that purpose. Therefore, it is of pivotal importance to conduct studies based on large cohorts to replicate previous reported findings and to discover potential new biomarker using different proteomics and metabolomics techniques.

In-vivo measurements of migraine biochemistry

Magnetic resonance spectroscopy is a non-invasive in-vivo method to detect biochemical compounds in the brain, without the need for radioactive tracers.[105] Resonances in MR spectra are identified primarily by their frequency, expressed as shift in frequency in parts per million (ppm) relative to a standard. ^{1}H-MRS (proton-MRS) allows quantification of brain metabolites that contain hydrogen and present in mM concentrations. The most relevant compounds that can be detected in vivo are alanine (Ala), choline (Cho), creatine (Cr), GABA, Glutamate (Glu), glutamine (Gln), lactate (Lac), myo-inositol (mI), N-acetylaspartate (NAA), N-acetylaspartylglutamate (NAAG), scyllo-inosotol (Scyllo), and taurine (Tau). [106]

Previous studies that used proton-MRS to investigate migraine pathophysiology suggested a disturbed energy metabolism, at least in a subgroup of migraine patients [107]. ATP levels in the occipital lobe of migraine without aura were found to be reduced and lactate levels were found increased in the occipital lobe of several migraine subtypes.[108,109] There are, however also heterogenous and contradictory results.[58] Possible reasons for these contradictory findings in proton-MRS studies are: (i) different types of migraine being studied; (ii) different timing of scanning in the migraine cycle; (iii) different scanning techniques between studies; (iv) different quality of obtained spectra between studies; and (v) difference in technical evolution between new and old studies. Furthermore, glutamate concentration have never been accurately measured in migraine patients, because glutamate and glutamine cannot be assessed individually at lower magnetic fields.[110] Therefore, there is a need for, quantitative brain MRS studies in migraine at higher magnetic field strengths, including patients with a single phenotype who experience a well-defined number of attacks and are not using prophylaxis, compared with a gender- and age-matched control group.

Biochemistry in cerebrospinal fluid

Due to the minimally invasive nature of its collection blood is the most commonly used biofluid for clinical chemistry diagnoses. However, its rich metabolome is a reflection of the metabolic state of the entire organism. Therefore, cerebrospinal fluid (CSF) may represent a better source of metabolites for diseases of the CNS since it is closer in proximity to the tissue of disease origin and may better represent its neurochemical state.[111]

CSF is the body fluid that fills the ventricles and the subarachnoid space of the brain and spinal column. Cells of the choroid plexus and ependymal cells that line the ventricular walls secrete CSF, and the exchange of molecules with blood plasma and nearby brain tissue contributes to its final composition.[112] A main mechanical function is protecting the brain from physical shock. However, CSF also has a function in circulating nutrients and chemicals filtered from the blood,[113] along with waste management by removing organic acids either by active transport or bulk flow from the extracellular fluid in the brain to the subarachnoid compartment, and ultimately into the venous blood stream.[114] Since the composition of CSF is directly dependent upon metabolite production rates in

the brain,[115] analysis of the CSF metabolome can offer biochemical insights into central nervous system disorders

High-throughput proteomics, peptidomics and metabolomics are relatively new fields of research.[116] With fast emerging technologies in mass spectrometry, thousands of proteins, peptides and metabolites can now be quantitatively measured from minimal amounts of biological material. However, there is no single platform in the world that is able to measure all biochemical compounds at once; therefore, we still have to rely on different analytical approaches with corresponding analytical platforms. The strength of metabolomics lies in the discovery phase: that is, the potential to detect and identify unanticipated changes in metabolite profiles which are characteristic of, for example, a disease state.[116] For diseases that are difficult to diagnose or for which the biochemical mechanism is unknown, this information can provide new insight, diagnostic biomarkers or therapeutic targets. These techniques are therefore very well suited to investigate the biochemistry of migraine, a brain disorder of which most of the underlying biochemical mechanisms are still unknown.

Scope and outline of this thesis

Apart for a few rare, monogenic, severe subtypes with known gene defects, our understanding of migraine biochemistry is still very limited and objective biomarkers for the common types of migraine are lacking. Since repeated migraine attacks are highly disabling for the patient and pose a great burden to society, and current medication is not adequate in many, more effective and well-tolerated preventative agents are urgently needed. To this extent, it is important to unravel the biochemical pathways involved in migraine pathophysiology. This thesis describes different studies on biochemical changes occurring in the brain and the cerebrospinal fluid (CSF) of migraine patients with the aim to learn more about the underlying biochemistry of mono-genetic and common migraine, and, if possible, to identify diagnostic biochemical profiles to distinguish migraine patients from non-migraine patients.

This thesis is divided in three parts. **Part one**, consisting of **Chapter 2 and 3**, describes the investigations of the biochemical constitution of the migraine brain *in-vivo* which were measured with proton Magnetic Resonance Spectroscopy (^{1}H-MRS). **Part two** of this thesis, consisting of **Chapters 4 and 5**, describes the

search for known and new – unknown – biochemical differences between migraine patients and healthy controls. For this several metabolomics technologies were applied to study the biochemical makeup of cerebral spinal fluid (CSF). **Part three** consists of two methodological studies, **Chapter 6 and 7**, in which the biochemical stability of CSF and the effect of contamination with ethanol is investigated.

Part 1: Biochemical profiling of the brain

Chapter 2 describes the measurements of biochemical changes in the brain with ^{1}H-MRS in patients with hemiplegic migraine in between attacks. Hemiplegic migraine is a rare subtype of migraine, characterized by hemiparesis during the aura phase. The aim of this study was to investigate regional biochemical changes with 7 tesla ^{1}H-MRS in the brains of hemiplegic migraine patients. This is the first time high-resolution 7 Tesla ^{1}H-MRS spectra were used to investigate interictal biochemical changes in the brains of familial and sporadic hemiplegic migraine patients.

The aim of **Chapter 3** was to determine the occipital glutamate concentration and its time-correlated compartment-specific characteristics in migraine patients in between attacks.. A case-control study was performed with interictal migraine patients (with and without aura), and age- and gender-matched healthy controls. With the use of ^{1}H-MRS and diffusion weighted spectroscopy (DWS) at 7 Tesla it was possible to accurately measure glutamate. This was technically challenging because it is difficult to measure glutamate separately from glutamine, and because glutamate is tightly regulated in the brain, differences were expected to be small.

Part 2: Biochemical profiling of cerebrospinal fluid

There is no clear overview of biochemical compounds that were previously measured in CSF in relation to migraine. **Chapter 4** describes a systematic review and meta-analysis with the aim to identify compounds with consistently altered CSF concentrations in migraine patients and to assess whether these changes are specific for CSF or are also present in blood. Sixty-two unique compounds were identified in 40 CSF studies. There were several important and consistent results in CSF and blood.

The aim of **Chapter 5** was to identify relevant biochemical or pathophysiological markers for migraine in CSF with an exploratory ^{1}H-NMR-based metabolomics approach. Patients with hemiplegic migraine, migraine with aura, migraine without aura, and healthy controls were included in this study.

Supervised multivariate modelling was carried in order to discriminate migraine patients from healthy controls based on their metabolic profile. Several metabolites showed to be useful in predicting the migraine diagnosis.

Part 3: Practical considerations for cerebrospinal fluid sampling

To ensure data quality in metabolomics studies, it is pivotal to standardize body fluid sampling, processing, and storage procedures. The mass-spectrometry techniques that are used in metabolomics are very sensitive, it is therefore important to make sure that the metabolic profiles are stable and influenced by sampling of pre-analytical handling of the samples. The aim of the study described in **Chapter 6** was to: (i) investigate the effect of five different sampling protocols and to determine which provides the most stable metabolic profile; and (ii) to assess the effects sampling and processing temperature, centrifugation and the use of ethanol as additive to stop enzymatic reactions. A novel strategy using isotopically labelled compounds was applied to trace any degradation during the sampling or sampling handling protocols.

Despite strict adherence to standard sampling guidelines, variable levels of ethanol were detected in the ^{1}H-NMR spectra of human cerebrospinal fluid (CSF) samples. **Chapter 7** describes the steps to retrace the exact origin of ethanol contamination in CSF samples. It also describes how ethanol affected the CSF sample matrix an how it obscures a significant part of the ^{1}H-NMR spectrum.

Finally **Chapter 8** provides a general discussion of the thesis, reviewing the results and discussing future possibilities for research of migraine biochemistry.

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Part I

Biochemical profiling of the brain

CHAPTER 2

Biochemical changes in the brain of hemiplegic migraine patients measured with 7 tesla ^{1}H-MRS

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Abstract

Aim: To assess biochemical changes in the brain of patients with hemiplegic migraine in between attacks.

Methods: Eighteen patients with hemiplegic migraine (M:F, 7:11; age 38 ±14 years) of which eight had a known familial hemiplegic migraine (FHM) mutation (five in the CACNA1A gene (FHM1), three in the ATP1A2 gene (FHM2)) and 19 age and sex matched healthy controls (M:F, 7:12; mean age 38 ± 12 years) were studied. We used single-voxel 7 tesla 1H-MRS (STEAM, TR/TM/TE=2000/19/21ms) to investigate four brain regions in between attacks: cerebellum, hypothalamus, occipital lobe, and pons.

Results: Patients with hemiplegic migraine showed a significantly lower total N-acetylaspartate/total creatine ratio (tNAA/tCre) in the cerebellum (median 0.73, range 0.59 - 1.03) than healthy controls (median 0.79, range (0.67 - 0.95); p = 0.02). In FHM1 patients with a CACNA1A mutation the tNAA/tCre was lowest.

Discussion: We found a decreased cerebellar tNAA/tCre ratio which might serve as an early biomarker for neuronal dysfunction and/or loss. This is the first high spectral resolution 7 tesla 1H-MRS study of interictal biochemical brain changes in hemiplegic migraine patients.

Introduction

Hemiplegic migraine is a rare subtype of migraine with aura, characterized by hemiparesis during the aura phase. There are two subtypes, familial hemiplegic migraine (FHM) which is autosomal dominant, and sporadic hemiplegic migraine (SHM).[1-3] Apart from the hemiparesis during the aura phase the symptoms of the migraine attacks are similar to the common forms of migraine. Therefore, it serves as a valid model to study migraine pathogenesis.[1,4]

So far, mutations in three different genes have been identified in FHM patients. FHM1 is caused by mutations in the *CACNA1A* gene, which encodes the $Ca_v2.1$ (P/Q-type) calcium channel and is important for neurotransmitter, mainly glutamate, release.[5] FHM2 is caused by mutations in the *ATP1A2* gene;[6] this gene encodes the alpha-2 subunit of a Na^+,K^+ ATPase pump which provides the steep Na^+ gradient essential for the transport of glutamate and Ca^{2+} over the cell membrane. The third gene is *SCN1A,* encoding the alpha-1 subunit of the neuronal sodium channel in FHM3.[7] Mutations in these three genes are thought to lead to an increase of glutamate levels in the cerebral synaptic cleft, making patients more vulnerable for cortical spreading depression, the underlying mechanism for the aura symptoms.[4] Besides the reduced threshold for development of cortical spreading depression, FHM patients may develop progressive permanent cerebellar impairment and atrophy,[2,8] especially in FHM1 patients with a mutation in the *CACNA1A* gene.

The pathophysiological mechanisms leading to migraine are complex and are only partially understood. Several brain structures are involved in different phases of a migraine attack. The phenomenology of premonitory symptoms suggest an early role for the hypothalamus in the onset of attacks.[9] The cortex is involved in the beginning of a migraine attack because of cortical spreading depression.[10] The brainstem is implicated through activation of the trigeminovascular system underlying the migraine headache.[11]

The aim of this study was to investigate biochemical changes in the cerebellum, hypothalamus, occipital cortex, and pons of hemiplegic migraine patients compared to healthy controls. Secondary, to investigate whether there are differences between FHM1 and non-FHM1 patients. We used 7 tesla proton magnetic resonance spectroscopy (^{1}H-MRS) which, in theory, leads to a higher spectral resolution resulting in a higher sensitivity and specificity to detect biochemical changes.

Methods

Subjects

Patients with hemiplegic migraine and healthy controls over 18 years of age were included. All patients had attacks which fulfilled the criteria for hemiplegic migraine according to the International Classification of Headache Disorders (ICHD-III).[3] Mutations in the FHM genes *CACNA1A*, *ATP1A2* and *SCN1A* had been proven or excluded by direct sequencing. Healthy volunteers were free of (a-)specific headaches and were age and sex matched. For both groups exclusion criteria were i) Body Mass Index (BMI) >28; ii) any oncological history; iii) contraindications to magnetic resonance imaging (MRI). Additionally, healthy controls had to be free of any neurological or psychiatric disorders. The study was approved by the local ethics committee. All subjects provided informed consent prior to study.

Study design

Scans were performed in the morning (before 12.00 a.m.). Subjects were not allowed to eat or drink anything but water at least eight hours before the scan. Patients were scanned in an attack-free period of at least three days prior and three days after the study. All subjects underwent a detailed standardized interview and neurological examination (by FB) prior to the scan. Frequency of migraine attacks and the typical symptoms during an attack were recorded. Cerebellar symptoms were scored using the Scale for the Assessment and Rating of Ataxia (SARA) which includes eight items reflecting neurologic manifestations of cerebellar ataxia.[12] SARA has eight items that yield a total score of 0 (no ataxia) to 40 (most severe ataxia). Gait (score 0 to 8), stance (score 0 to 6), sitting (score 0 to 4), speech disturbance (score 0 to 6), finger chase (score 0 to 4), nose-finger test (score 0 to 4), fast alternating hand movements (score 0 to 4) and heel-shin slide (score 0 to 4) were all separately rated. Cerebellar ataxia was defined as a SARA-score of 1 or more.

Data acquisition.

Subjects were scanned on an Achieva 7 tesla whole body MR system (Philips Healthcare, Best, The Netherlands), with a NOVA Medical quadrature transmit and 16 channel receive coil. For ^{1}H-MRS, four separate fixed sized volumes of interest (VOIs) were placed in the cerebellum (4.3 cc), hypothalamus (1.5 cc),

occipital cortex (4.9 cc), and pons (2.1 cc; see Figure 1). Planning was done on axial T2 weighted turbo spin echo images (15 slices; TR 7000, TE 100 ms; 0.5 x 0.6 x 2.5 mm3 resolution; 2.8 mm inter slice gap) covering the brain from base of the cerebellum up to the corpus callosum. For ^{1}H-MRS, single voxel STEAM was applied with TR/TM/TE = 2000/19/21 ms, bandwidth = 4 kHz, 2048 samples and second order pencil beam volume shimming. For water suppression, a frequency-selective RF pulse and gradient spoiling was used and six saturation bands, one on each side of the VOI, were applied to suppress signal from surrounding tissue. All scans included a reference scan without water suppression.

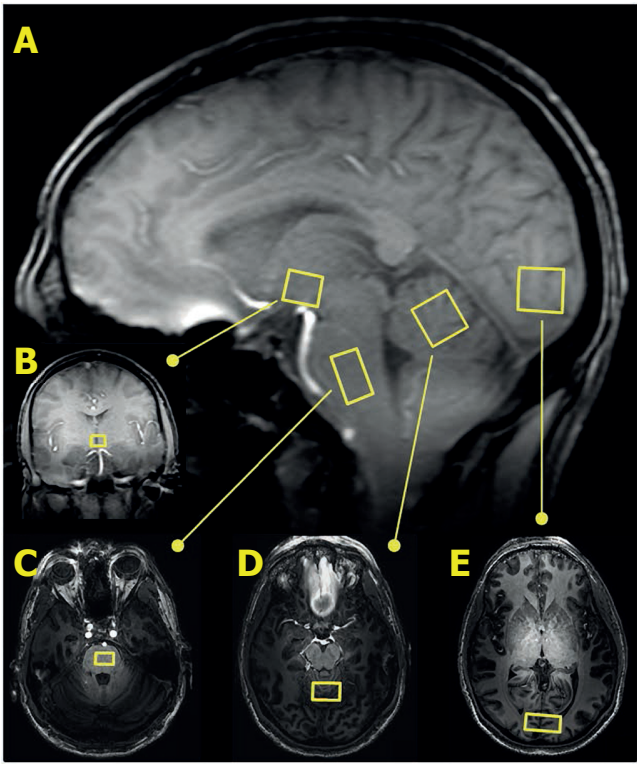


Figure 1: Representative localization of the regions of interest. T_2-weighted images in the mid-sagittal (image (a)), coronal (image (b)) and transverse planes (images (c) – (e)) in a male healthy control. Boxes indicate the locations and volume of interest (VOI) in the hypothalamus (image (b); voxel volume 1.5 cc), pons (image (c); voxel volume 2.1 cc), cerebellum (image (d); voxel volume 4.3 cc), and visual cortex (image (e); voxel volume 4.9 cc).

Data processing

MRS data were analyzed with LCModel (Provencher), using the unsuppressed water signal as an internal reference. For each anatomical region, an example of a ^{1}H-spectrum is shown in figure 2. A basis set simulated for all metabolites under investigation plus lipids and macro molecules was used. Since no data are available of T1 and T2 metabolite relaxation times at 7T, and the absolute concentration of the water in the regions selected is unknown, data are presented as ratio to total creatine (tCre) rather than concentrations. Metabolites under investigation were creatine plus phosphocreatine (tCre), choline (Cho), myo-inositol (Ins), combined N-acetylaspartate and N-acetylaspartylglutamate (tNAA) and combined glutamine and glutamate (Glx). Visual inspection of the spectra was performed retrospectively by two investigators (WMT and JvdG) who were blinded for the diagnosis.

Spectra showing clear artifacts, for example due to subject movement or inadequate water suppression, were a priori excluded from further analysis; the number of excluded measurements are indicated in table 2. On some locations the spectral quality for some metabolites was limited for a reliable estimation of the metabolite concentration. Measurements with a Cramér-Rao lower bounds (CRLB, ie. the estimated standard deviation expressed in percent of the estimated concentration) smaller than 15 %SD were considered reliable estimates of the metabolite concentration.

The T2 weighted images were evaluated by a neuroradiologist (MCK), who was blinded for the diagnoses, to identify any major structural anomalies.

Statistical analysis

Statistical analysis of LCModel output was performed with SPSS (SPSS 16 for Windows R16.0.2, SPSS Inc, Chicago). Data was checked on normality of distribution by means of a Kolmogorov-Smirnov test. Equality of variances was checked with the Levene's test as provided by SPSS and the corresponding p-value was registered. Because of non-normal distribution in some metabolite ratios, non-parametric tests were used to test for significance between groups. Group differences in metabolite/total creatine ratios between patients with hemiplegic migraine and controls were tested for significance with a Mann-Whitney test. Group differences in metabolite/total creatine ratios between FHM1, non-FHM1 and controls were tested for significance with a Kruskal-Wallis test, followed by a Mann-Whitney test. P-values less than 0.05 were

considered significant. We use Pearson correlation calculation of correlation coefficients between metabolite/total creatine ratios and time since last attack and attack frequency.

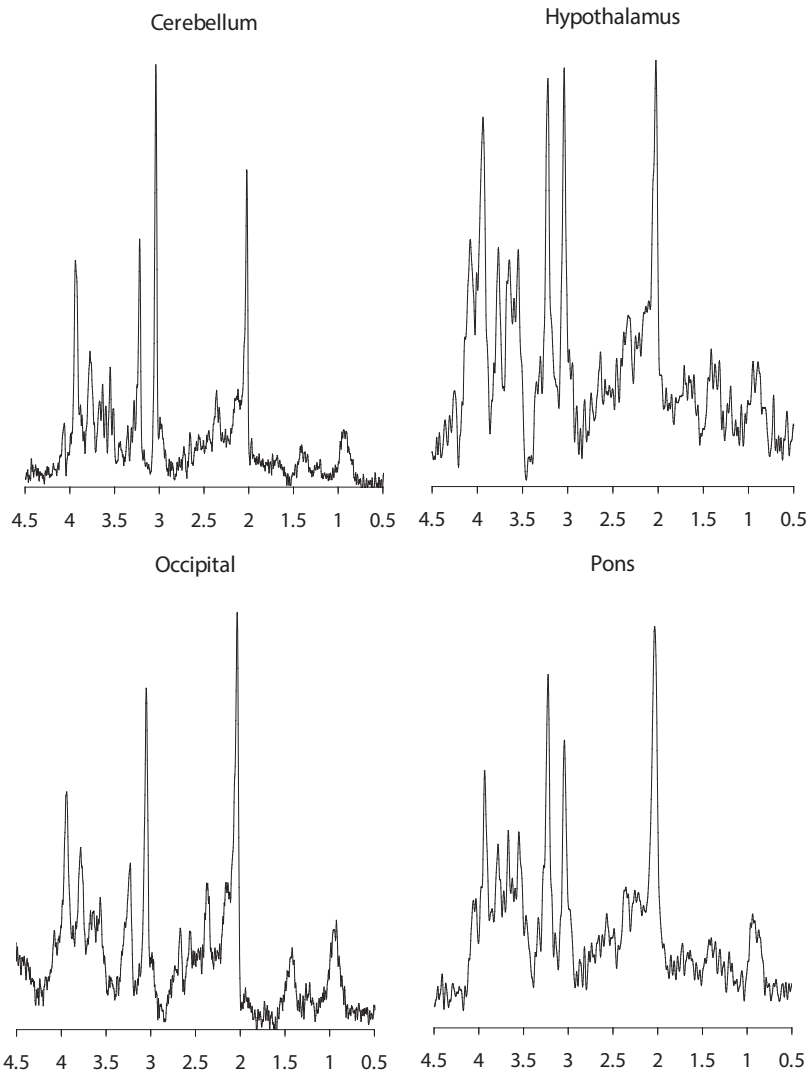


Figure 2: Example of 1H spectra acquired in the four regions of interest from a male healthy control. Vertical scale is in arbitrary units. Metabolites are total creatine (creatine plus phosphocreatine) (Cre-CH2 and -Ch3), Myo-inositol (Ins), Choline (Cho), combined N-acetylaspartate and N-acetylaspartylglutamate (tNAA) and combined glutamine and glutamate (Glx).

Results

Clinical and genetic characteristics of patients

Eighteen patients (7 males, 11 females; mean age 38.1 ± 14.4 years) with hemiplegic migraine were included in the study. Five patients from two distinct families had a pathogenic mutation in the *CACNA1A* gene (FHM1) and three patients from three distinct families had a pathogenic mutation in *ATP1A2* (FHM2). Eight patients were diagnosed with FHM with no known FHM mutation, and two patients with SHM also with no known FHM mutation (see table 1). Nineteen healthy volunteers served as age and sex matched controls (7 males, 12 females; mean age 38.5 ± 12.1 years). There were no differences in age (p=0.92) and sex (p=0.79) between the patients and controls groups because they were actively matched. Only one subject showed clear signs of cerebellar atrophy on the structural images (subject 5, with FHM1, *CACNA1A* mutation and clinical signs of cerebellar ataxia with a SARA score of 6) and one healthy control had a small infratentorial arachnoid cyst without mass effect, that did not interfere with planning of the VOI in the cerebellum. By visual inspection there were no clear signs of atrophy in other brain regions in patients or controls.

Metabolite changes

Patients with hemiplegic migraine showed a reduced tNAA/tCre ratio in the cerebellum compared to controls (median of 0.73 versus 0.80 respectively; p=0.02) (see table 2). The tNAA/tCre ratio was significantly different between FHM1 patients (median 0.71, range 0.59 – 0.75), non-FHM1 patients (median 0.74, range 0.62 – 1.03), and controls (median 0.80, range 0.67 – 0.95) (p<0.04) (figure 3). A post hoc analysis showed a significantly lower tNAA/tCre ratio in FHM1 patients compared to healthy controls (p<0.03), but not between FHM1 and non-FHM1 patients, nor between non-FHM1 patients and controls (figure 3). A relevant correlation between metabolite level and the presence of signs of cerebellar ataxia could not be calculated, because only 3/18 patients showed signs of cerebellar ataxia. There was no statistically significant correlation between tNAA/tCre and time since last attack (r=-0.078; p=0.76) and attack frequency (r=0.42; p=0.10). There were no differences found for the other metabolites in the cerebellum.

Table 1: Clinical and genetic characteristics of patients with hemiplegic migraine (n=18)

	Sex	Age (yrs)	Diagnosis	Attack frequency	Time since last attack	Onset *(yrs)*	Gene mutation	Visual aura	SARA	Headache other than HM	Other (neurological) disorders
1	M	57	FHM	1-2/yr	5 yrs, HM 1 yrs, MA	34	*CACNA1A* I1811L	Yes	14	MA	None
2	F	38	FHM	1-2/ mnth	2 yrs, HM	4	*CACNA1A* I1811L	Yes	0	MA	None
3	F	46	FHM	<1/ yr	9 yrs, HM	10	*CACNA1A* I1811L	No	0	No	None
4	F	28	FHM	1-2/ yr	1 yr, HM	8	*CACNA1A* I1811L	No	0	No	None
5	M	60	FHM	2/ wk	3 days, HM	9	*CACNA1A* E668A	Yes	6	MA	None
6	F	44	FHM	1-2/ mnth	2 wks, HM	5	*ATP1A2* R593W	Yes	2	MA	Depression
7	M	26	FHM	1/ 5yr	3 mnths, HM	16	*ATP1A2* R834X	Yes	0	TTH	None
8	F	24	FHM	1-2/ mnth	1 wk, HM	12	No	Yes	0	MA	Depression, SLE
9	M	22	FHM	1-2/ yr	1 yr, HM	6	No	Yes	0	MA	None
10	F	63	FHM	1-2/ mnth	2 mnth, HM 1 mnth, MA	10	No	Yes	0	MA	Bronchitis, hypertension
11	F	30	FHM	3-4/ yr	3 wks, HM	3	No	Yes	0	MA	Depression
12	F	51	FHM	1-2/ mnth	2 mnths, HM 1 mnth, MA	5	No	Yes	0	MA	Asthma, Lung emphysema
13	M	24	FHM	<1/ yr HM 1-2/ yr MO	5 yrs, HM 1 wk, MO	19	*ATP1A2* [a]	No	0	MO	Asthma
14	F	35	SHM	3/ yr HM 3-4/ mnth MA	3 mnths, HM 1 yr, MA	30	No	Yes	0	MA	None
15	M	19	FHM	1-2/ yr	1 yr, HM	10	No	Yes	0	MA	Epilepsy
16	F	51	FHM	1-2/ mnth	1.5 yr, HM	12	No	Yes	0	MA	Depression, epilepsy
17	M	23	FHM	2-4/ mnth	11 yrs, HM	8	No	Yes	0	MA	None
18	F	45	SHM	1-2/ yr	1 yr, HM 4 mnths, MA	20	No	Yes	0	MA	None

FHM = familial hemiplegic migraine, SHM = sporadic hemiplegic migraine, HM = hemiplegic migraine, MA = migraine with aura, MO = migraine without aura, TTH = tension type headache, *CACNA1A* = *CACNA1A* gene, which encodes the $Ca_v2.1$ (P/Q-type) calcium channel, *ATP1A2*= *ATP1A2* gene, which encodes the alpha-2 subunit of a Na^+,K^+ ATPase pump, SLE = systemic lupus erythematosus, M = male, F = female, wk(s) = week(s), mnth(s) = month(s), yr(s) = year(s). [a] Specific gene mutation to be published

Table 2: Comparison of metabolite/total creatine ratios in different brain regions of hemiplegic migraine patients and healthy controls.

Cerebellum	Healthy control (excluded spectra n=0)[a]			Hemiplegic Migraine (excluded spectra n=1)[a]			statistics
	Median	Range	N[b]	Median	Range	N[b]	p-value
Cho/tCre	0.20	0.16 - 0.25	19	0.18	0.16 - 0.25	17	0.25
Ins/tCre	0.76	0.45 - 1.33	19	0.74	0.46 - 0.95	17	0.46
tNAA/tCre	**0.80**	**0.67 - 0.95**	**19**	**0.73**	**0.59 - 1.03**	**17**	**0.02**
Glx/tCre	0.92	0.60 - 1.32	19	0.92	0.68 - 1.48	17	0.49

Hypothalamus	Healthy control (excluded spectra n=2)[a]			Hemiplegic Migraine (excluded spectra n=4)[a]			Statistics
	Median	Range	N[b]	Median	Range	N[b]	p-value
Cho/tCre	0.36	0.26 - 0.53	17	0.37	0.25 - 0.51	14	0.79
Ins/tCre	1.26	0.58 - 3.04	17	1.24	0.85 - 1.85	14	0.62
tNAA/tCre	1.08	0.74 - 1.91	17	1.19	0.84 - 1.77	14	0.52

Occipital	Healthy control (excluded spectra n=0)[a]			Hemiplegic Migraine (excluded spectra n=1)[a]			Statistics
	Median	Range	N[b]	Median	Range	N[b]	p-value
Cho/tCre	0.12	0.11 - 0.16	19	0.13	0.09 - 0.17	17	0.66
Ins/tCre	0.92	0.66 - 1.10	19	0.93	0.53 - 1.17	17	0.97
tNAA/tCre	1.40	1.14 - 1.58	19	1.42	1.09 - 1.73	17	0.40
Glx/tCre	1.16	0.97 - 1.78	19	1.19	0.87 - 1.64	17	0.80

Pons	Healthy control (excluded spectra n=10)[a]			Hemiplegic Migraine (excluded spectra n=4)[a]			Statistics
	Median	Range	N[b]	Median	Range	N[b]	p-value
Cho/tCre	0.43	0.38 - 0.66	9	0.47	0.31 - 0.64	14	0.88
tNAA/tCre	1.71	0.95 - 1.94	9	1.83	1.23 - 2.54	14	0.20

Metabolites under investigation were creatine plus phosphocreatine (tCre), choline (Cho), myo-inositol (Ins), combined N-acetylaspartate and N-acetylaspartylglutamate (tNAA) and combined glutamine and glutamate (Glx). Median and range of metabolite/creatine ratios and number of included spectra (N) in controls and hemiplegic migraine patients for anatomical regions of interest are shown. P-values refer to results of Mann-Whitney tests, significant results ($p < 0.05$) are in bold, not corrected for multiple testing.
[a] Number of excluded spectra based on visual inspection (by WMT and JvdG), spectra showing clear artifacts, for example due to subject movement or inadequate water suppression. [b] Number of spectra for which LCModel was able to etimate a metabolite/total creatine ratio.

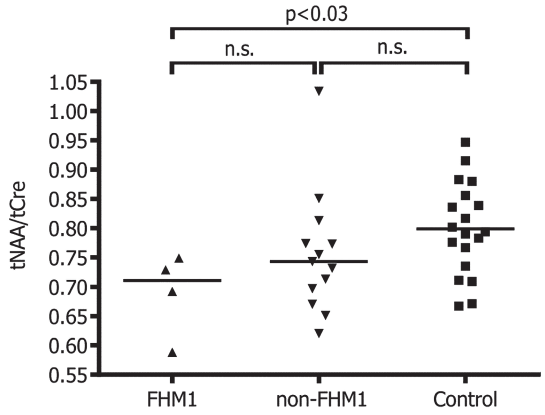


Figure 3: Comparison of cerebellar tNAA/total creatine ratios of *CACNA1A* positive (FHM1) and *CACNA1A* negative (NonFHM1) hemiplegic migraine patients and healthy controls. tNAA = combined N-acetylaspartate and N-acetylaspartylglutamate, tCre = creatine plus phosphocreatine, FHM1 = familial hemiplegic migraine type 1 (patients with a known *CACNA1A* mutation), non-FHM1= hemiplegic migraine patients without a *CACNA1A* mutation. Kruskal-Wallis test for three groups (p<0.04), p-values in this figure refer to post-hoc test with Mann-Whitney tests (uncorrected for multiple testing), the horizontal lines represent the median per group, n.s.= not significant.

In the hypothalamus, the occipital lobe, and pons no significant differences between patients and controls were found for the tNAA/tCre ratio or for other metabolites.

The spectral quality was highest with in the cerebellum and occipital lobe, and lower in the hypothalamus and pons. For a complete overview of spectral quality per region and per metabolite see supplementary table s-1.

Discussion

We report the first high field 7 tesla brain ^{1}H-MRS study that investigated biochemical changes in hemiplegic migraine. We found decreased cerebellar tNAA/tCre in hemiplegic migraine patients compared to controls. N-acetylaspartate (tNAA) is considered a marker for neuronal integrity, because it is synthesized and localized mainly in neuronal mitochondria.[13] Our finding suggests cerebellar neuronal loss or dysfunction in hemiplegic migraine regardless of signs of cerebellar dysfunction (like ataxia) or macroscopic atrophy. Therefore, tNAA may serve as an early biomarker for cerebellar dysfunction and/or neuronal loss in hemiplegic migraine.

FHM1 is caused by a mutation of the *CACNA1A* gene which encodes the $Ca_v2.1$ (P/Q-type) calcium channel, which is mainly present in the Purkunje cells[14] in the cerebellum. In FHM1 patients progressive cerebellar ataxia and atrophy may occur and one observation in a FHM1 case revealed Purkinje cell loss particularly in the cerebellar vermis.[15] In our study the subgroup of FHM1 patients with a CACNA1A mutation showed the lowest cerebellar tNAA/tCre ratio. We observed clinical signs of cerebellar ataxia in only three subjects; calculation of a correlation between cerebellar ataxia and tNAA/Cre would not be reliable. One other study investigated biochemical changes in the cerebellum of FHM1 patients and found decreased cerebellar tNAA levels, which exceeded macroscopic tissue loss and was correlated to gait ataxia.[16] So, a lower cerebellar tNAA is associated with hemiplegic migraine and probably even more pronounced in FHM1. The pathophysiological explanation for a lower cerebellar tNAA is probably neuronal loss, which can lead to macroscopic cerebellar atrophy and/or dysfunction, which results in ataxia.

Interestingly, the *CACNA1A* gene is also implicated in two other disorders, namely episodic ataxia type 2 (EA2) and chronic spinocerebellar ataxia type 6 (SCA6).[17,18] Both phenotypes also involve progressive cerebellar ataxia and atrophy.[19-21] In a small MRS study no differences were found in cerebellar tNAA/tCre ratios between EA2 patients and controls possibly due to lack of power[22], whereas MRS studies focusing on SCA6 showed a lower cerebellar tNAA/tCre ratio.[23,24]

We studied three other regions of interest implicated in different stages of a migraine attack. The hypothalamus is thought to play an important role in the control of nociception, the initiation of attacks, premonitory symptoms, and the seasonal, menstrual, and circadian periodicity of migraine attacks.[25,26] The hypothalamus has never been studied in hemiplegic migraine. Only Cho/tCre, Ins/tCre, and tNAA/tCre could be reliably measured and showed no differences between patients and controls. Because of its small volume and the central anatomical location near the air containing nasopharynx it is difficult to achieve a high SNR in the hypothalamus, even at high magnetic fields.

We also examined the occipital cortex, the anatomical source of visual migraine aura. Several ^{1}H-MRS studies in migraine also investigated the occipital cortex.[27] One ^{1}H-MRS study found decreased occipital NAA levels (which became even more pronounced upon visual stimulation) in migraine with aura patients but not in migraine without aura compared with controls.[28] Two recent studies in migraine without aura patients before and after visual stimulation did not show

any differences compared to controls.[29,30] Increased occipital lactate levels have been reported in migraine with aura,[31,32] but not in migraine without aura,[29,30] suggesting that a possible deficit in energy metabolism is more marked in migraine with aura patients. The only other ^{1}H-MRS study in FHM1 patients, did not show biochemical changes in the occipital cortex.[16] A multimodal imaging study on four FHM2 patients also did not find any significant biochemical differences when compared to controls.[33] We also did not find any differences in the occipital cortex, although 83 % of our hemiplegic migraine patients also experienced visual aura's during their attacks. The discrepancy with the earlier finding in migraine patients with visual aura might be explained by the fact that we performed the measurements interictally in patients with low attack frequency.

No studies have previously investigated the pons which harbors the trigeminal nucleus caudalis which plays a key role in the trigeminovascular system. Because of the small size of the VOI and the deep central location only Cho/tCre and tNAA/tCre could be reliably measured and showed no differences between patients and controls.

This study was performed in a unique group of hemiplegic migraine patients. Familial hemiplegic migraine can serve as a valuable monogenetic model for the common forms of migraine because, apart from the hemiplegia, the other symptoms during aura and headache phase are similar to the common forms, and patients often have non-hemiplegic attacks as well. The advantage of studying hemiplegic migraine patients is that they belong to a homogenous group with severe aura symptoms including hemiplegia. We included both patients with and without a known gene mutation because we assumed that the pathophysiological mechanisms leading to hemiplegic migraine would be more or less similar.

A possible limitation of our study is the low attack frequency of the included patients, more than half had an attack frequency of less than one or two per year. Most likely this obscured dynamic biochemical changes. Ictal measurements during different stages of the attack will solve this problem but can be challenging. A technical limitation of our study is the lack of a volumetric anatomical scan which prevented us to correct for partial volume effects which might potentially confound our results when we would use water as our internal reference. Therefore we chose to use total creatine as internal reference instead of water since concentration ratios are not affected by water scaling and are less sensitive to relaxation and partial volume effects. The metabolite/total creatine ratio is widely used and is considered a reliable indirect measure.

A strength of our study is that it is the first that used high field 7 tesla MRS to investigate the in-vivo biochemistry in the brain of migraine patients. Theoretically, it is possible to measure metabolites more accurately in smaller VOIs. However, the data presented in this study was acquired some years ago, at that time the 7 tesla MRI system was just operational and therefore we were not able to exploit all the theoretical advantages. Furthermore, the spectral quality was limited in the hypothalamus and pons but this seems inevitable for these deep cerebral structures with a small VOI, and location near the air containing nasopharynx which causes field inhomogeneity (a common issue in high field imaging). Because high field MRS is a relative new technique, it is subject to rapid development and ongoing technical improvements and will allow to distinguish for example glutamate.[34]

In conclusion, our study showed a decreased cerebellar total N-Acetylaspartate/total Creatine ratio in hemiplegic migraine which may serve as an early biomarker for cerebellar dysfunction and/or neuronal loss.

Acknowledgements

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Supplementary material

Table s-1: Overview of spectral quality per region and per metabolite

Cerebellum

	Healthy control (N=19/19) [a]			Hemiplegic Migraine (N=17/18) [a]		
	Mean	min-max	n Incl.	Mean	min-max	n Incl.
FWHM (ppm)	0.032	0.023 – 0.072	19	0.035	0.023 – 0.079	17
Cho (%SD)	4.3	3 - 6	19	5.0	3 - 9	17
Ins (%SD)	7.4	5 - 10	19	8.5	5 - 17	17
tNAA (%SD)	4.5	3 - 8	19	5.3	3 - 10	17
Glx (%SD)	9.0	5 - 17	19	8.5	5 - 15	17
tCre (%SD)	3.2	2 - 5	19	3.5	2 - 8	17

Hypothalamus

	Healthy control (N=17/19) [a]			Hemiplegic Migraine (N=14/18) [a]		
	Mean	min-max	n Incl.	Mean	min-max	n Incl.
FWHM (ppm)	0.045	0.026 – 0.052	17	0.040	0.020 – 0.052	14
Cho (%SD)	7.6	5 - 11	17	7.8	5 - 11	14
Ins (%SD)	11.8	7 - 26	17	13.1	9 - 20	14
tNAA (%SD)	8.6	6 - 14	17	9.0	6 - 15	14
tCre (%SD)	8.3	5 - 14	17	8.5	6 - 12	14

Occipital

	Healthy control (N=19/19) [a]			Hemiplegic Migraine (N=17/18) [a]		
	Mean	min-max	n Incl.	Mean	min-max	n Incl.
FWHM (ppm)	0.040	0.029 – 0.059	19	0.042	0.029 – 0.066	17
Cho (%SD)	8.4	5 - 14	19	8.5	5 - 15	17
Ins (%SD)	8.2	6 - 13	19	8.4	6 - 13	17
tNAA (%SD)	3.3	2 - 5	19	3.5	2 - 5	17
Glx (%SD)	7.5	4 - 12	19	7.4	4 - 11	17
tCre (%SD)	3.6	2 - 5	19	3.7	3 - 5	17

Pons

	Healthy control (N=9/19) [a]			Hemiplegic Migraine (N=14/18) [a]		
	Mean	min-max	n Incl.	Mean	min-max	n Incl.
FWHM (ppm)	0.069	0.039 – 0.105	9	0.064	0.039 – 0.098	14
Cho (%SD)	6.3	4 - 9	9	7.9	4 - 11	14
tNAA (%SD)	6.0	2 - 14	9	6.9	4 – 10	14
tCre (%SD)	7.9	4 - 11	9	10.1	6 – 14	14

[a] Number of included spectra after visual inspection (by WMT and JvdG), spectra showing clear artifacts were excluded, for example due to subject movement or inadequate water suppression.
FWHM (full width at half-maximum) is a rough estimate of the linewidth in the in vivo spectrum.
%SD's are the estimated standard deviations (Cramér-Rao lower bounds) expressed in percentage of the estimated concentrations. These %SD estimates are only lower bounds, but are the most useful reliability indicators. Multiply them by 2.0 results in a rough 95% confidence intervals; i.e., the range that would contain the true value about 95% of the time. Cramér-Rao lower bounds (CRLB) smaller than 15 %SD were considered reliable estimates of the metabolite concentration.

CHAPTER 3

Cortical glutamate in migraine

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Abstract

Objective: Cortical hyperexcitability due to enhanced glutamatergic activity has been implicated in migraine pathophysiology but direct evidence is lacking. Here we assessed glutamate levels and intracellular mobility in the visual cortex of migraineurs in-between attacks.

Methods: We included 50 migraineurs (23 with aura and 27 without aura) and 24 age- and gender-matched non-headache controls. We used proton magnetic resonance spectroscopy (1H-MRS) and diffusion weighted spectroscopy (DWS) at 7 tesla with a single volume of interest (VOI: 2x2x3cm) located in the primary and secondary visual cortex. For 1H-MRS we used a semi-LASER sequence with water referencing for absolute quantification. For DWS we used an adapted PRESS sequence with gradients applied in three directions and two different gradient amplitudes. Between groups differences were evaluated using ANCOVA with the gray matter fraction in the VOI as covariate and post-hoc comparisons with Bonferroni correction.

Results: Glutamate concentrations differed between groups ($p=0.047$) and were higher in migraineurs without aura (mean ± SD: 7.02 ± 0.50 mM) compared to controls (mean ± SD: 6.40 ± 0.78 mM, $p=0.042$). The apparent diffusion coefficient of glutamate was similar among groups ($p=0.129$) suggesting similar inter- and intracellular mobility of glutamate in all three study groups. No differences were observed for concentrations and diffusion constants of other metabolites.

Conclusion: Interictal glutamate levels are increased in the visual cortex of migraineurs without aura, supporting the hypothesis of cortical hyperexcitability in migraine.

Introduction

Migraine is a common[1] and highly disabling[2] episodic brain disorder, typically characterized by recurring attacks of severe headache and accompanying autonomic features for up to three days (migraine *without* aura).[3,4] In a third of patients, attacks may be associated with a, usually visual, migraine aura (migraine *with* aura)[5] which likely is caused by cortical spreading depolarization (CSD) (for review see[6]).

The mechanisms for the triggering, initiation and recurrence of migraine attacks are poorly understood. An important role has been postulated for increased brain glutamatergic activity leading to increased cerebral excitability and enhanced susceptibility to CSD, similar to what has been found for hemiplegic migraine.[6-8] Hemiplegic migraine is a rare subtype of migraine with aura[4] and considered a good monogenic model for pathogenetic mechanisms potentially involved in the common, multifactorial, forms of migraine.[6] Mutations in three different genes that have been associated with hemiplegic migraine all lead via different pathways ultimately to net increased cerebral glutamatergic neuroexcitatory activity and enhanced susceptibility to CSD. Whether similar mechanisms are involved in the common forms of migraine is uncertain. Indirect support for this intriguing hypothesis is coming from observations that glutamate levels were increased in cerebrospinal fluid of patients with chronic migraine and in blood of patients with episodic migraine.[9] In addition, anti-glutamatergic anti-epileptic drugs have shown prophylactic efficacy in migraine with and without aura.[10] Direct evidence for enhanced glutamatergic activity in the common forms of migraine is however still lacking.

Studies attempting to more directly demonstrate increased brain glutamate *in vivo* by using proton magnetic resonance spectroscopy (^{1}H-MRS) have consistently failed, most likely because they were using low magnetic field strengths and protocols which were not optimized to reliably distinguish glutamate from its major precursor glutamine.[11-18] These studies could thus only report the combined signal of glutamate and glutamine rather than the individual levels. This is a major limitation as both amino acids, although metabolically closely related via the glutamate-glutamine cycle, have very distinct functions and compartmental distributions within the brain. While glutamate is mainly located intracellular[19] in glutamatergic neurons,[20] glutamine is primarily found in astrocytes.[20]

When assessing glutamate in the brain, it is also important to consider its location within different tissue compartments. The glutamate signal in ^{1}H-MRS and DWS reflects the intracellular pool (about 80% neuronal and 20% astrocytic) of glutamate and not the extracellular or synaptic glutamate.[20] Not only the concentration but also the intracellular and subcellular compartmentalization of glutamate (e.g. in synaptic vesicles or in the cytosol) is important for normal brain function.[21] Insufficient compartmentalization might also be an important factor in migraine, because abnormally distributed intracellular glutamate may also have an effect on glutamate metabolism and function. Diffusion weighted spectroscopy (DWS) measures the diffusion properties of brain metabolites,[22,23] and has been shown to be sensitive to subtle microstructural changes of the intracellular compartments.[24,25] DWS might thus be helpful in identifying differences in compartmentalization of glutamate between migraineurs and controls.

In the present study, we assessed and compared glutamate levels in the visual cortex of interictal migraineurs with aura, migraineurs without aura, and age- and sex-matched non-headache controls. We used ^{1}H-MRS at 7 tesla and dedicated optimized techniques enabling measuring cerebral glutamate and glutamine separately.[26] Moreover, we employed DWS to measure the diffusion properties, mobility and, indirectly, intracellular compartmentalization of glutamate.[22,23] We pre-selected the visual cortex as the region of primary interest because previous studies have suggested that this brain structure is hyper-responsive in migraineurs and, in transgenic migraine mouse models, revealed enhanced susceptibility to CSD.[27,28]

Methods

Experimental design

We included three age-and-sex-matched study populations: migraine without aura (n=36), migraine with aura (n=27) and non-headache healthy controls (n=27). Migraine was diagnosed according to the International Classification of Headache Disorders third edition (beta-version).[29] Participants with migraine were included if they had have at least one migraine attack per month in the preceding six months and did not have chronic migraine or overuse of acute headache medication.[4] Participants with migraine with aura had to have visual auras in at least half of their attacks. Migraineurs were otherwise healthy without other known medical

conditions and refrained from using prophylactic medication for at least four weeks and from using acute migraine attack medication for at least three days prior to the investigation. Non-headache healthy controls could not have any form of primary or secondary headache, could not have more than one (non-specific) headache day per three months, and were otherwise healthy without known neurological or psychiatric disorders. MRS/DWS scans were to be performed interictal, defined as at least three days after a previous attack and at least two days before a subsequent attack (as checked afterwards). Scans performed within two days before a subsequent attack were defined as pre-ictal. All study participants did not use chronic medication other than oral contraceptives. The study was approved by the Leiden University Medical Center institutional ethics committee and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all subjects prior to the study.

MRS and DWS data acquisition

All MR scans were performed before 12.00 p.m. to minimize bias due to diurnal differences. To control for any dietary effect, subjects were asked not to eat or drink anything but water for at least eight hours before the scan. To reduce visual stimulation of the visual cortex, we instructed the subjects to keep their eyes closed throughout the whole scan session.

Subjects were examined at a 7 tesla MR system (Philips, Cleveland, USA) using a 32 channel receive array and a quadrature transmit coil (Nova Medical, Wilmington, MA) driven by 2 amplifiers (4 kW each) with a fixed phase setting between the two amplifiers to reach a local transmit field (B_1) of 20 μT in the regions of interest. An anatomical 3D T_1-weighted gradient echo image was acquired to allow accurate planning of the volume of interest (VOI) for MRS and DWS (Fig1A and B). Imaging parameters were: field of view: 248 x 246 x 174 mm^3, resolution 1 x 1 x 1 mm^3, repetition time (TR)/ echo time (TE)/ inversion time (TI) = 4.0/ 1.81 / 1300 ms.

For MRS we used a single-volume proton MRS (^{1}H-MRS) semi-localized by adiabatic selective refocusing (semi-LASER) sequence (TR/TE = 5000/30 ms, spectral width = 4 kHz, 2048 points, acquisition time ≈ 4 minutes), [30] preceded by a variable power and optimized relaxation delays (VAPOR) water suppression sequence. The transmitter frequency was set on 2.35 ppm (glutamate). The VOI (30 x 20 x 20 mm^3) was manually planned. The investigator (RZ) who included the participants during data-acquisition also operated the MRI scanner and

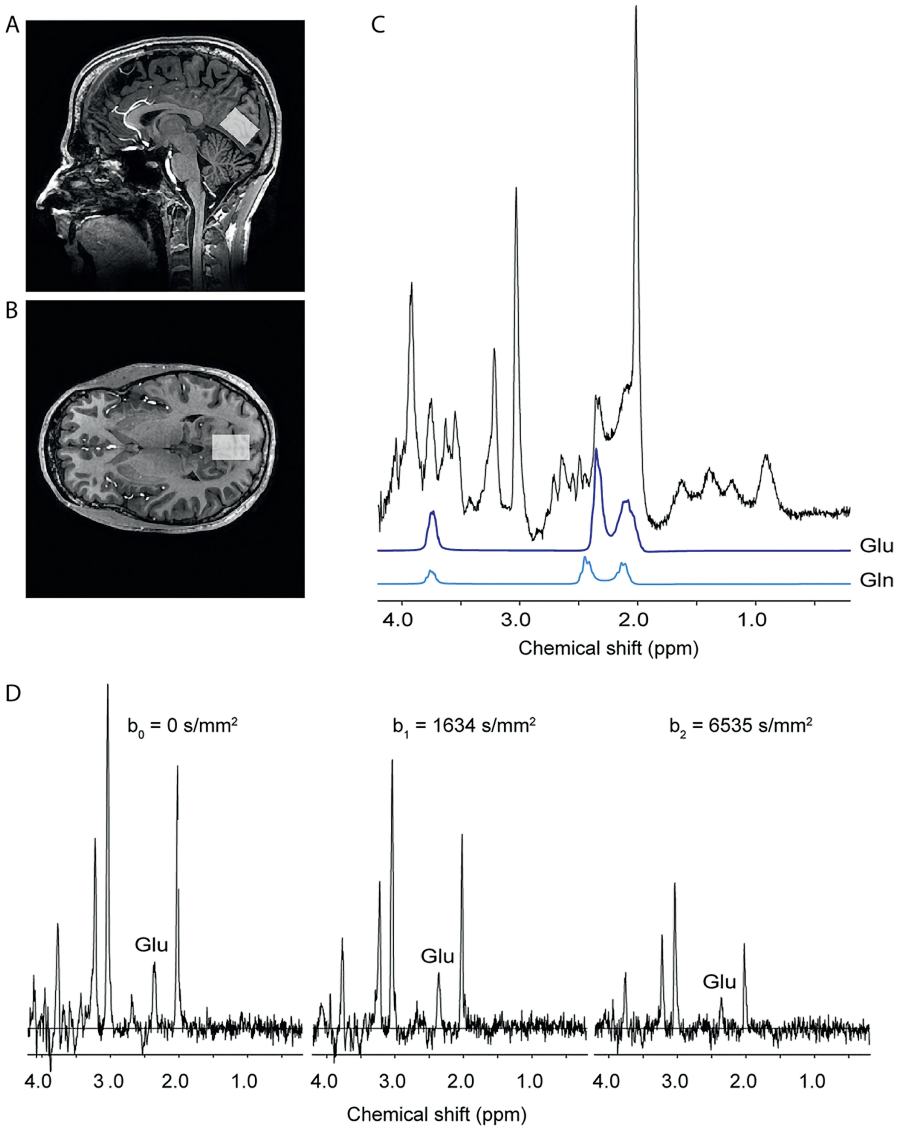


Figure 1: Example data from a male migraine without aura patient. A, B) T_1 weighted images in the mid-sagittal and transverse planes, the white box indicates the location of the volume of interest (VOI) in the occipital lobe with a size of 20 x 20 x 30 mm. C) Example of a ^{1}H-MR spectrum showing the individual fits for glutamate (Glu) and glutamine (Gln), illustrating the fact that glutamate and glutamine do not generate a prominent single peak but rather three distinct peaks; for visual representation the baseline fit was subtracted from the Glu and Gln fit. NAA, N-acetylaspartate; Cr, creatine; PCr, phosphocreatine; Ins, myo-inositol; Cho, choline; PE, phosphorylethanolamine; Asp, aspartate; MM, macromolecules. D) Averaged ^{1}H-MRS spectra at three different *b*-values for one diffusion-weighted direction.

was also responsible for placement of the VOIs. To avoid bias, clear anatomical landmarks for placement of the VOIs were used. The VOIs were centered along the calcarine fissure, symmetrically covering both hemispheres caudal of the parieto-occipital fissure and including the primary and secondary visual cortices (Brodmann areas 17 and 18; Figure 1A and B). After completing the study, an independent investigator (GJLO), who was blinded to subject status, confirmed correct placement of the VOIs in all participants. Shimming on the VOI was performed using first- and second-order shims based on a calculated B_0 map. The number of scan averages was 32. A non-water-suppressed spectrum was acquired from the same VOI, with the transmitter frequency set on the water resonance. MR spectra were pre-processed with a custom written script in Matlab® (version R2012a, The MathWorks, Inc., Natick, MA, USA) that yielded a weighted average of the individually-phased signals from all 32 receive channels and performed eddy current correction.

For DWS we used an adapted point resolved spectroscopy (PRESS) sequence (TR/TE = 2000/120ms, spectral width = 3 kHz, 1024 points, acquisition time ≈ 15 minutes) [23]. The number of scan averages was 56. Cardiac triggering was performed using the peripheral pulse unit. The transmitter frequency was set at 2.35 ppm (glutamate). Diffusion weighting was applied using bipolar gradients to reduce the effects of eddy currents on the MR spectra [31]. Diffusion-weighting was applied in three quasi-orthogonal directions and spectra were obtained at two different gradient strengths (14 and 28 mT/m), with gradient duration of 38 ms and a bipolar gap of 14 ms. The diffusion time was 60 ms. This resulted in *b*-factors of b_1 = 1634 s/mm^2 and b_2 = 6535 s/mm^2 in addition to a condition without diffusion weighting (b_0 = 0 s/mm^2). The choice of TE-value of 120 ms lies in the range of theoretically-predicted optimal values for quantification of glutamate [32]. The water signal was only partially suppressed in order to acquire a residual water signal for individual phasing of each spectrum. Data were acquired as single scans and averaged off-line after channel weighing, zero- and first-order phase correction, frequency drift correction and eddy current correction.[23] DWS data processing was performed using a custom written script in Matlab® as described previously.[33]

Tissue segmentation of VOI

In order to correct for inter-subject differences in grey matter fraction within the MRS VOI in the statistical analysis, we performed tissue segmentation with FSL (version 4.1.7, FMRIB Software Library, University of Oxford). Brain extraction

of the 3D T_1 weighted images was done with the Brain Extraction Tool (BET). Whole brain segmentation of grey matter, white matter and cerebrospinal fluid (CSF) was performed using FMRIB's Automated Segmentation Tool (FAST-) segmentation. The grey matter, white matter and CSF fractions in the VOI where calculated based on the segmented images and VOI size and coordinates with a custom written script in Matlab®.

Spectral fitting

The acquired ^{1}H-MR spectra were analyzed using LCModel (version 6.3-1B, Stephen Provencher, Inc., Oakville, ON, Canada), which calculates the best fit of the experimental spectrum as a linear combination of model spectra.[34] For the ^{1}H-MRS data we used a simulated basis set including 22 metabolites and for the DWS data a simulated basis set including 16 metabolites. Both basis sets were generated using NMRSIM (version 4.6.a., Bruker Biospin Inc). An acquired macromolecular spectrum was included in the basis sets. The parameter DKNTMN that controls the node spacing for the spline baseline fitting was set to the commonly-used value of 0.20.[35] LCModel fitting was performed over the spectral range of 0.2 - 4.2 ppm, appropriate for ^{1}H-MRS at 7 tesla.

Spectral quality assessment

Visual inspection of the spectra was performed by two investigators (RZ and JW) who were blinded for the diagnosis. Spectra showing clear artifacts, due e.g. to stimulated echoes, inadequate water suppression, or poor shimming were a priori excluded from further analysis. The quality of the DWS spectra was assessed in two stages. First, if the data quality was insufficient to perform individual phasing of each spectrum, the case was excluded. Second, after spectral fitting the individual DWS spectra were inspected and cases with spectra that showed clear artifacts were excluded from further analysis.

The signal to noise ratio (SNR) as given by LCModel is defined as the ratio of the maximum in the spectrum minus the baseline over twice the root-mean-square of the residuals between 0.2 – 4.2 ppm, and was used as a parameter to assess spectral quality. The full width at half-maximum of NAA (FWHM) is a measure of the linewidth in the spectrum. The Cramér-Rao lower bound (CRLB) of the metabolite concentrations represents the estimated standard deviation expressed in percentage of the estimated concentration; values smaller than 15 %SD on average were considered reliable estimates of the metabolite concentration; if the

CRLB of a given metabolite exceeded 15 %SD in more than 50% of the cases that metabolite was excluded from further analysis for all cases.

Quantification of MRS and DWS data

Spectral quantification was performed using the unsuppressed water signal obtained from the same VOI.[36] The relative densities of MR-visible water for grey matter, white matter and CSF were assumed to be 0.78, 0.65 and 0.97 respectively.[37] The T_1 and T_2 relaxation times of water used in the calculation of the water attenuation factors for the occipital VOI were: T_1(grey matter) = 2130 ms, T_1(white matter) = 1220 ms, T_1(CSF) = 4425 ms;[38] T_2(grey matter) = 50 ms, T_2(white matter) = 55 ms, T_2(CSF) = 141 ms.[39] The water attenuation was calculated for every subject separately based on the segmentation results of the VOI. As data were acquired with a long TR, partial saturation due to T_1 relaxation of the metabolites was not taken into account. The T_2 values of glutamate (T_2(Glu) = 93 ms) and other metabolites were taken from literature data.[40] The apparent diffusion coefficient (ADC) values were calculated for each direction from the DWS spectra the using a mono-exponential fit; the final ADC was then calculated by averaging the ADC values for the three directions.

Migraine symptoms and visual sensitivity measures

All participants with migraine kept a headache diary covering the seven days before and after examination and completed a questionnaire about their migraine characteristics, the migraine disability assessment scale (MIDAS),[41] and the headache impact test (HIT-6).[42] To assess any potential relation between glutamate concentrations in the visual cortex and visual sensitivity all participants also completed a 9-item validated visual sensitivity questionnaire (VSQ) which quantifies interictal sensitivity to light and visual patterns.[43] In addition, all participants underwent a pattern glare test immediately after the scan which quantifies visual perceptual distortions and visual illusions on viewing three striped patterns with different spatial frequencies.[44] For the pattern glare test, a general illusion index (GII) was calculated, which is the average number of distortions over the three different striped patterns.[45]

Statistical analysis

Power calculations for estimating the required number of subjects were based on estimates of glutamate concentration from literature.[40] In order to detect 10%

difference in glutamate concentration with a power of 80% in a two-sided t-test, at least 20 participants per group were needed. Statistical analysis was performed with SPSS (version 20.0, IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp). Data were checked for normality of distribution by means of a Kolmogorov-Smirnov test and equality of variances with Levene's test. To test for differences between groups in clinical and demographic characteristics we used a Chi-square test for proportions, a Kruskal-Wallis test or a Mann-Whitney test to test for differences in non-normal distributed continues variables, and a one-way analysis of variance (ANOVA) for normally distributed variables. The primary analysis assessed differences in metabolite concentrations between (only) interictal migraineurs with and without aura, versus healthy controls, using an analysis of covariance (ANCOVA) with the grey matter fraction within the VOI as covariate. Post-hoc pairwise comparisons were done by means of ANCOVA of the adjusted means with grey matter as covariate and adjusted for multiple comparisons using Bonferroni correction. Differences in apparent diffusion coefficient were tested with a one-way ANOVA. To assess whether the glutamate concentrations in the visual cortex are related to the time point in the migraine cycle we used Pearson's correlation to test for a relation between the glutamate concentration and number of days before the next attack. In addition we tested for differences in glutamate concentration between interictal and pre-ictal migraineurs with an ANCOVA with the grey matter fraction within the VOI as covariate. To increase the statistical power we pooled both interictal and pre-ictal migraineurs in a secondary analysis. To assess the potential relation between the glutamate concentration in the visual cortex and the clinical measures of visual sensitivity, i.e. sum score of the VSQ and the GII of the pattern glare test, we used Pearson's correlation. P-values less than 0.05 were considered significant.

Results

Spectral quality assessment

^{1}H-MRS and DWS data were obtained from 36 participants with migraine without aura (27 interictal and 9 pre-ictal), 27 with migraine with aura (23 interictal and 4 pre-ictal) and 27 non-headache controls (Figure 2). An example of a ^{1}H-MR spectrum and diffusion weighted spectra acquired from a single subject are shown in figures 1C and D, respectively. ^{1}H-MR spectra from three subjects were excluded from the analysis because of insufficient spectral quality and clearly visible artifacts (Figure 2).

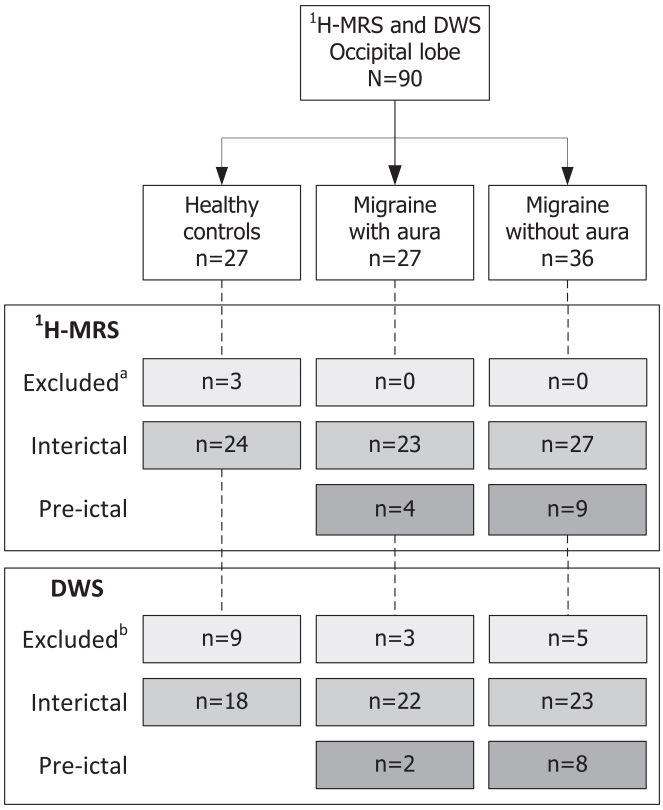


Figure 2: Flowchart of subject stratification for ^{1}H-MRS and DWS analysis. Interictal = attack free for 72 hours before and 48 hours after investigation; pre-ictal = attack free for 72 hours before investigation but with a migraine attack within 48 hours after investigation. [a] Subjects excluded because of insufficient spectral quality and clear artifacts. [b] Subjects excluded because DWS data were not (fully) acquired, DWS data-quality was considered insufficient after pre-processing, or because of insufficient spectral quality and clear artifacts.

Five DWS data sets were not fully acquired due to time constraints and therefore the data were excluded. The DWS data quality from six subjects was insufficient to perform individual phasing of the spectra; in addition, the spectral quality was insufficient or showed clear artifacts in six other subjects, these twelve subjects were therefore excluded from further analysis (Fig 2). In summary, after exclusion of the above data sets, there were for the primary analysis 27 MRS and 23 DWS scans available from participants with migraine without aura, 23 MRS and 22 DWS scans from participants with migraine with aura and 24 MRS and 18 DWS from non-headache controls. The exclusion of these data sets did not cause any between-group imbalance in age, gender or migraine characteristics.

The SNR of the ^{1}H-MR spectra that passed quality control was 41.6 ± 9.6 and the FWHM of the NAA peak was 13.4 ± 2.9 Hz. The CRLBs for glutamate and glutamine were 2.8 ± 1.0 and 8.8 ± 2.7 respectively. These values imply that LCModel was able to perform accurate fitting of glutamate and glutamine individually. The SNR of the diffusion weighted spectra was 13.6 ± 3.0 in the non-diffusion-weighted condition b_0 and decreased to 8.7 ± 2.5 in the highest diffusion-weighted condition b_2. The FWHM was 11.2 ± 2.2 in condition b_0 and 12.3 ± 2.7 in condition b_2 (no significant difference). The CRLB of glutamate increased from 6.3 ± 1.7 in condition b_0 to 9.4 ± 3.8 in the diffusion-weighted condition b_2, which is well within the conservative quality limit of CRLB less than 15% that we defined at the start of the study. There was full agreement between the original placements and the placements by an independent blinded investigator (GJLO) after the study had been completed in all but four cases (2 controls, 1 migraine with aura and, 1 migraine without aura). The differences in VOI placement in these four cases, however, were only minor (1-2 mm). The overlap in volume of the individual VOI placements was still 95%.

Clinical characteristics

The demographic and clinical characteristics of the participants included in the primary analysis are shown in Table 1 and of those included in the secondary analysis, where interictal and pre-ictal migraineurs were pooled, are shown in Table 2. Participants with migraine with and without aura did not differ in general migraine characteristics and migraine-related disability. Participants with migraine with aura experienced visual aura symptoms in 83.7% of their attacks. Participants with migraine had higher visual sensitivity as measured with the VSQ ($P < 0.001$) and experienced more visual illusions when looking at different striped patterns during the pattern glare test ($p = 0.001$) than non-headache controls. For both tests, there were no differences between participants with migraine with or without aura.

Glutamate concentrations and diffusion

Participants with migraine with or without aura had slightly higher grey matter fraction and lower CSF fractions in the VOI compared to controls (Table 1). Glutamate concentrations correlated with the grey matter fractions in the VOI (Pearson's $r = 0.51$, $p<0.001$); the grey matter fraction was therefore included as a covariate.

Table 1: Clinical and demographic characteristics of healthy controls and interictal migraineurs (n=74)

	Healthy Controls n=24	Migrainewithout aura n=27	Migrainewith aura n=23	p-value
General				
Female (n, %)	12 (50.0)	14 (51.9)	11 (47.8)	0.961[a]
Age	34.8 ± 8.7	35.1 ± 8.2	35.0 ± 9.3	0.992[b]
BMI	23.3 ± 2.5	22.6 ± 1.8	23.9 ± 2.6	0.325[b]
Migraine characteristics				
Age at onset	-	14.2 ± 7.7	14.4 ± 8.1	0.938[c]
Attack frequency (attack/month)	-	2.8 ± 2.5	2.3 ± 1.4	0.911[c]
Attack duration (hours)	-	36.6 ± 26.8	27.3 ± 21.0	0.235[c]
Headache days (days/month)	-	7.4 ± 5.0	5.4 ± 4.7	0.051[c]
Aura (% of attacks)	-	-	83.7 ± 22.6	-
Migraine related disability				
MIDAS	-	16.7 ± 15.2	15.1 ± 13.9	0.733[c]
HIT-6	-	62.6 ± 4.4	62.8 ± 6.3	0.614[c]
Visual sensitivity				
VSQ (sumscore)	3.2 ± 2.6	8.4 ± 4.8	12.4 ± 7.6	**<0.001**[b]
Pattern glare (GII)	2.7 ± 2.3	5.1 ± 2.1	5.1 ± 1.7	**0.001**[b]
Tissue segmentation of VOI				
GM fraction	0.62 ± 0.05	0.65 ± 0.03	0.65 ± 0.04	**0.020**[d]
WM fraction	0.28 ± 0.04	0.28 ± 0.04	0.28 ± 0.04	0.871[d]
CSF fraction	0.10 ± 0.06	0.07 ± 0.02	0.07 ± 0.04	0.059[d]

MIDAS: migraine disability assessment scale; HIT-6: Headache Impact Test; VSQ: Visual Sensitivity Questionnaire; GII: general illusion index; GM:gray matter; WM:white matter; CSF: cerebrospinal fluid. Values are expressed as mean ±SD. [a] Chi-square test. [b] Kruskal-Wallis Test. [c] Mann-Whitney test. [d] One-way ANOVA. P-values < 0.05 are depicted in bold.

In the primary analysis, glutamate levels were different when comparing the three primary study groups, i.e. interictal participants with migraine with aura (n = 23) or without aura (n = 27) and controls (n = 24, $F(2,70) = 3.20$; $p = 0.047$, Table 3 and Fig 3A). In a post-hoc analysis, glutamate levels were 6% higher in migraine without aura compared to controls ($p = 0.042$); however, no differences were found between migraine with aura and controls ($p = 0.72$) nor between migraine with and without aura ($p = 0.53$).

The glutamate concentrations did not significantly differ between migraine participants who were measured interictally and of those who were measured pre-ictal ($F(1,84) = 1.96$; $p = 0.16$).

Table 2: Clinical and demographic characteristics of healthy controls and inter plus pre-ictal migraineurs (n=87).

	Healthy Controls n=24	Migrainewithout aura n=36	Migrainewith aura n=27	p-value
General				
Female (n, %)	12 (50.0)	19 (52.8)	14 (51.9)	0.978[a]
Age	34.8 ± 8.7	35.4 ± 8.2	35.3 ± 9.2	0.984[b]
BMI	23.3 ± 2.5	22.8 ± 1.8	23.5 ± 2.7	0.818[b]
Smoking (n, %)	2 (8.3)	5 (13.9)	3 (11.1)	0.802[a]
Migraine characteristics				
Age at onset	-	14.1 ± 7.1	14.4 ± 7.6	0.906[c]
Attack frequency (attack/month)	-	3.0 ± 2.3	2.3 ± 1.4	0.415[c]
Attack duration (hours)	-	32.1 ± 26.5	28.7 ± 22.1	0.752[c]
Headache days (days/month)	-	7.1 ± 4.6	5.6 ± 4.5	0.092[c]
Aura (% of attacks)	-	-	83.9 ± 21.8	-
Migraine related disability				
MIDAS	-	16.4 ± 15.9	18.3 ± 19.5	0.701[c]
HIT-6	-	62.7 ± 4.3	63.2 ± 5.9	0.443[c]
Visual sensitivity				
VSQ (sumscore)	3.2 ± 2.6	8.7 ± 5.0	12.3 ± 7.2	<0.001[b]
Pattern glare (GII)	2.7 ± 2.3	5.1 ± 2.2	4.8 ± 1.8	0.001[b]
Tissue segmentation of VOI				
GM fraction	0.62 ± 0.05	0.65 ± 0.04	0.65 ± 0.05	0.013[d]
WM fraction	0.28 ± 0.04	0.28 ± 0.04	0.28 ± 0.04	0.831[d]
CSF fraction	0.10 ± 0.06	0.07 ± 0.03	0.07 ± 0.04	0.036[d]

MIDAS: migraine disability assessment scale; HIT-6: Headache Impact Test; VSQ: Visual Sensitivity Questionnaire; GII: general illusion index; GM:gray matter; WM:white matter; CSF: cerebrospinal fluid. Values are expressed as mean ±SD. [a] Chi-square test. [b] Kruskal-Wallis Test. [c] Mann-Whitney test. [d] One-way ANOVA.

There was no correlation between the number of days before a next attack and the glutamate concentration (Pearson's r = -0.18; p = 0.29; Fig 4). We pooled the inter- and pre-ictal concentrations in a secondary analysis (Table 3 and Fig 3B). The intergroup differences across pooled migraine with aura (n = 27), pooled migraine without aura (n = 36) and controls (n = 24) were similar, but statistically considerably stronger, compared to those observed in the primary analysis only including interictal concentrations. Thus, the glutamate concentration differed across groups (F (2,83) = 1.78; p = 0.004) and, in migraine without aura, was higher compared to controls (p = 0.006) and near-significantly higher compared to migraine with aura (p = 0.051).

No differences were found for the ADC of glutamate in both the primary and secondary analyses, nor for the concentration and ADC of the other metabolites (Table 3).

Table 3: Average metabolite concentrations and apparent diffusion coefficients in the visual cortex

	Healthy controls	Interictal migraine patients			Interictal plus pre-ictal migraine patients		
		Migrainewithout aura	Migraine with aura	p-value	Migrainewithout aura	Migraine with aura	p-value
Concentration (mmol/L)[a]							
Primary outcome	n=24	n=27	n=23		n=36	n=27	
Glu	6.40 ± 0.78	7.02 ± 0.50	6.77 ± 0.47	**0.047**	7.07 ± 0.56	6.76 ± 0.51	**0.004**
Secondary outcomes							
Gln	2.15 ± 0.42	2.40 ± 0.38	2.26 ± 0.51	0.194	2.43 ± 0.39	2.27 ± 0.48	0.109
Glu + Gln	8.55 ± 1.12	9.42 ± 0.76	9.03 ± 0.81	0.051	9.50 ± 0.86	9.03 ± 0.83	**0.008**
tNAA	10.29 ± 0.87	10.85 ± 0.52	10.69 ± 0.60	0.180	10.82 ± 0.56	10.71 ± 0.64	0.164
tCr	7.24 ± 0.69	7.72 ± 0.58	7.54 ± 0.45	0.101	7.69 ± 0.58	7.60 ± 0.49	0.105
Ins	4.08 ± 0.46	4.19 ± 0.38	4.30 ± 0.61	0.422	4.22 ± 0.39	4.30 ± 0.65	0.503
tCho	1.32 ± 0.26	1.35 ± 0.16	1.25 ± 0.17	0.220	1.35 ± 0.15	1.26 ± 0.19	0.179
PE	3.41 ± 0.98	3.58 ± 0.54	3.42 ± 0.62	0.782	3.54 ± 0.58	3.48 ± 0.64	0.877
Asp	3.73 ± 1.10	3.73 ± 0.65	3.58 ± 0.67	0.762	3.73 ± 0.65	3.61 ± 0.66	0.820
ADC ($\mu m^2/s$)[b]							
Primary outcome	n=18	n=23	n=22		n=31	n=24	
Glu	0.106 ± 0.018	0.108 ± 0.025	0.120 ± 0.027	0.129	0.110 ± 0.026	0.110 ± 0.027	0.204
Secondary outcomes							
Glu + Gln	0.106 ± 0.018	0.107 ± 0.025	0.118 ± 0.028	0.194	0.109 ± 0.027	0.117 ± 0.028	0.317
tNAA	0.107 ± 0.022	0.114 ± 0.026	0.127 ± 0.028	0.057	0.115 ± 0.026	0.124 ± 0.028	0.109
tCr	0.110 ± 0.015	0.117 ± 0.020	0.120 ± 0.019	0.247	0.118 ± 0.019	0.120 ± 0.019	0.220
tCho	0.099 ± 0.014	0.104 ± 0.018	0.108 ± 0.020	0.287	0.104 ± 0.018	0.109 ± 0.019	0.233

Interictal: no migraine attack in the three days before and the two days after examination. Pre-ictal: no migraine attack in the three days before examination but experiencing an attack within the two days following examination. Values are expressed as actual means ± SDs. [a] P-values from One-way ANCOVA between groups with GM fraction as covariate. [b] P-values from a One-way ANOVA. Glu, glutamate; Gln, glutamine; tNAA, total N-acetylaspartate; tCr, total creatine; Ins, myo-inositol; tCho, total choline; PE, phosphorylethanolamine; Asp, aspartate.

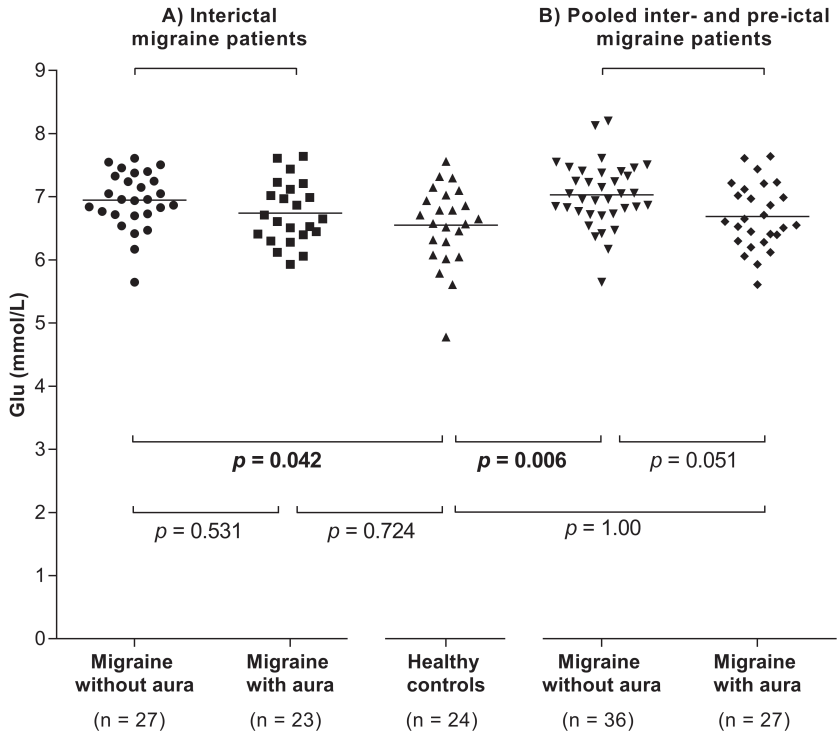


Figure 3: Glutamate (Glu) concentrations adjusted for grey matter fraction. A (Left side): primary analysis for group difference between the concentrations in the control group and the interictal values in migraineurs with or without aura ($F(2,70) = 3.20$; $p = 0.047$). B (Right side): secondary analysis for group differences between the concentrations in the control group and the pooled interictal and pre-ictal values of migraineurs with or without aura ($F(2,83) = 1.78$; $p = 0.004$). P-values from post-hoc tests with Bonferroni correction after one-way ANCOVA between groups with grey matter fraction as covariate.

Clinical parameters and glutamate

The glutamate concentration in the visual cortex did not correlate with the self-reported visual sensitivity (sumscore on the VSQ) (Pearson's $r = 0.17$, $p = 0.126$) or with the general illusion index from the pattern glare test (Pearson's $r = 0.01$, $p = 0.918$) when looking at all subjects, nor when looking at subgroups of interictal or pooled interictal and pre-ictal migraineurs separately.

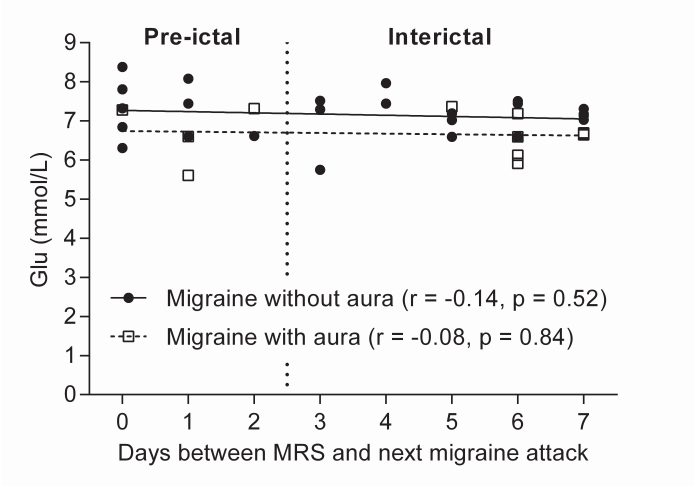


Figure 4: Relation between days until next attack and glutamate concentration. All subjects with attack within seven days after study day (n=37).

Discussion

In the present study, we used ^{1}H-MRS and DWS at 7 tesla to measure interictal levels of glutamate and glutamine in the visual cortex of migraine patients. Glutamate levels were increased in participants with migraine without aura but not in those with migraine with aura. Glutamine concentrations and intracellular mobility of glutamate were normal.

Although the main aim of the study was to assess baseline cortical glutamate and glutamine concentrations outside attacks, 13 migraine participants were accidentally investigated within two days before an attack. These pre-ictal concentrations did, however, not differ from interictal concentrations, suggesting that increased cortical glutamate is a permanent and rather stable feature of patients with migraine without aura.

^{1}H-MRS and DWS primarily measure intra-neuronal glutamate, which accounts for approximately 80% of glutamate present in the brain.[19,20] We believe our results indicate increased *intra*-cellular levels of glutamate rather than increased number of glutamatergic neurons as we failed to find any difference for the neuronal marker total N-acetyl-aspartate (tNAA)[46] (Tabel 2). Moreover, we also corrected for grey matter content of the VOI, which primarily consists of neurons.

Glutamate is distributed over different intracellular compartments, mainly the cytosol, cell organelles, and synaptic vesicles.[19,47] The mobility of glutamate within and between these compartments is influenced by the size of the compartments, obstructions in and between the compartments, and properties of the cytosol, such as viscosity.[22] The diffusion properties of metabolites indirectly provide information on the compartmentalization of metabolites within tissue compartments, on changes in cellular microstructure caused by disease or other factors, and on changes in cytosol properties, e.g. viscosity, tortuosity, and macromolecular crowding.[22] Although pathology-driven changes in compartmentalization of glutamate have been implicated in certain neurological diseases,[48-50] the normal intracellular mobility of glutamate in our study makes such mechanisms in migraine unlikely.

A major strength of our study is that we used ^{1}H-MRS at high magnetic field strength (7T) enabling accurate and separate assessment of glutamate and glutamine as confirmed by the low CRLB's of glutamate (2.8 ± 0.95) and glutamine (8.8 ± 2.7). This is a major advantage as both amino acids, although metabolically intimately related via the glutamate glutamine cycle, have very different functions. While glutamate acts as the main excitatory neurotransmitter, glutamine is essentially inactive and primarily functions as a precursor to glutamate.[19]

Previous ^{1}H-MRS studies in migraine have used techniques which were not optimised to accurately differentiate between glutamate and glutamine and consequently could only report the combined signal of glutamate and glutamine (Glx, in some studies simply reported as Glu).[11-13,15-18] One study reported an increased Glx/Creatine ratio in the occipital lobe of ten patients with migraine with aura [13] while five others studies failed to find any difference in Glx.[11,15-18] In one study the glutamate/glutamine ratio was increased in patients with migraine with or without aura[15] potentially suggesting a higher neuron to astrocyte ratio as glutamate is mainly present in neurons and glutamine in astrocytes. However, the CRLB for glutamine in that study was high (34.1% ± 19.2), indicating considerable margin of measurement error. In our study, the glutamate/glutamine ratio in the occipital lobe of migraine patients was normal. In a recent study using ^{1}H-MRS, primarily to measure lactate changes after hypoxia, cortical glutamate levels did not differ at baseline between patients with migraine and healthy controls.[18] However, the 3T MRS technique in this study was not optimised to separate glutamate from glutamine. As a consequence, Glx (glutamate and glutamine together) was probably estimated. Moreover, no information was provided on the

quality of the spectra LCModel fits. In the present study, we do not report lactate concentrations because lactate could not be fitted reliably. For robust detection of lactate, dedicated MRS editing sequences are needed.[51,52] These were, however, not operational at our department at the time of the present study. Moreover, adding such sequences would significantly have increased measurement time and thus burden for the participants.

As summarized in Table 3, we did not find differences between migraineurs and controls for the cerebral concentrations of tNAA, tCr, Ins, tCho, PE or Asp. Other studies using ^{1}H-MRS to measure brain metabolism in migraine have yielded disparate results.[14,53] This has most likely been due to a combination of a variety of methodological differences including heterogeneous migraine populations, different timings of the measurement (ictal versus interictal), and differences in MRS techniques, field strengths, signal processing, and quantification strategies. In a recent study, using 2D ^{1}H-MRS, no differences were found between interictal metabolite concentrations in the anterior cingulate cortex of healthy controls and patients with migraine with or without aura when applying univariate statistics.[54] However, when applying a quadratic discriminant analysis model, they found a complex of metabolite ratios (Asp/Cre, NAA/Cre, and Gln/Cre) which could discriminate migraineurs from controls. The exact direct of the changes, though, became not clear from the study results.

Increased glutamatergic activity has long been implicated in migraine pathophysiology through various putative mechanisms.[55] A major hypothesis has been that increased *extracellular* glutamate within the synaptic cleft would lead to cortical hyperexcitability and enhanced susceptibility to CSD.[6,56,57] As CSD is considered the underlying mechanism for migraine aura, one would have expected to find increased cortical glutamate in migraine with aura and possibly also migraine without aura.[6,8,58] Instead we found increased glutamate levels only in migraine without aura but not in migraine with aura.

Of relevance here is to realise that with the currently available measurement techniques one can only measure the total of the extracellular and the ten thousand fold higher intracellular glutamate.[20] At present, there is no method to distinguish these two glutamate compartments in humans, not even with high field ^{1}H-MRS and DWS. We therefore cannot confirm nor refute whether synaptic (extracellular) glutamate is changed in migraine with or without aura.

A second possible role for glutamate in migraine would be as part of the cortical glutamatergic excitation / GABA-ergic inhibition balance. Unfortunately, in our

study, GABA could not be fitted reliably because we didn't use dedicated MRS editing sequences which are required for robust detection of GABA.[51] In a recent study, in which a dedicated editing sequence for GABA was applied, increased levels of GABA+ (GABA plus substantial macromolecule contamination) were observed in the posterior cingulate cortex of patients with migraine with and without aura.[59] No sub-analyses were done separately for patients with migraine with and those without aura. GABA levels in that study positively correlated with pain and central sensitization scores.[60] In our study, we didn't observe any correlation between clinical parameters such as sensitivity to light and patterns and occipital glutamate concentrations.

Increase of glutamate might also be related to the role of glutamate in the energy metabolism of the brain. Glutamate is a central compound in cellular metabolism since its carbon skeleton is an input into several anabolic and catabolic pathways. The formation and degradation of glutamate is also part of the general energy metabolism of the brain, as it leaves and enters the tricarboxylic acid (TCA) cycle at the level of α-ketoglutarate. Glutamate can re-enter the TCA cycle as an alternative oxidizable substrate and as such can serve as an important energy reserve.[19,61] Since glutamate is such a key component of intermediary metabolism, the results of our study might also reflect changes in intracellular metabolism or an increase in alternative energy reserves. This would be well in line with previous observations with ^{31}P-MRS showing reduced interictal levels of high-energy phosphates such as adenosine triphosphate (ATP) and phosphocreatine (PCr) in migraine with and without aura. These observations would suggest disrupted energy metabolism and reduced energy reserve as a key mechanism in migraine. [14]

Maintaining low extracellular glutamate concentrations via glutamate uptake by neurons, astrocytes, and the blood-brain barrier (BBB) is also highly energy-dependent.[62] One could speculate that a slight impairment in glutamate up-take due to smaller energy reserves may lead to higher extracellular levels of glutamate[19], which in turn might increase cortical excitability and migraine susceptibility. The connection between glutamate as neurotransmitter - which compartmentalization is highly energy dependent - and glutamate as an intermediate in energy metabolism should be investigated in more detail in migraine patients. This could be done, e.g. by simultaneously using ^{1}H-MRS and ^{31}P-MRS techniques, or by using ^{13}C-MRS to study neuro-energetics and neurotransmitter cycling in the brain.[63] Future studies in migraine might also use functional MRS at 7 tesla to investigate neurotransmitter dynamics following visual stimulation. Recent

functional MRS studies at 7 tesla found a small but marked increase of glutamate (2-3% increase) and lactate (19-23%) concentrations in the occipital lobe after visual stimulation.[64-66] It will be interesting to investigate whether differences in cortical excitability, e.g. increased evoked potentials after visual stimulation, is associated with functional changes in brain metabolites.

Next to a number of evident strengths, such as: (i) use of 7 tesla ^{1}H-MRS and DWS, providing information on glutamate concentration and mobility; (ii) strict inclusion criteria to clearly distinguish participants with migraine with aura from those without aura; and (iii) inclusion of interictal and pre-ictal migraineurs, our study also has some limitations. Although our study population as a whole is larger than those investigated in other MRS studies, the subgroups are still small and might thus be underpowered to detect differences e.g. between migraine with and without aura or between interictal and pre-ictal migraineurs. Moreover, we could only measure the ADC of glutamate but not of glutamine because we selected a TE of 120ms which falls in the range of theoretically-predicted optimal values for quantification of glutamate and was chosen based on the need to accommodate the diffusion weighting gradients. The investigator who performed the measurements was not blinded for subject status. To avoid possible bias in VOI placement, the sizes of the VOIs were fixed and clear anatomical landmarks were used for placement of the VOIs. In addition, an independent investigator (GJLO) who was blinded to subject status verified correct placement of the VOIs in all participants. As the differences were only minimal and equally affected all three subgroups, a systematic (biased) error in VOI placement seems very unlikely. Finally, for scientific and methodological reasons we focused on the visual cortex as region of primary interest. We, therefore, cannot extrapolate our findings to other brain regions.

In conclusion, glutamate levels are increased in the visual cortex of patients with migraine without aura, strengthening the pathophysiological link of this amino acid with migraine, either through its neuroexcitatory effects, its role in energy metabolism, or a combination of both.

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Part II

Biochemical profiling of cerebrospinal fluid

CHAPTER 4

Migraine biomarkers in cerebrospinal fluid: A systematic review and meta-analysis

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Abstract

Objective: To perform a meta-analysis of migraine biomarkers in cerebrospinal fluid (CSF) and of corresponding blood concentrations.

Methods: We conducted a systematic search for studies that measured biochemical compounds in CSF of chronic or episodic migraineurs and non-headache controls. Subsequent searches retrieved studies with blood measurements of selected CSF biomarkers. If a compound was assessed in three or more studies, results were pooled in meta-analysis with standardized mean differences (SMD) as effect measures.

Results: Sixty-two compounds were measured in 40 CSF studies. Most important results include: increased glutamate (five studies, SMD 2.22, 95% C.I.: 1.30, 3.13), calcitonin gene-related peptide (CGRP) (three studies, SMD: 3.80, 95% C.I.: 3.19, 4.41) and nerve growth factor (NGF) (three studies, SMD: 6.47, 95% C.I.: 5.55, 7.39) in chronic migraine patients and decreased β-endorphin (β-EP) in both chronic (four studies, SMD: -1.37, 95% C.I.: -1.80, -0.94) and interictal episodic migraine patients (three studies, SMD: -1.12, 95% C.I.: -1.65, -0.58). In blood, glutamate (interictal) and CGRP (chronic, interictal and ictal) were increased and β-EP (chronic, interictal and ictal) was decreased.

Conclusions: Glutamate, β-EP, CGRP and NGF concentrations are altered in CSF and, except for NGF, also in blood of migraineurs. Future research should focus on the pathophysiological roles of these compounds in migraine.

Introduction

Migraine is a prevalent episodic brain disorder.[1] The WHO rates migraine as one of the most disabling chronic disorders.[2] Despite extensive research over the last decades migraine pathophysiology is not completely understood.[3] Although several compounds (e.g. CGRP, glutamate and serotonin) have been implicated in migraine pathophysiology, our understanding of the biochemistry of migraine is still limited.[4,5] Identification and validation of biochemical biomarkers might help us in uncovering pathophysiological processes involved in migraine, which in turn might lead to diagnostic tests or new therapeutic strategies.[6,7]

The field of biochemical biomarker research is expanding rapidly. Promising biomarkers have been discovered for brain disorders such as Alzheimer's disease, narcolepsy, and Parkinson's disease.[8–10] Cerebrospinal fluid (CSF) is believed to reflect biochemical changes in the brain and therefore the body fluid of primary interest for brain disorders.[11] Although many small studies have analysed biochemical changes in CSF from migraine patients, results were often inconsistent and have not led to pathophysiological and diagnostic biomarkers. However, the literature has never been systematically reviewed with quantitative synthesis of the evidence. With this first meta-analysis we aimed to identify biochemical migraine biomarkers which show consistent changes in CSF and to assess whether these changes are also present in blood.

Methods

Search strategy, study selection and eligibility criteria

We conducted and reported the review process in accordance with the PRISMA statement.[12] We performed an electronic search for published studies up to August 16, 2014 in MEDLINE, EMBASE and Web of Science on biochemical findings in CSF of migraine patients. Medical Subject Heading (MeSH) terms and free text terms were collated with the assistance of research librarians at the Leiden University Medical Centre. The full search string can be found in supplement 1.

Two investigators (R.M.D. and R.Z.) independently assessed titles and abstracts to determine potential eligibility. Disagreement was resolved by discussion. The same investigators independently assessed the full text articles of potentially relevant studies to verify if eligibility criteria were met, and to evaluate whether the results were adequately reported.

We included case-control studies and case-crossover studies (same patients studied in between and during migraine attacks), in which one or more endogenous compounds (metabolites, peptides, proteins) were quantified in CSF samples from migraine patients. Case reports were not considered eligible. Publications on pharmacological trials were excluded if no endogenous compounds were measured at baseline. Studies not written in English, conference abstracts, editorials and letters were also not eligible. Reference lists of articles eligible for full-text review and relevant reviews were additionally searched for potentially relevant studies.

Subsequent search for studies on blood concentrations of selected biomarkers

To assess whether CSF biomarkers show similar results in blood we performed in a second stage a literature search for published data on measurements in plasma and serum. We specifically searched for studies reporting blood concentrations of compounds which had shown consistent and significant differences in meta-analysis of CSF data. These additional blood studies were identified and selected by performing the same search and selection process as described for CSF studies. The full search string for blood is reported in supplement 1. After study selection data were extracted and subsequently included in study assessment and meta-analysis following the same methodology as for CSF.

Data extraction

Data extraction was performed by one investigator (R.M.D) using a standardized extraction form. A second investigator (R.Z.) was consulted if discussion was necessary. Information was extracted on: (1) study design, (2) study population characteristics (sample size, age, gender, medication, comorbidity and other potential confounders) and study groups definition (diagnostic criteria, presence of migraine attack during sampling, presence of chronic migraine component); (3) sampling methods (fasting, timing and storage temperature), measurement methods and data analysis; and (4) concentrations of endogenous compounds (metabolites, peptides, proteins) in study (sub)groups, including statistical parameters. To obtain relevant missing information of studies included for meta-analysis, we attempted to contact corresponding authors twice via email.

Risk of bias assessment

To assess risk of bias we adapted the Newcastle-Ottowa Scale[13] (supplement 1). We considered definition of cases and controls to be adequate when published criteria were used for diagnosis of migraine patients. Selection of cases was adequate when

patients were representative for the defined migraine type (no severe comorbidity or clinical reasons to sample body fluids). Selection of controls was adequate when controls were sampled from the same population as the cases. Comparability between cases and controls was assessed based on gender- and age-matching of study groups (either by design or analysis). Studies adequately describing sampling and measurement methods and performing measurements according to validated analytical methods were considered to have low risk of measurement bias. One investigator (R.M.D.) assessed selection and comparability, two investigators (R.M.D. and M.N.) assessed the description and validity of the measurements, and a third investigator (R.Z.) was contacted if discussion was necessary.

Group definition

We divided case-control comparisons into episodic migraine *versus* controls and chronic migraine *versus* controls. If there was no evidence that migraine patients had a chronic component, we classified them as episodic migraine. Findings in episodic migraine were further classified based on migraine state: interictal and ictal. Migraine with aura patients and migraine without aura patients were grouped, because results were often not reported separately for these groups. When applicable, ictal *versus* interictal findings, from case-control and from case-crossover studies, were compared.

Meta-analysis: pooling of results and statistical procedures

We used standardized mean differences (SMDs) with their 95% confidence interval (C.I.) as main effect measure. Compound concentrations were analysed in meta-analysis if data were available from three or more studies for one of the defined group comparisons. The way we have dealt with missing data, irregularities in the data, and pooling of data was in accordance with approaches described by the Cochrane Collaboration[14] (supplement 1).

For quantitative synthesis we used the inverse variance method. We applied a random-effects model by default given the expected clinical heterogeneity between studies. However, as the between-study variation cannot be estimated reliable in case of <5 studies, we applied a fixed-effects model in these instances. Homogeneity of effect sizes was assessed using the I^2 statistic and by visual inspection of forest plots. To examine the effect of inclusion of clear heterogeneous studies we applied a sensitivity analysis to assess their specific effect on the overall effect size. For statistical analysis we used RevMan 5.2 (Cochrane IMS, Baltimore, MD).

Results

Study selection and study characteristics

The selection of CSF studies is depicted in the flow chart (figure 1). A total of 1197 unique articles were identified of which 40 were considered eligible (38 case-control studies and 2 case-crossover studies). Investigator agreement on title and abstract screening, before consensus, was $\kappa = 0.72$. Episodic migraine patients were sampled for 22 case-control studies and chronic migraine patients for 16 case-control studies. The number of cases ranged from 4 to 60 (average: 24) and the number of controls from 5 to 108 (average: 24). Twelve CSF studies were finally included in meta-analyses on compounds that were measured in multiple studies. The subsequent search for blood studies on selected CSF biomarkers is illustrated in supplementary figure s-1.

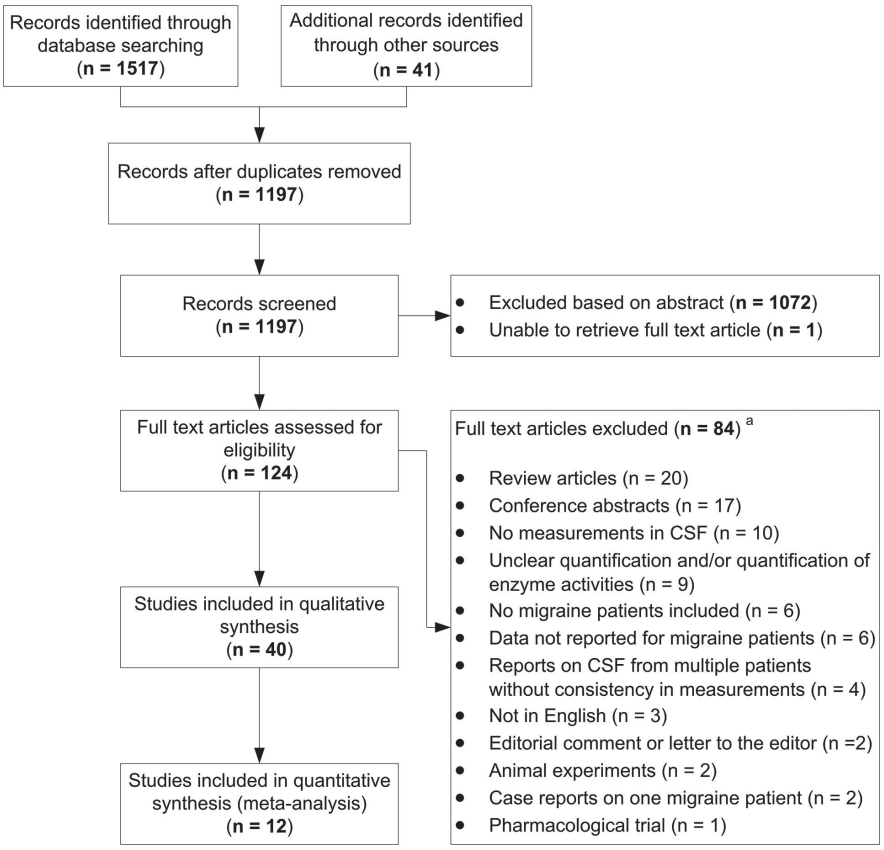


Figure 1: Flowchart of CSF study selection process. [a] Studies could be excluded because of more than one eligibility criteria. Therefore, overlaps exist between these categories.

Risk of bias assessment

Most CSF studies (73%) applied adequate diagnostic criteria (table 1); eleven studies that did not report the use of diagnostic criteria, were published before introduction of the ICHD-I.[15] Criteria for chronic migraine (Silberstein[16] and ICHD-II revision[17]) were applied by all but four studies on chronic migraine (75%).

Migraine cases were not always deemed representative for the diagnosed migraine type because lumbar punctures were performed to exclude other neurologic diseases (5 studies), migraine patients were admitted to the hospital for unstated reasons (4 studies) or because recruitment of cases was not clearly reported (11 studies) (table 1). Controls often had lumbar punctures for other purposes than migraine patients; either for other diagnostic purposes (13 studies) or before spinal anesthesia (4 studies). Based on available cohort descriptions only 6 studies recruited cases and controls from the same population, of which 4 studies sampled from the general population. For 15 studies it was explicitly stated that controls had no personal history of migraine (table 1). Furthermore, a minority of studies (8 studies) adjusted for age and gender.

Sampling and measurement methods were adequately described in 22 studies (55%; table 1). Especially the older publications lacked full and clear descriptions of methods. Measurement techniques were considered to be (partially) validated in 21 studies. Quantitation characteristics (precision, accuracy and limit of detection) were often not reported.

Table 1: Summary of study characteristics and risk of bias assessment of CSF studies.

Study characteristics	Studies	Risk of bias assessment	Studies
Publication year		**Selection**	Adequate
≤ 1960	1 (3%)	Definition of cases	29 (73%)
1961 – 1980	7 (18%)	Selection of cases	20 (50%)
1981 – 2000	15 (38%)	Definition of controls	15 (39%)*
≥ 2001	17 (43%)	Selection of controls	6 (16%)*
Study design		**Comparability**	
Case-control	38 (95%)	Matching for age and gender	8 (21%)*
Case-crossover only	2 (5%)	Matching for other factors	4 (11%)*
Migraine types and states		**Measurements**	
Episodic Migraine	22 (58%)*	Measurement description	22 (55%)
Ictal state	19 (50%)*	Validation of measurement technique	21 (53%)
Interictal state	13 (34%)*		
Mixed state	3 (8%)*		
Chronic Migraine	16 (42%)*		
Control types			
Healthy	7 (18%)*		
Spinal anesthesia	4 (11%)*		
Diagnostic lumbar puncture [a]	15 (39%)*		
Other neurological diseases	9 (24%)*		

Risk of bias assessment: number of studies which were assessed as adequate for the corresponding item, * = total of 38 studies (excluding two case-crossover studies since no controls were present). [a] Controls underwent a diagnostic lumbar puncture and, retrospectively, CNS disorders were excluded by original researchers after which samples were used as control samples.

Table 2: Overview of published biochemical measurements in CSF from migraine patients.

	Studies (n=)	**Chronic migraine**	**Episodic migraine** Interictal	**Episodic migraine** Ictal
NEUROTRANSMITTER SYSTEMS				
Glutamatergic system				
Glutamate	7	↑[18] ↑[19] ↑[20] ↑[21] ↑[22] u.d.[23]	u.d. [23]	↑[24] u.d. [23]
Glutamine	1	↑[23]	(↑) [23 a]	(↑) [23 a]
Glycine	1	↑[23]	(↑) [23 a]	(↑) [23 a]
Serotonergic system				
5-hydroxyindoleacetic acid	4	= [23]	(=) [25] = [26] (=) [23] (↑) [27 a]	(=) [25] = [26] (=) [23] (↑) [27 a]
Tryptophan	2		= [28] = [29]	↑[28] ↑[29]
5-hydroxytryptamine	1	u.d.[23]		
Dopaminergic system				
Homovanillic acid	4	= [30] = [23]	= [25] (=) [23] (=) [27]	= [25] (=) [23] (=) [27]
3,4-dihydroxyphenylacetic acid	1			↑[31]
Tyrosine	1			= [31]
Epinephrine	1			= [32]
Norepinephrine	1			= [32]
GABAergic system				
γ-Aminobutyric acid	4	= [33] u.d. [23]	u.d. [34] u.d. [35] u.d. [23]	(↑) [34] (↑) [35] u.d. [23]
Cholinergic system				
Acetylcholine	1			() [36]
NEUROPEPTIDES				
Endogenous opioids				
β-endorphin	5	↓ [37] ↓ [38] ↓ [39] ↓ [30]	↓ [37] ↓ [38] ↓ [40]	↓ [40]
β-lipotropin	2	↓ [37] ↓ [38]	= [37] = [38]	
Adrenocorticotropic hormone	2	= [37] = [38]	= [37] = [38]	
α-N-acetyl-β-endorphin	1	↑[30]		
'Enkephalins'	1		= [28 †]	↓ [28]
Met-enkephalin	1		↑[41 b]	
Tachykinin neuropeptides				
Substance P	2	↑[18] ↑[42]		
Neurokinin A	1	↑[18]		
Other neuropeptides				
Calcitonin gene-related peptide	3	↑[18] ↑[43] ↑[42]		
Neuropeptide Y	2		= [40]	= [40] ↑[44]
Somatostatin	2	↓ [45]	= [40 †]	↓ [40]
Orexin-A	1	↑[46]		
ENDOCANNABINOIDS				
Anandamide	1	↓ [43]		

Table 2: continued.

	Studies (n=)	**Chronic migraine**	**Episodic migraine** Interictal	**Episodic migraine** Ictal
Palmitoylethanolamide	1	↑[43]		
2-Arachidonoylglycerol	1	u.d. [43]		
NEUROTROPHINS				
Nerve growth factor	3	↑[20] ↑[21] [42]		
Brain-derived neurotrophic factor	2	↑[20] ↑[21]		
Glial cell-derived neurotrophic factor	1	↓ [45]		
CYTOKINES				
Tumor necrosis factor-alpha	2	↑[47]		u.d. [48]
Interleukin-1 receptor antagonist	1			↑[48]
Monocyte chemotactic protein-1	1			↑[48]
Transforming growth factor Beta 1	1			↑[48]
Interleukin-10	1			u.d. [48]
Interleukin-1β	1			u.d. [48]
Interleukin-4	1			u.d. [48]
METAL IONS				
Calcium (ionized)	1		= [49]	= [49]
Calcium (total)	1		= [49]	= [49]
Magnesium (total)	1		= [49]	= [49]
Potassium	1		= [49]	= [49]
Sodium	1		= [49 †]	↑[49]
OTHER				
Nitrite products (NO, NO_2^-, NO_3^-)	3	↑[18] ↑[43]		= [44 c]
Taurine	2	↑[23]	(↑) [23 a]	↑[50] (↑) [23 a]
Albumin	1		= [27]	= [27]
Aspartic acid	1			u.d. [24]
Chromogranin A	1			= [44]
Corticotropin releasing hormone	1	↑[46]		
Cortisol	1		↓ [51]	
Cyclic adenosine monophosphate	1			↑[35]
Follicle stimulating hormone	1		(↑) [51]	
Guanosine 2′,3′-cyclic phosphate	1	↑[18]		
Homocysteine (free)	1		= [52]	
Homocysteine (total)	1		↑[52]	
IgG	1		= [27]	= [27]
Luteinizing hormone	1		(↑) [51]	
Methionine	1		↓ [52]	
Neuron-specific enolase	1			(=) [53]
Phosphatidylcholine-specific phospholipase C	1		= [54 †] = [55]	↑[54]
Prolactin	1		(↑) [51]	

Published biochemical findings in migraine patients compared with controls. ↑ = significantly elevated concentrations, ↓ = significantly decreased concentrations, "=" = similar concentrations, between ( ) = no statistical analysis reported, u.d. = undetectable concentrations reported for studied migraine group, † =

significant difference between ictal and interictal migraine patients reported. [a] Significant when interictal and ictal groups were pooled. [b] Migraine state not reported. [c] Nitric oxide (NO) was measured; not reported if NO_2^- and NO_3^- were also quantified. Excluding glucose from the routine CSF measurements (nine studies reported normal glucose concentrations in migraine patients).

Biochemical findings

In total 62 unique compounds have been measured in CSF from migraine patients (table 2). Frequently measured compounds (in three or more studies) are glutamate, β-endorphin (β-EP), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), calcitonin gene-related peptide (CGRP) and nerve growth factor (NGF). Forty-four compounds were measured only once. Meta-analysis showed that glutamate (figure 2), β-EP (figure 3), CGRP (figure 4) and NGF (figure 5) concentrations were consistently altered in CSF from migraine patients compared to controls; results will be discussed below, together with results on blood concentrations. For HVA there was insufficient quantitative data available for meta-analysis (CSF concentrations not reported separately for ictal and interictal patients) and for 5-HIAA CSF studies showed inconsistent effects (supplementary figure s-2).

Glutamate (Figure 2)

Glutamate concentrations were increased in CSF from chronic migraine patients (SMD: 2.22, 95% C.I.: 1.30, 3.13).[18,19,21,22,20] In blood from interictal episodic migraine patients the pooled difference was not statistically significant (SMD: 1.08, 95% C.I.: -0.07, 2.22).[56–62] After exclusion of pediatric migraine patients[58], glutamate concentrations were increased in the remaining adult migraineurs (SMD: 1.61, 95% C.I.: 0.73, 2.49). Glutamate concentrations were increased in CSF from ictal patients (SMD: 2.01, 95% C.I.: 1.27, 2.75).[24] In blood, two studies showed clearly opposing results on ictal measurements and therefore we did not perform a meta-analysis.[56,24] There are no studies on glutamate concentrations in blood from chronic migraine patients and in CSF from interictal migraineurs.

β-Endorphin (Figure 3)

β-EP concentrations were decreased in CSF (SMD: -1.37, 95% C.I.: -1.80, -0.94)[37–39,30] and blood (SMD: -0.76, 95% C.I.: -1.17, -0.36)[38,63,64] from chronic migraine patients. Concentrations were also decreased in CSF (SMD: -1.12, 95% C.I.: -1.65, -0.58)[37,38,40] and blood (SMD: -0.40, 95% C.I.: -0.64, -0.16)[38,40,65–73] from interictal patients. Pooled estimates remained similar in sensitivity analysis. One blood study was excluded from meta-analysis because the assay that was used had a very high cross reactivity with β-lipotropin.[28] In ictal migraineurs β-EP

concentrations were decreased in CSF (SMD: -1.39, 95% C.I.: -2.40, -0.39)[40] and blood (SMD: -1.44, 95% C.I.: -2.11, -0.77).[40,66]

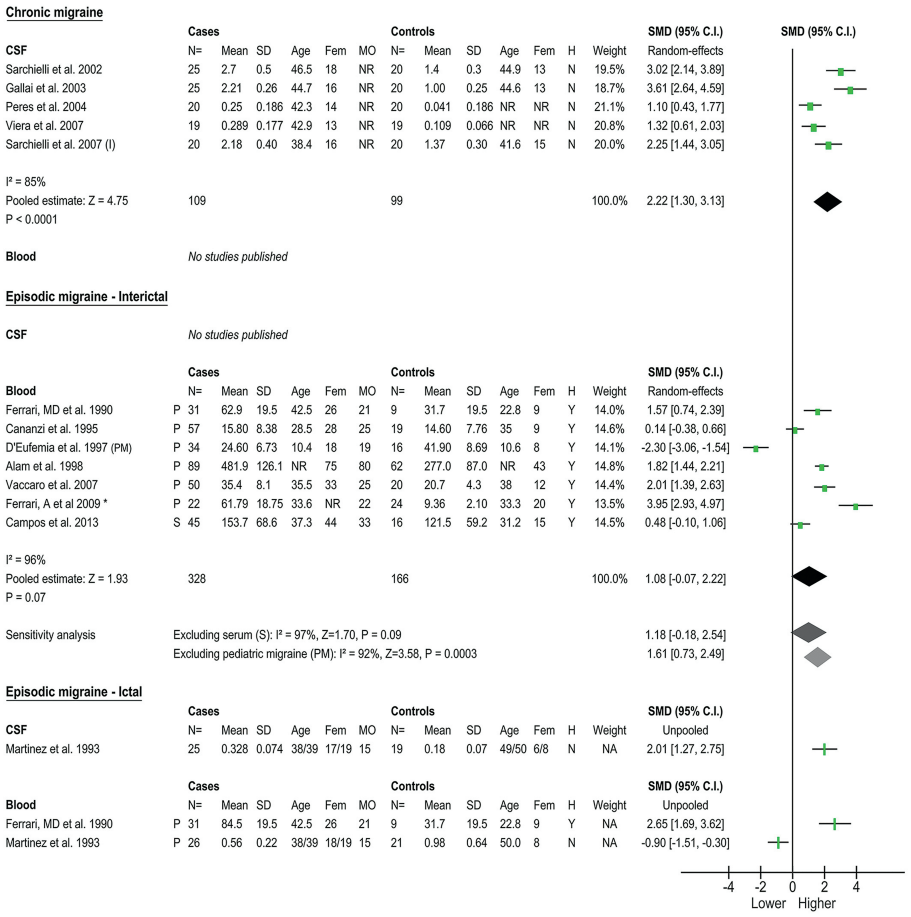


Figure 2: Forest plot of glutamate concentrations in migraine patients and controls. The squares represent effect sizes of the individual studies (size reflects the weight of the study) and the horizontal lines indicate the 95% confidence intervals (C.I.). The filled diamonds represent the overall effect size (horizontal width indicates the 95% C.I.). Age = mean age, Fem = number of females, MO = number of cases with migraine without aura, H= healthy controls, Y= yes, N = no, P = plasma concentrations, S= serum concentrations, * = migraine state is not explicitly reported for this study, the interictal state was assumed, (PM) = pediatric migraine patients and pediatric controls. Additional information on the handling of missing data (e.g. calculations, assumptions) can be found in the supplement.

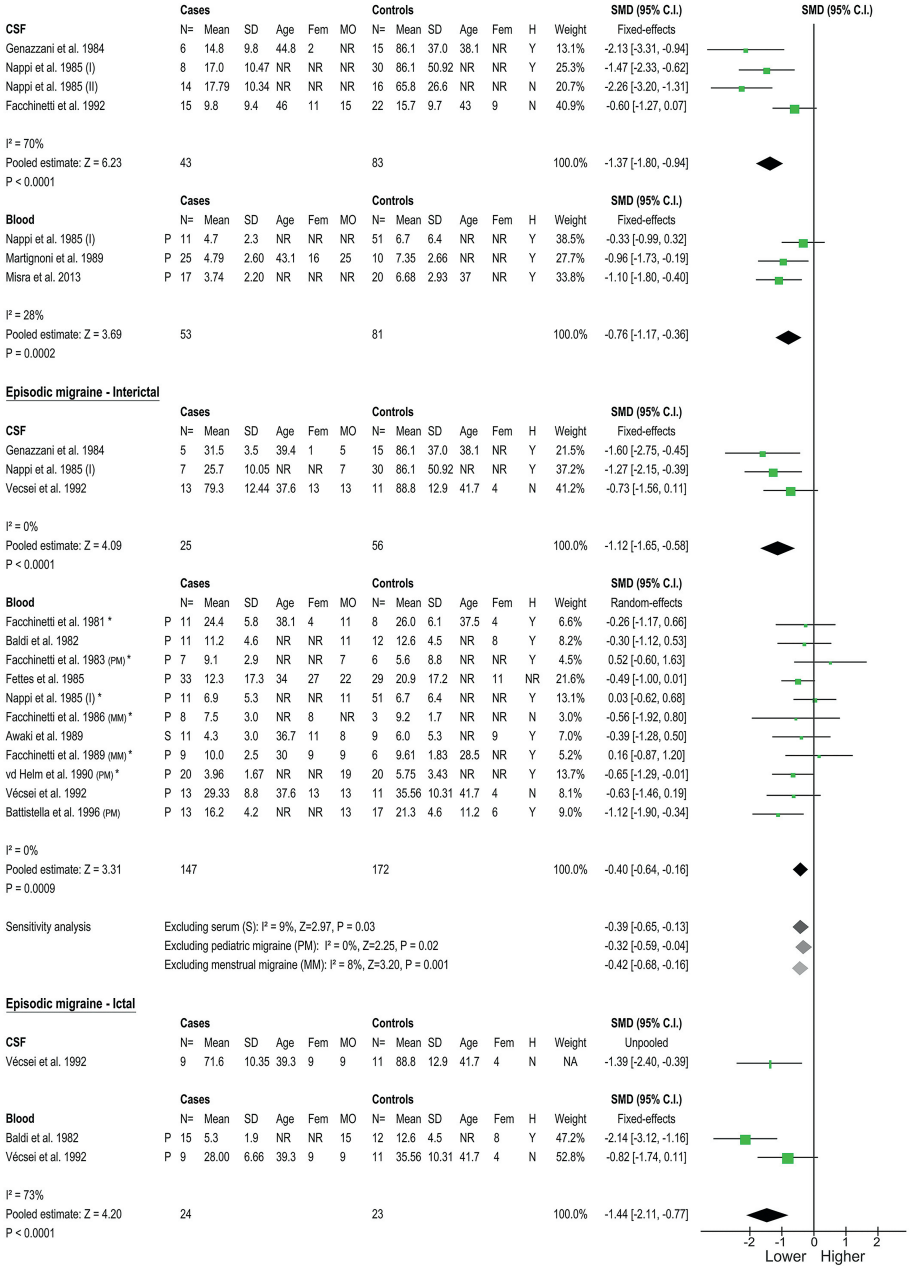


Figure 3: Forest plot of β-EP concentrations in migraine patients and controls. The squares represent effect sizes of the individual studies (size reflects the weight of the study) and the horizontal lines indicate the 95% confidence intervals (C.I.). The filled diamonds represent the overall effect size (horizontal width indicates the 95% C.I.). Age = mean age, Fem = number of females, MO = cases with migraine without aura, H= healthy controls, Y= yes, N = no, P = plasma concentrations, S= serum concentrations, * = migraine state is not explicitly reported for this study, the interictal state was assumed, (PM) = pediatric migraine patients

and pediatric controls, (MM) = menstrual migraine. Additional information on the handling of missing data (e.g. calculations, assumptions) can be found in the supplement.

Calcitonin gene-related peptide (Figure 4)

CGRP concentrations were increased in CSF (SMD: 3.80, 95% C.I.: 3.19, 4.41)[18,42,43] and blood (SMD: 0.70, 95% C.I.: 0.45, 0.95)[74–77] from chronic migraine patients, and in blood from interictal (SMD: 0.47, 95% C.I.: 0.20, 0.75)[74,76–83] and ictal (SMD: 0.79, 95% C.I.: 0.49, 1.09)[78,82–84] episodic migraineurs. Sensitivity analysis had small effects on pooled estimates. There are no studies on CGRP concentrations in CSF from episodic migraine patients.

Nerve growth factor (Figure 5)

Concentrations of NGF were increased in CSF (SMD: 6.47, 95% C.I.: 5.55, 7.39)[21,20,42] and blood (SMD: 1.08, 95% C.I.: 0.58, 1.59)[75] from chronic migraine patients. Blood concentrations were not significantly different in interictal patients (SMD: 0.06, 95% C.I.: -0.31, 0.42).[85] There are no studies published on ictal concentrations (CSF and blood) and interictal concentrations in CSF.

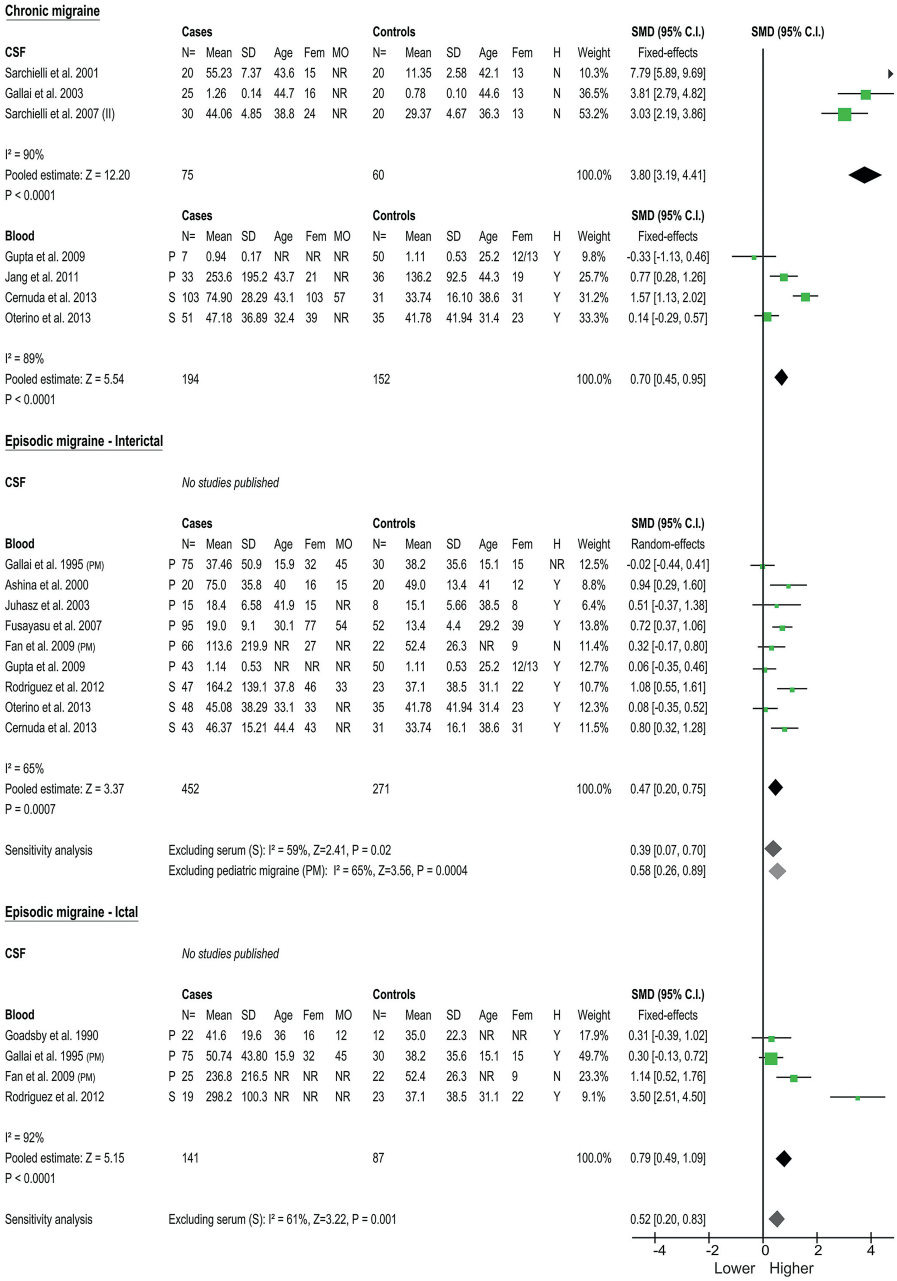


Figure 4: Forest plot of CGRP concentrations in migraine patients and controls. The squares represent effect sizes of the individual studies (size reflects the weight of the study) and the horizontal lines indicate the 95% confidence intervals (C.I.). The filled diamonds represent the overall effect size (horizontal width indicates the 95% C.I.). Age = mean age, Fem = number of females, MO = number of cases with migraine without aura, H= healthy controls, Y= yes, N = no, P = plasma concentrations, S= serum concentrations, * = migraine state is not explicitly reported for this study, the interictal state was assumed, (PM) = pediatric migraine

patients and pediatric controls. Additional information on the handling of missing data (e.g. calculations, assumptions) can be found in the supplement.

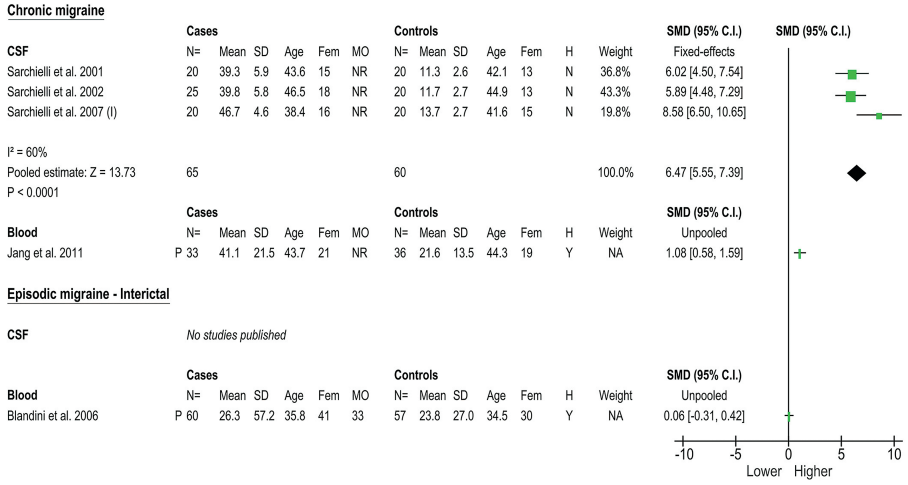


Figure 5: Forest plot of NGF concentrations in migraine patients and controls.The squares represent effect sizes of the individual studies (size reflects the weight of the study) and the horizontal lines indicate the 95% confidence intervals (C.I.). The filled diamonds represent the overall effect size (horizontal width indicates the 95% C.I.). Age = mean age, Fem = number of females, MO = number of cases with migraine without aura, H= healthy controls, Y= yes, N = no, P = plasma concentrations. Additional information on the handling of missing data (e.g. calculations, assumptions) can be found in the supplement.

Discussion

We conducted a systematic review and meta-analysis of biochemical measurements in CSF from chronic and episodic migraineurs. Meta-analysis showed increased concentrations of glutamate and CGRP and decreased concentrations of β-EP in CSF. These changes are also present in blood – a more accessible body fluid. Concentrations of NGF were increased in CSF from chronic migraine patients but blood data were limited.

Increases in glutamate and CGRP are in agreement with theories on pathophysiological mechanisms for migraine.[4,5] Glutamate is the principal excitatory neurotransmitter within the central nervous system and has been linked to neuronal hyperexcitability in migraine.[86] Glutamate has been implicated in the onset and generation of cortical spreading depression (CSD), which is believed to be the underlying cause of migraine aura. Increased synaptic glutamate concentrations lower the threshold for CSD.[5] CGRP has been implicated as a

mediator which activates and sensitizes peripheral meningeal nociceptors causing migraine headache.[4] Trigeminal fibers surrounding meningeal vessels release CGRP and other neuropeptides and there is increasing evidence that CSD can initiate this release in animal experiments.[5] Another rat model showed that CGRP released by primary trigeminal afferents impacts both CSF and blood concentrations and that the contribution of non-trigeminal structures to CSF concentrations is only minor.[87]

In CSF from chronic migraine patients NGF is increased with glutamate and CGRP. NGF is not only a well-known growth factor, but, following tissue injury, also an inducer of hyperalgesia via different peripheral mechanisms including mast cell degranulation.[88] After local injury or inflammation both peripheral (reactivated Schwann cells, non-neural cells) and central (neurons, astrocytes, microglia) sources upregulate NGF expression.[89] In contrast to CGRP, their relative contributions to CSF composition are still unknown. Additionally, NGF can upregulate CGRP expression in sensory and motor neurons.[90,91] In one included CSF study NGF and CGRP concentrations were indeed positively correlated.[42] Furthermore, by upregulating synthesis of brain-derived neurotrophic factor (BDNF) NGF can enhance synaptic transmission via N-methyl-D-aspartate receptors.[92,93] Primarily the latter is hypothesized to contribute to chronic sensitization of central neurons (i.e. in the nucleus trigeminus).[19,21,22,20] It is believed that this process also occurs in other chronic pain disorders such as fibromyalgia, where increased CSF concentrations of NGF, BDNF and glutamate have been found.[21] This indicates that the observed changes are possibly not specific for migraine and instead reflect exposure to chronic pain.

β-EP concentrations are decreased in CSF and blood from both interictal patients and chronic migraine patients. Low β-EP concentrations have been hypothesized to reflect low analgesic activity in individuals. However, recent evidence suggests chronic pain patients with low β-EP concentrations have stronger analgesic activity when in pain through rapid upregulation of β-EP.[94] In analgesic research with migraine patients β-EP could be a useful marker to study in more detail.

Study strengths and limitations

The main strengths of this study are the systematic approach to the identification, quality assessment and analysis of published data. However, our findings should be interpreted in the light of the limitations of the included evidence.

We found considerable clinical and methodological heterogeneity across studies. Statistical heterogeneity was also observed in meta-analysis of glutamate, β-EP and CGRP, but importantly all studies showed an effect in the same direction. Nonetheless, diversity in migraine patients was present due to differences in migraine frequency, timing of measurements and diagnostic criteria. Less diversity was present in CSF studies on CGRP and NGF, since the studies were performed at the same headache centre (new participants with approximately similar clinical characteristics (age, gender, disease history, headache frequency and medication overuse) were recruited for each study; confirmed with original investigators). Furthermore, migraine patients were not always representative for the diagnosed migraine type because samples were taken for diagnostic purposes (i.e. other neurological disorders were suspected). Diversity in controls seemed primarily related to the availability of samples as well. Control cohorts often consisted of non-healthy controls in which samples were collected for other diagnostic purposes than the migraine patients; this is especially the case for CSF studies where collection in healthy persons is often not possible.

Additionally we found that the quality of reporting was inconsistent. Studies particularly failed to specify the validation, sensitivity and monitoring of applied measurement technique. Additionally, group comparisons were not always clearly reported and applied statistical analysis was frequently not well explained. Therefore, despite our attempts to contact corresponding authors, we were not able to retrieve all required data and had to apply published methods to calculate or estimate these data.[14]

Publication bias is probably a major issue in the reporting of biomarker studies, because negative findings are less likely to get published.[95] However, we did not generate funnel plots to assess any publication bias because the power of this strategy is low with the relative small number of studies per compound.

Recommendations

Future research should further clarify the pathophysiological relevance of the altered glutamate, β-EP, CGRP and NGF concentrations in migraine. For better understanding of involved biochemical processes, and for potential application as diagnostic biomarkers, it is also important to know whether concentrations are altered in all migraine types and whether similar changes are present in other headache disorders and chronic pain disorders.

Acknowledgements

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Supplementary material

Search strings for PubMed, EMBASE and Web of Science

PubMed

Migraine AND Cerebrospinal fluid

("Migraine Disorders"[Mesh] OR "migraine"[all fields] OR "Sick Headache"[all fields] OR "Migraineurs"[all fields] OR "Migraineur"[all fields] OR "migrain*"[all fields]) AND ("Cerebrospinal Fluid"[Mesh] OR "Cerebrospinal Fluid"[all fields] OR "Cerebrospinal Fluids"[all fields] OR "CSF"[all fields])

Migraine AND Plasma/Serum

("Migraine Disorders"[Mesh] OR "migraine"[all fields] OR "Sick Headache"[all fields] OR "Migraineurs"[all fields] OR "Migraineur"[all fields] OR "migrain*"[all fields]) AND ("Plasma"[Mesh] OR "Plasma"[all fields] OR "Plasmas"[all fields] OR "Plasm"[all fields] OR "Serum"[Mesh] OR "Serum"[all fields] OR "Sera"[all fields] OR "Serums"[all fields] OR "Serologic"[all fields])

EMBASE

Migraine AND Cerebrospinal fluid

(exp "Migraine"/ OR "migraine".mp OR "Sick Headache".mp OR "Migraineurs".mp OR "Migraineur".mp OR "Migrain*".mp) AND (Cerebrospinal Fluid/ OR Cerebrospinal Fluid.mp OR Cerebrospinal Fluids.mp OR CSF.mp)

Migraine AND Plasma/Serum

(exp "Migraine"/ OR "migraine".mp OR "Sick Headache".mp OR "Migraineurs".mp OR "Migraineur".mp OR "Migrain*".mp) AND ((exp "Plasma"/ OR "Plasma".mp OR "Plasmas".mp OR "Plasm".mp) OR (exp "Serum"/ OR "Serum".mp OR "Sera".mp OR "Serums".mp OR "Serologic".mp))

Web of Science

Migraine AND Cerebrospinal fluid

TS=(Migraine OR Sick Headache OR Migraineurs OR Migraineur OR Migrain*) AND TS=(Cerebrospinal Fluid OR Cerebrospinal Fluids OR CSF)

Migraine AND Plasma/Serum

TS=(Migraine OR Sick Headache OR Migraineurs OR Migraineur OR Migrain*) AND TS=("Plasma" OR "Plasmas" OR "Plasm" OR "Serum" OR "Sera" OR "Serums" OR "Serologic")

Eligibility criteria for title and abstract selection

Inclusion criteria

1. Case-control and case-cross over studies that measure one or more compounds in CSF from more than one migraine patient.
2. To study references, review articles that report on biochemical measurements in migraine patients.

Exclusion criteria

1. Case-reports
2. Conference abstracts
3. Non-human study
4. HaNDLe (syndrome of transient headache and neurologic deficits with cerebrospinal fluid lymphocytosis)
5. Article not in English
6. Genetic studies
7. Pharmacological study where endogenous compound(s) is (are) measured as disease marker(s) without baseline measurements
8. Pharmacological study where exogenous compound(s) is (are) measured
9. Not relevant (not specified)

Risk of bias assessment

Selection

1. **Is the case definition adequate?**

Yes, if clear diagnostic criteria were applied. Although the ICHD criteria are the current golden standard, the criteria developed by the World Federation of Neurology and the Ad Hoc Committee on Classification of Headache Disorders

were also considered specific enough (both criteria focus on typical migraine symptoms). For chronic migraine the criteria developed by Silberstein et al. and the ICHD-II criteria were considered as adequate.

2. **Is the case selection adequate?**

Yes, if migraine patients were representative for their defined migraine type. The health status of patients has to be described and patients should not suffer from severe comorbidity; or clear exclusion criteria regarding comorbidity should be reported. Migraine patients which were sampled for clinical diagnostic purposes (i.e. to confirm or rule out other neurological disorders) were not considered representative.

3. **Is the control definition adequate?**

Yes, if controls had no personal history of migraine or other headache disorders (diagnostic criteria: see 1.). The health status of controls has to be explicitly mentioned in the article.

4. **Is the control selection adequate?**

Yes, if controls were recruited from the same population as the migraine patients (same risk factors and therefore would be cases if they had migraine). If controls were selected from a different population than the migraine patients, the selection of controls should not be related to the exposure (i.e. selection characteristics of controls should not influence concentrations of studied biochemical compounds; when a potential influence could not be excluded the study was considered to have moderate risk of selection bias).

Comparability

5. **Design controls for age and gender?**

Yes, if authors stated that cases and controls were matched for age and gender, or if statistical correction was applied.

6. **Design controls for any other factor?**

Yes, if study design controlled for any other factor (e.g. socio-economic status, special diet), or if statistical correction was applied.

Measurements

The original NOS checklist covers three aspects on Exposure.[1] We adjusted this part and formulated two questions to assess the quality of measurements based on recommendations from the analytical research field.[2–6]one limitation has been the lack of standards for presenting and exchanging such data. Here we present a proposal, the Minimum Information About a Microarray Experiment (MIAME

7. **Are the measurement methods adequately described?**

To evaluate whether the information about the analytical method was adequate. Based on the description other researchers should be able to reproduce the experiment(s).

Yes, if the analytical method was extensively described in the article or a reference, or in case of commercial kit – manufacturer and product name should have been specified.

8. **Was the performance of the analytical method validated?**

To determine whether the analytical performance of the method and the laboratory was tested and evaluated. The option to report moderate validation indicates measurements that underwent some, but not all steps necessary for full validation. If multiple compounds were measured and not all measurements were sufficiently validated for each compound, then we also considered validation to be moderate.

To qualify as validated the following characteristics of the method should have been reported:

i. Absolute quantitation:
   a. Precision and/or accuracy (RSD, SD, coefficient of variance) and
   b. Limits of detection
ii. Relative quantitation:
   a. Reproducibility/precision (RSD of measurement, etc.)

Exceptions:

a. If the measurements were done in a certified clinical chemistry lab measurements were considered validated.
b. If the measurements were performed using commercially available kits, but no validation data were present, validation was considered to be moderate.

Table s-1: Risk of bias assessment

	Selection	
1	Is the case definition adequate?	Yes/No
2	Is the case selection adequate?	Yes/No
3	Is the control definition adequate?	Yes/No
4	Is the control selection adequate?	Yes/No/Moderate
	Comparability	
5	Design controls for age and gender?	Yes/No
6	Design controls for any other factor?	Yes/No
	Measurements	
7	Are the sampling and measurement methods adequately described?	Yes/No
8	Was the performance of the analytical method validated?	Yes/No/Moderate

Applied formulas and assumptions for missing data

We used formulas and approaches described by the Cochrane Collaboration [7].
If needed we pooled data for subgroups:

(1) $N_1 + N_2$ Sample size (N)

(2) $\frac{N_1 M_1 + N_2 M_2}{N_1 + N_2}$ Mean (M)

(3) $\sqrt{\frac{(N_1-1)SD_1{}^2 + (N_2-1)SD_2{}^2 + \frac{N_1 N_2}{N_1+N_2}(M_1{}^2 + M_2{}^2 - 2M_1 M_2)}{N_1+N_2-1}}$ Standard deviation (SD)

If standard errors (SE) were reported instead of standard deviation (SD):

(4) $SD = SE * \sqrt{N}$

Despite application of aforementioned formulas and contacting of original investigators, some assumptions were needed for meta-analysis. Our specific approaches for missing data and other irregularities in data are described below.

Additional information on the handling of missing data

Glutamate meta-analysis (Figure 1)

(a) Gallai et al: statistical description in text does not match with figure. We used data from the figure.
(b) Peres et al.: SD and mean concentration are only reported for migraine patients and not for control group. We assumed control group SD to be the same as migraine group SD and calculated mean levels of the control group via the inverse of the statistical test used by the authors (student's t-test).
(c) Sarchielli et al. (2002): mean concentration reported for whole migraine group is higher than for both migraine subgroups.
(d) Sarchielli et al. (2007): statistical description in text does not match with figure. We used data from the figure.
(e) Vaccaro et al.: data extracted from bar plot.
(f) Ferrari, MD et al.: within-subjects SD reported. Separate SDs for cases and controls were not retrievable. We applied the reported SD for both cases and controls.
(g) Studies by Sarchielli et al. New participants were recruited for each study (correspondence with original investigators).
(h) Peres et al. and Vieira et al. New participants were recruited for each study (correspondence with original investigators).

β-EP meta-analysis (Figure 2)

(a) Vecsei et al.: data were extracted from a small bar plot, therefore it was difficult to estimate the SD's (even with digital enlargement).
(b) Facchinetti et al (1986 and 1989): women with menstrual migraine. Concentrations from the early-luteal phase were included in this analysis and not the late-luteal phase concentrations, because the latter might reflect ictal concentrations.
(c) Facchinetti et al. (1986): data were extracted from a small figure, therefore it was difficult to estimate the SD's (even with digital enlargement).
(d) Possible overlap between study cohorts of Genazzani et al. and Nappi et al. 1985 (I).

CGRP meta-analysis (Figure 3)

(a) Gallai et al. (2003): statistical description in text does not match with figure. We used data from the figure.
(b) Sarchielli et al. (2007): statistical description in text does not match with figure. We used data from the figure.
(c) Gupta et al.: mean and SD only published for migraine patients; concentrations in controls received from corresponding authors.
(d) Jang et al.: we assumed SD was reported and not the SE.
(e) Oterino et al.: CGRP data only published in figure on log scale; non-transformed data were received from corresponding authors.
(f) Studies by Sarchielli et al. New participants were recruited for each study (correspondence with original investigators).

NGF meta-analysis (Figure 4)

(a) Sarchielli et al. (2002): mean concentration reported for whole migraine group is higher than for both migraine subgroups.
(b) Sarchielli et al. (2007): statistical description in text does not match with figure. We used data from the figure.
(c) Studies by Sarchielli et al. New participants were recruited for each study (correspondence with original investigators).

Supplementary References

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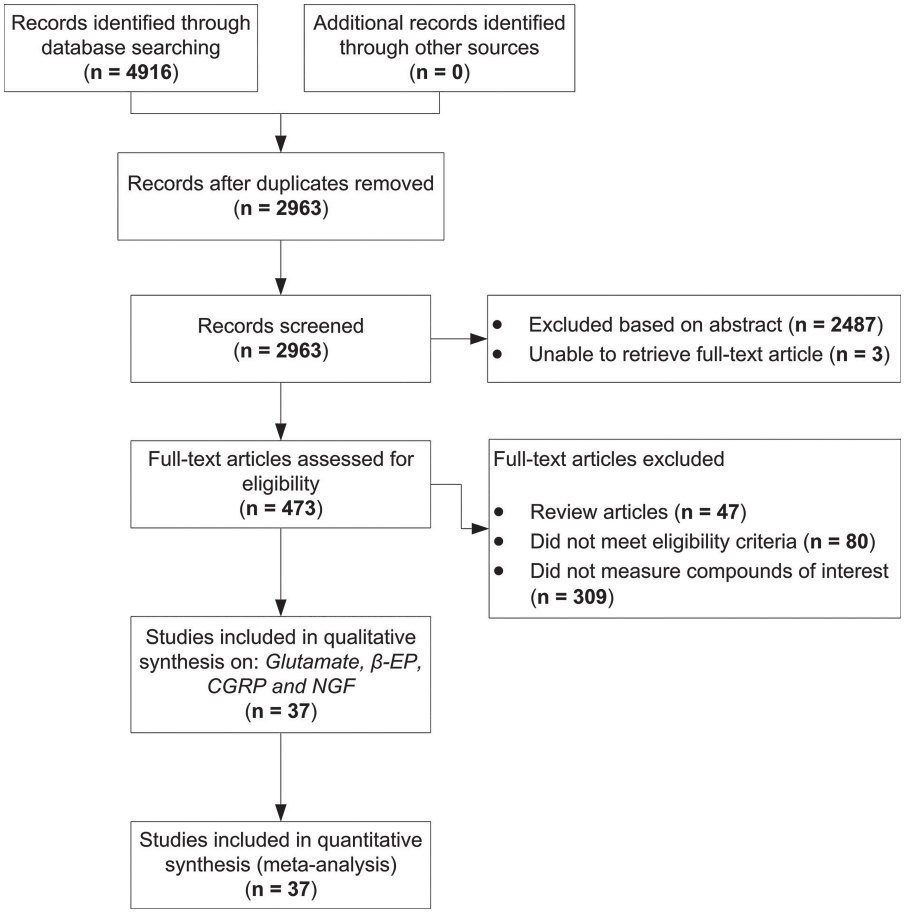


Figure s-1: Flowchart of blood study selection process. β -EP = β-endorphin, CGRP = calcitonin gene-related peptide, NGF = nerve growth factor.

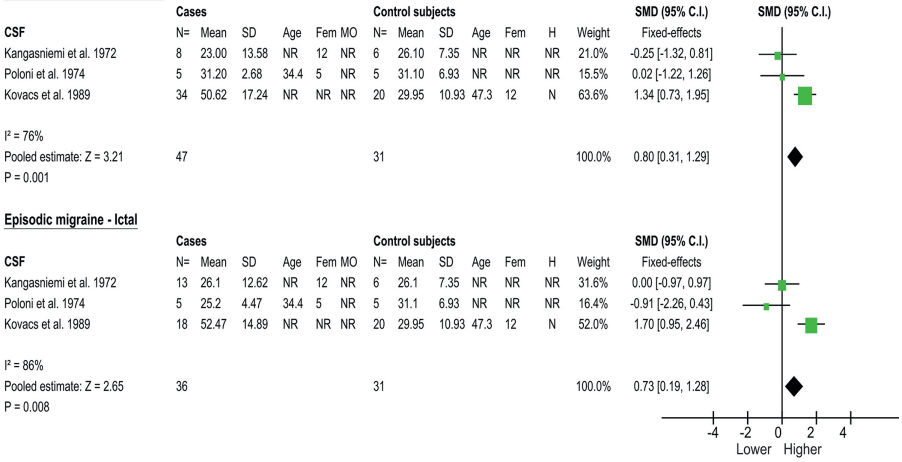


Figure S-2: Forest plot of 5-HIAA concentrations in migraine patients and controls. The squares represent effect sizes of the individual studies (size reflects the weight of the study) and the horizontal lines indicate the 95% confidence intervals (C.I.). The filled diamonds represent the overall effect size (horizontal width indicates the 95% C.I.). Age = mean age, Fem = number of females, MO = number of cases with migraine without aura, H= healthy controls, Y= yes, N = no. Citations are reported in the reference list of the main paper.

CHAPTER 5

Metabolic changes in the CSF of migraine patients – measured with ^{1}H-NMR spectroscopy

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Abstract

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.[1] In one-third of patients attacks are preceded by transient neurological symptoms, called aura.[2] Patients with and without aura form the two main migraine subtypes. When left untreated attacks can last from several hours to several days[3] and are very disabling for patients.[4] About 15% of the general population suffers from migraine; women three times more often than men.[5] 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[6] and has been proposed as a good model to study the common forms of migraine.[7] 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.[8,9]

Repeated migraine attacks are highly disabling for the patient and pose a great burden to society,[10] 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.[7,11] Although various biochemical mechanisms have been proposed,[11-13] biochemical knowledge has not let to a panel of biomarkers that could aid migraine diagnosis or predict treatment outcome.[14,15] For practical reasons, urine and blood (plasma or/and serum) are the most frequently used body fluids in biomarker discovery projects.[16] 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.[17-19] 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.[20] Thus, analysis of the biochemical composition of CSF offers a more direct insight into biochemical mechanisms in migraine. Here, we apply an exploratory ^{1}H-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)[21] were selected. Healthy volunteers were free of (a)specific headaches. All CSF samples of migraine patients were taken in between migraine attacks (i.e., interictally). Migraine patients had to be 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

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) x 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 aA 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. 366656, 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 D_2O containing 4 mM of sodium 3-trimethylsilyltetradeuteriopropionate (TSP) and 2.0 mM NaN_3 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.

^{1}H-NMR data acquisition

^{1}H-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) ^{1}H-NMR spectra were recorded at 300.0 K using the first increment of a nuclear overhauser effect spectroscopy (NOESY) pulse sequence [22] with presaturation (γB_1 = 50 Hz) during a relaxation delay of 4 s and a mixing time of 10 ms for efficient water suppression.[23] Duration of 90 degree pulses was automatically calibrated for each individual sample using a homonuclear gated nutation experiment[24] on the locked and shimmed samples after automatic tuning and matching of the probe head. Sixteen scans of 200,704 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).[25]

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.[26] 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.

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.[18] except 3-hydroxyisobutyrate. Public spectra of 3-hydroxyisobutyrate were used for its identification.[27] 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 (Supplementary Table s-1). 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.[28] Pyruvate and acetate were excluded due to an apparent batch effect, likely due to pyruvate instability (Supplementary Figure s-1). 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 (Supplementary Figure s-2), 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 "rrcov") 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") (Supplementary Figure s-3). 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 α=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 permutated at random, and used to construct and validate a model. By repeating this process 2,000 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 & 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.

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 (Supplementary Figure s-4). 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 supplemental information, Supplementary figure s-5.

Subsequently, this dataset was investigated for the presence of outliers using PCA (Supplementary Figure s-6). 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.

Table 1: Baseline characteristics of subjects (N=126) used in statistical data analysis.

	Healthy Controls (CO)	Hemiplegic migraine (HM)	Migraine with aura (MA)	Migraine without aura (MO)	*P*-value
Number of subjects	**43**	**18**	**38**	**27**	
Subject characteristics					
Females (%)	22 (51.2%)	10 (55.6%)	27 (71.1%)	19 (55.6%)	0.310
Years of age (±SD)	34.2 (±14.7)	39.5 (±14.9)	41.3 (±13.2)	46.6 (±13.2)	**0.004**
BMI (±SD)	23.9 (±3.0)	25.5 (±4.0)	24.6 (±2.8)	24.5 (±2.7)	0.349
Overnight fasting					
Fasting time in hours (±SD)	11.9 (±2.6)	11.5 (±0.9)	11.9 (±1.6)	12.2 (±1.4)	0.852
Migraine characteristics					
Migraine frequency (attacks/month)	-	2.3 (±5.0)	2.3 (±1.8)	3.2 (±2.5)	0.437
Headache days (days/ month)	-	5.3 (±8.5)	4.7 (±3.5)	4.9 (±3.4)	0.941
Migraine <3 days after LP					
No (%)	-	10 (55.6%)	35 (92.1%)	22 (81.5%)	
Yes (%)	-	1 (5.6%)	2 (5.3%)	4 (14.8%)	
Unknown (%)	-	7 (38.9%)	1 (2.6%)	1 (3.7%)	
Medication use					
Prophylactic medication (%)	-	4 (22.2%)	11 (28.9%)	7 (25.9%)	0.865
ß-blocker (%)	-	-	8 (21.1%)	4 (14.8 %)	
Antiepileptic drug (%)	-	3 (16.7%)	3 (7.9%)	2 (7.4%)	
Serotonine-antagonist (%)	-	1 (5.6%)	-	-	
Angiotensine II receptor antagonist (%)	-	-	-	1 (3.7%)	
Attack medication					
Triptan (%)	-	1 (5.6%)	17 (44.7%)	18 (66.7%)	**<0.001**
Sampling characteristics					
Opening pressure in mmH_2O (±SD)	17.1 (±3.6)	16.8 (±5.2)	18.0 (±3.8)	18.6 (±5.5)	0.492
Sampling time in seconds (±SD)	105.5 (±63.0)	88.9 (±46.3)	104.4 (±97.6)	95.0 (±64.7)	0.836
CSF characteristics					
Protein concentration in g/L (±SD)	0.33 (±.11)	0.31 (±.09)	0.41 (±.38)	0.40 (±.15)	0.290
Glucose in mmol/L (±SD)	3.0 (±.25)	3.2 (±.26)	3.1 (±.32)	3.1 (±.35)	0.077

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.

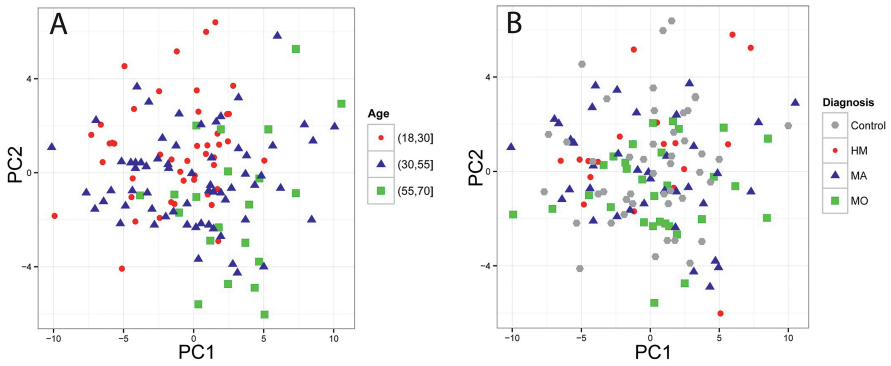


Figure 1: PCA analysis of [1]H-NMR migraine data. PCA analysis, showing that that there is no clear separation of groups. A: PCA plot showing an effect of age in the first component. B: PCA plot showing that there is no clear separation between groups in the first two components. HM: hemiplegic migraine, MO: migraine without aura, MA: migraine with aura.

Multivariate statistical analysis

PCA analysis was performed to detect the effects of main covariates, such as age, gender, and disease status in the data (Figure 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 (Figure 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 (Figure 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 (Supplementary Figure s-7). 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.

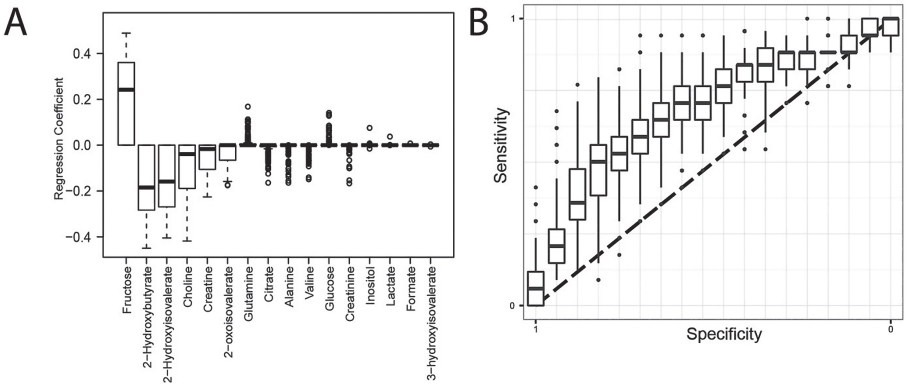
A targeted approach was used to determine if there were any differences between the migraine groups, and the matched control group, based on general trends in the set of 16 identified and quantified metabolites. Only identified metabolites were included in order to maximize the interpretability of the 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 colinearity 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 (Supplementary Figure s-8). 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 seperation 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 (Figure 2B). In addition, an ANOVA approach was used to model the cross-validated predictions. In comparison to variation around the mean, the average cross-validated predictions were marginally significant, at $p=0.08$ (Supplementary table s-2). 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 predication. 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 (Figure 2A).

The model outperformed over 95% of all randomly generated models, suggesting a low degree of additional structures in the dataset (Figure 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 (Figure 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-hydroxy-isovalerate. 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 (Figure 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-Hydroxy-isovalerate 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.



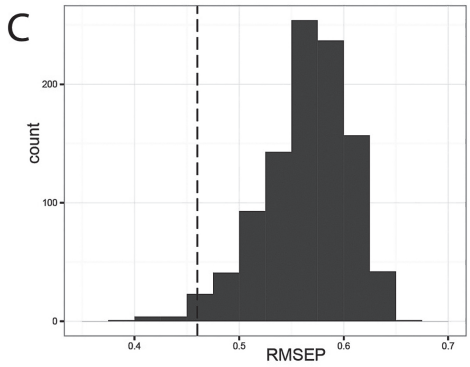


Figure 2: Multivariate statistical analysis of ^{1}H-NMR migraine data. **A:** distribution of metabolite regression coefficients of 5000 penalized logistic regression models, trained on age- and gender-matched hemiplegic migraine and control datasets. On average, 2-hydroxybutyrate, 2-hydroxyisovalerate and fructose are most important for prediction disease status. **B:** The ROC analysis of the cross-validated predictions showed models to have a moderate predictive ability, with an AUC average of 0.65 (95% C.I.: [0.54;0.75]). **C:** Histogram of RMSEP values acquired during randomization routine of the hemiplegic migraine dataset. Dotted line represents the RMSEP value of the original dataset. Original model outperforms 95% of the randomly generated models. AUC: area under the curve. RMSEP: root mean square error of prediction.

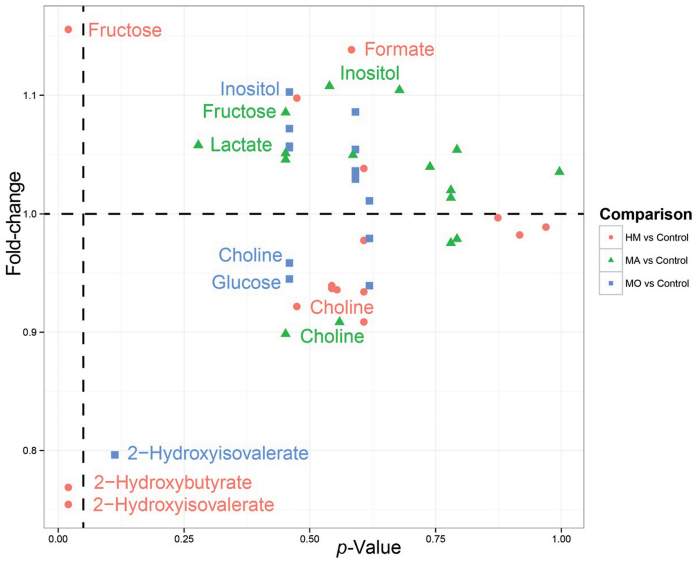


Figure 3: Univariate analysis of ^{1}H-NMR migraine data. Univariate comparison (Mann-Whitney U test) of 19 annotated metabolites in the three migraine groups (HM: hemiplegic migraine, MA: migraine with aura, MO: migraine without aura) *versus* the group of healthy controls. *P*-values from independent *t*-tests after adjustment with Benjamini-Hochberg FDR on the x-axis and fold-change on the y-axis. Dashed lines on the y-axis and x-axis represent equal metabolite levels between compared groups, and *p*-values (< 0.05), respectively.

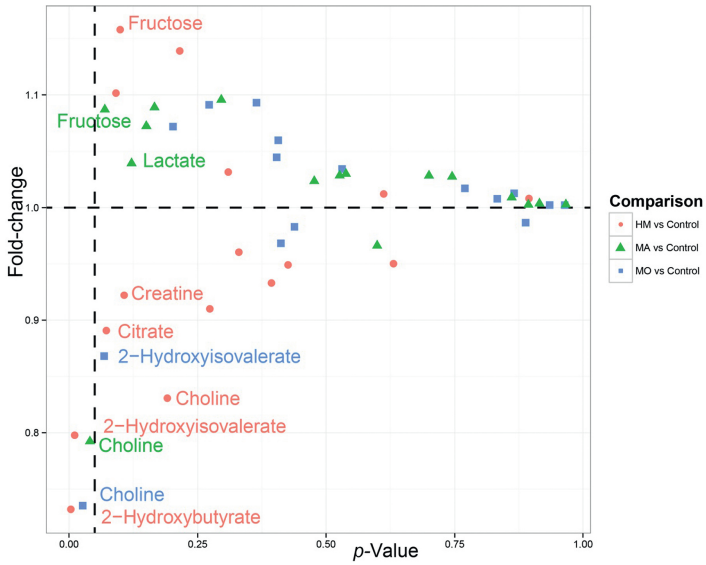


Figure 4: Age- and gender-adjusted analysis of ^{1}H-NMR migraine data. Univariate comparison of age and gender adjusted levels of 16 annotated metabolites in the three migraine groups (HM: hemiplegic migraine, MA: migraine with aura, MO: migraine without aura) *versus* the group of healthy controls by linear model. *P*-values were obtained by general contrasts. Dashed lines on the y-axis and x-axis represent equal metabolite levels between compared groups and significant *p*-values (< 0.05), respectively.

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 [18,27,30] with the potential to be used for routine diagnostics.[31] ^{1}H-NMR analysis of CSF has been used in neurological disorders,[32,33] such as complex regional pain syndrome,[34] multiple sclerosis,[35] Alzheimer's disease,[36] motor neuron disease,[37] and schizophrenia.[19] These studies seemed to imply that untargeted approaches, which make use of the spectral bins or peak integrals (i.e., features), are not optimal.[31,37] 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[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.[16] As part of routine CSF measurements glucose levels have been frequently reported to be normal in migraine patients.[38] 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.[40] These compounds could not be analyzed using the platform of the present study. Rothrock *et al.* have reported higher L-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.[41] However, the healthy controls in that study were significantly younger than the migraine patients and no correction for age effect was applied.[41] Our data show no significant differences in L-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),[40] 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.[18] Moreover, it was shown "borderline elevated" in CSF of patients with lactic acidosis and consistently elevated in CSF of patients with propionic acidemia,[42] 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.[43] 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.[44] 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* [45] who used ^{31}P magnetic resonance spectroscopy (^{31}P-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 ^{31}P-MRS study of patients with migraine with aura and migraine without aura reported similar findings of increased metabolic activity and a reduced energy reserve.[46] 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.[48] 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 ^{1}H-NMR metabolomics 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 migraine without aura from controls.

Acknowledgements

This study was supported by the Netherlands Organization for Scientific Research (VICI grant 918.56.601 and Spinoza 2009 to MDF), the Netherlands Organization for Health Research and Development (Clinical Fellowship 90700217 and Vidi 917-11-31 to GMT), and European Community (EC) FP7-EUROHEADPAIN - no. 602633 (to MDF and AMvdM).

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Supplementary material

Table s-1: Overview of peak annotations expressed in parts per million

	Name	HMDB	Peak							
			1	2	3	4	5	6	7	8
	TSP (internal standard)		0							
1	2-hydroxyisovalerate	HMDB00407	0.83	0.97						
2	2-hydroxybutyrate	HMDB00008	0.90							
3	Valine	HMDB00883	0.99	1.04						
4	2-oxoisovalerate	HMDB00019	1.13							
5	Ethanol	HMDB00108	1.19	3.66						
6	3-hydroxyisovalerate	HMDB00754	1.27							
7	Lactate	HMDB00190	1.33	4.12						
8	Alanine	HMDB00161	1.48							
9	Acetate	HMDB00042	1.92							
10	Acetone	HMDB01659	2.24							
11	Pyruvate	HMDB00243	2.38							
12	Glutamine	HMDB00641	2.43	2.45	2.47					
13	Citrate	HMDB00094	2.54	2.69						
14	Creatinine	HMDB00562	4.06							
15	Choline	HMDB00097	3.20							
16	Glucose	HMDB00122	3.24	3.40	3.49	3.54	3.72	3.73	3.775	5.24
17	Inositol	HMDB00211	3.63							
18	Creatine	HMDB00064	3.94							
19	Fructose	HMDB00660	4.03							
20	Formate	HMDB00142	8.46							

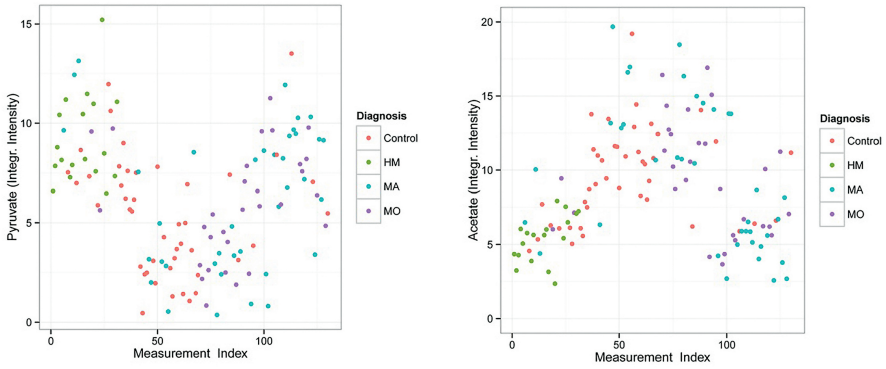


Figure s-1: Apparent batch effect of pyruvate an acetate. Pyruvate (A) and acetate (B) show an apparent batch effect in relation to their measurement index, i.e. the order in which the sample was collected, stored, prepared and measured. Reversed correlation between acetate and pyruvate levels can be explained by pyruvate conversion into acetate due to unknown cause.

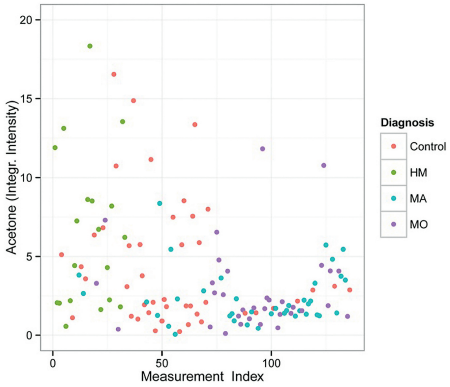


Figure s-2: Spread in acetone signal intensity correlates with the order in which the samples were collected, prepared and measured, i.e. the measurement index. As acetone concentration varies greatly in the first 80 samples and last 15 samples, but not within the samples in-between, it's likely an external source was present during their collection or immediate handling or that the acetone is a result of ketosis. Therefore, acetone was not taken into account during the analysis.

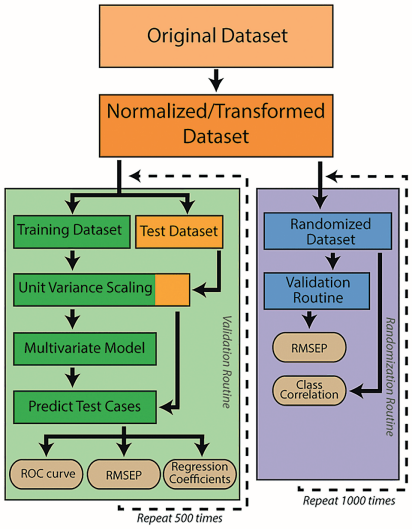


Figure s-3: Multivariate model building. Graphical representation of model building exercise by penalized logistic regression using elastic net. The complete dataset is normalized and transformed, prior to the manual selection of age- and gender-matched groups. Models are built and validated by a 10-fold cross-validation routine, which is repeated 500 times. During this routine, the receiver operator characteristic/root mean square error of prediction (ROC/RMSEP) statistics, and regression coefficients are extracted. To estimate the amount of overfitting, a randomization routine was used. By randomizing the dataset 1,000 times, and comparing the RMSEP statistic on each run to the RMSEP of the original dataset, we estimated the percentage of models which performed better at random. In this case, a low number means a low amount of over fitting.

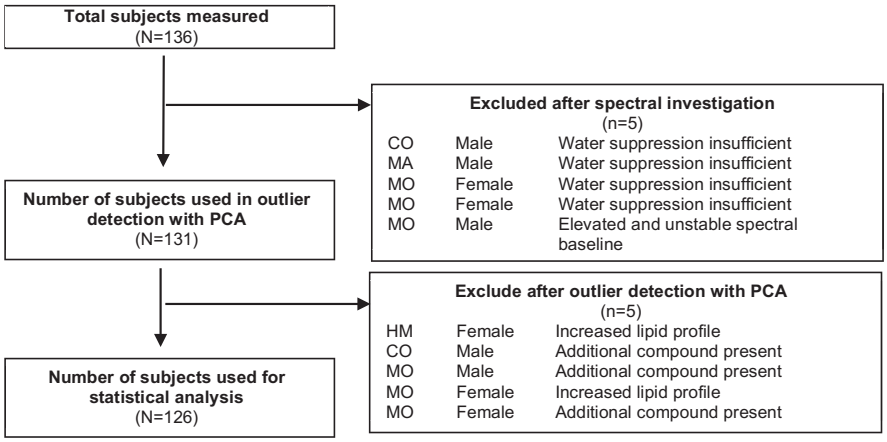


Figure s-4: Flow chart of case selection based on spectral quality and outlier detection. HM: hemiplegic migraine, MO: migraine without aura, MA: migraine with aura, CO: healthy controls.

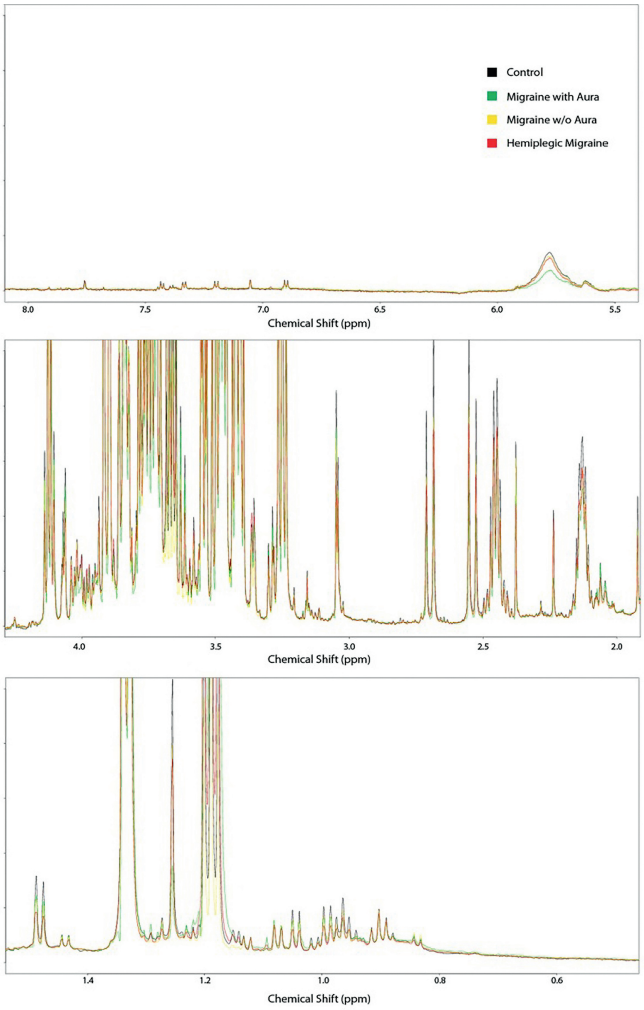


Figure s-5: Overlay of representative 1D-NOESY spectra, one for each diagnostic group. Spread in signal intensity is representative for the spread of metabolite concentration observed in the entire dataset. Separate parts of the spectrum are scaled differently, to fit peak height and spectral crowding.

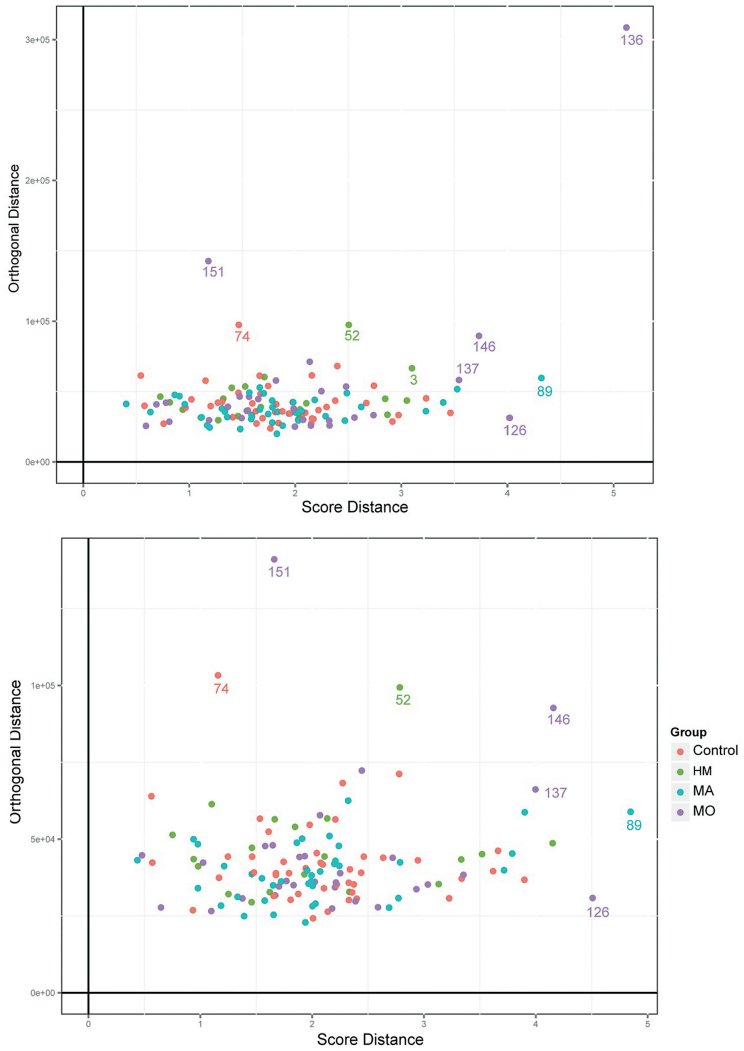


Figure s-6: PCA analysis on complete NMR spectral profiles to identify outliers. PCA analysis on complete NMR spectral profiles where the orthogonal distance to model and distance to score center are evaluated to identify outliers. Cases with a large distance indicates a spectral profile which differs greatly from the other cases. A: PCA plot including case 136 which shows both a large orthogonal and score distance. B: PCA plot without case 136 showing several other cases with a large orthogonal distance.

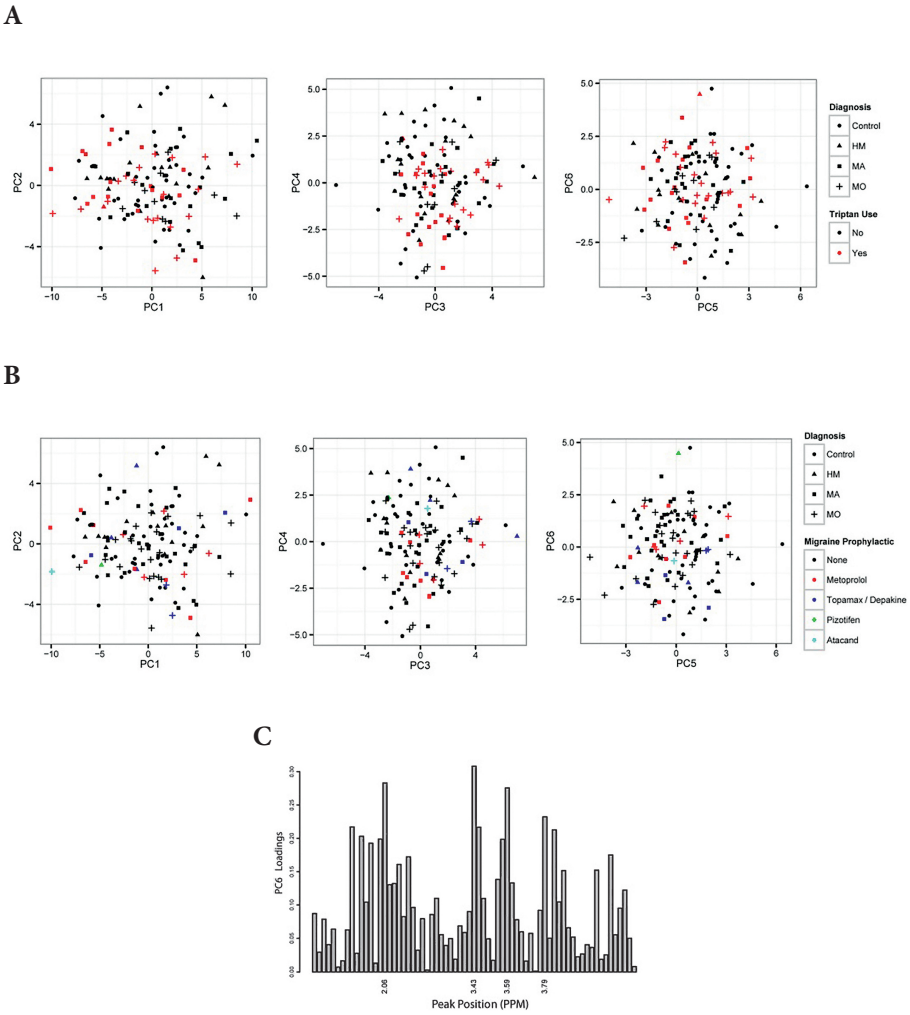


Figure s-7: PCA analysis shows no major effects of triptan (A) and migraine prophylactic (B) use. No clear separation can be seen for medicated subjects. Some clustering is observed in the 6th PC for subjects using Topamax or Depakine (B). However, the important loadings constituting the 6th PC do not involve any identified metabolites.

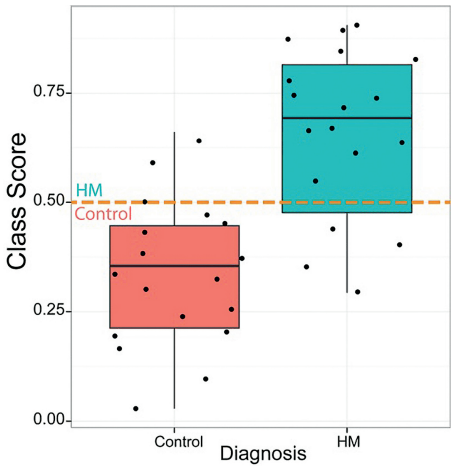


Figure s-8: Separation of controls and hemiplegic migraine patients in model training-data. Separation of the training-data according to migraine status by the penalized logistic regression (elastic net) model.

Table s-2: Result from CV-Anova of averaged cross-validation results.

	Df	SS	MS	F	p
Regression	1	0.815	0.81	3.39	0.08
Residual	32	7.68	0.24		



Part III

Practical considerations for cerebrospinal fluid sampling

CHAPTER 6

Labeling strategies to assess and optimize stability of endogenous amines during cerebrospinal fluid sampling

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Submitted

Abstract

Background: Metabolic profiling of cerebrospinal fluid (CSF) is a promising approach to study brain diseases. Metabolomics measurements should reflect the *in vivo* situation and therefore *ex vivo* metabolism should be avoided. This study investigated the effects of different CSF sampling protocols on the metabolic profile of endogenous amines.

Methods: CSF samples from 21 individuals were obtained via lumbar puncture. Five sampling protocols were used to assess effects of temperature, centrifugation and ethanol as additive to stop enzymatic reactions. Isotopically-labelled alanine, isoleucine, glutamine, glutamic acid and dopamine were added to sampling tubes prior to sampling to trace any degradation. Metabolomics analysis of endogenous amines, isotopically-labelled compounds and degradation products were performed with a validated LC-MS method.

Results: Thirty-six endogenous amines were quantified in CSF. After correction for volume effects caused by ethanol addition, there were no statistically significant differences between protocols in 31 out of 36 endogenous amines. There was an effect of temperature on GABA and an effect of ethanol on o-phosphoethanolamine and GABA concentrations. The degradation of isotopically-labelled compounds (e.g. glutamine to glutamate) was extremely small with no differences between protocols.

Conclusions: Our novel approach using isotopically-labelled metabolites allowed detection of degradation of endogenous metabolites during sampling with high sensitivity and selectivity. Although results were better when adding ethanol, the differences were small for amines in CSF. Amines can be considered stable during sampling, provided that samples are cooled immediately to 4 °C, centrifuged, and stored at -80 °C within two hours. The effect of ethanol addition for more unstable metabolites needs further investigation.

Introduction

Novel high-throughput biochemical analysis technologies used in proteomics and metabolomics allow for large-scale profiling of proteins and metabolites in body fluids.[1-3] With their advent there is growing interest in using cerebrospinal fluid (CSF) as a relatively easy accessible source for the discovery of biomarkers reflecting biochemical changes in the dysfunctional brain.[4] Wide-scale "CSF-omics" studies may reveal novel biochemical pathways for brain diseases[5,6], and may provide diagnostic[7,8] and prognostic biomarkers.[9,10]

In addition to the technological challenge to continuously increase coverage, sensitivity, specificity and through-put of the analytical methods, there are also several potential pre-analytical pitfalls which may confound the biological interpretation of results.[11,12] These include CSF contamination with blood and *ex vivo* changes of unstable compounds due to enzymatic or chemical reactions which occur during CSF sampling, processing and storage.[12-14] Many biochemical studies use centre-specific clinical protocols for sampling, processing and storage of CSF, thereby ignoring the risk of *ex vivo* biochemical changes that may occur after withdrawal of the CSF, and potentially reducing the comparability with samples from other studies.[15]

Previously published consensus guidelines for CSF sampling have already established the main pre-analytical factors that should be standardized.[14-16] However, the scientific basis for this guideline was mainly based on stability studies that focused on protein biomarkers for Alzheimer's (Aβ42, total tau and phosphorylated tau) and Parkinson's disease (α-synuclein). These guidelines do not recommend using special precautions for samples used for metabolomic studies. Major factors that can cause *ex vivo* biochemical changes of the CSF metabolome include the temperature during sampling and processing, additional procedures such as centrifugation to remove cells, and the use of additives to stabilize the metabolic profile [14].

The aim of this study was therefore to investigate the effect of these major factors (low sampling and processing temperature, centrifugation to remove cells, and addition of ethanol to stop enzymatic reactions) on the stability of the metabolic profile of primary and secondary endogenous amines. To this end we compared five different sample handling protocols for CSF. In addition, we aimed to quantify the degradation of allegedly unstable endogenous metabolites such as L-glutamine and L-glutamic acid by adding isotopically-labelled versions of these metabolites to sampling tubes before CSF sampling.

Methods

Sampling procedures

We obtained human CSF samples from 21 individuals (16 migraine patients and 5 healthy controles) as part of a program on migraine pathophysiology. The study was approved by the Medical Ethical Committee of Leiden University Medical Centre (LUMC). All subjects gave written informed consent prior to collection.

CSF sampling was performed in the morning (before 12.00 a.m.) via lumbar puncture. All subjects were subjected to overnight fasting and were only allowed water in the 8 hours preceding lumbar puncture. For disinfection of the skin Chlorhexidine (5g/L)/denatured ethanol 70% (art. no. 909602; Pharmacy LUMC, Leiden, the Netherlands) was used. CSF was sampled between the L3/L4, L4/L5 or L5-S1 interspace with a Pajunk 20G or 22G a-traumatic Sprotte® needle (Pajunk GmbH, Geisingen, Germany). For routine CSF diagnostics 3 mL CSF was sampled in a 12-mL polystyrene tube (art. no. 160172; Greiner, Kremsmünster, Austria); for migraine research purposes approximately 12 mL of CSF was collected. After this, we collected five times 1 mL CSF in five separately prepared 15-mL polypropylene falcon tubes (art.no. 188271; Greiner) which were used for the current study.

Five different sample handling protocols

For each of the five tubes there was a unique sample handling protocol to study the effects of temperature, centrifugation and the addition of ethanol. The five protocols are illustrated in Figure 1.

For protocol 1, CSF collection and centrifugation were performed at room temperature; for the four additional protocols sampling tubes were kept on ice, before and after sampling, and during sample handling. For protocol 2, we prepared the sampling tube by adding 2 mL cold ethanol (ethanol absolute prod. no. 8098; J.T.Baker, Avantor Performance Materials, Center Valley, U.S.A). Subsequently, CSF was sampled directly into this tube and inverted several times to mix thoroughly with the ethanol. For protocol 3, after sampling, 0.25 mL of CSF was added to cryotubes containing 0.5 mL of ethanol. This mixture was also inverted several times. For protocol 4 sampled, we performed no additional sample handling steps. For protocol 5, we centrifuged the sample.

All centrifugation steps (protocols 1, 3, and 5) were performed for 5 minutes (2000 rpm, 747 g) directly after sampling. Centrifugation in protocol 1 was performed at 21 ˚C and for protocol 3 and 5 at 4 ˚C. After centrifugation, the

supernatant was transferred to another 15-mL polypropylene falcon tube, and divided in aliquots. Samples from protocols 1, 4 and 5 were divided in 0.25-mL aliquots (into 0.5-mL cryotubes (Eppendorf, Hamburg, Germany)), samples from protocols 2 and 3 in 0.75-mL aliquots (into 1.5-mL cryotubes (Eppendorf)). All aliquots were immediately placed on dry ice after processing, within 30-60 minutes from sampling, and transferred to -80 °C for storage within 120 minutes from sampling. All samples remained at -80 °C until sample preparation, no extra freeze-thaw cycles were allowed.

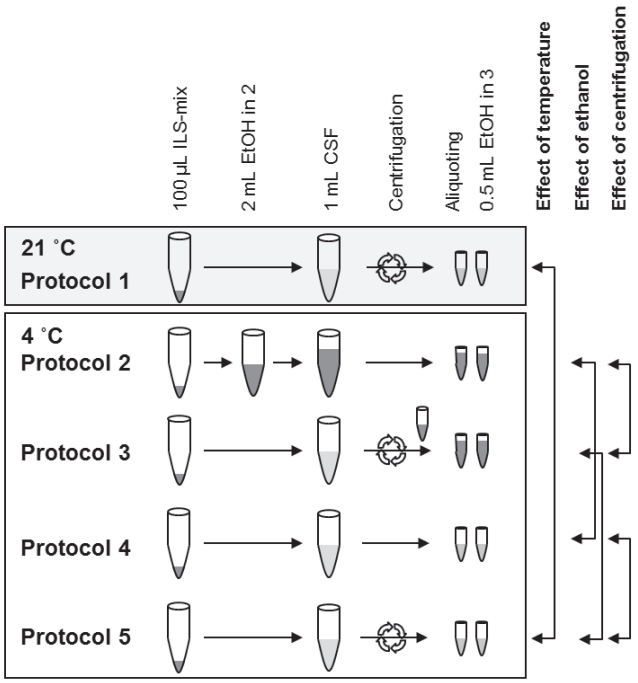


Figure 1. Scheme of different CSF handling protocols.

Compound labeling strategy

To assess whether potential differences between protocols were due to metabolic degradation, and not due to other factors, we added a mix of isotopically-labeled compounds (ILC-mix) containing two stable and three potentially unstable compounds to the tubes. Stable isotopically-labeled compounds were: 2,3-$^{13}C_2$-alanine (ILC-mix: 300 µmol/L; Cortecnet, Voisins-Le-Bretonneux, France) and $^{13}C^{15}N$-isoleucine (ILC-mix: 30 µmol/L; Cambridge isotope laboratories,

Tewksbury, U.S.A.).[17] Potentially unstable compounds were: 1,3-$^{13}C_2$-glutamine (ILC-mix: 5000 µmol/L; Cambridge isotope laboratories), U13C-U15N-UD-glutamic acid (ILC-mix: 125 µmol/L; Cambridge isotope laboratories), and 1,2,3,4,5,6-$^{13}C_6$-dopamine (ILC-mix: 1.25 µmol/L; CDN Isotopes, Pointe-Claire, Canada). Concentrations of isotopically-labeled compounds were chosen such that they approximate physiological concentrations after addition of CSF. The ILC-mix was prepared in one batch, then divided into aliquots, and stored at -80 ˚C until the day of sampling. Maximal 1 hour before sampling the ILC-mix was defrosted at room temperature. For each subject, we prepared five 15-mL polypropylene falcon tubes (art.no. 188271; Greiner) for sampling by adding 100 µL of the ILC-mix.

Measurements of endogenous amines

For targeted analysis of primary and secondary amines we employed a LC-MS method using AccQ-Tag derivatization as described before.[18] Cell-free U-^{13}C-U^{15}N-labeled amino acids mix (Cambridge isotope laboratories), D_4- β-alanine, D_6-ornithine, D_4-histamine, D_6-2-Aminobutyric acid, D_3-3-methyl-histidine, D_3-3-Methoxytyrosine and D_4-3-Methoxytyramine (CDN Isotopes) were used as internal standards. Acquired data were evaluated using MassHunter software (Agilent, Santa Clara, U.S.A.) by integration of assigned SRM peaks and normalization using proper internal standards (ISTDs). For analysis of amino acids, their U-^{13}C-U^{15}N -labeled analogues were used as ISTDs. For other amines – the closest-eluting ISTD was used. Relative concentrations of endogenous compounds were reported as response ratios to their respective ISTDs. Data quality was monitored and compensated for shifts in sensitivity of the mass spectrometer over time using additionally measured quality control (QC) samples as described before.[19] Measurement variation was evaluated by calculating the relative standard deviation (RSD) per amine from replicate samples and QC samples.

Measurements of isotopically-labelled stability markers

Isotopically-labeled stability markers and degradation products were measured with a modified version of the method used for endogenous compounds. Sample preparation and LC settings remained unchanged and the MRM transitions of MS were set for specific masses of isotopically-labeled compounds, including spiked compounds and potential degradation product of $^{13}C_2$-glutamine namely $^{13}C_2$-glutaminc acid. For normalization, the same ISTDs were used as for the endogenous

equivalents. Similarly, the referred QC approach was applied. Calibration samples were prepared with pure standards of isotopically labeled compounds, spiked at 7 consecutive 2-fold dilutions into aliquots of CSF pooled from all the study samples. Covered concentration ranges were: 3.75-240 µM for $^{13}C_2$-alanine, 0.875-56 µM for $^{13}C^{15}N$-isoleucine, 65.5-4000 µM $^{13}C_2$-glutamine, 1.56-100 µM for U-$^{13}C^{15}ND$-glutamic acid and 0.0156-1 µM for $^{13}C_6$-dopamine HCl. Samples of CSF pool without any standard were used as zero point for the calibration. Absolute concentrations in µmol/L were calculated using linear calibration lines as previously described and implemented in R package 'chemCal'.[20]

Correction for volume differences

In protocols 2 and 3 the sampling procedure involved mixing CSF and ILC-mix with ethanol. Potential inaccuracies in mixing of different volumes can lead to an increased variability of metabolites concentrations or a systematic bias in further analysis. Especially in protocol 2 the CSF volume is less controlled because CSF drips directly into a relatively large sampling tube already containing ethanol. In CSF samples from protocol 2 concentrations of almost every endogenous and isotopically-labeled metabolite were approximately 20% higher in comparison to samples from the other protocols (Figure 1a and c). The fact that both endogenous (from CSF) and isotopically-labeled metabolites (from ILC-mix) were increased indicated that there was less ethanol in samples from protocol 2 than anticipated. This systematic error could result from evaporation of ethanol before, during and after sampling, or might be caused by inconsistencies in pipetting of cooled volatile ethanol.

In order to test for differences in endogenous metabolite levels between all five protocols, without the influence of the systematic error described above, we standardized concentrations of all endogenous metabolites to the concentrations of endogenous L-alanine and L-isoleucine (Supplementary Figure S-1A and B). L-alanine and L-isoleucine are considered stable compounds[17] and differences in these metabolites were assumed not to be caused by degradation. Concentrations of all isotopically-labeled compounds were standardized to the concentrations of $^{13}C_2$-alanine and $^{13}C^{15}N$-isoleucine in a special reference sample (ILC-mix with 1 mL of water instead of CSF, sampled and processed on the same day as clinical samples; Supplementary Figure s-1C and D). See Supplementary Methods for full descriptions and examples of the applied corrections.

Statistical analysis

Relative concentrations of endogenous compounds and absolute concentrations of stability markers were log-transformed prior to statistical analysis.[21,22] To test for differences between the five protocols, we applied one-way repeated measures ANOVA. Reported p-values from repeated measures ANOVA were not corrected for multiple testing. P-values below 0.05 were considered significant. When significant, post-hoc pairwise t-test comparisons were applied with Bonferroni's correction. The same ANOVA strategy was used to compare degradation percentages of $^{13}C_2$-glutamine to $^{13}C_2$-glutamic acid (volume correction and log-transformation were not necessary for these data). Repeated measures ANOVA was performed with R software (package "ez" version 4.2-2).

Results

Sample set

Between April 2013 and January 2014, we obtained CSF from 12 females and 9 males (mean age of 38.2 ± 12.2). Routine CSF diagnostics were performed and were all within reference limits. Median erythrocyte count was 2.5 cells/3µL (range 0 – 200); mean leucocyte count 3.50 ± 2.50 cells/3µL; mean total protein 0.33 ± 0.11 g/L; and mean glucose levels 3.24 ± 0.19 mmol/L. With LC-MS we identified and quantified 36 amino acids and 7 isotopically-labeled compounds.

Endogenous metabolites

There were no significant concentration differences between protocols for 31 out of 36 (86.1%) amines. Five amines showed significant differences: o-phosphoethanolamine (PE), gamma-aminobutyric acid (GABA), L-glutamic acid, L-methionine sulfoxide and L-aspartic acid (Table 1). Protocol 1, in which sample handling was performed at room temperature, showed significantly higher PE, L-glutamic acid and L-methionine sulfoxide concentrations compared to protocol 3 and significantly higher GABA concentrations compared to protocols 2-5 (Figure 2A-D). PE concentrations were significantly lower in the protocols in which ethanol was used as additive (protocols 2 and 3) compared to protocols without ethanol (protocols 4 and 5; Figure 2A). GABA concentrations were also lower in samples from protocols 2 and 3 (with ethanol) compared to samples from protocols 1 and 5 (without ethanol; Figure 2B). L-aspartic acid showed no

significant differences in the post-hoc analysis (data not shown in Figure 2). To exclude potential confounding effects of the volume correction, we performed an additional analysis in which we excluded protocol 2 and did not perform volume correction. This analysis showed the same effects for GABA, PE and L-glutamic acid (Supplementary Table s-1).

Stability markers

There were no significant differences between protocols in concentrations of the stable ($^{13}C_2$-alanine, and $^{13}C^{15}N$-isoleucine) and the potentially unstable isotopically-labeled markers ($^{13}C_2$-glutamine, $U^{13}C$-$U^{15}N$-UD-glutamic acid, and $^{13}C_6$-dopamine) (Table 2). We observed very low concentrations for $^{13}C_2$-glutamic acid, the degradation product of $^{13}C_2$-glutamine. Degradation ranged from 0.158 to 0.166 % of the original $^{13}C_2$-glutamine levels with no difference between protocols. We did not detect degradation products of $^{13}C_6$-dopamine. An additional analysis in which we excluded protocol 2 also showed no significant differences between protocols (Supplementary Table s-2).

Table 1: Relative concentrations of endogenous amines per protocol after volume correction

Metabolites	Relative concentration					p-value
	Protocol 1	Protocol 2	Protocol 3	Protocol 4	Protocol 5	
o-phosphoethanolamine	0.078 ± 0.02	0.071 ± 0.015	0.064 ± 0.014	0.078 ± 0.018	0.079 ± 0.017	**<0.001**
gamma-aminobutyric acid	0.0071 ± 0.0026	0.0049 ± 0.002	0.0052 ± 0.0024	0.0055 ± 0.0022	0.0057 ± 0.0024	**<0.001**
L-glutamic acid	0.022 ± 0.009	0.02 ± 0.01	0.018 ± 0.007	0.020 ± 0.007	0.020 ± 0.009	**0.017**
L-methionine sulfoxide	0.016 ± 0.005	0.015 ± 0.006	0.014 ± 0.005	0.015 ± 0.004	0.014 ± 0.005	**0.033**
L-aspartic acid	0.055 ± 0.051	0.052 ± 0.068	0.051 ± 0.048	0.054 ± 0.043	0.050 ± 0.051	**0.047**
L-methionine	0.537 ± 0.123	0.544 ± 0.126	0.554 ± 0.132	0.534 ± 0.121	0.544 ± 0.121	0.124
SDMA	0.0039 ± 0.0009	0.0045 ± 0.0013	0.0044 ± 0.0016	0.0040 ± 0.0011	0.0041 ± 0.0012	0.168
L-isoleucine	0.013 ± 0.004	0.013 ± 0.003	0.013 ± 0.003	0.014 ± 0.004	0.013 ± 0.004	0.172
ADMA	0.0005 ± 0.0002	0.0006 ± 0.0003	0.0007 ± 0.0003	0.0006 ± 0.0003	0.0006 ± 0.0003	0.177
L-alanine	0.068 ± 0.016	0.069 ± 0.018	0.070 ± 0.018	0.068 ± 0.016	0.068 ± 0.017	0.208
L-threonine	1.359 ± 0.359	1.356 ± 0.368	1.383 ± 0.385	1.366 ± 0.363	1.396 ± 0.387	0.248
L-asparagine	0.322 ± 0.073	0.332 ± 0.078	0.327 ± 0.081	0.323 ± 0.074	0.326 ± 0.075	0.286
L-serine	2.574 ± 0.665	2.590 ± 0.776	2.625 ± 0.679	2.620 ± 0.636	2.660 ± 0.679	0.362
L-valine	0.037 ± 0.011	0.037 ± 0.011	0.037 ± 0.011	0.036 ± 0.011	0.037 ± 0.011	0.406
L-arginine	1.896 ± 0.458	1.931 ± 0.471	2.005 ± 0.56	1.898 ± 0.472	1.969 ± 0.492	0.432
L-lysine	3.221 ± 0.787	3.283 ± 0.806	3.305 ± 0.913	3.248 ± 0.746	3.306 ± 0.8	0.436
ethanolamine	3.595 ± 0.562	3.726 ± 0.787	3.784 ± 0.873	3.739 ± 0.69	3.818 ± 0.642	0.447
L-proline	0.025 ± 0.018	0.025 ± 0.018	0.025 ± 0.016	0.025 ± 0.016	0.024 ± 0.016	0.455
taurine	0.302 ± 0.085	0.320 ± 0.108	0.323 ± 0.132	0.307 ± 0.092	0.315 ± 0.096	0.465
L-kynurenine	0.0023 ± 0.0012	0.0025 ± 0.0016	0.0023 ± 0.0011	0.0024 ± 0.0011	0.0025 ± 0.0009	0.473
N6N6N6-trimethyl-L-lysine	0.0056 ± 0.0008	0.0056 ± 0.0013	0.0059 ± 0.0014	0.0058 ± 0.0011	0.006 ± 0.001	0.474
putrescine	0.0082 ± 0.0025	0.0085 ± 0.0031	0.0087 ± 0.0035	0.0078 ± 0.0028	0.0084 ± 0.0029	0.526
L-leucine	0.018 ± 0.004	0.018 ± 0.004	0.018 ± 0.004	0.017 ± 0.004	0.018 ± 0.004	0.552
L-2-aminoadipic acid	0.0011 ± 0.0004	0.0011 ± 0.0004	0.0011 ± 0.0004	0.0011 ± 0.0004	0.0011 ± 0.0004	0.563
L-tryptophan	0.310 ± 0.064	0.323 ± 0.085	0.326 ± 0.082	0.312 ± 0.068	0.318 ± 0.065	0.588
glycylglycine	0.021 ± 0.008	0.021 ± 0.005	0.020 ± 0.009	0.023 ± 0.015	0.022 ± 0.008	0.595

L-histidine	0.090 ± 0.016	0.094 ± 0.02	0.094 ± 0.025	0.092 ± 0.018	0.094 ± 0.018	0.651
L-glutamine	11.877 ± 2.061	12.231 ± 2.401	12.471 ± 2.977	12.217 ± 2.571	12.320 ± 2.696	0.665
L-alpha aminobutyric acid	0.072 ± 0.021	0.073 ± 0.023	0.074 ± 0.025	0.072 ± 0.021	0.074 ± 0.023	0.685
L-tyrosine	0.725 ± 0.154	0.751 ± 0.189	0.756 ± 0.219	0.73 ± 0.154	0.747 ± 0.169	0.790
sarcosine	0.0010 ± 0.0003	0.0009 ± 0.0004	0.0010 ± 0.0005	0.0011 ± 0.0006	0.0011 ± 0.0006	0.852
ornithine	0.200 ± 0.071	0.198 ± 0.087	0.199 ± 0.069	0.198 ± 0.066	0.199 ± 0.067	0.885
L-phenylalanine	0.023 ± 0.004	0.023 ± 0.005	0.023 ± 0.006	0.022 ± 0.004	0.023 ± 0.005	0.912
L-homoserine	0.0061 ± 0.0014	0.0060 ± 0.0011	0.0059 ± 0.0014	0.0060 ± 0.0017	0.0060 ± 0.001	0.937
citrulline	0.04 ± 0.014	0.04 ± 0.015	0.04 ± 0.014	0.039 ± 0.014	0.04 ± 0.016	0.941
L-4-hydroxyproline	0.023 ± 0.013	0.024 ± 0.014	0.024 ± 0.014	0.023 ± 0.014	0.024 ± 0.015	0.947

Relative concentrations of endogenous compounds reported as response ratios to their respective internal standards. P-values from one-way repeated measures ANOVA. Metabolites sorted based on p-value (small to large). P-values < 0.05 are depicted in bold. SDMA = symmetric dimethylarginine, ADMA = asymmetric dimethylarginine

Table 2: Stability markers per protocol after volume correction

Metabolites	**Absolute concentration (µmol/L)**					**p-value**
	Protocol 1	**Protocol 2**	**Protocol 3**	**Protocol 4**	**Protocol 5**	
Stable						
2,3-$^{13}C_2$-alanine	28.89 ± 0.3	28.77 ± 0.39	28.84 ± 0.41	28.88 ± 0.32	28.77 ± 0.22	0.564
$^{13}C^{15}N$-isoleucine	6.22 ± 0.07	6.24 ± 0.09	6.23 ± 0.09	6.22 ± 0.07	6.24 ± 0.05	0.567
Unstable						
1,3-$^{13}C_2$-glutamine	453.6 ± 18.17	467.1 ± 53.57	455.99 ± 50.93	454.01 ± 11.79	450.37 ± 16.62	0.613
$^{13}C_2$-glutamic acid	0.72 ± 0.06	0.75 ± 0.08	0.72 ± 0.08	0.75 ± 0.07	0.75 ± 0.07	0.101
$U^{13}C$-$U^{15}N$-UD-glutamic acid	11.44 ± 0.36	11.64 ± 0.66	11.32 ± 0.89	11.56 ± 0.30	11.57 ± 0.33	0.125
1,2,3,4,5,6-$^{13}C_6$-dopamine	0.11 ± 0.01	0.11 ± 0.02	0.11 ± 0.02	0.11 ± 0.01	0.10 ± 0.01	0.508
Degradation [a]	**Conversion from glutamine to glutamate (%)**					
1,3-$^{13}C_2$-glutamine to $^{13}C_2$-glutamic acid (%)	0.158 ± 0.012	0.162 ± 0.026	0.160 ± 0.027	0.165 ± 0.015	0.166 ± 0.017	0.346

P-values from one-way repeated measures ANOVA. [a] Volume correction was not necessary since both glutamine and glutamate were measured in the same volume.

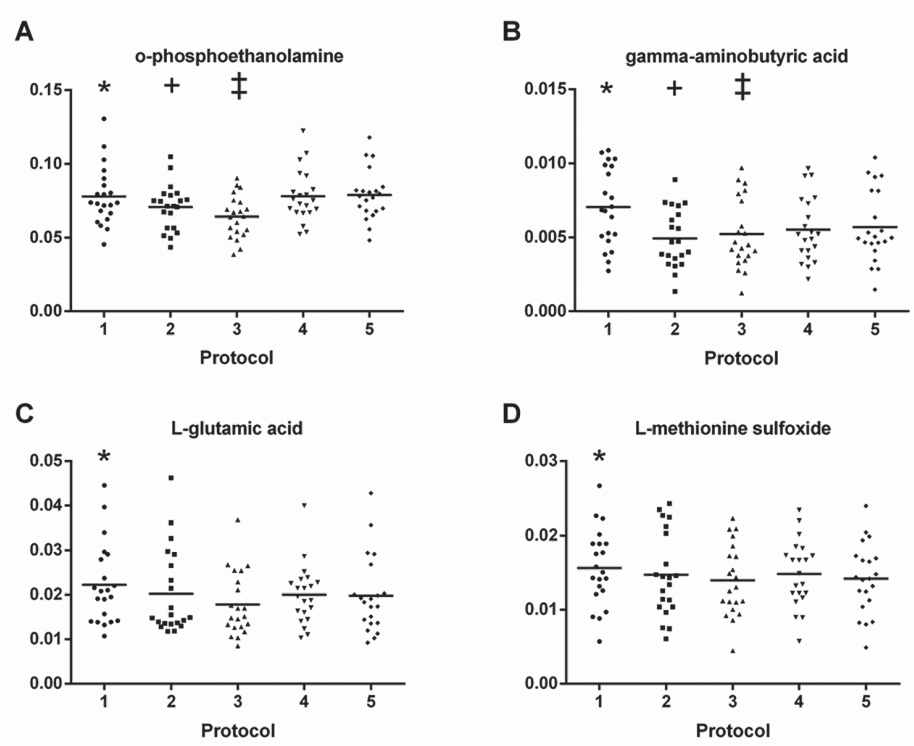


Figure 2. Scatter plots of metabolites with significant protocol differences. Relative concentrations reported as response ratios to their respective internal standards. (A) o-phosphoethanolamine: significant differences between protocol 1 (*) and protocol 3 (p-value = 0.007); between protocol 2 (*) and protocols 4 (p-value = 0.041) and 5 (p-value = 0.006); and between protocol 3 (‡) and protocols 4 (p-value = 0.001) and 5 (p-value <0.001). (B) Gamma-aminobutyric acid: significant differences between protocol 1 (*) and protocols 2-5 (p-value < 0.001 for all comparisons); between protocol 2 (+) and protocols 4 (p-value = 0.027) and 5 (p-value = 0.014); and between protocol 3 (‡) and protocol 5 (p-value <0.001). (C) L-glutamic acid: significant difference between protocol 1 (*) and protocol 3 (p-value = 0.004). (D) L-methionine sulfoxide: significant difference between protocol 1 (*) and protocol 3 (p-value = 0.016).

Discussion

When performing metabolomics in CSF we want our measurements to reflect the *in vivo* situation as close as possible. It is therefore important to avoid *ex vivo* metabolism. The aim of this study was to investigate the effect of three major factors (low sampling and processing temperature, centrifugation to remove cells, and the use of ethanol as additive to stop enzymatic reactions) on the stability of primary and secondary endogenous amines. To this end we compared five different sample

handling protocols for CSF. In addition, we aimed to quantify the degradation of allegedly unstable metabolites such as L-glutamine and L-glutamic acid by adding isotopically-labelled versions of these metabolites to sampling tubes before CSF sampling. We showed that the effects of different protocols were minimal, except for a clear effect of temperature on GABA, and effects of ethanol on PE and partly on GABA. In addition, our labeling strategy with isotopically-labeled compounds allowed us to successfully monitor degradation of L-glutamine to L-glutamic acid and showed that the amount of degradation was extremely small, with no difference between protocols.

Our observation that most endogenous amines are stable under controlled sampling conditions with minimal delay in sample storage is in line with other large scale stability studies which have measured multiple amino acids in CSF.[23-26] In addition, the fact that the reported CSF concentrations of most amines are quite similar over different studies also indicates that most endogenous amines in CSF are stable, despite varying methodologies for sample handling and measurement.[23] However, glutamic acid is an important exception because reported concentrations in CSF vary widely between studies[23,27]; additionally, studies investigating the stability of glutamic acid report varying results which suggests that glutamic acid concentrations might be unstable in CSF.[12,27]

Previous studies showed that *ex vivo* non-enzymatic (chemical) degradation of L-glutamine to L-glutamic acid is negligible.[28,29] Studies measuring L-glutamine degradation in water showed that there is only a small amount of non-enzymatic degradation at room temperature (<1% if left at room temperature for more than 15 days) and no observable decrease at -80 °C.[28,29] More importantly, degradation did not result in the formation of glutamic acid; instead it presumably resulted in formation of pyroglutamic acid. Only after heating to 100 °C, small amounts of glutamic acid were detected [29]. The *ex vivo* enzymatic conversion of L-glutamine and L-glutamic acid is likely the most important factor leading to the alleged instability of these compounds.[26] When left at room temperature, glutamic acid levels in untreated CSF will steadily increase and double within 24 hours.[24-27] The time-related glutamic acid changes in CSF suggests enzymatic processes that mediate the slow formation of new glutamic acid from glutamine or proteins.[27] Different additives have been tried to deproteinize CSF, such as by trichloroacetic acid[12], sulfosalicylic acid[30], or perchloric acid[23], however, this led to conflicting results because acidic conditions also cause other chemical reactions and release of bound amino acids.

The labeling strategy applied in this study allowed the selective monitoring of the conversion of L-glutamine to L-glutamic acid. We observed almost no degradation from $^{13}C_2$-glutamine to $^{13}C_2$-glutamic acid. Because the concentration of L-glutamine in CSF is approximately thousand times higher than L-glutamic acid, it is theoretically still possible that minimal degradation of L-glutamine is affecting L-glutamic acid concentrations. However, since we did not observe significant protocol differences for isotopically-labeled compounds ($^{13}C_2$-glutamine, $^{13}C_2$-glutamic acid and U^{13}C-U^{15}N-UD-glutamic acid) the impact will be similar for all protocols.

The rate of chemical and enzymatic activity is highly dependent on the sample temperature, which combined with a delayed storage determines the actual degree of change.[26,27] The effect of delayed storage was minimal in our study because all samples were placed on dry ice within one hour and stored at -80 ºC within two hours. We did observe a clear effect of temperature on GABA concentrations, which were higher at room temperature (protocol 1) compared to samples that were processed at 4 ºC (other protocols). The increase of GABA at room temperature is in line with previous reports on GABA stability[26,31] and is thought to be secondary to enzymatic hydrolysis of GABA-containing peptides [32]. We also observed significant higher levels of endogenous L-glutamic acid and L-methionine sulfoxide in samples at room temperature (protocol 1) compared to cooled samples containing ethanol (protocol 3). Taken together, the effect of temperature seems limited for most of the metabolites provided that the CSF samples are processed quickly, cooled to -20 °C within one hour and stored at -80 ºC within two hours. In conclusion, it is advisable to quickly cool CSF samples and perform sample processing at 4 °C to reduce enzymatic activity, which seems especially relevant for GABA.

Immune cells, either native to CSF or artificially introduced due to blood contamination during sampling may be present in CSF[24]. Removing cells is also expected to limit effects of enzymatic and metabolic activity in CSF, and in addition reduces the risk of contamination of CSF with cytoplasmic metabolites due to cell lysis during storage at -80 ºC[24]. In our study we did not observe a clear impact of centrifugation. This might be due to the fact that contamination of CSF samples with blood was minimal, as evidenced by the low red blood cell count in our samples. In addition, samples used for this study were collected after collection of 15 mL of CSF for other purposes; it may be that there is a gradient in blood contamination in which the CSF that was collected at the end is least affected. We

would argue that blood contamination is not always clearly visible as red-colored CSF. As even minor contamination can have a major impact on the metabolic profile[24], we advise a centrifugation step immediately after withdrawal of CSF.

Ethanol was used in this study because it has a non-specific denaturation effect on all enzymes and will reduce or stop their activity, this inactivates most of the metabolic reactions and thereby, in theory, should stabilize the CSF metabolome [33,34]. However, the effect of ethanol for the metabolites analyzed in this study was small; the two protocols containing ethanol (protocols 2 and 3) showed slightly lower concentrations of PE (decrease of 19%) and GABA (decrease of 9%) compared to the other protocols (Table 1). *Ex vivo* PE and GABA are known as degradation products of phospholipids and GABA-containing peptides, respectively (ref, 31, 32). Sampling into pre-cooled ethanol (protocol 2) causes rapid cooling and inactivation of enzymes, however, this procedure had only small advantages compared with protocol 3. Still, addition of ethanol allows in principle aliquoting of samples below 0 °C, as the melting of water/ethanol mixtures is below 0 °C. A downside to the addition of stabilizing agents such as ethanol is an additional step in the procedure and it may lead to distortions in metabolomics measurements such as reported for NMR [35]. So despite the theoretical benefits of adding ethanol its positive effects seem minimal under these controlled sampling conditions and therefore not considered necessary for the class of metabolites studied. However, for more unstable metabolite classes the addition of ethanol could still be beneficial and needs further investigation.

The major strength of this study was that we were able to monitor *ex vivo* degradation of selected metabolites by adding them as isotopically-labeled markers to sampling tubes. Because of this addition we were also able to detect volume effects, which otherwise might have been mistaken for stability effects. A possible limitation of this labeling strategy is that some of the degradation of isotopically-labeled compounds occurred before CSF was added; additionally there might be some impurity of the isotopically-labeled standards that is within the limits of industrial quality. However, this would not affect the validity of our study, because preparation of the ILC-mix was performed in one batch, and for each subject we used one aliquot of the ILC-mix, which was thawed just before sampling. Furthermore, preparation of the sampling tubes with ILC-mix was highly standardized, and there were no differences between protocols in time between preparation of sampling tubes and CSF sampling. Nonetheless, if degradation of the ILC-mix had occurred prior to CSF sampling, this would have resulted in an

overestimation of degradation; the observed degradation of 1,3-$^{13}C_2$-glutamine to $^{13}C_2$-glutamic acid can therefore be considered an upper limit of degradation. The unique labeling strategy used in this study could benefit future studies addressing stability issues of other metabolites, because it allows detection of degradation during sampling and sample handling with a higher sensitivity and selectivity than just studying endogenous metabolites.

In summary, we have evaluated five different CSF sampling protocols, including an innovative sampling protocol which uses ethanol as preservative, on stability of endogenous amines. The effects of centrifugation, temperature and addition of ethanol were minimal for most amines, with the exception of PE, GABA and to a lesser extent L-glutamic acid. In addition, we showed that *ex vivo* degradation of L-glutamine to L-glutamic acid under controlled conditions is very limited. Taken together, we conclude that the consensus guideline for sampling CSF as was previously published [14] is adequate for metabolomics studies (at least for metabolites included in this study), provided that samples are cooled to 4 °C immediately after collection, centrifuged, and stored at -80 °C within 2 hours.

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Supplementary material

Supplementary methods: volume correction strategy

We standardized isotopically-labeled compounds according to protocol differences observed for $^{13}C_2$-alanine and $^{13}C^{15}N$-isoleucine because these compounds were considered to be stable.

1. Reference concentration was calculated as follows:
$^{13}C_2$-alanine = average concentration of $^{13}C_2$-alanine across n=21 reference samples (ILC-mix with 1 mL of water instead of CSF).
$^{13}C^{15}N$-isoleucine = average concentration of $^{13}C^{15}N$-isoleucine across n=21 reference samples (ILC-mix with 1 mL of water instead of CSF).
2. For each sample a correction factor was calculated as follows:
$^{13}C_2$-alanine = sample concentration of $^{13}C_2$-alanine / reference concentration of $^{13}C_2$-alanine.
$^{13}C^{15}N$-isoleucine = sample concentration of $^{13}C^{15}N$-isoleucine / reference concentration of $^{13}C^{15}N$-isoleucine.
The mean of both numbers is the correction factor.
3. For each sample the correction factor was applied as follows:
Corrected signal = observed concentration of compound X in sample Y / correction factor of sample Y.

Example:
Reference concentrations of $^{13}C_2$-alanine and $^{13}C^{15}N$-isoleucine were 0.5996 and 0.3350, respectively.

Sample Y of protocol 2 has concentrations of 0.7089 for $^{13}C_2$-alanine and 0.4078 for $^{13}C_2$-^{15}N-isoleucine. The correction factor would then be (0.7090/0.5996 + 0.4078/0.3350)/2 = 1.120. Corrected concentrations of $^{13}C_2$-alanine and $^{13}C^{15}N$-isoleucine would be 0.7089/1.120 = 0.632 and 0.4078/1.120 = 0.364, respectively. Similarly, for $^{13}C_2$-glutamine, with a concentration of 510.7 in sample Y, the corrected concentration would be 510.7 /1.120 = 456.0

For endogenous amines we applied the same calculation steps as above with L-alanine and L-isoleucine as reference markers. For each sample protocol 4 concentrations were chosen as reference concentrations.

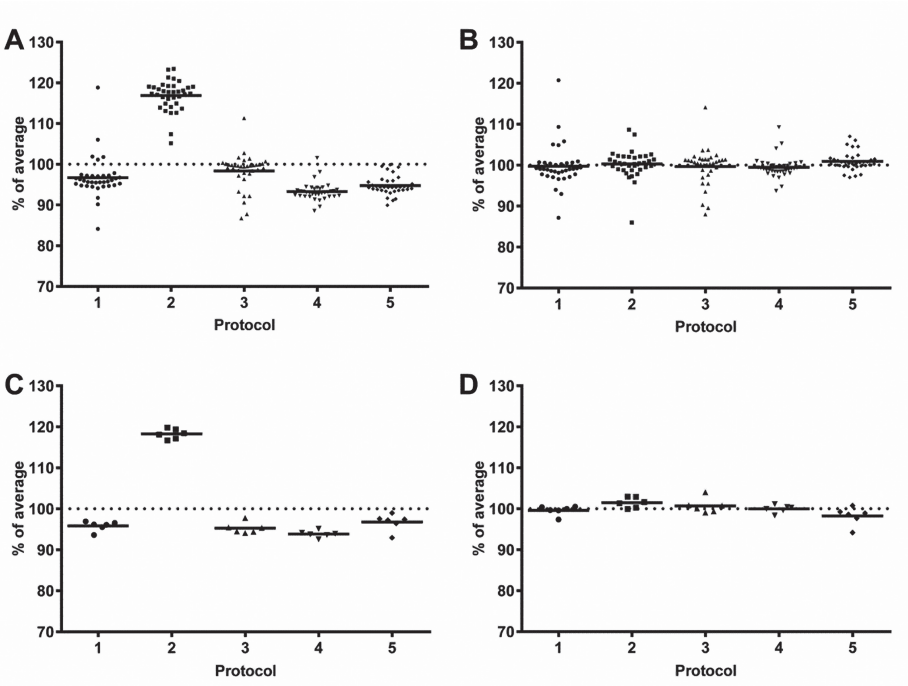


Figure s-1: Differences between protocols before and after volume correction. Every point represents the average of one compound (n=21 subjects) per protocol, relative to the average concentration per compound over all five protocols (dotted lines at 100%). (A) Endogenous primary and secondary amines (n=36) before volume correction and (B) after volume corrections. (C) Isotopically-labelled compounds before volume correction and (D) after volume correction.

Table s-1: Endogenous amines without volume correction and without protocol 2

Metabolites	Relative concentration				p-value
	Protocol 1	Protocol 3	Protocol 4	Protocol 5	
gamma.aminobutyric.acid	0.0071 ± 0.0026	0.0051 ± 0.002	0.0053 ± 0.0021	0.0056 ± 0.0023	**<0.001**
o.phosphoethanolamine	0.078 ± 0.02	0.066 ± 0.019	0.076 ± 0.019	0.078 ± 0.019	**0.010**
L.glutamic.acid	0.022 ± 0.009	0.018 ± 0.009	0.019 ± 0.006	0.02 ± 0.01	**0.017**
L.arginine	1.896 ± 0.458	2.004 ± 0.459	1.828 ± 0.447	1.918 ± 0.455	**0.048**
putrescine	0.0082 ± 0.0025	0.0085 ± 0.0031	0.0077 ± 0.003	0.0082 ± 0.003	0.092
ADMA	0.0005 ± 0.0002	0.0007 ± 0.0003	0.0006 ± 0.0003	0.0006 ± 0.0003	0.097
L.phenylalanine	0.023 ± 0.004	0.023 ± 0.005	0.022 ± 0.005	0.022 ± 0.005	0.144
SDMA	0.004 ± 0.001	0.004 ± 0.001	0.004 ± 0.001	0.004 ± 0.001	0.159
L.tryptophan	0.31 ± 0.064	0.329 ± 0.077	0.302 ± 0.071	0.313 ± 0.07	0.159
L.methionine.sulfoxide	0.016 ± 0.005	0.014 ± 0.005	0.015 ± 0.005	0.014 ± 0.005	0.178
L.methionine	0.537 ± 0.123	0.565 ± 0.161	0.517 ± 0.124	0.532 ± 0.119	0.189
L.tyrosine	0.725 ± 0.154	0.758 ± 0.177	0.708 ± 0.166	0.734 ± 0.181	0.213
ethanolamine	3.595 ± 0.562	3.786 ± 0.726	3.611 ± 0.688	3.745 ± 0.63	0.279
taurine	0.302 ± 0.085	0.319 ± 0.092	0.296 ± 0.085	0.306 ± 0.088	0.289
L.glutamine	11.877 ± 2.061	12.552 ± 2.582	11.754 ± 2.256	12.094 ± 2.693	0.326
L.valine	0.037 ± 0.011	0.038 ± 0.013	0.035 ± 0.011	0.037 ± 0.012	0.328
L.alanine	0.068 ± 0.016	0.07 ± 0.019	0.066 ± 0.018	0.067 ± 0.016	0.375
L.kynurenine	0.0023 ± 0.0012	0.0023 ± 0.0009	0.0023 ± 0.0011	0.0025 ± 0.0009	0.438
L.leucine	0.018 ± 0.004	0.018 ± 0.005	0.017 ± 0.005	0.017 ± 0.005	0.444
L.asparagine	0.322 ± 0.073	0.331 ± 0.081	0.314 ± 0.088	0.319 ± 0.073	0.448
L.4.hydroxyproline	0.023 ± 0.013	0.024 ± 0.015	0.022 ± 0.013	0.023 ± 0.015	0.454
citrulline	0.04 ± 0.014	0.041 ± 0.017	0.038 ± 0.015	0.04 ± 0.017	0.462
L.histidine	0.09 ± 0.016	0.094 ± 0.019	0.089 ± 0.021	0.092 ± 0.018	0.477
L.aspartic.acid	0.055 ± 0.051	0.054 ± 0.057	0.052 ± 0.043	0.051 ± 0.056	0.508
L.alpha.aminobutyric.acid	0.072 ± 0.021	0.077 ± 0.034	0.07 ± 0.022	0.073 ± 0.025	0.532
L.Lysine	3.221 ± 0.787	3.337 ± 0.793	3.137 ± 0.754	3.244 ± 0.793	0.541
N6.N6.N6.trimethyl.L.lysine	0.0056 ± 0.0008	0.0059 ± 0.0013	0.0057 ± 0.0013	0.0059 ± 0.0011	0.549
L.threonine	1.359 ± 0.359	1.414 ± 0.45	1.324 ± 0.399	1.361 ± 0.357	0.614
glycylglycine	0.021 ± 0.008	0.02 ± 0.009	0.022 ± 0.015	0.022 ± 0.008	0.722
L.serine	2.574 ± 0.665	2.66 ± 0.7	2.534 ± 0.658	2.607 ± 0.67	0.740
L.proline	0.025 ± 0.018	0.026 ± 0.02	0.024 ± 0.015	0.024 ± 0.017	0.760
Ornithine	0.2 ± 0.071	0.202 ± 0.08	0.192 ± 0.069	0.196 ± 0.072	0.818
L.2.aminoadipic.acid	0.0011 ± 0.0004	0.0011 ± 0.0004	0.0011 ± 0.0004	0.001 ± 0.0003	0.830
L.isoleucine	0.013 ± 0.004	0.013 ± 0.004	0.013 ± 0.004	0.013 ± 0.004	0.833
sarcosine	0.001 ± 0.0003	0.001 ± 0.0005	0.0011 ± 0.0005	0.0011 ± 0.0005	0.850
L.homoserine	0.0061 ± 0.0014	0.006 ± 0.0016	0.0058 ± 0.0018	0.0059 ± 0.0012	0.853

Relative concentrations of endogenous compounds reported as response ratios to their respective internal standards. P-values from one-way repeated measures ANOVA. Metabolites sorted based on p-value (small to large). P-values < 0.05 are depicted in bold.

Table s-2: Stability markers per protocol without volume correction and without protocol 2

Metabolites	Absolute concentration (µmol/L)				p-value
	Protocol 1	Protocol 3	Protocol 4	Protocol 5	
Stable					
2,3-$^{13}C_2$-alanine	25.67 ± 4.21	25.23 ± 4.6	25.04 ± 3.84	26.28 ± 3.97	0.480
$^{13}C^{15}N$-isoleucine	5.53 ± 0.97	5.46 ± 1.07	5.4 ± 0.86	5.7 ± 0.89	0.476
Unstable					
1,3-$^{13}C_2$-glutamine	404 ± 75	398 ± 80	394 ± 67	412 ± 70	0.564
$^{13}C_2$-glutamic acid	0.64 ± 0.11	0.63 ± 0.14	0.65 ± 0.13	0.68 ± 0.11	0.233
$U^{13}C$-$U^{15}N$-UD-glutamic acid	10.19 ± 1.84	10.02 ± 2.44	10.04 ± 1.65	10.59 ± 1.75	0.397
1,2,3,4,5,6-$^{13}C_6$-dopamine	0.09 ± 0.02	0.1 ± 0.02	0.09 ± 0.01	0.09 ± 0.01	0.799
Degradation [a]	Conversion from glutamine to glutamate (%)				
1,3-$^{13}C_2$-glutamine to $^{13}C_2$-glutamic acid (%)	0.158 ± 0.012	0.160 ± 0.027	0.165 ± 0.015	0.166 ± 0.017	0.191

P-values from one-way repeated measures ANOVA. [a] Volume correction was not necessary since both glutamine and glutamate were measured in the same volume.

CHAPTER 7

Ethanol contamination of cerebrospinal fluid during standardized sampling and its effect on ^{1}H-NMR metabolomics

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Abstract

Standardization of body fluid sampling, processing, and storage procedures is pivotal to ensure data quality in metabolomics studies. Yet, despite strict adherence to standard sampling guidelines, we detected variable levels of ethanol in the ^{1}H-NMR spectra of human cerebrospinal fluid (CSF) samples (range: $9.2x10^{-3}$ – 10.0 mM). The presence of ethanol in all samples and the wide range of concentrations clearly indicated contamination of the samples of some sort, which affected the ^{1}H-NMR spectra quality and the interpretation. To determine where in the sampling protocol the ethanol contamination occurs, we performed a CSF sampling protocol simulation with 0.9% NaCl (saline) instead of CSF and detected ethanol in all simulation samples. Ethanol diffusion through air during sampling and preparation stages appeared the only logical explanation. With a bench study we showed that ethanol easily diffuses into *ex vivo* CSF samples via air transmission. Ethanol originated from routinely used skin disinfectants containing ethanol and from laboratory procedures. Ethanol affected the CSF sample matrix at concentrations above ~9.4 mM and obscured a significant part of the ^{1}H-NMR spectrum. CSF sample preparation for ^{1}H-NMR-based metabolomic analyses should therefore be carried out in a well-ventilated atmosphere with laminar flow, and use of ethanol should be avoided.

Introduction

Cerebrospinal fluid (CSF) is generally believed to reflect brain physiology and is therefore frequently used to assess biomarkers for brain disorders.[1-3] CSF sampling, processing, and storage procedures are pivotal to prevent biochemical *ex vivo* changes which may invalidate the observed profiles. Recently, a consensus protocol was published[4] to standardize CSF sampling and storage for CSF biobanks. However, despite rigorous adherence to this and other protocols we detected substantial amounts of ethanol disturbing the ^{1}H-NMR spectra of human CSF samples. Other studies reporting the presence of ethanol in CSF have interpreted this either as a contaminant[5,6] or due to the disease process.[7,8]

In the present study we set out to identify the origin of ethanol contamination and to assess the effect is has on the sample matrix.

Methods

Human CSF samples

The cohort in which we initially discovered ethanol contamination consisted of human CSF samples that were collected for research purposes, namely to investigate the pathophysiology of migraine. The study protocol was approved by the Medical Ethical Committee of Leiden University medical Center (LUMC), and all subjects gave written informed consent prior to collection. For disinfection of the skin Chlorhexidine (5g/L)/denatured ethanol 70% (Pharmacy LUMC, art.no.: 909602) was used. CSF samples were taken via lumbar puncture. Three different sample handling protocols were used simultaneously for the preparation of samples for MS-based metabolomics (methods 1 and 2) and for ^{1}H-NMR-based metabolomics (method 3) (see figure 1). Method 3 was used for the initial ^{1}H-NMR measurements in which we detected ethanol contamination. For sampling of method 3, 4.8 mL of CSF dripped through air into a 15-mL polypropylene falcon tube. Directly after sampling the CSF was centrifuged at 4°C for 5 minutes (2000 rpm, 747 g). Following centrifugation, the supernatant was transferred to a 15-mL polypropylene falcon tube, and divided in 0.5-mL aliquots, placed on dry ice within 30 minutes of sampling and transferred to -80°C for storage within 60 minutes of sampling. In sampling methods 1 and 2, cold ethanol was added to the CSF during the sample handling to denature proteins and to be

able to thaw the samples before measurements at lower temperatures. Because this was a likely source of ethanol contamination we also analysed three CSF samples obtained for clinical diagnostic purposes to check whether ethanol is also present in these samples. Disinfection of the skin with Chlorhexidine (5g/L)/denatured ethanol 70%, sample collection, and sample processing of these clinical samples was similar to the research samples with the exception that ethanol was not used anywhere in the handling of these samples. For additional details on sampling and processing of the research and clinical CSF samples see the Supplementary Material.

Simulation of CSF sampling protocol with saline

To determine the origin of the ethanol during our sample processing procedure we performed a CSF sampling protocol simulation. A 0.9% NaCl solution (saline) was used instead of CSF in an exact simulation of the CSF sampling protocol (see figure 1). Different pipetting methods were used for Mass Spectrometry (MS)-based metabolomics (methods 1 and 2) and for ^{1}H-NMR-based metabolomics (method 3). In methods 1 and 2, one pipette was used throughout the entire sampling protocol, including pipetting of ethanol. In method 3, a different pipette was used which had never been exposed to ethanol; only these samples were measured with ^{1}H-NMR as described below.

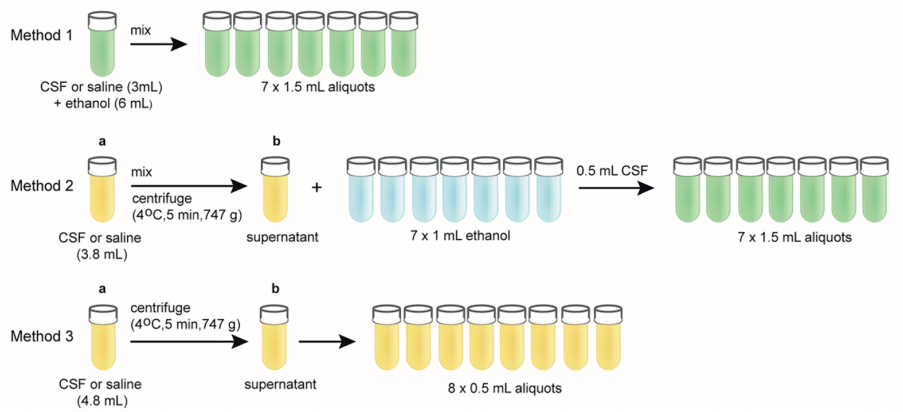


Figure 1: In-house sample processing protocol used for preparation of samples for Massa-Spectrometry (MS) based metabolomics (methods 1 and 2) and for ^{1}H-NMR-based metabolomics (method 3). All aliquots were immediately placed on dry ice within 30 minutes of sampling, and then transferred to -80°C for storage within 60 minutes.

Air to sample diffusion of ethanol

To test how fast ethanol can diffuse into CSF via air we created a work area simulation. Ethanol (1 mL, Biosolve, 96% analytical grade) was placed into 2 x 1.8-mL cryotubes (Nunc, art. no. 368632). An up-turned 500-mL glass beaker was placed over two open cryotubes containing 1 mL of saline and one open cryotube containing ethanol (see figure 2a). After 5 minutes one saline cryotube was removed and capped firmly until ^{1}H-NMR analysis. The second cryotube was removed after 30 minutes. This process was repeated without the beaker in the same well-ventilated work space. All samples were then prepared for and analyzed by quantitative ^{1}H-NMR spectroscopy as described below.

Effect of ethanol on sample matrix

A serial dilution was made to test at which level of ethanol contamination the CSF matrix and thereby the whole ^{1}H-NMR spectrum is affected. 200 mL of CSF was aliquoted into 10 x 1.5-mL eppendorf tubes containing ethanol (Biosolve, 96% analytical grade), decreasing in concentration in regular stepped increments so that the final ethanol concentrations were $9.1x10^{-3}$ – 500.0 mM. Samples were prepared for ^{1}H-NMR analysis as described below.

^{1}H-NMR sample preparation.

In 1.5-mL eppendorf tubes that were placed on ice, 225 µL of CSF, 0.9% NaCl, or CSF/ethanol, was added to 25 µL of pH 7.0 phosphate buffer (50 mM) in D2O containing 4 mM of sodium 3-trimethylsilyltetradeuteriopropionate (TSP) and 2.0 mM NaN_3. Following thorough mixing by repeated inversion, 190 µL of the sample was transferred into 3-mm NMR tubes in a cooled rack at 6 °C.

^{1}H-NMR data acquisition and processing

^{1}H-NMR data were obtained using a Bruker 600 MHz AVANCE II spectrometer equipped with a 5-mm TCI cryoprobe 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) ^{1}H-NMR spectra were recorded at 300.0 K using the first increment of a NOESY pulse sequence [9] with presaturation (γB1 = 50 Hz) during a relaxation delay of 4 s and a mixing time of 10 ms for efficient water suppression.[10] Duration of 90 degree pulses was automatically calibrated for each individual sample using a homonuclear gated nutation experiment [11] on the locked and shimmed samples after automatic tuning

and matching of the probe head. 16 scans of 200,704 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 artifacts of the residual water resonance were manually corrected using a polynomial of degree 5 least square fit filtering of the free induction decay (FID).[12]

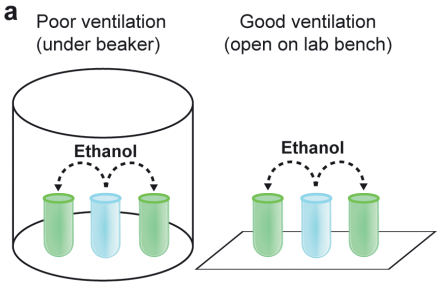
^{1}H-NMR Data Analysis

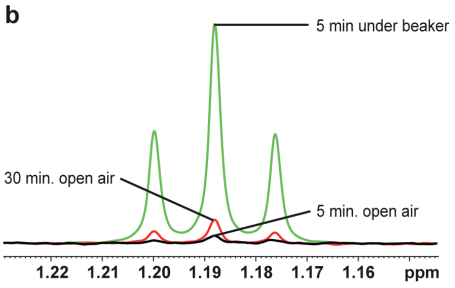
Quantification of ethanol peaks was carried out by iterative line-fitting in the frequency domain (Topspin version 2.1, Bruker Biospin; MDCON command).

Results

Ethanol in human CSF samples

Ethanol was detected in ^{1}H-NMR spectra of all research CSF samples but also in three clinical CSF samples where ethanol is only used during disinfection and not during sample processing (see figure 2c). In the research samples the ethanol levels varied between 0.08 and 10.0 mM and in in the three clinical samples the ethanol levels were: 9.2 x10^{-3} mM, 0.023 mM, and 0.063 mM.





c

Experiment	EtOH conc. (mM)
Air to sample diffusion	
5 min under beaker	0.82
30 min under beaker	2.58
5 min open air	7.2 x10^{-3}
30 min open air	0.055
Simulation of CSF sampling protocol with saline	9.4 x10^{-3} - 0.52
Clinical CSF samples	9.2 x10^{-3} - 0.063
Research CSF samples	0.08 -10.0

Figure 2: Cross contamination of CSF by diffusion of ethanol through air. (**a**) Schematic representation of experimental set-up to test for air diffusion of ethanol. (**b**) ^{1}H-NMR overlay spectrum showing the methyl signal of ethanol; here it is used to visualise the relative amount of ethanol found in test samples from the air diffusion experiment. (**c**) Table comparing the amount of ethanol quantified in the air to sample diffusion test with the simulation of CSF sampling protocol with saline, clinical CSF samples, and research CSF samples.

Simulation of CSF sampling protocol with saline

In the simulation experiment ethanol was detected in all samples, even in method 3 (figure 1) in which no ethanol was used. Furthermore, we observed a trend of increasing ethanol concentration from 0.070 to 0.203 mM (mean) in the aliquots of method 3, which was related to the time the cryotubes were uncapped during aliquoting. These findings suggested that the ethanol arose from cross-contamination by air diffusion during sampling and processing procedures.

Air to sample diffusion of ethanol

To test the hypothesis of ethanol diffusion through air we created a work area-simulation. Ethanol readily diffused into an open vessel containing saline. After 5 minutes under a beaker, ethanol was detected at 0.82 mM compared to 7.2 $x10^{-3}$ mM after 5 minutes open on the lab bench. After 30 minutes, ethanol had increased to 2.5 mM and 0.055 mM, respectively (see figure 2b and 2c). These levels are in the same range as the CSF and saline samples prepared using sampling handling method 3 (see figure 2c).

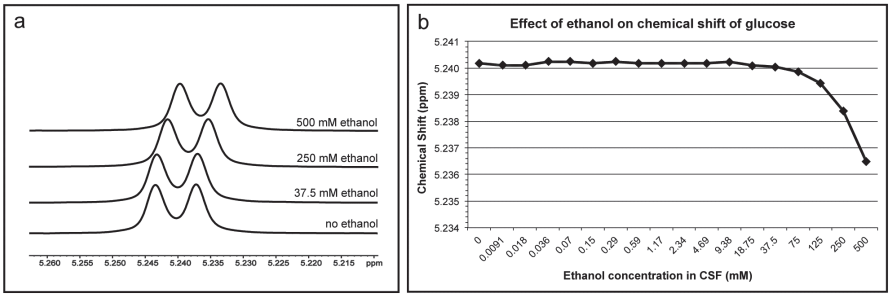


Figure 3: ^{1}H-NMR spectra overlay (**a**) and graph (**b**) showing effect of ethanol on chemical shift of the α-anomeric proton of D-glucose as an example.

Effect of ethanol on sample matrix

An effect of high ethanol concentrations on CSF matrix was demonstrated in a spiking experiment. A distinctive upfield shift of small metabolite signals is observed at ethanol concentrations of 9.38 mM and higher (figure 3).

Discussion

We showed that CSF samples can be contaminated with ethanol during routine sampling and processing steps via diffusion through air. Most likely sources of ethanol are (i) disinfection of the skin with chlorhexidine/70% ethanol prior to lumbar puncture and (ii) use of ethanol in the same room as where the CSF samples were prepared for ^{1}H-NMR measurements. Ethanol affected the CSF sample matrix at concentrations above ~9.4 mM and obscured a significant part of the ^{1}H-NMR spectrum.

The most important effect of ethanol contamination is on the interpretation of the ^{1}H-NMR spectra. The two signal groups of ethanol at 1.18 and 3.66 ppm can mask metabolites that lie in the same chemical shift region of ^{1}H-NMR spectra. The methylene quartet at 3.66 ppm will itself be masked, as it lies in the heavily crowded glucose region of the spectrum. The methyl signal of the small metabolite 3-hydroxybutyric acid, however, normally occurs in the same region of the CSF spectrum as the ethanol methyl triplet (1.18 ppm). In the presence of ethanol the detection and subsequent quantification of this metabolite is heavily compromised.

There are several solutions to minimize the risk of ethanol contamination and to mitigate its effects on metabolomics analysis. 1) Ethanol regions can sometimes be removed from ^{1}H-NMR spectra prior to multivariate analysis. 2) Spectrum alignment might be required prior to analysis in cases of severe matrix distortion. 3) Processing of CSF samples for ^{1}H-NMR analysis in an ethanol-free zone or well-ventilated area such as a laminar flow cabinet. 4) Use of non-ethanol containing disinfectant instead of chlorhexidine/ethanol.

We believe that the recommendations resulting from this study should be considered when collecting CSF for biomarker research in brain disorders.

Acknowledgements

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Supplementary material

Sampling protocol for research CSF samples

Research CSF sampling was performed in the morning (before 12.00 a.m.) via lumbar puncture. All subjects were subjected to overnight fasting from at least 8 hrs before the lumbar puncture and were only allowed water prior to the lumbar puncture. Subjects underwent a neurological examination prior to the lumbar puncture to check for contra-indications for lumbar puncture. The procedures were performed by six, highly experienced Resident doctors in Neurology. Subjects were positioned in the left lateral recumbent knees-to-chest position. For disinfection of the skin Chlorhexidine (5g/L)/denatured ethanol 70% (Pharmacy LUMC, art. no. 909602) was used. CSF samples were taken via lumbar puncture between the L3/L4 or L4/L5 interspace with a Medi Plast 0.9(20G) x 90-mm traumatic needle. After penetrating the subarachnoid space the intracranial pressure was measured with a CSF manometer (Medisize B.V., art. no GDO02*S-A). For routine CSF diagnostics 3 mL CSF was sampled in a 12-mL polystyrene tube (Greiner, art. no. 160172). Three different sample handling protocols were used for preparation of samples for MS-based metabolomics (methods 1 and 2) as well as for NMR-based metabolomics (method 3). All research samples were handled exactly the same way after sampling and all sample handling steps were carried out on ice.

For sampling method 1, 6 mL of cold ethanol (J.T.Baker, ethanol absolute, prod. no. 8098) was added to a 15-mL polypropylene falcon tube (Greiner, art.no. 188271) and placed on ice. Subsequently, 3.0 mL of CSF was sampled directly into this tube and inverted several times to mix thoroughly with the ethanol. Directly after sampling the CSF/Ethanol mixture was divided in 1.5-mL aliquots into 7 x 1.8-mL labeled cryotubes (Nunc 1.8-ml cryotubes – art. no. 368632), and placed on dry ice.

For sampling method 2, 3.8 mL of CSF was sampled in a 15-mL polypropylene falcon tube and centrifuged at 4°C for 5 minutes (2000 rpm, 747 g) directly after sampling. Following centrifugation, the supernatant from this tube was transferred to a 15-mL polypropylene falcon tube, and divided in 0.5-mL aliquots, into 7 x 1.8-mL cyrotubes (Nunc, 1.8-ml cryotubes – art.no. 368632) already containing 1 mL of cold ethanol. The aliquots were repeatedly inverted to mix thoroughly and placed on dry ice.

For sampling method 3, 4.8 mL of CSF was sampled directly in a 15-mL polypropylene falcon tube and centrifuged at 4°C for 5 minutes (2000 rpm, 747 g)

directly after sampling. Following centrifugation, the supernatant was transferred to a 15-mL polypropylene falcon tube, then divided in 0.5-mL aliquots into 8 x 1.0-mL cryotubes (Nunc 1.0-mL cryotubes – art. no. 366656), and placed on dry 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 extra freeze-thaw cycles were allowed.

Sampling protocol for clinical CSF samples

Clinical CSF samples were collected at the Neurology outpatient clinic at the Leiden University Medical Center via lumbar puncture. The procedures were performed by three well-experienced residents in Neurology. For disinfection of the skin Chlorhexidine (5g/L)/denatured ethanol 70% (Pharmacy LUMC, art. no. 909602) was used. CSF samples were taken via lumbar puncture between the L3/L4 or L4/L5 interspace with a Medi Plast 0.9(20G) x 90-mm or 0.7(22G) x 70-mm traumatic needle. CSF was collected for routine CSF diagnostics and was sampled in a 12-mL polystyrene tube (Greiner, art. no. 160172). CSF was processed within 30 minutes after CSF collection. A small portion of CSF was used for cell count, and the remainder centrifuged at 21°C for 5 minutes (2000 rpm, 500 g). The supernatant was placed in a polyethylene tube (Roche, Twintube, art. no. 12029774001) and used for routine CSF measurements. The remaining CSF was stored in 2.0-ml polyethylene cryotubes (Sarstedt, Micro tube, art. no. 72.609), capped with a polyethylene screwcap (Sarstedt, Screw cap, green, art. no. 65.716.005), and transferred to a -20 °C freezer within 60 minutes from sampling. All clinical samples remained at -20 °C until sample preparation, no extra freeze-thaw cycles were allowed.

CHAPTER 8

General discussion

General discussion

Although we have a reasonable pathophysiological explanation for most symptoms associated with migraine,[1-4] we are still missing important pieces of the puzzle. Foremost, we do not understand why and when a migraine strikes, nor do we understand why a patient can have different types of migraine attacks. There is increasing evidence that the start of a migraine attack coincides with distinct intra- and extra-cerebral biochemical changes,[5] but our understanding of these changes is far from complete.

The aim of this thesis was to investigate biochemical mechanisms involved in migraine pathophysiology. This is challenging as the biochemistry of a living organism is a dynamic process with many regulating mechanisms that try to keep levels of biochemical compounds within functional limits.[6] Especially when studying an episodic disorder such as migraine, it is important to realize that the biochemical constitution will likely change over the course of a migraine cycle.[7] In addition, biochemical profiles will likely differ between anatomical regions of the brain and between different body fluids, such as blood and cerebrospinal fluid (CSF).[8,9] By combining multiple approaches, e.g. investigating the biochemical brain composition with ^{1}H-magnetic resonance spectroscopy (^{1}H-MRS) to obtain spatially localized information of compound concentrations inside the brain and applying various types of metabolomics technologies on CSF to study various classes of compounds, we expect to obtain a better insight in biochemical processes relevant to migraine pathophysiology.

The clinical studies described in this thesis were all performed outside migraine attacks, i.e. in the interictal phase. This approach provided valuable baseline information on the biochemical constitution of the brain and CSF, and whether there are biochemical compounds with significantly different levels in migraine patients compared with healthy controls, and may give new insights in migraine pathophysiology.

Biochemical profiling of the brain in migraine patients

In **Chapters 2 and 3** we investigated the *in vivo* brain biochemistry of patients with different types of migraine. We used ^{1}H-MRS, a very powerful and non-invasive tool that can target specific anatomical brain regions to measure a limited number of compounds.

In vivo *differences in brain biochemical profiles of hemiplegic migraine patients*

In **Chapter 2** we used ^{1}H-MRS to investigate brain biochemical profiles in a group of eighteen hemiplegic migraine patients: two sporadic patients (SHM) and sixteen familial patients (FHM), eight of which had a known FHM mutation (five with a *CACNA1A* (FHM1) and three with a *ATP1A2* (FHM2) mutation). When compared with a group of nineteen age- and sex-matched healthy controls, the main observation was a lower concentration of total N-acetyl aspartate (tNAA) in the cerebellum of hemiplegic migraine that was most pronounced in the subgroup of FHM1 patients. The lower levels of tNAA in the cerebellum of patients with hemiplegic migraine are indicative for neuronal loss and dysfunction because tNAA is considered a marker for neuronal integrity.[10] Though, only two of five FHM1 patients showed signs of cerebellar ataxia, one of which with cerebellar atrophy. A previous ^{1}H-MRS study in fifteen FHM1 patients also observed decreased cerebellar NAA levels but also found reduced levels of Glx (glutamate + glutamine) and elevated myo-inositol levels, which are all signs of neuronal loss and glial proliferation resulting in gliosis.[11] Together these observations point to neuronal loss in the cerebellum in FHM1, which can explain the ataxia and cerebellar atrophy in these patients.[12,13] Although statistically underpowered, observations in **Chapter 2** suggest that neuronal loss in the cerebellum is not only present in FHM1 patients with cerebellar ataxia but also occurs in hemiplegic migraine patients without a *CACNA1A* mutation or with a *CACNA1A* mutation without manifested cerebellar ataxia and therefore may serve as an early biomarker for cerebellar dysfunction and/or neuronal loss in all hemiplegic migraine patients.

In **Chapter 2** we also investigated three other brain regions because of their implication in different stages of a migraine attack, namely the hypothalamus (prodrome), the occipital cortex (aura), and the pons (headache initiation), but found no biochemical difference between hemiplegic migraine patients and control subjects. A ^{1}H-MRS study in FHM1 patients also did not detect disease-related biochemical differences in other brain regions, i.e. the visual and parietal cortex[11]. It should be noted however, that none of the patients was examined during a migraine attack and that the attack frequency of the patients with hemiplegic migraine was low, so biochemical changes may have been missed in the interictal phase. In this respect it is also noteworthy that in our study was the first to use high-field 7 tesla MRS for migraine research. As high-field imaging is a relatively new technique, we may not have exploited all its theoretical advantages and biochemical changes may have remained undetected. For example, we were

not able to accurately measure intracerebral glutamate concentrations which is thought to play an important role in (hemiplegic) migraine.

In vivo differences in brain biochemical profiles in migraine with and migraine without aura

For our ^{1}H-MRS study in migraine patients with and without aura, described in **Chapter 3**, we optimized the measurement technique to separate glutamate from glutamine that allowed accurate measurement of intracerebral glutamate concentrations. Glutamate is intimately connected to migraine pathophysiology, and despite the fact that glutamate and glutamine have distinct functions inside the brain, these molecules are metabolically closely related to each other via the glutamate-glutamine cycle. We focussed on measurements in the primary and secondary visual cortex, as these regions have been shown to be hyper-responsive in migraine patients outside a migraine attack.[14,15] In addition, the visual cortex is the anatomical location in the brain with the highest susceptibility to develop CSD.[14,15] We also used diffusion-weighted spectroscopy (DWS) to obtain information on the mobility of glutamate because both its concentration and subcellular compartmentalization (e.g. whether it is in synaptic vesicles or in the cytosol) is important for normal brain function.

Glutamate concentrations were higher in the visual cortex of migraine without aura patients compared with controls, but was not different between migraine with and migraine without aura nor between migraine with aura and healthy controls. Based on diffusion characteristics there was no observable difference in glutamate mobility and indirectly no differences were observed for the compartmentalization of glutamate between healthy controls and migraine patients. So we were not able to further pinpoint the intracellular location of the increased glutamate levels in migraine without aura patients. Most studies that used ^{1}H-MRS to investigate migraine found no biochemical difference between migraine patients and controls.[16] Some studies did observe reduced NAA/tCre in the occipital lobe,[17,18] but other studies[19,20] including our study in **Chapter 3** could not replicate this finding. In addition, none of the previous studies was able to accurately measure glutamate; some do report on Glx (glutamate + glutamine),[21-23] but only one found elevated levels of Glx/tCre in the visual cortex of ten migraine with aura patients compared with controls.[24]

Intracerebral glutamate concentrations in migraine with (hemiplegic) aura

The observation that we did not detect a difference, more precisely an increase, in intracerebral glutamate concentrations in patients with hemiplegic migraine and/or migraine with aura was rather unexpected. This may be due to insufficient statistical power or differences in the methodology used in **Chapters 2 and 3**. Another reason might be that our expectation is too simplistic, or more likely too optimistic to be detected with currently available technology. Cellular and animal studies of FHM mutations predict an increase in glutamate level in the synaptic cleft, perhaps already at basal level. During CSD the extracellular glutamate concentration further increases and glutamate diffuses into the extracellular space which is an important factor for propagation of the CSD wave.[25] Microdialysis techniques showed a 2- to 10-fold increase of extracellular glutamate during CSD compared with baseline.[26-28] The highest reported extracellular glutamate concentration in humans is 2 μmol/L.[29] Therefore, a 10-fold increase during a CSD theoretically results in an extracellular glutamate concentration of 20 μmol/L. This translates to only 0.03% of the total amount of glutamate measured in the visual cortex as reported in **Chapter 3**. Notably, the tiny increase is much smaller than the current measurement variability of MRS. Therefore, the extracellular increase of glutamate expected during a CSD, let alone at basal levels outside an attack, is not within the detection limit of MRS imaging.[30,31]

Biochemical profiling of cerebrospinal fluid

Because there was no clear overview of biochemical compounds measured in CSF from migraine patients, we performed a systematic review and meta-analysis described in **Chapter 4**. We aimed to identify compounds that consistently show altered concentrations in CSF from migraine patients and to assess whether these changes are also present in blood. We showed that sixty-two unique compounds have been measured in CSF from migraine patients. These compounds span a great variety of different functional classes including neurotransmitter systems, neuropeptides, endocannabinoids, neurotrophins, and cytokines. Several compounds show consistent results and have a clear implication in migraine pathophysiology as discussed below. In **Chapter 5** we performed an exploratory ^{1}H-NMR-based metabolomics study of CSF from a large group of patients with hemiplegic migraine, migraine with aura, migraine without aura, and healthy controls. We were able to identify nineteen different metabolites, of which only two (glutamine and glucose) were previously measured in CSF of migraine patients.

Glutamate

Our meta-analysis (**Chapter 4**) showed that glutamate concentrations are significantly increased in CSF of patients with chronic migraine. However, whether glutamate concentrations are also elevated in CSF of episodic migraine patients outside a migraine attack is unknown. Only one study showed increased CSF glutamate levels during migraine attacks in patients with episodic migraine.[32] Our meta-analysis did show that glutamate levels are elevated in blood of these patients outside attacks. Elevated blood glutamate concentrations were also observed in a case-crossover study in both migraine with and migraine without aura.[33] It has not been investigated whether elevated glutamate concentrations is a specific trait of migraine or that it is also present in other primary headache disorders. Regardless, since glutamate has so many different functions in the central nervous system (CNS) it is probably not suitable to use as a diagnostic biomarker for migraine, however, it will be important to further unravel its role in the pathophysiological mechanisms of migraine.

Calcitonin gene-related peptide

Our meta-analysis (**Chapter 4**) showed that CGRP levels are significantly higher in CSF of patients with chronic migraine. CGRP levels in blood are also elevated in patients with episodic migraine, both interictal and ictal, which possibly also is the case in patients with chronic migraine.[34] At the moment, CGRP levels in CSF have only been measured in patients with chronic but not episodic migraine. Elevated CGRP levels have also been reported in serum and saliva both during spontaneous and nitric oxide (NO)-induced migraine attacks.[35,36] Despite this apparent clear picture of elevated CGRP levels, there is, however, one study that did not observe an elevation of CGRP in the jugular blood during a migraine attack;[37,38] there is debate whether the considerable delay in sample processing in the latter study could explain these negative findings. One study correlated increased CGRP levels in chronic migraine to the clinical response to onabotulinumtoxin type A (onabotA) and showed that interictal CGRP levels can predict the response.[34,39] Responders, unlike non-responders, showed a significant decrease in blood CGRP levels. Elevation of CGRP, however, is not specific for migraine since CGRP is also released during cluster headache attacks,[40] trigeminal neuropathic pain with neurovascular features,[41] and chronic paroxysmal hemicranias.[42]

β-endorphin

Our meta-analysis (**Chapter 4**) showed that β-endorphin levels are reduced in episodic migraine outside attacks. Concentrations of β-endorphin were significantly lower in CSF and blood in both chronic and episodic migraine patients compared with controls. Individual studies regarding plasma β-endorphin level in episodic migraine, however, are conflicting;[43-45] a case-crossover study did not show a difference between ictal and interictal β-endorphin levels.[45] So the lower β-endorphin level in patients is likely more related to migraine frequency than an indicator of migraine state (e.g. ictal versus interictal). Lower β-endorphin levels are, however, not specific for migraine as they have also been reported in other chronic pain conditions such as trigeminal neuralgia, rheumatoid arthritis,[46] neuropathic pain,[47] and, interestingly, in blood from cluster headache[48] but not chronic tension type headache[49] patients.

Nerve growth factor (NGF)

Our meta-analysis (**Chapter 4**) showed that concentrations of nerve growth factor (NGF) were increased in CSF and blood of patients with chronic migraine. Blood NGF levels were, however, not elevated in patients with episodic migraine.[50] Of the four compounds that surfaced from our meta-analysis, the evidence for NGF being relevant to migraine pathophysiology, is least convincing also because there were only five studies that measured this compound in CSF or blood of patients.[50-54] Levels of NGF are elevated in patients with a variety of acute and chronic pain states including fibromyalgia,[51] arthritis and spondyloarthritis,[55] endometriosis,[56] degenerative intervertebral disc disease,[57] and allergic diseases and asthma.[58] Plasma NGF levels appear not to be elevated in other primary headache disorders, such as tension type headache[59] and cluster headache.[50] Since finding an increased level of NGF in CSF or blood is not specific for chronic migraine, it is likely not a useful diagnostic biomarker. However, as is the case with CGRP, elevated levels of NGF could be used as potential biomarkers of disease evolution or prediction of treatment outcome.

Biochemical mechanisms in migraine

The role of glutamate in migraine pathophysiology

Glutamate levels in the occipital cortex are higher in patients with migraine without aura (**Chapter 3**). Our systematic literature review and meta-analysis in **Chapter 4** also showed higher glutamate levels in blood and CSF of migraine patients. Glutamate is the major excitatory neurotransmitter in the central nervous system and has since long been suggested to play an important role in migraine pathophysiology but its role in the various migraine subtypes and various phases of a migraine attacks is less clear.

Migraine aura is thought to be caused by cortical spreading depression (CSD)[60,61] that is believed to originate in metabolically unimpaired tissue upon excessive release of potassium and glutamate into the extracellular space at a level that exceeds the buffering and removal capacity of the tissue.[62] In experimental animals, CSD can be induced by topical application of glutamate and blocked by NMDA antagonists.[28,63] The extracellular glutamate concentration increases during CSD, as shown by microdialysis experiments in rats.[28] Genetic evidence for the involvement of glutamate in migraine came from the discovery of mutations in the three genes for FHM that point towards increased glutamatergic neurotransmission and an excess of glutamate in the synaptic cleft.[25] The importance of neuronal glutamate release was further proven in a transgenic mouse model with a human pathogenic FHM mutation in a voltage-gated $Ca_V2.1$ calcium channel subunit[64] that showed a normalisation of the propensity for CSD when glutamatergic neurotransmission in the cortex was normalised by a $Ca_V2.1$-specific calcium channel blocker.[2,65]

Several other brain structures are relevant to understanding the role of glutamate (and its receptors) in migraine. For instance, pain-relay structures, such as the trigeminal ganglion, trigeminal nucleus caudalis and thalamus all contain glutamatergic neurons.[66] Moreover, glutamate receptors are present in superficial laminae of the trigeminal nucleus caudalis, neurons in the trigeminal nucleus caudalis, thalamus and other pain-related areas.[67-69] Glutamate levels were shown to increase in the trigeminocervical complex after stimulation of dural structures and in the ventroposteromedial thalamic nucleus following experimentally noxious stimulation along the trigeminal nerve in rats.[70-72] Notably, glutamate receptor antagonists (especially targeting the Kainate iGluR) reduce nociceptive input in animal studies[73] and have proven as effective as sumatriptan in aborting migraine attacks in humans.[74]

Glutamate and its relation to energy metabolism

Most of the links between migraine and glutamate focus on glutamate's role as an excitatory neurotransmitter. However, glutamate is also closely related to cellular metabolism in two important ways.

Firstly, as an alternative oxidizable substrate, glutamate serves as an important energy reserve.[75,76] Glutamate is a central compound of cellular metabolism and its carbon skeleton is diverted to many diverse anabolic and catabolic pathways. This can only occur after glutamate is sequestered from the TCA cycle at the level of alpha-ketoglutarate.[29] On average a glutamate molecule is recycled one or two times in the glutamate–glutamine cycle before it is degraded in the TCA cycle.[77] *De novo* synthesis of glutamate accounts for approximately 20% of cerebral glucose metabolism, all of which initially occurs in astrocytes. Another argument that glutamate is involved in energy metabolism comes from recent functional MRS (fMRS) studies at 7 tesla that found a small, but marked, 2-3% increase of glutamate concentrations in the occipital lobe of individuals after visual stimulation.[78-80] This glutamate increase coincided with a decrease in aspartate, which directly links to the transamination reaction between glutamate and aspartate, which is a critical step in the malate-aspartate shuttle. An increased flux in this shuttle is indicative for an increased oxidative metabolism.[81] Therefore, elevated levels of glutamate observed during fMRS experiments are thought to reflect foremost an increase in oxidative metabolism.[81,82]

Given that glutamate is a key component of the above mentioned metabolism, high levels in brain (**Chapter 3**) and CSF (**Chapter 4**) do not necessarily reflect altered glutamatergic neurotransmission but may instead reflect changes in metabolic demand. The latter explanation would be more in line with studies using ^{31}P-MRS that consistently showed that different types of migraine seem to share a multi-systemic impairment of energy metabolism in both skeletal muscle[83] and brain.[84] The finding of an interictal deficit of brain and muscle bioenergetics has led to the hypothesis that a bioenergetics defect may be an intrinsic feature of migraine.[85] A compromised bioenergetics state may enhance the probability for developing an attack, especially when brain energy demand is increased and/or the supply of oxidizable substrates and oxygen is temporarily diminished.

Secondly, the excitatory function of glutamate as neurotransmitter is dependent on maintaining a strict compartmentalization of glutamate with high concentrations inside the cell and low concentrations outside the cell. This compartmentalization is maintained by uptake of glutamate by neurons, astrocytes,

and the blood-brain barrier (BBB), this process is highly energy-dependent.[86] So the distribution of glutamate is in dynamic equilibrium and highly sensitive to changes in the energy supply. Because of the high rates of glutamate release, inhibition or impairment of glutamate uptake can lead to high extracellular levels of glutamate within seconds.[75] High extracellular glutamate can activate glutamate receptors causing further release of glutamate, this activation increases energy consumption and Na^+ and Ca+ influx, which in turn can impair adequate glutamate uptake by glial excitatory amino acid transporters, and possibly even reverse these transporters causing further increase.[75] Such a circular process is also thought to play an important role in the initiation of CSD where high extracellular glutamate can activate glutamate receptors causing further glutamate release.[3]

The role of calcitonin gene-related peptide in migraine pathophysiology

CGRP is elevated in migraine patients and may serve as a biomarker to predict the treatment response to botulinum toxin (more precise onaboA) in chronic migraine (**Chapter 4**). CGRP is a neuropeptide that is widely expressed in the peripheral and central nervous system.[87-89] CGRP is mainly localized in thin unmyelinated nerve fibres (C-fibres) in the cerebral vasculature, middle meningeal artery, and dura matter, which originate from the trigeminal ganglion.[90,91] Its vascular and neuronal effects have been implicated in different aspects of migraine pathophysiology.[35] Activation of the trigeminovascular system (TGVS) releases several vasoactive neuropeptides, especially CGRP, from presynaptic nerve terminals.[92] The trigeminal CGRP nerves trigger a protective, reflex vasodilation that is thought to counteract prolonged vasoconstriction of cerebral arteries supplying blood to the brain.[93] In addition, given the hypothesis that repeated TGVS activation sensitize peripheral and central pain pathways which might lead to migraine chronification,[92] CGRP is a very important compound in migraine pathophysiology. Notably, this has resulted in the creation of CGRP antagonists which are very effective in aborting migraine attacks.[94]

The role of beta-endorphin in migraine pathophysiology

Levels of β-endorphin are lower in CSF and blood from both chronic and episodic migraine patients compared with controls (**Chapter 4**). The neuropeptide β-endorphin has morphine-like effects.[95,96] Plasma and CSF levels of β-endorphin reflect activity of two functionally separate opioid systems.[97] Peripheral β-endorphin is produced in the pituitary and is circulated via the blood stream

to interact with specific opioid receptors located throughout the body where it produces analgesia by inhibiting the firing of peripheral somatosensory fibres.[95] Central β-endorphin is produced by hypothalamic pro-opio-melanocortin (POMC) neurons for release inside the CNS and the CSF. β-endorphin in CSF uses synaptic (and non-synaptic paracrine) communication via volume transport mechanisms provided by the flowing CSF[98] and plays a complicated role in controlling pain and other body functions. It is puzzling how lower β-endorphin levels may link to migraine aetiology, since β-endorphin levels do not seem to decrease during a migraine attack.[45] Although the endogenous opioid system has downstream effects on pain modulation, there is no evidence to suggest that it has an important role in the pathogenesis of the attack itself. The intriguing finding that the evolution of episodic to chronic migraine is concomitant with progressively reduced levels of β-endorphin CSF led to the hypothesis that chronification of the disease is linked to a deterioration of the anti-nociceptive system, biochemically sustained by a progressive reduction of CSF β-endorphin levels.[99]

The role of neurotrophic factors in migraine pathophysiology

NGF is increased in CSF from chronic but not episodic migraine patients (**Chapter 4**). NGF is a member of the neurotrophin (NT) superfamily that also includes brain-derived neurotrophic factor (BDNF), NT-3, and NT-4/5.[100] NGF levels are elevated in a variety of acute and especially chronic pain states and is thought to modulate pain and hypersensitivity due to its effects on nociceptive neuron function, either directly or indirectly via an action on mast cells.[100] NGF receptor TrkA is expressed on nociceptive neurons [101] that also express TRPV1.[102] TRPV1 is a non-selective ligand-gated cation channel that reacts to mechanical, thermal and chemical stimuli derived from extra- and intracellular sources. An increase in NGF expression affects the function of TRPV1 in two ways, first via NGF binding to the TrkA receptor on nociceptive neurons, which activates phospholipase C that in turn leads to TRPV1 sensitization,[103] and second by increasing TRPV1 expression.[104] In addition it was also shown that the P2X purinergic receptor sensitivity may be affected by mediators such as CGRP, NGF, and BDNF.[105] More specifically, the sensitization of pain-transducing P2X3 receptors is modulated by the migraine mediators CGRP and NGF.[106] So increased levels of NGF tend to decrease the threshold for action potentials in nociceptive neurons, which may be a relevant factor in pain chronification.

How can we biochemically understand a complex disease like migraine?

From a clinical perspective, migraine is a very complex syndrome because of its episodic nature and the many symptoms that can be present during different phases of the migraine cycle. Currently, there are several well-established pathophysiological mechanisms that are able to explain most of the migraine symptoms. However, how these different mechanisms are connected is still largely unknown. From a biochemical perspective, migraine is also very complex because there are numerous compounds that may interact to produce the different migraine symptoms and phases of the migraine cycle. Our current understanding of the underlying biochemistry is very incomplete. The systematic review in **Chapter 4** clearly showed that biochemical research in migraine until now has been unstructured and scattered over different chemical classes, anatomical locations and body fluids, subtypes of migraine, and phases of the migraine cycle. Only few of the findings have been replicated and information on the sensitivity and specificity of potential diagnostic biomarkers is scarce.

To better understand the biochemistry underlying migraine pathophysiology and to identify useful biochemical biomarkers, there are two general approaches that can be used. The first approach is to perform hypothesis-driven biochemical research, which requires a pathophysiological framework consisting of compounds known to be involved in migraine pathophysiology. Ideally, compounds are ordered in a pathophysiological relevant way related to their specific place in the migraine cycle. The second approach is to perform non-hypothesis-driven biochemical research, in a structured way, exploring different compound classes in different phases of the migraine cycle. A more structured approach is necessary in order to identify relevant molecules and to be able to attribute these to (a) specific phase(s) of the migraine cycle and the underlying pathophysiological mechanism(s). Regardless of the approach taken, a theoretical framework of migraine pathophysiology is needed in order to make sense of identified biochemical changes.

One such framework for migraine pathogenesis is based on accumulating evidence that shows that the interplay between brain parenchyma and the trigeminovascular system (TGVS) being important for the initiation of migraine attacks.[107] From experimental animal research on CSD we obtained detailed information on biochemical changes that occur during a CSD.[3] For instance, various molecules are released into the interstitial space (e.g. K^+, protons, arachnidonic acid, nitric oxide, adenosine, ATP and glutamic acid), many of them

have noxious potential.[3,107] There is compelling evidence that if these noxious molecules reach sufficiently high levels, they diffuse to pial vessels where they are able to activate a network of trigeminal axons (the visceral afferent system of the brain), which leads to lateralized pain.[108-111] Subsequent discharge of second-order neurons causes local neurogenic inflammation and mast cell degranulation within the dura mater layer of the meninges.[110] A recent study in wildtype mice revealed that CSD causes opening of neuronal Pannexin1 (Panx1) mega channels that lead to neuroinflammatory responses that involve neuronal caspase 1 activation and neuronal release of high-mobility group box 1 (HMGB1) into the extracellular space, nuclear factor κB activation in astrocytes, and ultimately trigeminovascular activation.[112] Studies like this one emphasize that migraine is likely to be caused by interactions of cells in the brain parenchyma and the meninges, which are more intertwined and chemically connected than previously thought. It seems that a 'brain in stress' is able to communicate with the meningeal vasculature and that this is important for the pathogenesis of migraine attacks. Notably, these biochemical changes in the brain parenchyma can (theoretically) be measured using different biochemical imaging modalities such as ^{1}H-MRS (glutamine and glutamate; applied in **Chapters 2 and 3**), ^{31}P-MRS (energy metabolism), ^{13}C-labelled ^{1}H-MRS (metabolic flux) and PET. And since a lot of biochemical compounds in the brain parenchyma are able to diffuse from the interstitial space into the CSF compartment,[113] it would also be very interesting to identify changes in biochemical profiles of CSF over the course of a migraine cycle.

Practical considerations for biofluid sampling in migraine

Cerebrospinal fluid to study the biochemistry of migraine pathophysiology

The studies described in **Part 2 and 3** of this thesis were all aimed at investigating the composition of CSF. From a bio-analytical point of view, CSF is an ideal body fluid because it is less complex in its biochemical constitution than blood, has a smaller biological variability[8], and is metabolically stable (as shown in **Chapter 6**) due to the fact that there are fewer enzymes presents and hardly any cells that might have unwanted biochemical interactions.[114] Still CSF contains hundreds of molecules.[115] There are several important arguments why CSF also is an ideal body fluid to study migraine and CNS disorders in general.

The CSF compartment lies between the endothelial blood-brain barrier (BBB) and the epithelial blood-cerebrospinal fluid barrier (BCSFB); the CNS parenchyma is only separated from the CSF by the glia limitans, which is composed of astrocytic foot processes and a parenchymal basement membrane.[116] Because astrocytes of the glia limitans are connected via gap junctions it is not considered part of the BBB, so material can readily pass between the foot processes from the brain parenchyma into CSF.[113] The traditional view is that CSF is produced by the choroid plexus, circulates through the ventricles, the cisterns, and the subarachnoid space to be absorbed into the blood by the arachnoid villi.[117,118] However, it was recently discovered that a pulsatile to and from movement throughout the entire brain is present with local fluid exchange between blood, interstitial fluid, and CSF.[119] This additional CSF circulation around blood vessels that penetrates from the subarachnoid space into the Virchow Robin spaces seems to provide both a drainage pathway for waste molecules from the brain and a site for interaction of the systemic immune system with the brain, and as such seems to function as a cerebral lymphatic system.[118,120] These new insights in CSF physiology and function are of major importance to migraine pathophysiology because if the CSF functions as vehicle for chemical messaging, this is where biochemical changes relevant to migraine pathophysiology may more readily be detected.

Practical considerations for CSF collection

A technical limitation to the use of CSF as a bio-fluid for biochemical research is the open sampling system that is normally used, which has the risk of unwanted contamination of CSF samples (as described in **Chapter 5**). It is, therefore, advised not to use volatile substances such as ethanol in the surroundings of CSF sampling, which is challenging in a hospital setting where ethanol-containing substances are widely used as disinfectant. Another practical limitation of collecting CSF via lumbar puncture is that there are misconceptions among the general lay public and even (non-neurological) physicians, leading to fear for the procedure. This makes the recruitment of study participants a challenging task. However, if executed by an experienced clinician, the burden of a lumbar puncture to patients is minimal and the risk of (serious) complications is almost negligible when patients were appropriately pre-screened for contra-indications.[121] Admittedly, there remains the risk for post-dural puncture headache (PDPH) which is alike for migraine patients and healthy controls; the risk is markedly reduced with the use of a-traumatic needles.[122-124] Fortunately, there is a noticeable change in the way researchers and

ethics committees judge the collection of CSF which will make the procedure more feasible. Since the importance of CSF to study the pathophysiology of CNS disorders has become more evident, there is less reticence, and several large CSF studies in for example Alzheimer's and Parkinson's disease are ongoing, some collecting multiple CSF samples over several years from the same subject aiming to find biochemical markers for disease progression.[125,126]

Biomarkers in blood: advantages and disadvantages

Although CSF may represent a better source for biomarker discovery for diseases of the CNS, as it is in closer proximity to the tissue of disease origin and may better represent its neurochemical state than other body fluids (e.g. saliva, blood, urine)[8], its collection is still cumbersome and more invasive than the collection of for instance blood, especially from control individuals.

A major advantage of blood samples is that patients can be followed up and screened over multiple time points, for example at different phases of a migraine cycle. Blood is the most commonly used bio-fluid in clinical chemistry due to the minimally invasive nature of collection, its rich metabolome[127] and proteome[128], and its reflection of the metabolic state of the entire organism. However, intracerebral compounds, in brain disorders like migraine, that are released in blood are diluted in 4 L as compared to 150 mL when entering CSF. Consequently, biochemical changes need to be profound to allow detection in blood. All in all, biomarker data from sampling blood will represent diluted steady state concentrations of molecules that are affected by complex production and numerous matrix effects, transport and clearance kinetics. Regardless, it is of importance to know how metabolic profiles of CSF and blood correlate, which is challenging in itself given the various brain-fluid barriers (blood-brain, blood-CSF, brain-CSF) that have a profound impact on identifying differences in metabolic profiles between CSF and blood. Whether choosing CSF or blood is better as bio-fluid for biochemical research of migraine remains unclear. After all, a disease biomarker initially discovered in CSF will probably cause difficulties to get translated in a broadly used blood-based test, and a disease biomarker initially discovered in blood may be a weak reflection of the disease process in the brain.

Future perspectives

Future research efforts based on spectroscopy

Because high-field MRS is a relative new technique, it is subject to rapid development and ongoing technical improvements. Potential future research may comprise of further robust, quantitative and multinuclear brain MRS studies. Multinuclear brain MRS studies, combining ^{1}H-MRS and ^{31}P-MRS, could address the question whether there is a relation between the previous found energy metabolism and the higher glutamate concentrations reported in **Chapter 3**. Performing such studies before and during the onset of for instance an NTG-provoked migraine attack will give valuable information on biochemical changes in migraine during different stages of the migraine cycle. This could specifically test the hypothesis whether changes in glutamate concentration are related to the onset of migraine attacks. To investigate the link between CSD and *in vivo* glutamate it would be interesting to investigate whether glutamate levels are changing during the course of an aura. This is practically challenging, but the scientific gain is high because that would provide direct evidence in humans whether and to what extent the glutamatergic system is actually involved in the aura phase in humans. To further investigate the link between elevated glutamate concentrations and altered cortical excitability in migraine patients a combination of visual stimulation, visual-evoked potentials and functional MRS would also be interesting. Recent advances in fMRS make this kind of research feasible and hopefully will provide direct insight into brain metabolism by investigating the metabolic responses of the brain to physiological stimuli. This could provide objective proof that an altered cortical excitability in migraine is also reflected at the biochemical level.

Future research efforts based on body fluid biomarker discovery

Biomarker discovery studies have an exploratory setup with numerous compounds measured in a single run. Although several candidate neurochemical biomarkers have been identified for different neurological diseases, including migraine, translation into clinical application is hindered by the small sample size of current studies, multiplicity issues, and the lack of independent verification of promising findings. In order to push knowledge of migraine biochemistry forward, it will be extremely important for future biochemical studies to combine hypothesis-driven and non-hypothesis-driven studies. Hypothesis-driven studies in larger study populations can be aimed at replicating previous findings from

smaller studies, which is desperately needed. Non-hypothesis-driven studies can basically screen as many biochemical compounds as possible to provide valuable novel clues for migraine disease mechanisms that may have escaped our attention. There is increasing evidence that noxious compounds originating from the brain are causing activation of the trigeminovascular system via activation of primary afferent nociceptive neurons that innervate cephalic tissues, primarily the cranial meninges and their related blood vessels.[3,107,129] Despite this compelling evidence from animal studies, direct evidence in humans is still lacking.[130,131] However, it might be possible to test the hypothesis of noxious stimuli directly in humans. The recent discovery, that the CSF also functions as the brain's lymphatic system and flows freely from the arachnoid space along blood vessels into brain tissue, makes the CSF a very important body fluid to study in the context migraine. Because CSF reflects, in part, the biochemical constitution of het ECF of the brain, it might be possible to detect the noxious compounds from the brain in CSF. It will therefore be important to study different phases of the migraine attack, especially during a migraine attack because that is where the biggest changes are expected. To know whether potential neurochemical biomarkers are specific for migraine, or whether they are generic for headache disorders, it will also be important to collect CSF and blood from patients suffering from other primary headaches. These new research approaches are likely to substantially enhance our knowledge of the pathogenesis of migraine which should result in the identification of dearly needed novel prophylactic treatment targets, predictive biomarkers for treatment response, and objective diagnostic biomarkers to facilitate the reliable differentiation of migraine from other primary headache syndromes that require different treatment approaches.

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Appendix

Summary
Nederlandse samenvatting
Dankwoord
List of publications
Curriculum Vitae

Summary

The aim of this thesis was to investigate the underlying biochemistry of different types of migraine. Migraine is highly disabling for the patient and there is an urgent need for more effective and well-tolerated preventative agents. It is therefore important to unravel the biochemical pathways underlying migraine pathophysiology with the hope of finding new drug targets.

The research described in this thesis is divided in three parts. The **first part** reports on investigations of the biochemical constitution of the migraine brain *in vivo* using proton magnetic resonance spectroscopy (^{1}H-MRS) and diffusion-weighted spectroscopy (DWS). The **second part** describes efforts in body fluids to identify biochemical differences relevant to migraine. To this end metabolomics technologies were applied to assess the biochemical make-up of, primarily, cerebral spinal fluid (CSF). The **third part** consists of methodological studies in which the biochemical stability of CSF and the effect of contamination with ethanol are investigated.

Part 1: Biochemical profiling of the brain

Magnetic resonance spectroscopy (MRS) is a non-invasive *in vivo* method to detect biochemical compounds in the brain. MRS was used to investigate the biochemical constitution of different brain areas in various groups of migraine patients (**Chapters 2 and 3**). For **Chapter 2** we performed biochemical measurements with ^{1}H-MRS in the brain in patients with hemiplegic migraine in between attacks. Hemiplegic migraine is a rare subtype of migraine, characterized by hemiparesis during the aura phase. These patients may develop progressive permanent cerebellar impairment and atrophy, especially when they have the disease due to a mutation in the voltage-gated calcium channel subunit gene *CACNA1A* (FHM1). We searched for biochemical changes in the cerebellum, hypothalamus, occipital cortex, and the pons of hemiplegic migraine patients and healthy controls; and compared patients with and without a *CACNA1A* mutation. A decreased cerebellar total N-acetyl aspartate (tNAA) was observed in hemiplegic migraine patients compared with controls. The subgroup of FHM1 patients showed the lowest cerebellar tNAA level. Since tNAA is considered a marker for neuronal integrity, this finding suggests cerebellar neuronal loss or

dysfunction in hemiplegic migraine. In the other three regions of interest, which are implicated in different stages of a migraine attack, no biochemical differences were observed. At the time of the study it was not possible to measure glutamate and glutamine accurately; therefore, the total concentration of the combined glutamate and glutamine was reported.

Glutamate has been implicated in migraine pathophysiology but it is unknown whether and to what extent intra- and extracellular cerebral concentrations of glutamate are different in migraine patients. In **Chapter 3** we determined the occipital glutamate concentration and its time-correlated compartment-specific characteristics in a case-control study with migraine patients with and without aura (in between attacks – i.e. interictal) and age- and gender-matched healthy controls. With an improved ^{1}H-MRS sequence at a high magnetic field strength or 7 tesla it was possible to accurately measure glutamate separate from glutamine. Glutamate concentrations in the occipital lobe were higher in migraine without aura patients outside an attack compared with healthy controls. However, no difference was seen for migraine with aura patients. Furthermore, there was no difference in the diffusion of glutamate – so there is no clear differences in glutamate mobility and, indirectly, compartment-specific characteristics of glutamate. The occipital glutamate concentration showed no relation with clinical parameters such as migraine severity and frequency, visual sensitivity, and time until the next migraine attack. This result reinforces the concept that the glutamatergic system is involved in migraine. It is somewhat puzzling that we did not find increased glutamate concentrations in migraine with aura patients as typically the link between glutamate and migraine is thought to occur via cortical spreading depression, the underlying mechanism of migraine aura. However, the formation and degradation of glutamate is also part of the brain's general energy metabolism, since glucose is converted into glutamate before being oxidized further via the tricarboxylic acid (TCA) cycle, which may explain better our observed effect in migraine without aura.

Part 2: Biochemical profiling of cerebrospinal fluid

A clear overview of biochemical compounds measured in CSF in relation to migraine was lacking. Therefore, in **Chapter 4**, we conducted a systematic review and meta-analysis aimed at identifying compounds that consistently revealed altered CSF concentrations in migraine patients. In addition, we assessed whether these changes are also present in blood. Sixty-two unique compounds were identified in 40 CSF studies. Most important and consistent results for CSF were increased concentrations of glutamate, calcitonin-gene related peptide (CGRP), and nerve growth factor in chronic migraine patients and decreased concentrations of β-endorphin (β-EP) in both chronic and interictal episodic migraine patients. Blood concentrations were increased for glutamate (interictal migraine) and CGRP (chronic, interictal and ictal migraine) and decreased for β-EP (chronic, interictal and ictal migraine). Unfortunately, sample sizes in most studies were relatively small and the quality and reporting of applied measurement methodology was often limited. This study emphasized that the biochemical puzzle underlying migraine is still far from complete. Future research should, therefore, replicate findings in larger study populations and explore a wider biochemical profile.

The aim of **Chapter 5** was to identify relevant biochemical pathophysiological markers for migraine in CSF using an exploratory ^{1}H-NMR-based metabolomics approach. Patients with hemiplegic migraine, migraine with aura, migraine without aura, and healthy controls were included in the study. Supervised multivariate modelling was carried out and revealed that hemiplegic migraine patients can be discriminated from healthy controls based on their metabolic profile. Metabolite 2-hydroxybutyric acid was shown to be the most important metabolite for the discrimination; both for male and female hemiplegic migraine patients. Univariate analysis, with a Generalized Linear Models (GLM), showed 2-hydroxy-butyric acid and 2-hydroxyisovalerate to be lower in patients with hemiplegic migraine than in healthy controls. Notably, 2-Hydroxybutyrate is involved in energy metabolism and abnormal energy metabolism has been observed in FHM patients in both brain and muscle. 2-hydroxyisovaleric acid originates mainly from ketogenesis and from the metabolism of valine, leucine and isoleucine.

Part 3: Practical considerations for cerebrospinal fluid sampling

For the study of biochemical profiles in body fluids different metabolomics techniques can be applied, of which mass-spectrometry-based techniques are very sensitive and capable of measuring compounds at micro-molar levels. It is therefore, theoretically, very important to minimize sources of variation in the measurements by standardization of CSF sampling, processing and storage procedures. The aim of the study in **Chapter 6** was to investigate the effect of five different sampling protocols to determine which provides the most stable metabolic profile. In addition, we assessed the effects sampling and processing temperature, centrifugation and the use of ethanol as additive to stop enzymatic reactions. Isotopically labelled compounds were used to trace degradation during the sampling or handling procedures. There were no clear observable difference between protocols and the amount of degradation of metabolites was very small. This study showed that the theorized negative effects of different processing steps are minimal. This reassured us that the CSF samples that we have collected in a standardized and controlled manner over the past years are of high quality and that the variation added to the metabolic profile is minimal.

Despite strict adherence to standard sampling guidelines, variable levels of ethanol were detected in the ^{1}H-NMR spectra of human cerebrospinal fluid (CSF) samples. **Chapter 7** describes research aimed to retrace the exact origin of ethanol contamination in CSF samples. This study showed that ethanol easily diffuses into *ex vivo* CSF samples via air transmission. Ethanol affected the CSF sample matrix at high concentrations and obscured a significant part of the ^{1}H-NMR spectrum. It is therefore advised that CSF sample preparation for ^{1}H-NMR-based metabolomics analyses should be carried out in a well-ventilated atmosphere with laminar flow, and use of ethanol should be avoided.

Finally, **Chapter 8** places our biochemical findings in a broader perspective. The relevance of these biochemical changes in migraine, their role in pathophysiological mechanisms, and future directions for research are discussed.

Nederlandse samenvatting

Migraine is een ernstige aandoening die vaak voorkomt in de algemene bevolking met een prevalentie van ongeveer 15%. Er is een duidelijke behoefte aan effectievere en beter te verdragen preventieve medicijnen. Om nieuwe en betere aangrijpingspunten te vinden voor nieuwe medicijnen is het belangrijk om meer te weten te komen over de biochemische processen die een rol spelen in de migraine pathofysiologie. Het doel van dit proefschrift was daarom de onderliggende biochemische processen gerelateerd aan migraine te onderzoeken.

De verschillende onderzoeken die in dit proefschrift beschreven worden zijn onderverdeeld in drie delen. Het **eerste gedeelte** (**Hoofdstukken 2 en 3**) beschrijft twee onderzoeken waarin gekeken is naar de biochemische constitutie van de hersenen van migraine patiënten door middel van proton magnetische resonantie spectroscopie (^{1}H-MRS) en diffusie gewogen spectroscopie (DWS). In het **tweede gedeelte** (**Hoofdstukken 4 en 5**) wordt een systematische review met meta-analyse beschreven om een overzicht te geven van eerdere inspanningen om in verschillende lichaamsvloeistoffen biochemische verschillen te detecteren die relevant zijn voor migraine. Daarnaast wordt er een studie beschreven waarin is gekeken naar biochemische verschillen in liquor van migraine patiënten en gezonde controles. In het **derde gedeelte** worden twee methodologische studies beschreven waarin (**Hoofdstukken 6 en 7**) de biochemische stabiliteit van liquor en het effect van vervuiling met ethanol werden onderzocht.

Deel 1: Biochemische profielen van de hersenen

Magnetische resonantie spectroscopie (MRS) is een techniek waarmee op een niet-invasieve manier de concentratie van verschillende biochemische stoffen in de hersenen gemeten kan worden. Deze techniek is gebruikt om de biochemische constitutie van meerdere hersengebieden te onderzoeken in mensen met verschillende migraine subtypes (**Hoofdstukken 2 en 3**). In Hoofdstuk 2 wordt een studie beschreven waarin biochemisch metingen zijn gedaan met ^{1}H-HMR in de hersenen van patiënten met hemiplegische migraine. Hemiplegische migraine is een zeldzaam subtype van migraine met aura en wordt gekarakteriseerd door een hemiparese tijdens de aura fase van de migraine aanval. Deze patiënten kunnen daarnaast ook een progressieve cerebellaire ataxie en atrofie ontwikkelen,

dit is met name het geval in patiënten met familiaire hemiplegische migraine type 1 (FHM1). FHM1 wordt veroorzaakt door een mutatie in het *CACNA1A* gen dat codeert voor een onderdeel van spannings-afhankelijk calcium kanalen. Er is gezocht naar biochemische verschillen tussen patiënten met hemiplegische migraine en gezonde controle personen in vier hersengebieden: het cerebellum, de hypothalamus, de occipitale cortex en de pons. Daarnaast is er ook gekeken naar verschillen tussen patiënten met hemiplegisch migraine met en zonder *CACNA1A* mutatie.

In patiënten met hemiplegische migraine was het totaal N-acetyl aspartaat (tNAA) in het cerebellum verlaagd in vergelijking met gezonde controles. De subgroep van patiënten met FHM1 had de laagste hoeveelheid tNAA in het cerebellum. tNAA wordt beschouwd als een indicator voor neuronale integriteit en een verlaging van tNAA in het cerebellum suggereert daarom een verlies van neuronen met als gevolg een dysfunctie van het cerebellum in hemiplegische migraine patiënten. In de andere drie hersengebieden, welke elk betrokken zijn bij verschillende fases van een migraine aanval, vonden we geen verschillen in biochemische stoffen tussen patiënten met hemiplegische migraine en gezonde controles.

De neurotransmitter glutamaat speelt waarschijnlijk een belangrijke rol in de migraine pathofysiologie, in het bijzonder in FHM. Gebaseerd op dierexperimenteel onderzoek is de hypothese ontstaan dat de intra- en extracellulaire glutamaat concentratie in de hersenen van patiënten met migraine mogelijk verhoogd is. Op het moment dat de MRS studie in patiënten met hemiplegische migraine werd uitgevoerd (**Hoofdstuk 2**) was het technisch niet mogelijk om glutamaat van glutamine van elkaar te onderscheiden. Daarom werd de totale concentratie van glutamaat plus glutamine (Glx) gerapporteerd, echter, we vonden geen verschillen in Glx tussen patiënten met hemiplegische migraine en gezonde controles.

In **Hoofdstuk 3** hebben we specifiek de occipitale glutamaat concentratie en diffusie karakteristieken bepaald in migraine patiënten met en zonder aura buiten een aanval en vergeleken met gezonde controles. Met een technisch verbeterde ^{1}H-MRS sequentie op een scanner met een hoge magnetische veldsterkte (7 tesla) was het mogelijk om glutamaat en glutamine op een nauwkeurige wijze van elkaar te onderscheiden. De glutamaat concentratie in de visuele cortex was hoger in patiënten met migraine zonder aura in vergelijking met gezonde controles. Er was echter geen verschil in de glutamaat concentratie tussen de migraine patiënten met aura en gezonde controles. De diffusie karakteristieken van glutamaat verschilden

niet tussen migraine patiënten en gezonde controles – wat suggereert dat er geen duidelijke verschillen zijn in intracerebrale mobiliteit van glutamaat tussen migraine patiënten en gezonde controles en, zij het indirect, geen verschillen in de distributie van glutamaat over verschillende intracellulaire compartimenten. We vonden geen relatie tussen de occipitale glutamaat concentratie en verschillende klinische parameters zoals migraine ernst en frequentie, visuele gevoeligheid en de afstand in tijd tot een volgende migraine aanval.

Deze studie versterkt het concept dat het glutamaterge systeem betrokken is in mechanismen die leiden tot migraine. Het blijft echter onduidelijk waarom de glutamaat concentratie niet hoger was in patiënten met aura, ondanks het feit dat excitatoire functie van glutamaat een belangrijke rol lijkt te spelen in cortical spreading depression, het onderliggende mechanisme van de migraine aura. De formatie en degradatie van glutamaat heeft echter ook een belangrijke rol in het energie metabolisme van de hersenen. Glucose wordt voor een deel omgezet in glutamaat voordat het verder wordt geoxideerd in de citroenzuur cyclus. Het zou dus ook kunnen dat de verhoogde glutamaat concentratie in patiënten met migraine zonder aura meer gerelateerd is aan metabole processen in de hersenen dan aan de excitatoire functie van glutamaat. Dit is een interessant onderwerp voor verdere studie.

Deel 2: Biochemische profielen van liquor

Liquor is een belangrijke lichaamsvloeistof voor het bestuderen van biochemische processen die betrokken zijn bij verschillende aandoeningen van de hersenen zoals bijvoorbeeld migraine. Er zijn in het verleden verschillende stoffen gemeten in het liquor van patiënten met migraine, echter, een helder overzicht van deze verschillende stoffen en een beoordeling van de relevantie voor migraine ontbrak in de migraine literatuur. Daarom hebben we een systematisch review met meta-analyse uitgevoerd, beschreven in Hoofdstuk 4, met als doel het identificeren van biochemische stoffen die door meerdere studies consistent veranderde concentraties laten zien in het liquor van migraine patiënten. Aanvullend hebben we gekeken of de stoffen die consistent afwijkend waren in het liquor ook hetzelfde patroon lieten zien wanneer ze in bloed werden gemeten.

In totaal werden er 63 unieke stoffen geïdentificeerd in 40 verschillende studies. De belangrijkste en meest consistente bevindingen in liquor waren

verhoogde concentraties van glutamaat, calcitonin gene-related peptide (CGRP) en nerve growth factor (NGF) in chronische migraine patiënten en een verlaagde concentratie van β-endorphin (β-EP) in zowel chronische als episodische migraine patiënten. De concentraties van glutamaat (interictaal) en CGRP (interictaal, ictaal, en in chronische migraine) waren ook verhoogd in bloed en de concentratie van β-EP was ook verlaagd (interictaal, ictaal, en in chronische migraine. Beoordeling van de methodologische kwaliteit van de geïncludeerde studies liet ook zien dat de steekproefgrootte van de meeste studies relatief klein waren en dat de kwaliteit van rapportage en gebruikte meetmethoden vaak beperkt was. Onze review met meta-analyse heeft duidelijk laten zien dat ons begrip van de biochemische processen die betrokken zijn bij migraine nog steeds verre van compleet zijn. Toekomstig onderzoek zou daarom gericht moeten zijn op het repliceren van eerder gerapporteerde bevindingen in grotere studie populaties en daarna moet er gekeken worden naar bredere biochemische profielen om zo mogelijk nieuwe biochemische stoffen te identificeren die belangrijk zijn in de pathogenese van migraine.

Het doel van **Hoofdstuk 5** was het identificeren van biochemische stoffen in het liquor die mogelijk relevant zijn in de pathofysiologie van migraine. We hebben daarvoor gekozen voor een expolerende metabolomics benadering met behulp van ^{1}H-NMR. Patiënten met hemiplegische migraine, migraine met en zonder aura en gezonde controles werden geïncludeerd in deze studie. Multivariate statistiek liet zien dat het mogelijk was om hemiplegische migraine patiënten te onderscheiden van gezonde controles op basis van hun metabole profiel. De stof 2-hydroxybutyric acid was het meest bepalende metaboliet voor het maken van dit onderscheid. Univariate statistiek liet zien dat de concentratie van 2-hydroxybutyric acid lager was in patiënten met hemiplegisch migraine vergeleken met gezonde controles. 2-Hydroxybutyric acid is betrokken bij energie metabolisme. Een veranderd energie metabolisme is eerder geobserveerd in FHM patiënten in zowel de hersenen als in spieren, een verandering in 2-hydroxybutyric acid zou hier bij kunnen passen.

Deel 3: Praktische overwegingen voor liquor afname

Voor het bestuderen van biochemische profielen in liquor kunnen verschillen metabolomics technieken gebruikt worden. Massaspectrometrie technieken

zijn het meest sensitief en in staat om stoffen te meten die aanwezig zijn in micromolaire concentraties. Het is daarom erg belangrijk om verschillende externe bronnen die variatie in de metingen kunnen introduceren te minimaliseren door de afname, verwerking en langdurige opslag van liquor zoveel mogelijk te standaardiseren. Het doel van **Hoofdstuk 6** was om te onderzoeken wat het effect was van vijf verschillende afname en verwerkingsprotocollen op de concentratie van verschillende endogene amines in CSF en om te bepalen welk protocol resulteert in het meest stabiele biochemische profiel. Daarnaast hebben we ook specifiek gekeken naar de effecten van de temperatuur tijdens afname en verwerking, het centrifugeren van samples en het gebruik van ethanol als additief om enzymatische reacties te stoppen. Om enige degradatie van stoffen te kunnen monitoren hebben we isotoop gelabelde stoffen toegevoegd aan de afnamebuizen voorafgaand aan de liquor afname. Er was geen duidelijk verschil in de stabiliteit van de metabolieten tussen de protocollen. De degradatie van de isotoop gelabelde stoffen was zeer laag, in het bijzonder de omzetting van glutamine naar glutamaat, en niet verschillend tussen de protocollen. In deze studie hebben we laten zien het effect van verschillende verwerkingsstappen minimaal is en dat het metabole profiel van endogene amines stabiel is mits de liquormonsters direct na afname gekoeld en gecentrifugeerd worden en binnen twee uur worden opgeslagen bij -80 °C. Dit betekend dat de bijna 300 liquormonsters van migraine patiënten en gezonde controles die wij op een gecontroleerde manier hebben verzameld in de afgelopen jaren een stabiel metabool profiel hebben.

Ondanks strikte naleving van richtlijnen voor liquorafname zagen we variërende hoeveelheden ethanol in de ^{1}H-NMR spectra van het liquor van migraine patiënten en gezonde controles. In **Hoofdstuk 7** wordt een studie beschreven die erop gericht was om de oorsprong van deze ethanol contaminatie te achterhalen. Ethanol bevattende desinfectantia worden veel gebruikt in ziekenhuizen voor het ontsmetten van handen, objecten en oppervlaktes, maar ook voor desinfectie van de huid voorafgaand aan de lumbaal punctie. Van elke deelnemer aan onze studie naar biochemische veranderingen in liquor werd liquor verzameld in drie verschillende afnamebuizen die elk op verschillende manieren zijn verwerkt. In twee van deze verwerkingsprotocollen, maar niet in de monsters gebruikt voor ^{1}H-NMR analyse, werd ethanol gebruikt als additief om proteïnes te denatureren. In Hoofdstuk 7 hebben we laten zien dat ethanol makkelijk via de lucht in liquor monsters terecht kan komen. Liquor wordt afgenomen met behulp van een open afnamebuis waar het liquor rechtstreeks van de naald in druppelt,

dit in tegenstelling tot bloed waarvoor vaak een gesloten (vacuüm) systeem gebruikt wordt. Dit open afname systeem zorgt ervoor dat ethanol makkelijk in CSF terecht kan komen wanneer ethanol aanwezig is in de ruimte waar liquor wordt afgenomen. De ethanol contaminatie heeft een duidelijk effect op de matrix van het monster als het in hoge concentraties aanwezig is en daarnaast wordt een deel van het ^{1}H-NMR spectrum overschaduwd door het signaal van ethanol. We hebben daarom geadviseerd om liquor afname en verwerking te verrichten in een goed geventileerde ruimte en dat het gebruik van ethanol beperkt moet worden als de monsters gemeten moeten worden met ^{1}H-NMR.

In de algemene discussie in **Hoofdstuk 8** worden onze biochemische bevindingen in breder perspectief geplaatst. De relevantie van de verschillende biochemische veranderingen die een rol spelen bij migraine wordt bediscussieerd en er worden suggesties gedaan voor toekomstig onderzoek naar de biochemische processen die betrokken zijn bij migraine.

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Curriculum Vitae

Ronald Zielman was born on February 19th 1983 in Hasselt, the Netherlands. He attended the Greijdanus College in Zwolle for pre-university education where he graduated in 2001. In the same year he moved to Groningen to study medicine at the Rijksuniversiteit Groningen. During his study he received a studentship from the Junior Scientific Master Class to perform research on the interaction between force production and cognitive performance in humans. He held the position of student chairman of the participation council and was member of the exams committee of the medical faculty. During his study he worked as a medical research assistant at the Pharma Bio-Research Group, Zuidlaren. In 2008 he performed his master's research at the Department of Neurology at the Isala Clinics in Zwolle studying the pharmacological treatment of migraine patients before and after referral to a neurologist in a retrospective study. He did his final clinical internship at the Department of Neurology at the Isala Clinics in Zwolle and obtained his medical doctor degree in 2009.

Subsequently, he started working as a PhD candidate at the Department of Neurology at the Leiden University Medical Center under supervision of professor M.D. Ferrari, professor A.M.J.M. van den Maagdenberg, and Dr. G.M.Terwindt. The aim of his research was to find migraine related biochemical profiles and potential biomarkers in CSF of migraine patients using metabolomics and proteomics in collaboration with the Netherlands Metabolomics Centre and the Biomolecular Mass Spectrometry unit of the Leiden University Medical Centre. He also conducted two magnetic resonance spectroscopy studies to investigate the biochemistry of the migraine brain in collaboration with the C.J. Gorter Center for High-field MRI at the Leiden University Medical Center.
During his PhD period he did two clinical internships as a resident physician in neurology to obtain more experience in clinical neurology. Since 2016 he is working at GlaxoSmithKline in Zeist as a medical scientific liaison at the medical affairs department

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