Biochemistry in different phases of the migraine attack

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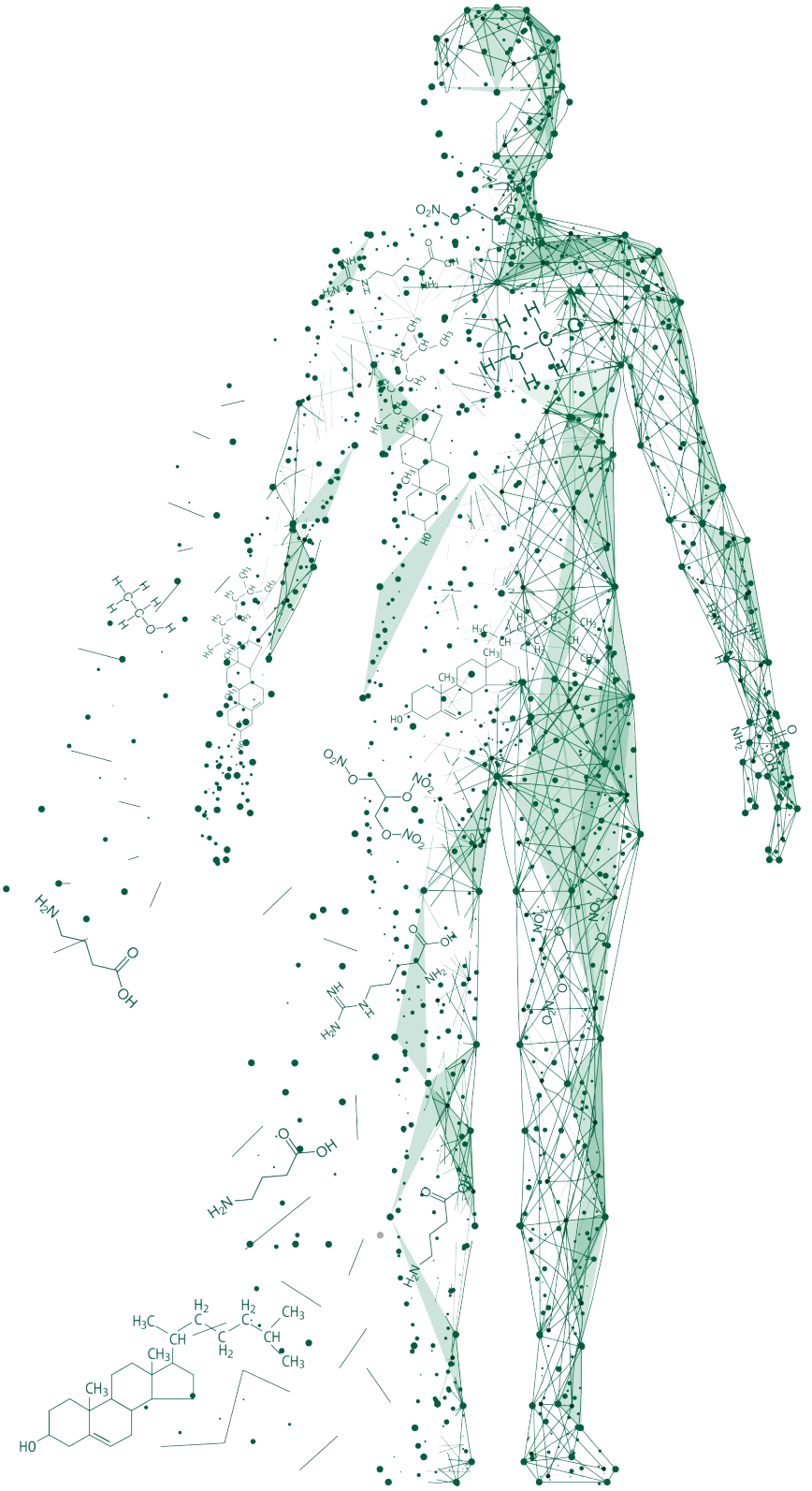

Chapter 7

General Discussion & Future Perspectives

Migraine attacks strike unexpected. Despite that much has been accomplished in expanding knowledge regarding what is happening in the brain during the migraine aura and the headache, there is still much work to do in order to understand why attacks are provoked and which pathophysiological processes are implicated in attack provocation and early phases of attacks. In addition, for clinical care important comorbidities associated with migraine,[1] such as cardiovascular disease (e.g. stroke, and myocardial infarction),[2,3] and psychiatric disorders (e.g. depression and anxiety),[4] need to be taken into account. However, knowledge regarding the biochemical basis of the migraine aura, the headache, and the associated comorbidities is lacking, in particular, the mechanisms behind the triggering and initiation of attacks remain to be unraveled.

By expanding knowledge on migraine biochemistry and identifying biomarkers we may gain further insight regarding migraine pathophysiology and its associated comorbidities. These insights might in time be used to either predict or monitor treatment response, may serve for diagnostic purposes for migraine or its complications (such as risk for stroke or cardiovascular disease), or provide guidance for new treatment approaches. In migraine, as it is a paroxysmal neurovascular brain disorder, most of the biochemical research has been conducted outside attacks (interictal) and in easily accessible biofluids such as blood and urine. However, likely more specific findings will be done when investigating the brain itself and associated cerebrospinal fluid (CSF), and preferably at time slots closely before (preictal) and during (ictal) attacks. However, although triggers[5,6] and premonitory symptoms[7–9] have been reported, investigating the preictal and ictal phase with elaborate and detailed measurements is very challenging as only few triggers consistently provoke attacks and in most patients premonitory symptoms also are not consistent enough to be used as an indication of when to measure. With this in mind, the aim of this thesis to measure during the different phases of migraine can be regarded as challenging. The studies were set up to measure metabolites in different matrices (plasma/CSF/brain). These studies used a range of techniques (proton nuclear magnetic resonance (^{1}H-NMR), ultra-performance liquid chromatography mass spectrometry (UPLC-MS), and proton magnetic resonance spectroscopy (^{1}H-MRS)) to explore possibilities of measuring biochemical profiles during the interictal, preictal and ictal phases of migraine attacks in patients.

Interictal biochemical migraine biomarkers

Interictal biochemical biomarkers in plasma

The search for biochemical biomarkers in this thesis focused on multiple biomarker types across the different studies and chapters. In **Chapter 2**, we used a ^{1}H-NMR-based metabolomics platform, order to quantify 146 individual metabolites (mainly

lipoproteins, lipids, and fatty acids) and 79 metabolite ratios in plasma of migraine patients from various Dutch cohorts encompassing in total 10,153 individuals. These mostly vascular metabolite measures are of interest because especially in women migraine has been linked to an increased risk for cerebrovascular and cardiovascular diseases.[2,3,10,11] The underlying mechanism for the association has been suggested to be systemic (micro)vascular dysfunction,[12,13] instead of atherosclerosis.[14,15] A decreased apolipoprotein A1 (apoA1) level and free cholesterol to total lipid ratio present in small high-density lipoprotein subspecies (HDL) were found to be associated with migraine status. Of note, a decreased omega-3 fatty acid level was found associated with migraine status, however, only in male participants. A pathway analysis further supported that HDL traits (medium (M-) to very large (XL-)HDL subclasses) generally associated with migraine status across the majority of cohorts, but not other lipoproteins appear associated with migraine status.

HDL is composed of lipids and proteins, each one roughly representing 50% of the total mass (figure 1). Major proteins are apoA1 (70%) and apoA2 (20%) that together with proteins, such as apoA4, apoE, apoJ, lecithin cholesterol acyltransferase, paraoxanase, haptoglobin, and α2-macroglobulin make up the protein content of HDL particles.[16] The proteins play a role in the various functions of HDL, among other things the reverse cholesterol transport pathway and anti-oxidative, anti-inflammatory, and anti-thrombotic effects.[17] While atherogenic lipoproteins, such as low-density lipoprotein (LDL), intermediate-density lipoprotein (IDL) and very-low-density lipoprotein (VLDL), carry apolipoprotein B instead. Combining the various analyses described in **Chapter 2**, we identified an association between migraine and lowered HDL measures, in the absence of clear LDL, IDL, or VLDL involvement supported by no clear association of apoB with migraine. This suggests that migraine is associated with alterations in aforementioned specific HDL functions but not a general dyslipidemia profile as is characteristic for cardiovascular conditions. Hypothesizing our data may provide some biochemical evidence for a link with endothelium dysfunction in migraine as HDL with its anti-inflammatory, anti-oxidative, and anti-thrombotic effects plays a role in endothelial function.[16–18] HDL has been shown to stimulate nitric oxide release from human endothelial cell cultures and increase endothelial nitric oxide synthase (eNOS). It also it suppresses the expression of adhesion molecules (i.e. vascular cell adhesion molecule 1), and inhibits the adhesion of white blood cells. Furthermore, through reducing platelet activation and tissue factor expression in endothelial cells exposed to cytokines HDL also has anti-thrombotic effects.[19] Whether or not migraine is associated with endothelial dysfunction remains to be elucidated with research yielding mixed results.[18,20,21] As the study in **Chapter 2** was designed for biomarker detection rather than to provided pathophysiological insight it is at present only speculation whether reduced protective actions of HDL by means of endothelial dysfunction may explain the

association with migraine. On the other hand, it has been suggested that omega-3 fatty acids and certain HDL subclasses can travel across the blood-brain barrier, and may have effects at the neuronal level.[22–24] In order to yield robust results eight different cohorts from across the Netherlands were included in **Chapter 2** and results were analyzed using a meta-analysis. However, this approach limited the specificity in migraine diagnosis and no separate results for migraine with and without aura could be obtained.

In **Chapter 3** we measured three study populations composed of interictal migraine patients diagnosed with migraine with and without aura (N = 99 and N = 98, respectively) and healthy volunteers (N = 96). As this was a single-cohort-study, participants were matched for sex, age, and timing of sampling in order to limit the influence of diurnal fluctuations. Apart from plasma, CSF was also sampled at the same patient visit to the Leiden Headache Center. A technique called UPLC-MS was used to assess 31 individual amine levels, allowing the study of global amine profiles, and specific amine pathways. Amines were measured because well-known amines, such as glutamate, glutamine, gamma-aminobutyric acid (GABA) and serotonin, have been frequently implicated in migraine pathophysiolology.[25–27] In **Chapter 2** a limited selection of eight amines was included that revealed no individual metabolite measures associated with migraine in CSF. In **Chapter 3** we also found no individual amine differences in plasma between study groups and therefore no specific amine associated with either migraine with or without aura. Confirming our results for the eight amines (alanine, phenylalanine, leucine, isoleucine, valine, histidine, tyrosine, and glutamine) previously measured in **Chapter 2**. However, the larger number of amines measured in plasma in **Chapter 3** allowed for a more elaborate multivariate analysis. This analysis revealed that global amine profiles (taking into account all 31 amines measured) associated with migraine with aura and without aura were different from the profile of healthy controls, while migraine with and without aura profiles did not differ from each other. When tested, unfortunately these profiles lacked sufficient power to discriminate between controls and the two migraine subtypes and cannot be used as a diagnostic biomarker at the individual patient level. When investigating different pathways the strongest associated pathways were ‘Arginine biosynthesis’ (i.e. comprised of trans-4-hydroxy-L-proline, proline, glutamate, GABA, ornithine, and putrescine (measured in **Chapter 3**)) and ‘Arginine and proline metabolism’ (i.e. comprised of glutamine, glutamate, ornithine, citrulline, and arginine (measured in **Chapter 3**)) with migraine with aura and migraine without aura, respectively. In **Chapter 3**, apart from plasma, CSF was also sampled and measured using the same technique, outcomes of which are presented below.

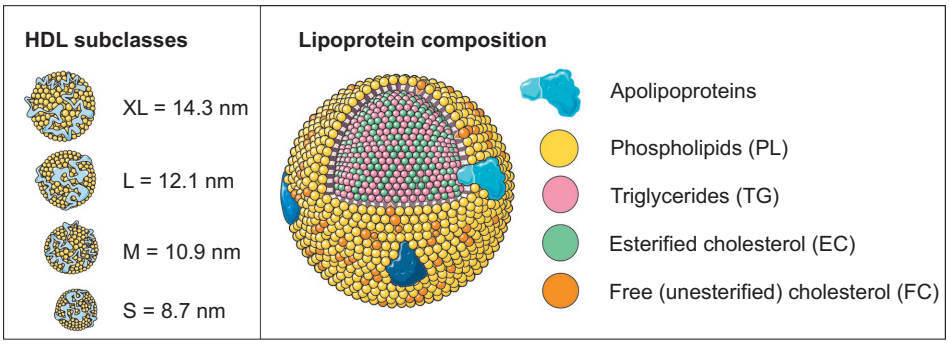

Figure 1. High-density lipoprotein subclasses and composition. The ^{1}H-NMR platform differentiated four subclasses of HDL: very large (XL; 14.3 nm), large (L; 12.1 nm), medium (M; 10.9 nm), and small (S; 8.7 nm) HDL. Furthermore, ratios were provided for the contribution of each particle component. C=total cholesterol, EC=esterified cholesterol, FC=free cholesterol, HDL=high-density lipoprotein, L=lipids, PL=phospholipids, TG=triglycerides.

Interictal biochemical biomarkers in CSF

In contrast to plasma, CSF is in direct contact with the brain interstitial fluid and likely better reflects the biochemical changes that occur in the (diseased) brain, instead of being a reflection of the overall metabolic state of the participant (as is the case for plasma/serum), making it a more appropriate biofluid to examine metabolic consequences in central nervous system disorders.[28–30] In CSF, we found a 10.4% lower arginine level in migraine with aura and a 5.0% lower level in migraine without aura, when compared to healthy volunteers. In addition, phenylalanine levels were 6.9% lower in migraine with aura and 8.1% lower in migraine without aura compared to healthy controls when the less strict False Discovery Rate (FDR) correction was used. Like in plasma, the global CSF amine profiles associated with migraine with aura and without aura were different from the profile of healthy controls. While migraine with and without aura profiles did not differ from each other, similar to arginine and phenylalanine levels which also did not differ between migraine subtypes. Implying that the pathophysiological mechanism giving rise to these effects may apply to migraine with and without aura. Unfortunately, again, the individual amines (arginine and phenylalanine) and CSF amine profiles lacked sufficient power to discriminate between controls and the two migraine subtypes, and therefore cannot be used as a diagnostic biomarker. In CSF, strongest associated pathways were the same as found in plasma ('Arginine biosynthesis' for migraine with aura and 'Arginine and proline metabolism' for migraine without aura), however, these were now significantly associated while in plasma this as not the case.

Although arginine has been measured in relation to migraine in serum, plasma, platelets, saliva, and urine, with inconsistent findings across and within biofluids, it had never been measured in CSF.[26,31–35] The greatest pool of arginine in the central nervous system

(CNS) is present in astrocytes, less prominent in neuronal tissue, while not present at all in oligodendrocytes.[36] Numerous functions have been attributed to arginine, such as endocrine activity, immune system modulation, regulation of vascular tone, and as a proteinogenic amino acid it is also used for peptide and protein production in the CNS.[36] Metabolism of arginine is closely connected with the metabolism of citrulline and ornithine, which can be synthesized and degraded into eachother.[36] Pathway analysis revealed that although the results seem primarily driven by arginine, there was still an association between migraine and ´Arginine biosynthesis´, which includes citrulline and ornithine, when arginine was excluded from the analysis. Arginine can be catabolized by to arginine decarboxylase to agmatine, by arginine:glycine amidonotransferase to guanidonoacetic acid (ultimately forming creatine), or to by arginase forming urea and ornithine.[36] However, these metabolites could not be measured on the platform used in **Chapter 3** and were, therefore, not included in the pathway analysis.

In peripheral tissues, the urea cycle takes a central place in arginine metabolism that deals with an excess of nitrogen and the detoxification of ammonia. In neuronal and glial tissues arginine is able to be synthesized through argininosuccinate from the citrulline- nitric oxide cycle, an urea cycle shortcut. Nitric oxide production is catalyzed by nitric oxide synthase (NOS), for which arginine serves as the only available nitrogen-containing substrate.[36] There are three isoforms of NOS: endothelial (e), neuronal (n), and inducible (i) NOS.[36] The observed lower arginine levels in CSF could potentially reflect an overactivity of nNOS. Blocking NOS with a non-selective inhibitor was shown to be effective against spontaneous migraine attacks,[37,38] while trials with specific inhibition of iNOS failed and those with nNOS inhibition and combined 5-hydroxytryptamine 1B/1D (5-HT1B/1D) receptor agonist showed mixed results.[38–40] As extracellular CSF arginine was measured in **Chapter 3**, and since arginine is involved in a variety of functions, we cannot conclude on the intracellular status of nitric oxide (whether elevated or decreased) in the present study. However, our findings offer support that nitric oxide signaling is involved in migraine pathophysiology, also outside attacks. This is of interest as nitric oxide released by glyceryl trinitrate (GTN), a nitric oxide donor, can provoke migraine-like headaches in migraineurs (both in migraine with aura and migraine without aura), but not in healthy controls.[40] Nitric oxide released by GTN activates, among other things, intracellular soluble guanylate cyclase which catalyzes the formation of cyclic guanosine monophosphate (cGMP). cGMP is an important second messenger involved in the activation of various protein kinases, implicated in smooth muscle relaxation and vasodilatation.[6,40] Migraine without aura patients that develop a migraine-like attack after infusion with GTN have been shown to develop stronger systemic cardiovascular responses during infusion compared to healthy controls (more rapid and longer-lasting decreased cardiac output and stroke volume while heart rate was increased, mean arterial pressure and total peripheral resistance were higher and decreased steeply

after an initial increase).[41] Another study into response to GTN between migraine without aura, episodic tension-type headache patients, and healthy controls, found more noticeable arterial blood velocity (V_{mean}) responses following infusion in migraine patients compared to the other groups.[42] So even well before the onset of a migraine response to a strong vasodilator was shown to be more pronounced in migraine without aura patients. This suggest an increased systemic sensitivity to GTN, although the reason behind this still remains to be elucidated. Speculating, several distinct or combination of mechanisms might be in play: (1) insufficient autonomic compensatory mechanisms, (2) higher perivascular concentrations of NOS, and/or (3) chronically disturbed nitric oxide signaling. Of note, other compounds with vasodilative properties have been shown to be effective in provoking migraine-like headache such as: calcitonin gene-related peptide (CGRP), prostaglandins (Prostaglandin (PG) D_2, -$I_{2,}$ and -E_2), pituitary adenylate cyclase activating polypeptide-38 (PACAP38), histamine, and cilostazol. However, another well-known pharmacological provocation model with sildenafil provokes migraine-like headache without preceding vasodilatation,[40,43] and vasoactive intestinal peptide (VIP) giving extensive vasodilation proved which unsuccessful in provoking attacks in one study[44] and provoked attacks in 14% of cases in another study.[45] This evidence suggests a complex relationship between migraine-like attack onset and vasodilation, where vasodilation is perhaps an introductory component or a side-effect of a pathway modulating a final common pathway leading to migraine-like attack onset.

Transitioning from interictal to preictal and ictal

A consistent provocation model with migraine-like attacks resembling spontaneous attacks

Clinical manifestation of migraine is thought to be made up by non-modifiable genetic factors (up to 60%)[5,46–48] and modifiable non-genetic factors (around 40%) such as internal (e.g. hormonal fluctuations and comorbid diseases) and external (sleep pattern, alcohol and food consumption, and fatigue) risk modulation factors.[27,49,50] The role of these external risk modulation factors, also called trigger factors, is quite variable within a particular trigger and between different triggers.[5,51–55] Complicating the association between triggers and provoked attacks are premonitory symptoms, which are numerous and include concentration difficulties, tiredness/fatigue, food cravings, irritability, yawning, photophobia, and neck stiffness, and are regularly mislabeled by patients as triggers.[5,9,56–59] In **Chapter 4** we investigated alcoholic beverages as a self-reported migraine trigger factor and in **Chapter 5** we studied reported premonitory symptoms following attacks triggered using the pharmacologic trigger GTN in migraineurs compared to symptoms reported by healthy controls.

In **Chapter 4** we observed that alcoholic beverages were reported by 35.6% of migraineurs as a trigger for their attacks. When given the option between wine (red/white), champagne, beer, whisky, vodka, rum or another type of alcoholic beverage; wine, especially red wine, was recognized as the most common trigger (77.8%) by the migraineurs and vodka the least common (8.5%). Time of onset to attack was rapid (within three hours) in one-third of patients and almost 90% had an onset within ten hours independent of beverage type (red wine or vodka). This rapid onset in one-third of patients is in-line with several smaller studies,[51,60] which suggests a mechanism distinct from hangover headache (termed delayed alcohol-induced headache in the ICHD-3) in which pain typically occurs when the blood ethanol level is declining,[61,62] and may represent a true trigger. However, the potency of these beverages to provoke an attack consistently (every time) was reported to be rather low and comparable between red wine (8.8%) and vodka (10.7%). This triggering inconsistency is not limited to our study as it is also reported by prospective studies.[51,53–55,63] This low consistency does not have to contradict the potential triggering effect of alcoholic beverages because this may depend also on the variable susceptibility status that make migraineurs less or more prone to having an attack. Internal risk modulation factors, such as fluctuations in neuronal excitability creating a fluctuating trigger threshold,[56,64] likely influence susceptibility status as well, as does the co-occurrence of other external triggers.[63,65]

Opposed to alcoholic beverages with a reported triggering consistency of around 10% in selected patients we used the pharmacologic trigger GTN with a reported triggering potential of around 80% in selected patients to study reported premonitory symptoms in migraineurs compared to symptoms reported by healthy controls in **Chapter 5**. Indeed, GTN was found a be a potent trigger with attacks provoked in 84.2% of migraineurs participating in our study. Soon after infusion with GTN symptoms like yawning, fatigue, thirst, neck stiffness, concentration difficulties, nausea, and photophobia, also sometimes deemed triggers, were frequently reported. However, focusing on migraineurs who developed migraine-like headaches, yawning, nausea, photophobia, and concentration difficulties were frequently mentioned and thus most specific. While fatigue, thirst, and neck stiffness were also regularly reported healthy controls. When relating the specific symptoms to brain areas, this may indicate involvement of the hypothalamus (yawning, nausea), brainstem (nausea), thalamus (photophobia) and certain cortical areas (photophobia, concentration difficulties) in the early stages of an attack, prior to the onset of migraine-like headache.[57,66] These symptoms, as we also found in our study, persist during ictal and postictal phases.[8,67,68] Thereby, substantially lengthening the duration of attacks and making migrainous headache only one of the symptoms in the intricate cascade. This gave rise the hypothesis of an oscillating system of complex brain-networks, perhaps involving the hypothalamus, brainstem, occipital cortex and certain cortical areas identified in **Chapter 5**, which influence the susceptibility threshold of

sensory signals and bodily functions towards the onset of migrainous headache.[69–71] Evidence from natural[55] and pharmacological triggers[6,43] in provocation studies may suggest external risk modulation factors, such as also reported by migraineurs for alcoholic beverages in **Chapter 4**, work either parallel with or on their own. These factors may influence aforementioned brain areas to modify the susceptibility threshold, or as previously postulated their effectiveness may be determined by the susceptibility threshold itself.

At the neurotransmitter level, the dopaminergic system might well be a driving force, as dopaminergic agonists have been reported to increase symptoms as yawning and nausea in migraine patients found in **Chapter 5**, while dopaminergic antagonists are used to treat nausea.[70] Unfortunately, similar to other CSF migraine studies,[72] dopamine could not be measured in CSF or plasma using the techniques employed in **Chapter 3**. Our research in **Chapter 3** did however identify lowered phenylalanine CSF levels for both migraine subtypes. Of note, phenylalanine can be hydrolyzed into tyrosine that can subsequently be formed into dopamine. However, as humans cannot synthesize phenylalanine *de novo*,[36] concentrations are fully dependent on dietary protein intake. Although all participants were fasting, we cannot exclude long-term dietary intake differences. Furthermore, no difference was observed in tyrosine, which, like phenylalanine, also has a dietary component.[36] Therefore, these findings must be interpreted with caution and no conclusion on dopamine can be drawn.

Preictal and ictal biochemical migraine biomarkers

Studying the ictal and especially the preictal phase of spontaneous migraine attacks is very challenging. Attacks strike unexpectedly and related disability frequently inhibits patients from traveling to the hospital or if they manage, at this stage the attack is already in the ictal phase[43], so the transition from the interictal to the preictal phase is missed. Fortunately, experimental human migraine models, such as intravenous GTN infusion[6,43] can be used to investigate attack initiation under precisely monitored and controlled conditions.[40,43] In both GTN-provoked (see **Chapter 5**) and spontaneous attacks patients experienced premonitory symptoms, and activation of hypothalamus, occipital cortex and brainstem have been demonstrated,[27,73–78] which illustrates the applicability of this model to study the early processes involved in the onset of attacks.

In **Chapter 6** we used this model, in a subpopulation of migraineurs (N = 23) and healthy controls (N = 14) of **Chapter 5** to measure the concentration of glutamate, GABA, glutamine in the visual cortex. We measured using single-volume ^{1}H-MRS at 7 Tesla before and over the course of a provoked attack to detect possible involvement of the

glutamatergic system in the onset of attacks. Glutamate and glutamine levels did not change from interictal to the preictal and ictal state. Importantly, GABA levels increased from interictal towards the preictal state for migraine patients compared with healthy controls. Increased GABA levels in interictal migraineurs have been reported, while no elevation was found in musculoskeletal pain and other chronic pain syndromes in a meta-analysis.[79] In **Chapter 3** no elevation was found in CSF of plasma of interictal migraine patients, while ictally GABA levels appear to be elevated in CSF.[72] This suggest a role of GABA in the migraine pathophysiology. The detected increased preictal GABA levels, in **Chapter 6**, may reflect a compensating mechanism to reduce a brain hyperexcitatory state and/or may reflect a protective role for GABA in suppressing headaches.[80,81]

There are several possible explanations why changes in glutamate levels were not detected during attack initiation. Although indirect biochemical evidence revealed elevated CSF glutamate levels in patients with chronic migraine and elevated blood glutamate levels in patients with episodic migraine.[72] In **Chapter 3**, interictally no change in glutamate or its precursor glutamine levels were detected in interictal migraine with and without migraine patients compared of healthy controls in CSF and plasma. Two studies measured Glx (combined glutamine and glutamate signal and concentration), in the visual cortex (migraine with aura) and pons (migraine without aura) during provoked migraine-like attacks through hypoxia, CGRP and sildenafil, revealed no change in Glx levels.[82,83] Similar to our observation that glutamate and glutamine levels did not change in the preictal or ictal state of GTN-provoked migraine-like attacks.

Furthermore, glutamate level changes in migraine with aura attacks still cannot be fully excluded since only female migraine without aura patients were included in the research of **Chapter 6.** However provocation studies with GTN have only rarely been able to provoke aura symptoms in patients with migraine with aura,[84,85] and even migraine-like headache in patients with hemiplegic migraine.[40] This suggests that the effect of the nitric oxide donor GTN is through another pathophysiological cascade of events, able to reliable trigger/initiate the onset of premonitory symptoms as seen in **Chapter 5** and migraine-like headache (**Chapters 5 and 6**) but not migraine aura, which is likely caused by CSD.[27] The following pathway to provoke migraine-like headache has currently been proposed for GTN. Nitric oxide released by GTN activates intracellular soluble guanylate cyclase, which catalyzes the formation of cGMP, which is an important second messenger involved in the activation of various protein kinases, implicated in smooth muscle relaxation and vasodilatation.[40] In CSD induction and/or propagation glutamate is suspected to bind to the *N*-methyl-D-aspartate (NMDA) receptor that may cause an increase in intracellular calcium.[86,87] Calcium in turn binds to calmodulin and activates neuronal nitric oxide synthase (NOS), which is also able to produce and increase nitric oxide levels.[86,87] The rise in nitric oxide level, may result in cGMP formation thought to

be involved in migraine-like headache, through activated intracellular soluble guanylate cyclase. This chain of thought may suggest that triggering migraine-like headache with GTN bypasses or modulates the glutamate-nitric oxide-cGMP pathway, which is suspected to play a role in activation through CSD, as the artificial attack triggering method directly engages nitric oxide. Likewise, sildenafil, which breaks down cGMP through selective inhibition of the enzyme phosphodiesterase 5 that is expected to cause cGMP accumulation,[40] did lead to a transient rise in brainstem Glx levels, however, in healthy controls independent of provoked headache.[88] While CGRP, another method to pharmacologically trigger migraine-like attacks, this time via the cyclic adenosine monophosphate (cAMP) pathway, did not induce Glx changes in the brainstem or thalamus.[88] Since in **Chapter 6** results were corrected for metabolite changes occurring in healthy controls, not related to migraine-like attack onset, these would not be detected and are in line with aforementioned hypothesis.

Future perspectives

The Five Ws in biochemical migraine research

Throughout this thesis different patient populations were explored at various times during the migraine cycle using different techniques to measure different metabolite measures in a range of matrices. This was done in order to gain experience and knowledge of migraine pathophysiology and to use this insight to make recommendations for future research. Lessons for future research learned during research performed for this thesis are best listed using the Five Ws: "Who, What, Where, When, and Why".[89]

Who?

When doing biochemical research into migraine it is important to define the research population as precisely as possible, resulting in a highly homogenous group, in order to be able to detect subtle metabolite differences associated with migraine status. This is especially important when looking into pathophysiological mechanisms of migraine. In order to be sure results are specific to migraine, especially when studying the ictal phase, inclusion of one or more control group(s) composed of other pain conditions is an option. Later, results can be verified in a more heterogenous population in order to be able to generalize findings. What ICHD-3 migraine diagnosis is to be investigated, e.g. migraine with aura, migraine without aura, or chronic migraine? As shown in this thesis, in **Chapter 2**, due to different cohorts being investigated, the most exact diagnosis used was "migraine" while in **Chapters 3 – 6** migraine with and without aura were used. While amines measured in **Chapter 3** revealed similar profiles between migraine with and without aura, individual results arginine and phenylalanine levels did not differ between migraine subtypes, although this unlikely will be the case for every metabolite studied.

When looking at CSF studies in migraine conducted so far only a small minority (<25%) were matched for age and sex.[72] **Chapters 2, 3 and 6** also revealed metabolites that are heavily influenced by age and sex in blood, CSF and the cortex. Frequently, like in **Chapter 3**, the effects of age and sex on the metabolite level are stronger than the effect of migraine status. This stresses the need for carefully matching for these variables and to also correct for these variables in the statistical analysis.

What?

Prior to sampling it is important to clearly define the purpose of the study, for instance whether it is a non-hypothesis-driven biomarker discovery study, like was the case in **Chapters 2 and 3**, in which the goal was to measure large numbers of compounds on one or a few platforms in a single study. Or whether it is a hypothesis-driven study, like was the case in **Chapter 6**, in which a limited number of compounds was measured based on a predefined hypothesis based on literature or previously conducted research. In line with this, what compound, for instance DNA, mRNA, protein or metabolite is the researcher interested in?[90] Certain conditions such a fasting status and time of day may influence modifiable compounds such as mRNA, proteins and metabolites but will not influence DNA. Furthermore, as different compounds require different sampling protocols also within a particular class depending on technical approach used, e.g. metabolites measured with ^{1}H-NMR (**Chapter 2**) and UPLC-MS (**Chapter 3**). Therefore, in case of sample collection for biobanking purposes robust sampling protocols also composed of various collection approaches should be employed.[91]

Where?

Defining the sample matrix in which measurements will be performed is very important as each will have its up- and downsides. Urine and saliva are perhaps the most easily attainable, with saliva being the most readily-available thereby allowing for frequent longitudinal sampling, paradoxically development of metabolomic analysis of saliva has been lagging.[92] While serum and plasma, for instance, are minimally invasively collected, allow for repeated sampling, with readily available sample processing and sample storage methods and protocols, can likely be used in multicenter studies (**Chapter 2**). Due to the rich blood metabolome, various platforms that have already been developed, possible biomarkers are likely more readily translated and validated to a clinical setting. Making this type of sample well-suited for large scale robust biomarker discovery studies. While the rich, more peripheral metabolome of blood measuring only known compounds with an industrial platform, might cloud and leave undetected more subtle pathophysiological aspects of migraine that may be investigated with hard to obtain CSF (**Chapter 3**) or even brain tissue. Compounds related to pathophysiological aspects of migraine may also be detected noninvasively, such as in **Chapter 6**, but by using complicated and expensive technological applications such as MRS.

When?

There is an apparent temporal fluctuation of the clinical phenotype in a paroxysmal disorder, as migraine. This means that patient may experience different types of attacks during lifetime (e.g. chronic versus episodic migraine, migraine with and without aura attacks). But is also due to the paroxysmal nature of migraine that patients are at different timepoints in different phases, either interictal, preictal, ictal, postictal. Besides that there is a continuous change of the internal threshold (e.g. due to menstruation in women when the threshold is likely lowered) and fluctuating external trigger factors. With all these changes it is likely that biochemical processes occurring in the background also fluctuate. In this thesis, the biochemical processes in the interictal (**Chapters 2 and 3**)**,** preictal (**Chapters 5 and 6**) and ictal (**Chapters 5 and 6**) phases were investigated. Fluctuations in the presence of premonitory symptoms between interictal, preictal and ictal phases were shown in **Chapter 5**, while fluctuations in neurotransmitter GABA were observed in **Chapter 6.** Therefore, clearly defining the migraine phase is not only essential when comparing research with available literature,[72,93] but also when forming a research question and designing a study and therefore should be carefully monitored prior to, during, and even after the study day.

Why?

If a study aims to discover pathophysiological mechanisms behind a trait such as migraine, for instance with the goal to distinguish subgroups of primary headache disorders from one another or from controls, or to dissect the molecular mechanism behind the onset of attacks, the main challenge is when to take biospecimens as it may be important to quantify the degree of change over time. This is different when the research question relates to finding a diagnostic biomarker or a marker to predict or monitor risk for chronification, or complications, or treatment response. All these different biochemical biomarker possibilities, influence the other aforementioned W's and it is therefore good to define as accurately as possible early in the design of study what is the actual goal of the study.

Future opportunities

Throughout this thesis diverse groups, using various methodological approaches in different phases of the migraine attack have been studied, which greatly enhanced the reach of the exploring the biochemical signature of migraine as a hole, but as a consequence limited the in-dept scrutiny and search for a more narrow and specific biomarker. As platforms and possibilities of multimodal examination continue to become more enhanced and extensive this will aid in-dept study using the aforementioned W's to find biomarkers to fill in knowledge gaps which may ultimately expand treatment possibilities.

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