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Oosterhout, W.P.J. van

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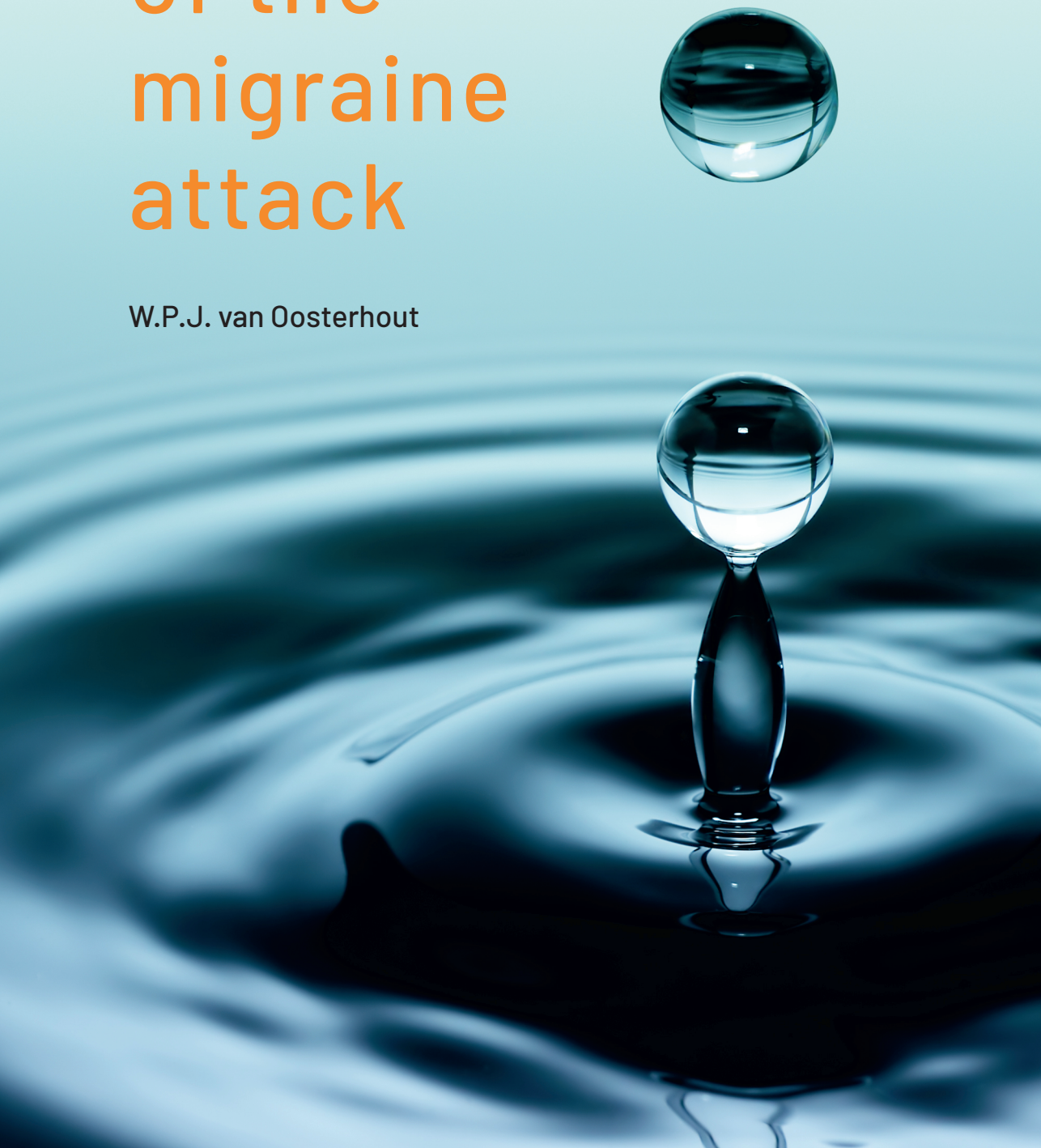

The onset of the migraine attack

Willebrordus Petrus Johannes van Oosterhout (roepnaam: Ron)

Colophon

Willebrordus Petrus Johannes van Oosterhout
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Chapter 1.

General introduction and aims of this thesis

Migraine

Migraine is a common, multifactorial neurovascular disorder characterized by recurrent, disabling attacks of severe and often unilateral headaches, accompanied by symptoms of photophobia, phonophobia, nausea and vomiting [1, 2]. In one third of patients, transient neurological symptoms, called migraine aura, precede the headaches [3]. When untreated, migraine attacks can last for several hours up to several days, and pose a large burden on patients especially when the attack frequency is high [4-6]. Migraine is a multiphasic disorder. The premonitory phase is the phase preceding the headache phase and - if present - the aura phase, and is considered to be the first phase of a migraine attack. Although the mechanisms behind the migraine headache and aura symptoms are reasonably well understood, the triggering mechanisms for the initiation of migraine attacks are unknown [7]. In recent decades, accumulating evidence has shifted the emphasis away from the vascular theory of migraine towards mechanisms of central nervous system activation originating from deeper brain regions such as the hypothalamus and the brainstem. These regions may play a pivotal role in the early phases of the migraine attack, as is suggested by evidence from preclinical, clinical, biochemical and imaging studies [8, 9].

Clinical symptoms during the four phases of a migraine attack

During the course of a migraine attack cycle, up to four different phases can be distinguished: i) the premonitory (or prodromal) phase; ii) the aura phase; iii) the headache phase; and iv) the postdromal phase. Clinically, there is great variability in phenotype between patients as is reflected by the large clinical heterogeneity over all the different phases of the migraine cycle. A more detailed overview of each of these four phases I will describe below.

The premonitory phase

The duration of the premonitory phase varies between patients and ranges from 2-72 hours before the aura and/or migraine headache starts [10]. In this phase a variety of general, non-specific, non-headache symptoms can occur, which are usually rather consistent within patients. These symptoms may provide an early warning signal for the upcoming migraine headache. The most frequently reported premonitory symptoms include fatigue, mood and cognitive changes, gastrointestinal symptoms, neck pain, yawning, temperature change, smell and taste distortion, food craving and appetite changes. Many of these symptoms are assumed to be of hypothalamic origin [11-14].

Despite being recognised in the literature for decades [15, 16], the pathophysiological relevance of premonitory symptoms and their clinical implications have been largely neglected [15]. It is unknown when and where in the brain functional or metabolic changes occur in the premonitory phase nor what these exactly are. In addition, there are no validated screening instruments for premonitory symptoms and they have a very subjective character, all hampering studying them properly. Exact prevalence rates therefore remain difficult to assess. Based on the scarce literature, it is estimated that over 80% of migraine patients experience a minimum of one premonitory symptom [3, 17].

The aura phase

The aura phase is present in approximately 30% of migraine patients [1, 18, 19] and is characterized by transient neurological deficits with a duration ranging from 5 until 60 minutes, of which at least one has a unilateral localization. In the majority of patients (75%), aura symptoms last for less than 30 minutes, and only 5% has auras lasting longer than 4 hours [20-22]. In migraine with aura patients, not every migraine headache needs to be preceded by an aura. Detailed prospective diary study work has shown that at least one of three auras last over an hour in up to 26% of patients [23].

The aura is considered the clinical correlate of cortical spreading depolarisation (CSD: formerly known as cortical spreading depression; see also section Neurobiology of migraine). Based on the specific cortical regions involved, several different aura symptoms can be distinguished: i) visual symptoms; ii) sensory symptoms; iii) aphatic speech disturbances; and iv) motor symptoms. The visual aura is the most common aura symptom, reported by 80-90% of patients with migraine with aura. Visual symptoms can vary from simple flashes or dots to fortification spectra (positive phenomena), scotomas (negative phenomena) or complex hallucinations with metamorphopsias [3]. Sensory symptoms usually are paraesthesias, which occur in 30% of patients with migraine with aura and show a preferential cheiro-oral distribution. Aphatic speech disturbances (17%) and motor weakness (10%) are less common. When patients experience multiple aura symptoms, their occurrence follows a specific temporal sequence that can be explained by the pathway the wave of depolarisation spreads over the cortex: visual, sensory, aphatic speech and then motor symptoms [24, 25]. Although the aura phase usually directly precedes the headache phase, a short delay between the end of the aura phase and the beginning of the headache can occur: a recent study has demonstrated that the overlap of aura and headache phases is more common rather than the exception [26]. The classification of migraine into subtypes is based on the presence of aura symptoms (see Table 1).

The headache phase

The migraine headache phase is characterized by a moderate to severe, often unilateral headache with a throbbing or pulsating character with a duration from several hours up to several days when untreated [2]. The headache is considered disabling by patients, who usually need to lie down during the headache [2, 27]. Accompanying gastrointestinal symptoms, such as nausea, vomiting, abdominal pain and diarrhea, are very common. Nausea and vomiting are being reported by 70-90% of patients. These symptoms are very consistent over time in different migraine attacks and interfere with the patients' ability to take their oral medication in 30-40% [11]. Symptoms of sensory hyperexcitability include photophobia, phonophobia and osmophobia and are experienced frequently as well.

The postdromal phase

After the headache phase has subsided, postdromal symptoms can be experienced that can last from several days up until one week [28]. More than 80% of patients report at least one postdromal symptom: a stiff neck, tiredness, weakness, mood changes and cognitive difficulties are the most common symptoms [13, 29, 30]. Mild residual head discomfort, light-headedness, and gastro-intestinal symptoms are frequently reported as well [29, 30]. As there

is some overlap in premonitory and postdromal symptomatology, it is suggested that these symptoms may have been present during the entire attack, but were overshadowed by the aura symptoms, the severe headache, nausea and vomiting [28]. Most patients (93%) return to normal within 24 hours after the headache resolves. The duration of the postdromal phase is not associated with migraine headache severity [13].

Migraine classification

Defining migraine patients and migraine attacks has been difficult, because of both the variability of migraine symptoms between patients, and the variability between recurrent attacks within the same patient. The introduction of the International Classification of Headache Disorders (ICHD) in 1988 has standardised the diagnosis of migraine, enabling more accurate epidemiologic studies, more accurate patient selection for clinical trials, scientific research, and for diagnosis in healthcare. Currently, the third edition is the prevailing classification system [2]. According to the ICHD, two subtypes of migraine can be distinguished based on the presence of aura symptoms (Tables 1.1 and 1.2).

Table 1: Classification of migraine without aura (1.1) and migraine with aura (1.2) according to the International Classification of Headache Disorders 3 criteria [2].

1.1 Migraine without aura			
A.	At least 5 attacks [1] fulfilling criteria B-D		
B.	Headache attacks lasting 4-72 hours (untreated or unsuccessfully treated) [2,3]		
C.	Headache has at least 2 of the following 4 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		

Notes

1. One or a few migraine attacks may be difficult to distinguish from symptomatic migraine-like attacks. Furthermore, the nature of a single or a few attacks may be difficult to understand. Therefore, at least five attacks are required. Individuals who otherwise meet criteria for 1.1 *Migraine without aura* but have had fewer than five attacks, should be coded 1.5.1 *Probable migraine without aura*.
2. When the patient falls asleep during a migraine attacks and wakes up without it, duration of the attack is reckoned until the time of awakening.
3. In children and adolescents (aged under 18 years), attacks may last 2-72 hours (the evidence for untreated durations of less than 2 hours in children has not been substantiated).

1.2 Migraine with aura		
A.	At least 2 attacks [1] fulfilling criteria B and C	
B.	One or more of the following fully reversible aura symptoms [2]:	1. Visual 2. Sensory 3. Speech and/or language 4. Motor [3] 5. Brainstem 6. retinal
C.	At least 2 of the following 4 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[1] 3. At least one aura symptom is unilateral[4] 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 has been excluded	

Notes

1. When, for example, three symptoms occur during an aura, the acceptable maximal duration is 3 x 60 minutes.
2. Usually, a headache with the features of migraine without aura follows the aura symptoms. Less common, the headache is without migraineous features, or even is absent. Most patients who have attacks of migraine with aura also report attacks of migraine without aura [2]
3. The very rare hemiplegic migraine, a form of migraine with aura characterized by a transient hemiplegia that may last from several minutes to hours or even days, is considered to be the most severe subtype of the migraine spectrum [31].
4. Aphasia is always regarded as a unilateral symptom; dysarthria may or may not be.

Especially for the purpose of large-scale epidemiologic and genome-wide association studies, large numbers of migraine patients (and non-migraine individuals) are to be included in order to obtain reliable study data. Accurate and easy-to-use screening instruments, based on the International Headache Society's criteria [2], are of utmost importance. Screening questionnaires or web-based tools can be feasible for this purpose, as has been shown in the past in the Genetic Epidemiology of Migraine study [32]. They, however, necessitate validation within every population in which they are used.

Epidemiology and burden of disease

Migraine is a public health problem of great impact on both the patient and society. In western countries, the overall one-year migraine prevalence in the general population is at least 12 percent, and its epidemiologic profile has remained stable over the past decades [27, 32-34]. Two-thirds of migraine patients are women, in which the one-year prevalence is 16-18% in comparison to a prevalence of 6-8% among men [35]. The lifetime prevalence of migraine

is up to 33% in women and up to 13% in men [32]. The median attack frequency is 1-2 attacks per month and the median attack duration is one day. More than 10% of migraine patients have weekly attacks lasting for 2-3 days each. The burden of migraine therefore is high. It is associated with deteriorated health-related quality of life and lost productivity, with a large impact on migraine patients and their families [6, 33, 34]. The World Health Organization (WHO) has rated migraine as the sixth most prevalent disorder globally, the condition with the second most years lived in disability, and the most disabling of neurologic disorders [6, 36, 37]. Furthermore, WHO has also ranked migraine as the most costly neurologic disorder in the European Union [37]. Although the epidemiological profile has remained stable, the rankings increase over time[7].

Migraine attack susceptibility, modulating and trigger factors

Every person can have a migraine attack. An individual is considered a migraine patient according to the criteria of the International Headache Society only after five attacks of migraine without aura, or after 2 attacks of migraine with aura [2]. Both between and within patients, attack frequency varies and depends on genetic susceptibility, intrinsic and extrinsic factors that modulate the so-called excitability threshold, and trigger factors that can initiate a new migraine attack[7, 9].

Attack susceptibility

As suggested by clinical practice, there is a strong genetic component in migraine [7]. Population-based family studies have shown that direct family members of migraine patients have an increased risk of having migraine in comparison to relatives of matched controls [38], with the risk being the highest for relatives of migraine with aura patients [7]. In addition, twin studies have revealed significantly higher pairwise concordance rates of migraine in monozygotic versus dizygotic twins [39]. In the small subset of patients with familial hemiplegic migraine, mutations in ion channel genes cause a lifelong susceptibility to migraine in this monogenic form [40]. In the majority of patients with migraine without aura or migraine with aura (excluding the hemiplegic migraine subset), genome wide association studies have revealed several distinct genomic loci associated with migraine, which show enrichment for genes expressed in vascular and smooth muscle tissue [41-43]. The exact pathways involved are still topic of extensive research[7].

Cortical and non-cortical hyperexcitability

In recent years it has been suggested that an altered neuronal excitability of the cortex, subcortical structures and/or trigeminal neurons are underlying mechanisms for migraine susceptibility. There is accumulating evidence for increased glutamergic transmission as one of the underlying pathways. Glutamate is the major excitatory neurotransmitter in the Central Nervous System and is implicated in several mechanisms related to migraine, including trigeminovascular activation, central sensitization and cortical spreading depolarization [44]. The hypersensitivity to light (photophobia), to sounds (phonophobia), odours (osmophobia) and touch (allodynia) are considered to result from central sensitisation, a condition in which dorsal horn nociceptive neurons exhibit enlargement of

their receptive fields, increased synaptic strength and increased excitability [45]. For allodynia, there is large body of evidence endorsing this hypothesis. For the other hypersensitivities, this mechanism is suggested [46]. It has been suggested that migraine may be considered as a brain state of altered excitability [45]. A clear biochemical correlate for the neurobiological cascade leading from enhanced excitability via modulating or triggering factors to a new migraine attack has not been elucidated yet and is dearly needed.

Modulating and triggering factors

Intrinsic and extrinsic modulating factors include stress, relaxation, fatigue, prophylactic treatment [47] and hormonal fluctuations [48,49]. Collectively these factors may lead to a state in which the brain is more susceptible to developing a new migraine attack [50]. Trigger factors are defined as any factor that on exposure or withdrawal leads to a development of a migraine attack [51]. It can nevertheless be difficult to clearly distinguish between modulating factors and trigger factors. Extensive lists of potential modulators / trigger factors for the onset of a new migraine attack have been described in literature, including stress, nutritional factors, sleep changes, changes in circadian rhythm, atmospheric factors, sex hormones and pharmacological compounds (such as glyceryl trinitrate, PACAP, sildenafil) [52-67]. Methodological issues in assessing trigger factors, premonitory symptoms and self-prediction have hampered correct interpretation of the association with occurrence of migraine attacks [67, 68]. Observational (mainly questionnaire) studies have often suggested strong associations between possible trigger factors and the attack onset, but these correlations have rarely been confirmed in prospective or interventional studies. I will briefly discuss some of these factors below.

Stress

Migraine patients report stress and negative emotions both as modulators and trigger factors for a new attack [13, 69, 70]. There is some evidence suggesting headache severity and duration in migraine patients are correlated to the cortisol response to a stressor [71]. Interestingly, the sudden absence of perceived stress might also be relevant, as timing of migraine headaches in the weekend is a clear clinical observation [72].

Sleep and circadian rhythmicity

Sleep and sleep deprivation play a role in migraine: sleep is considered an effective means to alleviate the migraine headache; attacks of migraine may occur during or shortly after either nocturnal or diurnal sleep; sleepiness may emerge during various phases of the migraine attack [73, 74], and sleep deprivation is associated with the onset of migraine attacks [28, 75]. Polysomnographic findings of sleep disturbances can be found in nights preceding migraine attacks [76]. Several studies have suggested that migraine attacks show seasonal and circadian periodicity [77-81], implicating chronobiological, probably hypothalamic-mediated mechanisms in the triggering and initiation of migraine attacks.

Sex hormones

Migraine prevalence and the frequency, duration and severity of migraine attacks are highly dependent on age, gender and, in women, events which are associated with marked fluctuations in female reproductive hormones [32, 82, 83]. The prevalence of *active* migraine,

defined as at least one attack in the previous year, shows a bell-shaped pattern across lifetime in both sexes. In the fertile period, three times more women (24%) than men (8%) have active migraine and their attacks are on average more frequent, longer, and more severe [32, 83]. Additional evidence that sex hormones might modulate migraine risk and activity comes from a range of other clinical and experimental observations. Higher migraine prevalence rates were reported among obese individuals [84], when starting oestrogen therapy in male-to-female transsexuals [85], or when having certain polymorphisms in sex hormone receptor genes [86]. Migraine is less prevalent after testosterone administration in women with migraine [87]. Attack frequency or prevalence rates vary after starting or stopping of oral contraceptives [88], with menstrual cycle-related changes in oestrogen levels [40, 89], and due to gender-related differences or experimental manipulation of sex hormone levels in rat transgenic mouse models [40, 90].

Pharmacological factors

A variety of pharmacological compounds are known for their ability to provoke migraine or migraine-like headache attacks and can be considered pharmacological models of migraine [91]. These compounds include glyceryl trinitrate (nitroglycerin), PACAP, and sildenafil [92, 93]. They can be used as models to study mechanisms responsible for migraine in humans, and to explore the mechanisms of action of existing and future anti-migraine drugs. The most often used is the nitroglycerin provocation model which has sufficient information on reproducibility and reliability available [94]. Infusion of nitroglycerin results in an immediate type of headache unrelated to aura symptoms in both healthy volunteers and migraine patients and a 4-6 hours delayed migraine attack in about 50% of migraine patients (range 20% - 80%), but not in healthy volunteers [95-97]. The nitroglycerin model is commonly used, as the induced attacks are very similar to the genuine attack, including the premonitory symptoms and response to acute [94] and prophylactic [98] treatment [91, 96, 99]. It is also a reliable and reproducible model that has been critically evaluated by several independent groups.

Neurobiology of migraine

As laid out in the International Classification of Headache Disorders 3 criteria, migraineous symptoms are not just restricted to headache pain, but include a wide variety of sensory and homeostatic symptoms throughout the course of a migraine attack [2]. Sensory symptoms include hypersensitivity to light stimuli (photophobia), acoustic stimuli (phonophobia), olfactory stimuli (osmophobia) or tactile stimuli (allodynia). Symptoms that reflect a disruption of the normal homeostasis include altered sleep, altered feeding behaviour, changes in mood and in water homeostasis. These different clinical symptoms seem to be driven by particular underlying pathophysiological mechanisms [7].

The interictal period

Evidence from electrophysiological and imaging studies has suggested that the migraine brain differs from the non-migraine brain in function and structure also in the interictal period, i.e. between two migraine attacks. Interictal studies with auditory evoked potentials

[100] or with the nociceptive blink reflex [101] suggested that the migraine brain over-responds [7]. Interictal imaging studies showed alterations in grey matter volume and hypometabolism in pain processing areas [102-108]. Biochemically, abnormal patterns of hypothalamic hormonal secretion were found interictally in the serum of chronic migraine patients, including decreased nocturnal prolactin peaks, increased cortisol concentrations, a delayed nocturnal melatonin peak and lower melatonin concentrations [109]. Several other studies also found increased interictal levels of prolactin, LH and FSH levels and decreased interictal cortisol levels [110]. In CSF studies increased levels of LH, FSH and prolactine and hypocretin were detected [110-112]. Overall, both increased and decreased levels of hormones were detected.

In summary, the brain of migraine patients differs subtly from non-migraine individuals in structure and functioning even outside attacks.

The premonitory phase

The clinically heterogeneous pattern of premonitory symptoms has led to the hypothesis that some of these symptoms, such as yawning, frequent urination, thirst, mood changes food intake, craving and alterations in the sleep-wake cycle [7, 12], might be hypothalamic in nature, in which the neurotransmitters dopamine [113], vasopressin [114] and the orexins [115] play a key role. The underlying pathophysiologic mechanisms of the premonitory phase have only been studied scarcely, mostly due to the technical and logistic challenges of predicting spontaneous migraine attacks. Neuroimaging studies have provided some insight in timing and anatomical localisation of functional changes, but relevant mechanisms still need to be elucidated. In patients with provoked premonitory symptoms, Maniyar et al. found blood flow increases in the posterior hypothalamus [116]. Later, Stankewitz et al. found pre-ictal normalisation of the interictally reduced activation of spinal trigeminal nuclei, that further increased during the headache phase. A correlation with clinical premonitory symptoms was not found [117]. Finally, in a fMRI study scanning a single patient every morning for 30 days, altered hypothalamic blood flow coupled with brain stem nuclei was found 24 hours before pain onset in three captured spontaneous migraine attacks [118]. Although Denuelle et al. were the first to capture hypothalamic involvement in acute migraine attacks, they only focussed on the headache phase [119].

The aura phase

Aura symptoms, defined by transient neurological deficits preceding or just overlapping with the headache phase in migraine, have a believed experimental correlate which is the cortical spreading depolarisation (CSD), a steady wave of depolarising neuroglial membranes. This was first described in 1944 by Leão, who studied the rabbit cortex and suggested CSD to be the neurobiological substrate of the clinical migraine aura [120], mainly since CSD and aura have similar rates of propagation (3mm/minute) [121]. Later studies detecting blood flow changes and blood oxygenation levels confirmed this assumption [120-123]. Taken together, these studies point to CSD as the underlying mechanism in the visual aura in migraine [7]. Whether aura symptoms can occur independently (and induce further symptoms, including headache) or are related to earlier changes elsewhere in the brain as part of a bigger cascade of events, remains unresolved.

The headache phase

The headache in migraine is considered to be due to activation of the trigeminovascular system (TGVS). The brain itself is insensate, but pial, arachnoidal and dural vessels are richly innervated by nociceptive large non-myelinated (C) and thinly myelinated (Aβ) fibers from mainly the ophthalmic division of the trigeminal nerve [7, 124]. The axon terminals of these fibers contain vasoactive neuropeptides such as calcitonin gene-related peptide (CGRP), substance P, neurokinin A and pituitary adenylate cyclase-activating peptide (PACAP) [125, 126], which upon release after stimulation cause vasodilatation of dural and pial vessels [125, 127]. The convergence of sensory inputs from intracranial and extracranial structures explains the distribution of migraine headache pain over the frontal and temporal regions as well as involvement of occipital and high cervical areas [128]. All nociceptive information is relayed via ascending projections to brainstem and diencephalon areas involved in pain processing. Activation of these regions is considered to contribute to the perception of headache pain in migraine, and to account for autonomic, cognitive, endocrine and affective symptoms occurring over the course of a migraine attack [7].

More recent is the appreciation that the hypothalamus also is involved in the control of pain [129] and the attack initiation of primary headaches [77, 130, 131]. As early as 1989, Lance already hypothesized that both internal and external stimuli may initiate a migraine attack via hypothalamic activation and its downstream connections with brainstem nuclei [16]. The hypothalamus has reciprocal connections with many structures involved in nociceptive processing: descending projections to the superior salivatory nucleus, where they affect autonomic regulation, and modulate trigeminovascular nociceptive processing at the spinal level [7]. Orexinergic peptides [115] and somatostatin [132] from the posterior hypothalamus, $GABA_a$-ergic projections from the paraventricular hypothalamic nucleus [133], as well as inhibitory dopaminergic projections from the A11 hypothalamic nucleus [134, 135] can modulate dural- and cutaneous evoked trigeminovascular transmission. A changed function of any of these hypothalamic regions may result in altered processing of nociceptive inputs that sustain or modulate migraine headache. It might also trigger activation of previously silent trigeminovascular neurons correlating with attack initiation. The hypothalamus also regulates many other important processes involved in homeostasis, such as feeding, sleep/wake and stress. An altered function of the hypothalamus may also result in many of the accompanying symptoms in a migraine attack [136].

Together with data from concomitant hypothalamic activation in both nitroglycerin-triggered [116] and spontaneous [119] migraine attacks, the evidence mentioned above suggests that brainstem and hypothalamic structures are crucial for migraine pathophysiology and might also reflect the clinical heterogeneity in migraine attacks [7].

The postdromal phase

This phase follows the end of the headache phase and can persist for hours or days when the patient is free of headache but has not fully returned to feeling normal. As mentioned before, there is some overlap in premonitory and postdromal symptomatology [28], but up to date no imaging of neurobiological studies have been reported focusing on this phase of the migraine cycle.

The onset of the migraine attack; a multi-modal approach into modulating and trigger factors

As presented, there are many different modulating and triggering factors involved in migraine attack susceptibility. Some of these are, on both clinical and pre-clinical grounds, hypothesised to be associated with possible altered functioning of hypothalamic nuclei during the premonitory phase and possibly early headache phase of the migraine attack. This thesis describes the onset of the migraine attack and its modulating and trigger factors. Since it is not feasible to elaborate on all, I will focus on some modulating or triggering mechanisms in both spontaneous and nitroglycerin-induced migraine attacks in this thesis using a multi-modal approach. I will distinguish the three different parts 'Clinical aspects and modulators', 'Imaging aspects' and 'Biochemical aspects'.

Clinical aspects and modulators (Part I)

Large scale epidemiological and genetic studies require inclusion of large numbers of migraine patients and non-migraine control subjects. In chapter two, the validity of a new self-administered migraine questionnaire is described.

In the third and fourth chapter, the focus is on several clinical aspects of modulating or triggering factors in migraine. The distribution of chronotypes, circadian timing of migraine attacks, sleep quality and effect of sleep disturbances on migraine are assessed in chapter three. As is unclear whether the severity of restless legs syndrome (RLS) in migraine differs from non-migraine RLS patients and whether sleep quality is affected differently by RLS in this group, we assess this in a study described in the fourth chapter.

Whether or not migraine patients have a higher risk of getting post-dural puncture headache than controls is an unanswered question. In a prospective, CSF biochemical profiling study, the differences in the occurrence of post-dural puncture headache between migraine patients and non-migraine individuals are studied and presented in the fifth chapter. The effects of perceived stress (for the planned lumbar puncture in an experimental setting) on migraine attack frequency in the migraine patients are assessed as well.

In chapter six the cardiovascular effects of nitroglycerin in migraine patients and non-migraine control subjects are studied. Nitroglycerin is a compound known to be able to trigger migraine-like headache in susceptible individuals [94]. The chapter describes an experimental study assessing the possible differences in effects on systemic cardiovascular parameters between migraine patients and non-migraine individuals after intravenous nitroglycerin infusion.

Imaging aspects (Part II)

As mentioned earlier, data from imaging studies have suggested hypothalamic involvement in the premonitory phase of the migraine attack [116, 117, 119]. Part II describes an experimental setting in which hypothalamic metabolites are studied, both interictal and during nitroglycerin-induced attacks. We compare hypothalamic activation after oral ingestion of a glucose solution in the interictal and pre-ictal phase between migraine patients and non-migraine control subjects using functional MRI in chapter seven.

Biochemical aspects (Part III)

Part III focusses on several biochemical factors that are involved in migraine attack susceptibility. Migraine prevalence and the frequency, duration and severity of migraine attacks are highly dependent on age, gender and, in women, events which are associated with marked fluctuations in female reproductive hormones [32, 82, 83]. In males with migraine, however, this has never been studied before. Therefore, in the first chapter of Part III baseline levels of female sex hormones are compared between male migraine patients and male non-migraine control subjects (chapter eight). Furthermore, the levels of these hormones are longitudinally measured within the migraine group in the days prior to the next migraine attack, including the premonitory and headache phases as described in chapter nine. In chapter ten, the development and validation are described of a human capsaicin model to enable assessment of salivary CGRP secretion.

Aims of this thesis

There are several different modulating or triggering factors that play a role in migraine attack susceptibility and I will focus on several of these mechanisms in both spontaneous and nitroglycerin-induced migraine attacks using a multi-modal approach. Therefore, the aims of the thesis are as follows:

1. To assess the validity of a web-based questionnaire to diagnose migraine and migraine aura (Part I; chapter 2)
2. To study the distribution of chronotypes and circadian timing of attacks in migraine patients, and to assess sleep quality and the effect thereof on migraine attack susceptibility (Part I; chapter 3)
3. To study the prevalence and severity of restless legs syndrome in migraine patients, and assess the interference with sleep quality (Part I; chapter 4)
4. To study the incidence of post-dural puncture headache in migraine patients, and the possible role of perceived stress on new migraine attacks (Part I; chapter 5)
5. To assess migraine-specific effect of intravenous nitroglycerin infusion on systemic cardiovascular parameters (Part I; chapter 6)
6. To assess hypothalamic activation after an oral glucose challenge in migraine patients interictally, and during the premonitory phase of both nitroglycerin-induced and spontaneous attacks (Part II; chapter 7)
7. To assess levels of sex hormones in relation to migraine susceptibility in males, and to study changes in these levels prior to the migraine attack (Part III; chapter 8)
8. To study trigeminovascular cyclicity in women with menstrually related migraine (Part III; chapter 9)
9. To develop a human capsaicin model to quantitatively assess salivary calcitonin gene-related peptide (CGRP) secretion (Part III; chapter 10)

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Part I.

Clinical aspects and modulators

Chapter 2.

Validation of the web-based LUMINA questionnaire for recruiting large cohorts of migraineurs

van Oosterhout WPJ[1]
Weller CM[2]
Stam AH[1]
Bakels F[1]
Stijnen T[3]
Ferrari MD[1]
Terwindt GM[1]

[1]Department of Neurology, Leiden University Medical Center, Leiden, the Netherlands
[2]Department of Human Genetics, Leiden University Medical Center, Leiden, the Netherlands
[3]Department of Medical Statistics, Leiden University Medical Center, Leiden, the Netherlands

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Abstract

Objective
To assess validity of a self-administered web-based migraine-questionnaire in diagnosing migraine aura for the use of epidemiological and genetic studies.

Methods
Self-reported migraineurs enrolled via the LUMINA website and completed a web-based questionnaire on headache and aura symptoms, after fulfilling screening criteria. Diagnoses were calculated using an algorithm based on the International Classification of Headache Disorders (ICHD-2), and semi-structured telephone-interviews were performed for final diagnoses. Logistic regression generated a prediction rule for aura. Algorithm-based diagnoses and predicted diagnoses were subsequently compared to the interview-derived diagnoses.

Results
In 1 year, we recruited 2397 migraineurs, of which 1067 were included in the validation. A seven-question subset provided higher sensitivity (86% vs. 45%), slightly lower specificity (75% vs. 95%), and similar positive predictive value (86% vs. 88%) in assessing aura when comparing with the ICHD-2-based algorithm.

Conclusions
This questionnaire is accurate and reliable in diagnosing migraine aura among self-reported migraineurs and enables detection of more aura cases with low false-positive rate.

Introduction

Migraine is a common brain disorder characterized by recurrent, disabling attacks of headache, autonomic features (migraine without aura; MO), and, in one third of patients, transient neurological aura symptoms (migraine with aura; MA). In western countries, the overall migraine prevalence in the general population is at least 12 percent, two-thirds of which concerns females [1-4]. Since no biomarker for migraine exists, diagnosis according to the headache classification of the International Headache Society (IHS) [5] relies exclusively on the headache history. A careful history taken by a headache specialist is the gold standard for making a valid migraine and aura diagnosis.

Large-scale studies with several thousands of participants are important to obtain information for epidemiological and genetic migraine research and may yield important insights in migraine pathophysiology. Migraine is a complex genetic disorders, i.e. multiple genetic and environmental factors contribute to migraine susceptibility.

Twin and population-based family studies showed that genetic factors play an important role in migraine susceptibility, especially in the MA subtype [6-12]. However, genetic linkage studies using migraine subtypes as an end diagnosis did not yield gene variants thus far. Clinical heterogeneity in migraine and aura diagnosis may have hampered the identification of such variants. Recently, in a large genome wide association analysis (GWA) with a large set of clinic-based migraineurs, a first-ever genetic risk factor was identified associated with common types of migraine, in patients that were largely recruited from specialist headache clinics with a clinic-based migraine diagnosis [13]. However, population-based large-scale studies exclude the possibility of a face-to-face examination, and, therefore, a less time-consuming and less costly diagnostic strategy has to be chosen. A web-based questionnaire represents an attractive and inexpensive alternative for a clinic interview. Several groups have reported on the use of internet to recruit headache and other patients for clinical research [14-18]. However, reliably diagnosing aura remains an issue.

The availability of a validated, aura-specific questionnaire is important when large numbers of cases are needed, especially in studies with self-reported migraineurs from the general population [19, 20]. We developed the LUMINA (Leiden University MIgraine Neuro-Analysis) website and designed and validated a self-reporting, web-based questionnaire to reliably diagnose migraine headache and aura symptoms, using only a limited number of questions. In this paper, we will present the validation of this web-based migraine and aura questionnaire.

Methods

Subjects

Participants were Dutch adults aged 18 to 74 years with migraine (MA and MO), who were informed via the lay press nationwide to enrol via the especially designed LUMINA website. Additionally, patients from our outpatient headache clinic were invited by a letter. In this

clinic-based study, all participants were self-reporting migraineurs, of which approximately 90% had previously been diagnosed with migraine by a physician.

Study flow

Study flow is depicted in Figure 1. Patients who visited the website were informed about the study and could enrol directly. The first step was to fulfil the screening criteria, using a simple screening questionnaire that was validated previously in the population-based GEM-study [3]. This screening questionnaire included five questions asking whether the patient i) had severe headaches in the past 12 months; ii) what the headache severity was; iii) had suffered from headaches which were preceded by visual disturbances; iv) had been diagnosed with migraine by a physician; and v) had ever used anti-migraine medication. After fulfilling these criteria, cases received a unique user ID-code via e-mail to log on to the study website, where they could participate in an extended, web-based questionnaire study. Having completed the extended questionnaire, a number of randomly selected participants were contacted by telephone by WPJvO, CMW, and AHS, who are experienced in diagnosing migraine. This semi-structured telephone interview detailed questions on headache and aura characteristics including ICHD-2 migraine and aura criteria [5] with special attention for visual, sensory, motor and speech symptoms, was used as the gold standard. Median interview duration was 10-15 minutes, ranging up to 30 minutes if necessary. Afterwards, a final diagnosis was made: in case of ambiguity, a headache specialist (GMT) was consulted. Patients were excluded from the analysis if they could not be reached by telephone after five failed telephone contact attempts. The study was approved by the local medical ethics committee. All participants provided written informed consent.

Construction of questionnaire

The extended questionnaire (accessible via www.lumc.nl/hoofdpijn) was based on the ICHD-2 [5] and incorporated 127 items on migraine headache and aura characteristics, premonitory symptoms, trigger factors, allodynia, and medication use and was presented to participants as a digital web-form. The questions were to be answered by choosing from categorical alternatives. On the web-form multicolour exemplary illustrations were shown with the most characteristic visual aura features (hemianopsia, scotoma, fortification spectra, visual blurring) and sensory aura features (anatomical distribution).

ICHD-2 based algorithm

After completion of the extended questionnaire, an algorithm based on ICHD-2 [5] migraine criteria was run and individual diagnosis was determined. The algorithm had the following possible outcomes: 'no migraine'; 'migraine without aura'; and 'migraine with aura'. In the analysis, the algorithm outcomes were dichotomised into 'aura' and 'no aura' (Supplementary Figure e-1).

Statistical analysis

Descriptive statistics

Descriptive statistics were performed on demographic and clinical variables, on the algorithm based diagnoses and on the interview-derived diagnoses. Results are reported as mean ± SD or as percentage. Differences in between-groups means were analyzed with

independent sample t-tests and ANOVAs. Proportions were compared using Chi-square tests. All items from the extended questionnaire that concerned ICHD-2 migraine criteria were evaluated separately. Likelihood ratios were calculated using standard formulas for positive likelihood ratio (LR+, sensitivity/ 1 – specificity) and negative likelihood ratio (LR-, [1 – sensitivity]/ specificity).

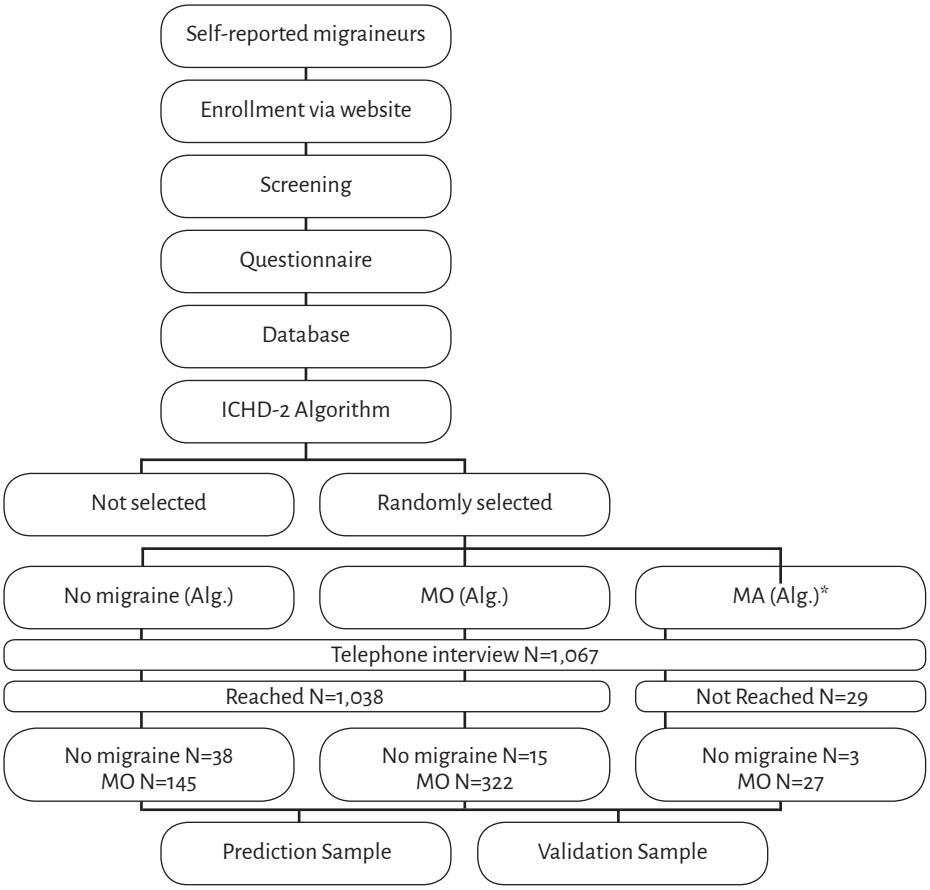

Figure 1. Flowchart of (semi-)automated study flow. Screening = Screening Questionnaire; Questionnaire = Extended Questionnaire; MO = Migraine without Aura; MA = Migraine with Aura; Alg.= ICHD-2 based Algorithm Diagnosis; Int.= Interview Diagnosis. * In the total MA group, 91.6% (447/488) reported visual aura symptoms.

Questionnaire validation process

The questionnaire validation process was divided into two phases and was aimed at identifying a combination of items that were better predictors for diagnosing migraine aura than the ICHD-2 based algorithm, with the interview-derived diagnosis as the gold standard. In phase I, a sample of 838 self-reported migraineurs (approximately 80% of

total group) was randomly selected and used as a training sample (see Figure 1) to derive a predictive model. These patients fulfilled set screening criteria from the five-item LUMINA screener before they could enter the extended questionnaire. Logistic regression (see below) was used to develop the predictive model that included questionnaire items most contributing to predict subcategories 'aura' and 'no aura'. Subsequently, we compared both the ICHD-2 based algorithm diagnoses and the diagnoses predicted by the logistic model, to the gold standard. In phase II, we validated this derived predictive model in an independent validation sample, consisting of 200 patients, approximately 20% of our sample (see Figure 1).

Phase I: Development of prediction rule

In phase I, a prediction rule for the aura subcategories 'aura' vs. 'no aura' was developed using a multivariate logistic regression analysis. Relevant, individual, dichotomized items (n=33) were selected from the extended questionnaire and were used as predictor variables for aura in the model. Selection of items was made by the authors (WPJvO; CW; GMT) and was based on clinical relevance to migraine aura, and sensitivity, specificity, PPV, NPV en likelihood ratios of individual items. Inter-item correlation was assessed for relevant items using Spearman's rank coefficients and when items correlated with coefficients >0.9, one of these items was excluded from the analysis. A forward selection strategy using the likelihood ratio test was performed to identify items that were significant (p<0.05) predictors for the outcome of aura. For each subject in this sample (n=838), a prediction score was calculated using these items. Subsequently, a receiver operator characteristics (ROC) curve was generated to assess the optimum cut off point for this prediction score. Using the method proposed by Halpern et al.[21], an optimum cut-off (highest sensitivity and specificity) was determined from the ROC curve. Therefore, the logistic model resulted in a selection of the 33 items with significant (p<0.05) contribution in the aura prediction.

Phase II: Validation of prediction rule

The derived predictive rule was subsequently validated in the second sample (validation sample; n=200; see Figure 1). Validity of this predictive model was assessed by checking whether the selected items contributed significantly (p<0.05) for the prediction in the second sample too. Subsequently, the sensitivity and specificity from the ROC optimum in the training sample were compared with these parameters in the validation sample, using the same cut-off value.

Overall outcome measures

Sensitivity, specificity, positive and negative predictive values were calculated to compare the fit of the three different models with the interview-derived aura diagnosis as the gold standard. These models were: 1) ICHD-2 based algorithm; 2) predictive model from phase I; and 3) validation of predictive rule in phase II.

All data analyses were performed using SPSS 16.0.2 (SPSS inc., IBM, USA). p values less than 0.05 were considered significant. When appropriate, categorical items were dichotomized into binary variables for the analysis in an attempt to simplify the instrument.

Receiver Operator Characteristics (ROC) curve
From the data in the training sample, we generated an ROC curve by plotting the sensitivity of the questionnaire against one minus the specificity. As a graphical representation of the trade-off between false negative and false positive rates for every possible cut-off point, the ROC curve reflects the trade-offs between sensitivity and specificity, and plots the false positive rate on the X axis and the true positive rate on the Y-axis. The area under the curve is a measure of correlation between the prediction of the questionnaire and the gold standard diagnosis. The closer the area under the curve (AUC) is to 1, the better the test is. To validate the derived logistic model, we compared the ROC from the prediction sample (n=838) to the ROC of the validation sample (n=200).

Results

General results

Over a 1-year period, from April 2008 until April 2009, 2,397 subjects fulfilled the set screening criteria and completed the extended questionnaire (Figure 1). During this time period, a total of 1,067 subjects (44.5%) were randomly selected for the semi-structured telephone interview, of which 1,038 (97.3%) were reached and could be used in the analysis. A total of 29 subjects (2.7%) were not included in the analysis because they could not be reached by telephone, after having tried at least five times. From these 1,038 subjects, 838 (79.4%) were randomly selected and used for the prediction model and the remaining sample of 200 subjects (18.9%) was used for validation (Figure 1).

Baseline characteristics of the total study population and separate prediction and validation samples are depicted in Table 1. Almost 90% of self-reported migraineurs had previously been diagnosed with migraine by a physician. Age, gender, prevalence of previous migraine diagnosis and use of anti-migraine medication did not differ significantly between selected subjects and non-selected subjects, nor between subjects that were reached compared to those that could not be reached for telephone interview (see Table 1). In the selected subjects (n=1,067; with special attention to patients which fulfilled ICHD-2 migraine criteria except for attack duration), the algorithm diagnosis of 'no-migraine' was more prevalent (28.6% [305/1,067] vs. 2.7% [36/1,330]; $p<0.001$) compared to non-selected subjects (n=1,330).

Screening questionnaire

In total, 94.6 percent of subjects (982/1,038) fulfilling the screening criteria, fulfilled ICHD-2 migraine criteria in the telephone interview. We considered everyone fulfilling the screening criteria to be migraineur. We used a logistic model to predict individual aura vs. no aura status.

Algorithm diagnosis

From the total sample of 1.038 subjects, the ICHD-2 based algorithm classified 488 subjects as MO patients, 251 as having MA, and 299 subjects as non-migraineurs (Figure 1). Of these, 243 were misclassified as non-migraineurs due to reporting of longer than actual attack duration. Table 2 summarizes the sensitivity, specificity, positive and negative

predictive values as well as the corresponding likelihood ratios for the ICHD-2 based algorithm diagnosis of migraine aura in the total sample (n=1,038). Similar values for this classification in the training sample (n=838) suggest this sample is a good representation of the whole group. In both the total group and the training sample, sensitivity for aura was approximately 0.45, specificity 0.95, positive predictive value (PPV) 0.88 and negative predictive value (NPV) 0.70 (Table 2). Additionally, we calculated characteristics of all individual questionnaire items that reflect migraine headache and migraine aura criteria and summarized those in Supplementary tables e-1 and e-2. The results show individual sensitivity ranging up to 0.97 (photophobia; nausea) and PPV up to 0.98 (headache severity; headache duration).

Table 1. Baseline characteristics of total study population and separate study samples. SD = standard deviation; M = migraine; * indicating $p<0.001$ (χ^2-test).

	Total	Selection for study		Telephone interview		Sample	
		Not selected	Selected	Not reached	Reached	Training	Validation
Number	2,397	1,330	1,067	29	1,038	838	200
Age (years: mean; SD)	42.8 (11.9)	41.6 (12.0)	44.3 (11.6)	43.9 (11.1)	44.4 (11.6)	44.6 (11.7)	43.3 (11.5)
Gender (% female)	84.8%	83.9%	85.8%	89.7%	85.6%	85.0%	88.5%
Ever M diagnosis	88.9%	87.8%	90.2%	100%	89.9%	90.2%	89.0%
Use of anti-M drugs	82.8%	80.3%	85.8%	93.1%	85.6%	85.2%	87.5%
Algorithm diagnosis M	87.1%	97.3%*	71.4%*	79.3%	72.4%	72.1%	73.5%

Phase I: Derivation of predictive model

Using logistic regression, 7 questions (from the 33 included; none showed Spearman rank correlation >0.9) showed a significant impact on the likelihood of having a migraine aura in accordance to the gold standard derived from the telephone interview. These questions are summarized in Table 3, which also shows significance levels and regression coefficients derived from the logistic model. The questions show partial overlap with the questions used in the ICHD-2 based algorithm. This model explained between 35.4% (Cox and Snell R Square) and 47.3% (Nagelkerke adjusted R Squared) of variance, and correctly classified 651/838 (77.8%) of subjects.

ROC curve

From the data in the predictive cohort, we generated an ROC curve by plotting the sensitivity of the questionnaire against one minus the specificity (Figure 2a). This analysis resulted in an optimal cut off point for the used logistic model at 0.35 with AUC of 0.85 (95% C.I. 0.83-0.88), yielding a 7 item questionnaire with a sensitivity of 0.83 and a specificity of 0.74. Compared to the ICHD-2 based algorithm outcome, this approach therefore resulted in a vast increment in sensitivity, with only small decrement of specificity (Table 2).

Figure 2a.

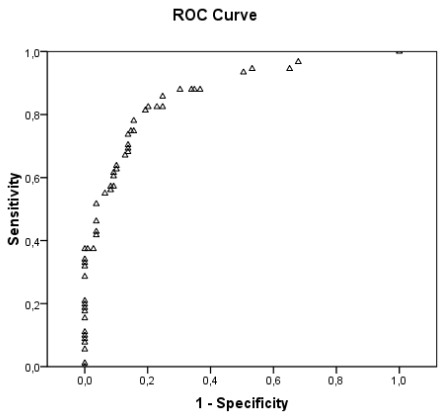

Figure 2b.

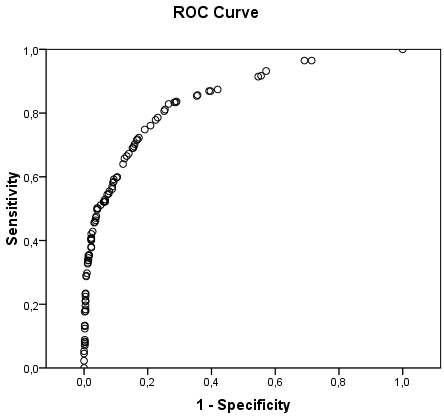

Figure 2. Receiver operator characteristics curves. Receiver operator characteristics (ROC) curves for the derived prediction rule in the initial training sample (n=838) (Figure 2a) and in the validation sample (n=200) (Figure 2b). The area under the ROC curve (C-statistic; AUC) for the prediction rule was 0.85 (95% C.I. 0.83-0.88) in the training sample and 0.87 (95% C.I. 0.82-0.92) in the validation sample.

Phase II: Validation of derived prediction rule

Using the predictive model and cut-off point (0.35) derived from the training sample (n=838), we validated this model in a second, independent sample (n=200) of subjects who also fulfilled the set screening criteria. This analysis showed the model to have approximately similar sensitivity and specificity in this validation sample (Table 2). In the validation cohort, the ROC curve yielded an AUC of 0.87 (95% C.I. 0.82-0.92), which is comparable to the output from the training cohort (Figure 2b). When using this cut off from the training cohort, migraine aura diagnosis was predicted correctly in 160/200 (80.0%) of subjects.

Test-retest reliability

For a random selection of 44 patients who completed the extended questionnaire a second time, with a mean test-retest interval of 155 days (median 89 days, range 1-422 days), test-retest reliability was found to be good with a test-retest kappa for algorithm diagnostic group of 0.59 (95% CI 0.38-0.80). Test-retest interval did not influence agreement (linear regression, p=0.852).

Table 2. Sensitivity, specificity, positive and negative predictive values as well as the corresponding likelihood ratios for diagnosis of migraine aura based on: 1) the IHCD-II based algorithm (in both the total group and training sample); and 2) the derived 7 item prediction model (in both the training sample and in the validation sample). PPV = positive predictive value; NPV = negative predictive value; MA = migraine with aura; MO = migraine without aura.

	ICHD-2 based algorithm Total sample (n=1,038)	ICHD-2 based algorithm Predictive sample (n=838)	Model Training sample (n=838)	Model Validation sample (n=200)
Sensitivity	45%	44%	83%	86%
Specificity	95%	95%	74%	75%
PPV MA	88%	89%	74%	74%
PPV MO (=NPV MA)	70%	64%	83%	86%
Positive likelihood ratio	8.2	8.7	3.1	3.5
Negative likelihood ratio	0.6	0.6	0.2	0.2

Table 3. Significantly correlated questions (n=7) are shown with their significance levels (95%C.I.) and regression coefficients derived from the logistic regression model (training sample; n=838). B = regression coefficient; OR = odds ratio; 95%C.I. = 95% Confidence interval.

	OR	(95%C.I.)	*p*
Did you have visual disturbances before headache in the past 12 months?	2.07	(132-3.26)	0.002
Did the visual disturbances last 5-60 minutes?	5.25	(3.08-8.96)	<0.001
Have you had scintillating lines before or during your headache in the past 12 months?	3.35	(2.06-5.45)	<0.001
Have you had loss of vision before or during your headache in the past 12 months?	2.49	(1.63-3.80)	<0.001
Did you suffer from numbness or a tingling feeling in your face/ unilateral arm/ leg that started prior to headache in the past 12 months?	1.88	(1.07-3.29)	0.027
Did you use nonsense words prior or during your headache in the past 12 months?	1.97	(1.22-3.19)	0.005
Did you use a triptan in the past 12 months?	0.57	(0.39-0.83)	0.003

Discussion

Our study has been the first one to validate a web-based questionnaire for purposes of diagnosing aura cases using a large sample of self-reported migraineurs. Few previous studies on migraine screeners and questionnaires have focussed on migraine aura, and the numbers of MA cases used to validate the questionnaire instruments in these studies were limited to n=8-186 (17, 19, 22-24) respectively, in comparison to the large number of 488 aura

cases in our study. Physicians frequently rely on aura as a cardinal symptom of migraine, as suggested by the 1.9 fold higher rate of medical diagnosis in interview settings when comparing MA cases to cases of MO [25]. Our study shows that, in self-reported migraineurs, a distinction between MA and MO can be made via a self-administered web-based questionnaire, with a focus on visual aura symptoms. The difficulty in diagnosing other aura types might be explained by the lack of perceptions and recognition of verbal and other non-visual auras by patients [26]. For diagnosing patients with these specific aura symptoms a clinical interview is needed. However, since the vast majority of the self-reported aura cases suffer from visual auras and only a small minority suffers from non-visual auras [27], we believe this number is neglectable when recruiting aura cases from a population of self-reported migraineurs. Perhaps the most helpful item identifying aura cases is the duration of the aura phenomena, since this question enables to distinguish visual aura symptoms from non-specific visual disturbances. Additionally, our data show aura patients are less likely to use triptans for rescue medication, which might be an indicator of lower headache severity.

We show that the question addressing the duration of the headache may hamper correct identification of migraine cases in a web-based questionnaire setting because some migraineurs overestimate the duration of an attack. Conversely, a question addressing headache severity should be included because this is helpful in distinguishing aura cases with migraineous headache from patients with non-specific headache.

The strength of our study includes the large samples of both the training (n=838) and validation sample (n=200), which are representative for the population studied. Both out-clinic patients and other patients (most of whom are treated by their own GP or neurologist elsewhere) were included via the same web-based flow. We found no clinical or demographic differences between these populations that could have affected the predictive model. Secondly, the use of a telephone interview as a gold standard by well-trained physicians with consultation of a headache specialist assured precise categorisation of migraineurs. Although we did not have a face-to-face interview as gold standard, we feel that our thorough semi-structured telephone interview safeguarded a very reliable migraine and aura diagnosis. Thirdly, the use of a validated screening instrument prior to our new questionnaire resulted in a group of self-reported migraineurs in which 95% could in fact be diagnosed with migraine. Fourth, we used a web-based questionnaire that was easy to fill out and send in for participants. With this approach, we successfully recruited large samples of migraineurs and contributed to the identification of the first genetic risk factor for the common forms of migraine [13]. We included a selected population of self-reported migraineurs, that had already been diagnosed with migraine by a physician, or otherwise thought they suffered from migraine, in which our questionnaire shows a high reliability in diagnosing aura. Our study did not aim to validate the questionnaire as a screening instrument for migraine in a naïve, general population.

The World Wide Web as a tool for recruiting patients and conducting research has several advantages. First, a large and diverse subject population can be reached at low cost [16]. Secondly, internet research imposes fewer burdens on participants, compared to

non-internet research [15]. Thirdly, available software permits data entry and analysis in a secure Web database. Fourth, investigators may be able to increase patient awareness and participation on clinical research. However, there might be certain challenges too [28]. Internet users tend to be younger and better educated than the patient population as a whole; visually impaired and minority groups may be underrepresented; and the symptoms expressed by participants may be more severe than is typical. We feel, however, these potential biases haven't pivotally influenced our data. Additionally, the so-called 'virtual Munchhausen syndrome', i.e. individuals referring themselves for studies for which they are not truly eligible, may compromise the validity of results [29]. In our study, we have no evidence that data have been influenced by subjects masquerading electronically as patients. This is in accordance with previous migraine research [15]. Even with such biases, altogether, the internet represents an appropriate aid to conduct research aimed at collecting clinical headache data from large numbers of patients.

We conclude that our web-based recruitment system in combination with an automated study flow is a very successful instrument to truly distinguish MA and MO in self-reported migraine patients. We propose to use our identified seven questions that have a higher accuracy in identifying aura cases from a population of self-reported migraineurs than an ICHD-2 based algorithm.

Acknowledgements

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Supplementary material

Table e-1. Sensitivity, specificity, predictive values and likelihood ratios of individual questionnaire headache items vs. the interview diagnosis of migraine headache. Sens. = sensitivity; Spec. = specificity; PPV = positive predictive value; NPV = negative predictive value; LR+ = positive likelihood ratio; LR- = negative likelihood ratio.

Variable	Question	Interview		Sens.	Spec.	PPV	NPV	LR+	LR-
		Migraine	No migraine						
Duration 4-72 hrs	Yes	**721**	19	0.74	0.72	0.97	0.16	2.64	0.36
	No	249	**49**						
Throbbing	Yes	**670**	232	0.94	0.29	0.74	0.71	1.32	0.21
	No	40	**96**						
Unilateral	Yes	**863**	57	0.95	0.56	0.94	0.61	2.16	0.89
	No	46	**72**						
Increase by activity	Yes	**878**	57	0.93	0.41	0.94	0.39	1.58	0.17
	No	63	**40**						
Severe	Yes	**516**	11	0.53	0.84	0.98	0.11	3.31	0.56
	No	455	**56**						
Nausea	Yes	**867**	63	0.96	0.53	0.93	0.67	2.04	0.08
	No	36	**72**						
Vomiting	Yes	**627**	87	0.91	0.75	0.88	0.80	3.64	0.12
	No	64	**260**						
Photophobia	Yes	**859**	91	0.97	0.41	0.90	0.72	1.64	0.07
	No	25	**63**						
Phonophobia	Yes	**809**	128	0.96	0.36	0.86	0.70	1.50	0.11
	No	30	**71**						

Table e-2. Sensitivity, specificity, predictive values and likelihood ratios of individual questionnaire aura items vs. the interview diagnosis of migraine aura. Table 2a comprises visual aura symptoms, Table 2b sensory aura symptoms, Table 2c motor aura symptoms and Table 2d disturbances respectively.

Table e-2a. Sensitivity, specificity, predictive values and likelihood ratios of individual questionnaire visual aura items vs. the interview diagnosis of migraine aura. Other specific visual disturbances could be filled out by patients in words and does not comprise any type of visual aura symptom mentioned. Sens. = sensitivity; Spec. = specificity; PPV = positive predictive value; NPV = negative predictive value; LR+ = positive likelihood ratio; LR- = negative likelihood ratio.

Aura	Question.	Interview		Sens.	Spec.	PPV	NPV	LR+	LR-
		Yes	No						
Visual aura symptoms									
Suffer from visual disturbances?	Yes	**436**	235	0.91	0.54	0.65	0.87	1.98	0.17
	No	42	**278**						
Shitters	Yes	**335**	117	0.70	0.77	0.74	0.74	3.04	0.39
	No	143	**396**						
Stars	Yes	**201**	71	0.42	0.86	0.74	0.62	3.00	0.67
	No	277	**442**						
Flashes	Yes	**178**	42	0.37	0.92	0.81	0.61	4.63	0.68
	No	300	**471**						
Scintillating lines	Yes	**223**	25	0.47	0.95	0.90	0.66	9.40	0.56
	No	255	**488**						
Figures	Yes	**111**	29	0.23	0.94	0.79	0.57	3.83	0.82
	No	367	**484**						
Coloured spots	Yes	**153**	70	0.32	0.86	0.69	0.58	2.29	0.79
	No	325	**443**						
Trembling air sensations	Yes	**488**	412	0.14	0.95	0.73	0.54	2.80	0.91
	No	25	**66**						
Wet window glass	Yes	**118**	71	0.25	0.86	0.62	0.55	1.79	0.87
	No	360	**442**						
Loss of vision	Yes	**283**	62	0.59	0.88	0.82	0.70	4.92	0.47
	No	195	**451**						
Diplopia	Yes	**146**	72	0.31	0.86	0.67	0.57	2.21	0.80
	No	332	**441**						
Other specific visual disturbances	Yes	**87**	67	0.18	0.87	0.57	0.53	1.38	0.94
	No	391	**446**						

Table e-2b. Sensitivity, specificity, predictive values and likelihood ratios of individual questionnaire sensory aura items vs. the interview diagnosis of migraine aura. Sens. = sensitivity; Spec. = specificity; PPV = positive predictive value; NPV = negative predictive value; LR+ = positive likelihood ratio; LR- = negative likelihood ratio.

Aura	Question.	Interview		Sens.	Spec.	PPV	NPV	LR+	LR-
		Yes	No						
Sensory aura									
Sensory	Yes	**114**	268	0.90	0.70	0.30	0.98	3.00	0.14
Numbness/ tingling	No	13	**623**						
Unilateral	Yes	**111**	236	0.87	0.73	0.32	0.98	3.22	0.18
	No	16	**655**						
5-60 min	Yes	**49**	50	0.39	0.94	0.50	0.92	6.50	0.65
	No	78	**841**						
Start before headache	Yes	94	**154**	0.74	0.83	0.38	0.96	4.35	0.31
	No	33	**737**						

Table e-2c. Sensitivity, specificity, predictive values and likelihood ratios of individual questionnaire motor aura items vs. the interview diagnosis of migraine aura. Sens. = sensitivity; Spec. = specificity; PPV = positive predictive value; NPV = negative predictive value; LR+ = positive likelihood ratio; LR- = negative likelihood ratio.

Aura	Question.	Interview		Sens.	Spec.	PPV	NPV	LR+	LR-
		Yes	No						
Motor aura symptoms									
Muscle weakness	Yes	20	**203**	0.77	0.80	0.09	0.99	3.85	0.29
	No	6	**802**						
Unilaterality	Yes	14	**59**	0.54	0.94	0.19	0.99	9.00	0.49
	No	12	**946**						
Duration 5-60 minutes	Yes	6	**47**	0.23	0.95	0.11	0.98	4.60	0.81
	No	20	**958**						
Starts prior to headache	Yes	14	**128**	0.54	0.87	0.10	0.99	4.15	0.53
	No	12	**877**						
Pinching	Yes	13	**117**	0.50	0.88	0.10	0.99	4.17	0.57
	No	13	**888**						
Arm lifting problem	Yes	10	**62**	0.39	0.94	0.14	0.98	6.50	0.65
	No	16	**943**						
Crippled walking	Yes	9	**51**	0.35	0.95	0.15	0.98	7.00	0.68
	No	17	**954**						
Facial asymmetry	Yes	8	**26**	0.31	0.97	0.24	0.98	10.33	0.71
	No	18	**979**						

Table e-2d. Sensitivity, specificity, predictive values and likelihood ratios of individual questionnaire speech disturbance items vs. the interview diagnosis of migraine aura. Sens. = sensitivity; Spec. = specificity; PPV = positive predictive value; NPV = negative predictive value; LR+ = positive likelihood ratio; LR- = negative likelihood ratio.

Aura	Question.	Interview		Sens.	Spec.	PPV	NPV	LR+	LR-
		Yes	No						
Speech disturbances									
Speech problems	Yes	132	**366**	0.94	0.57	0.27	0.98	2.19	0.11
	No	8	**489**						
Stiff mouth/ tongue	Yes	66	**103**	0.47	0.88	0.39	0.91	3.92	0.60
	No	74	**752**						
Wrong words	Yes	80	**96**	0.57	0.89	0.46	0.93	5.18	0.48
	No	60	**759**						
Expressive aphasia	Yes	119	**311**	0.85	0.64	0.28	0.96	2.36	0.23
	No	21	**544**						
Dysarthria	Yes	73	**98**	0.52	0.89	0.43	0.92	4.73	0.54
	No	67	**757**						
Prior to headache	Yes	102	**154**	0.73	0.82	0.40	0.95	4.06	0.33
	No	38	**701**						

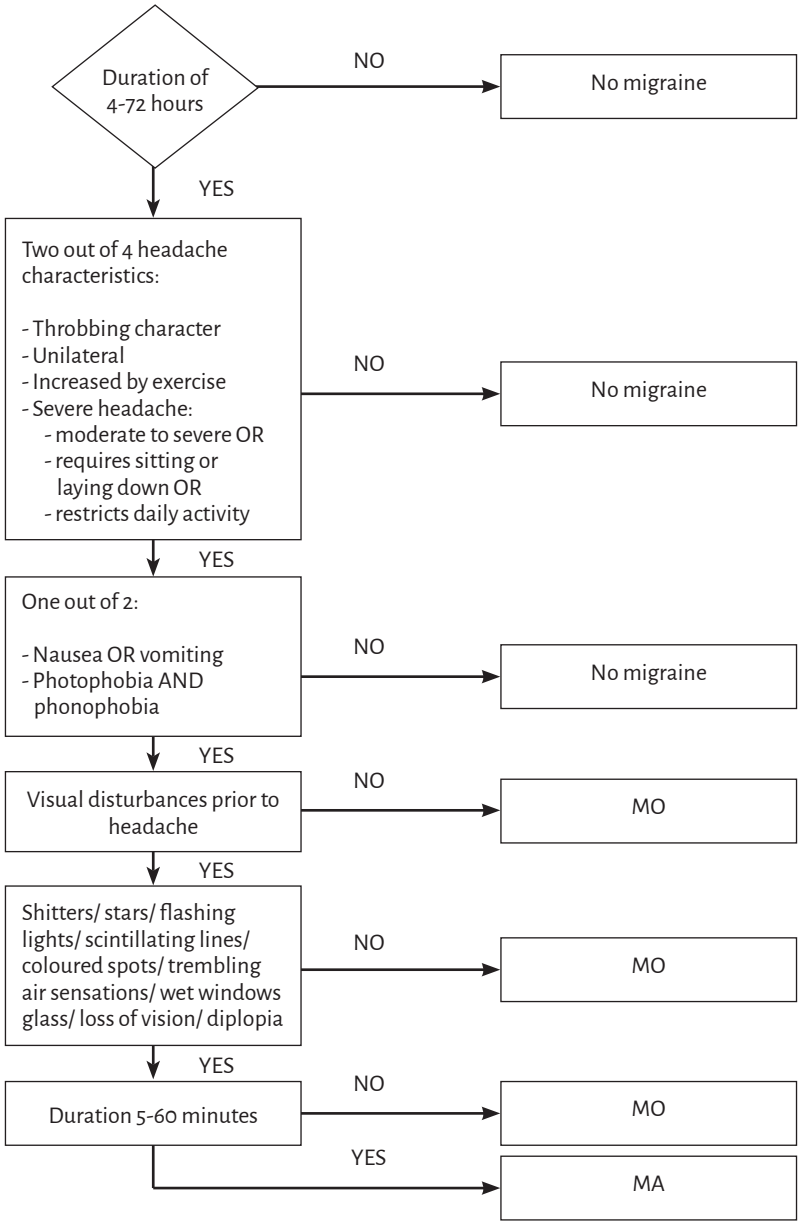

Figure e-1. Structure of ICHD-II based algorithm used in LUMINA study. MO = migraine without aura; MA = migraine with aura;

Chapter 3.

Chronotypes and circadian timing in migraine

van Oosterhout WPJ[1]
van Someren EJ[2,3]
Schoonman GG[1,4]
Louter MA[1,5]
Lammers GJ[1,6]
Ferrari MD[1]
Terwindt GM[1]

[1]Department of Neurology, Leiden University Medical Center, Leiden, the Netherlands
[2]Department of Sleep and Cognition, Netherlands Institute for Neuroscience, an Institute of the Royal Netherlands Academy of Arts and Sciences, Amsterdam, the Netherlands
[3]Depts. of Integrative Neurophysiology and Medical Psychology, Center for Neurogenomics and Cognitive Research (CNCR), VU University and Medical Center, Amsterdam, the Netherlands
[4]Department of Neurology, Elisabeth-Tweesteden Hospital Tilburg, Tilburg, the Netherlands
[5]Department of Psychiatry, Leiden University Medical Center, Leiden, the Netherlands
[6]Sleep Wake Center SEIN Heemstede, Heemstede, the Netherlands.

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Abstract

Background
It has been suggested that migraine attacks strike according to circadian patterns, and that this might be related to individual chronotype. Here we evaluated and correlated individual chronotypes, stability of the circadian rhythm, and circadian attack timing in a large and well-characterized migraine population.

Methods
In 2,875 migraine patients and 200 non-headache controls we assessed differences in: (i) distribution of chronotypes, (Münich Chronotype Questionnaire); (ii) the circadian rhythm's amplitude and stability, (Circadian Type Inventory); and (iii) circadian timing of migraine attacks. Data were analysed using multinomial and linear regression models adjusted for age, gender, sleep quality and depression.

Results
Migraineurs more often showed an early chronotype compared to controls (48.9% vs. 38.6%; adjusted OR 2.42 95% C.I. 1.58-3.69; p<0.001); as well as a late chronotype (37.7 vs 38.0%; adjusted OR 1.69 (95% C.I.: 1.10-2.61; p=0.016). Migraineurs, particularly those with high attack frequency, were more tired after changes in circadian rhythm (i.e. more languid; p<0.001) and coped less well with being active at unusual hours (i.e. more rigid; p<0.001) than controls. 961/2,389 (40.2 %) migraineurs reported early morning attack onset.

Conclusion
Migraine patients are less prone to be of a normal chronotype than controls. They are more languid and more rigid when changes in circadian rhythm occur. Most migraine attacks begin in the early morning. These data suggest that chronobiological mechanisms play a role in migraine pathophysiology.

Introduction

Several studies have suggested that migraine attacks show seasonal and circadian periodicity with attacks more likely to occur in the early morning, implicating chronobiological mechanisms in attack triggering and initiation [1-3]. Candidate mechanisms include those involved in (i) the later stages of the sleep cycle and/or the sleep/wake transition; (ii) various intrinsic circadian cycles and (iii) mechanisms functioning as *zeitgebers*, environmental signals such as the light modulating the biological clock [1-3].

Chronotype refers to an individual's endogenous circadian clock rhythm and how it synchronizes (entrains) to the 24h day. Chronotypes depend on sex, age, genetic and environmental factors [4], and are distributed normally in a given population. Some are very late (evening) people ('owls') while others are very early (morning) people ('larks') [5]. Early and late chronotypes have been associated several diseases. Early chronotypes have been associated with depression [6] and epilepsy [7], paroxysmal brain disorders with strong bidirectional comorbidity with migraine [8]. Late chronotypes have been associated with suicide attempts [9] and bipolar disorder [10], psychiatric disorders showing unidirectional comorbidity with migraine [9, 11]. Only a few small studies have investigated chronotype and migraine, with inconsistent results [2, 12]. Whether early or late chronotypes are associated with specific circadian timings of the onset of migraine attacks is unknown. Besides circadian phase (i.e. chronotype), low amplitude and high flexibility of circadian rhythm enable better coping with changes in sleep/wake pattern [13].

The present study has three aims. First, to analyse chronotypes in a large and well-defined migraine population. Second, to assess circadian rhythm amplitude and stability in relation to migraine. Finally, to study whether chronotype and circadian timing of migraine attacks are associated.

Material and methods

Subjects

Our study was conducted as a part of the Leiden University Medical Center Migraine Neuro Analysis (LUMINA) programme [14]. Participants were Dutch adults aged 18 to 74 years of age, both migraine patients and healthy controls. Patients with migraine with and without aura fulfilled the International Classification of Headache Disorders (ICHD-3b) criteria [15]. Controls did not suffer from migraine, cluster headache, chronic tension type headache or medication overuse headache. Both migraine patients and controls were recruited via public announcement, advertising in lay press and via the research website, and were considered eligible after a two-step inclusion process using validated questionnaires (see Supplementary Text 1 for details).

Respondents and non-respondents in this study

Eligible subjects (both migraine patients and non-headache controls) within the LUMINA study were sent an invitation to participate in this study into chronotype by e-mail. A

reminder was sent twice. Subjects not having participated after two reminders were considered non-respondents. Baseline and demographic data of the non-respondents were available in the LUMINA study.

Standard protocol approvals, registrations and patient consents

The study had been approved by the local medical ethics committee. All subjects provided written informed consent prior to the procedure.

Design

In this observational and cross-sectional study, eligible subjects were sent an invitation to a digital questionnaire on sleep habits and sleeping problems. This included questions on phase, rhythm and stability of circadian chronotype as well as items on circadian timing of migraine attack onset. Questionnaires were filled out between September 2010 and September 2011. Non-responders were reminded twice per e-mail, and once per telephone.

Chronotype assessment

Circadian chronotype phase

Chronotype was assessed using a Dutch translation of the Munich Chronotype Questionnaire (MCTQ)[16,17]. The MCTQ obtains one's subjective self-reported chronotype (early, normal, or late). From the MCTQ, the 'timing of mid sleep on free days' is calculated. This timing is an objective measure of chronotype derived from the timing of mid-sleep on free days (MSF), the point of time exactly in the middle of the total sleep time on free days (individual sleep timing and duration are independent traits). For participants who indicated they were on shift-work at the moment of filling the questionnaire, additional questions on timing of sleep, going to bed etc. - separately for each of the different shifts - were visible and obliged to fill out. At the moment of our study the specific MCTQ that is validated for shift work[18] was not yet available.

Chronotype, sleep duration, age and gender

Both sleep duration and sleep timing on free days are influenced by the sleep-debt accumulated over the workweek[17]. These parameters were therefore corrected for the confounding effect of sleep-debt during the workweek, which were used in the analyses[17]. Analyses between migraineurs and controls were adjusted for gender and age, given its age and gender-dependency[17].

Circadian rhythm amplitude and stability

Amplitude and stability were assessed using the Circadian Type Inventory, a scale measuring individual capabilities and preferences regarding changes in sleep pattern[19]. The scale consists of two subscales. The languid-vigour scale reflects the individual capability to recover from a change in sleep-pattern and is linked to the amplitude of the circadian rhythm. The flexible-rigid scale reflects preferences regarding sleep pattern and is linked to the rhythm's stability. Each subscale contains 15 items, with 5 answer options (ranging from 1: 'practically never' to 5: 'practically always'), and total scores range from 15-75 per subscale. A higher score on languid-vigour indicates that an individual is more tired after changes in circadian rhythm (i.e. more languid: difficulty to overcome drowsiness and lethargy after

reduced sleep). A lower score on flexible-rigid indicates that an individual is coping less with being active or sleep at unusual hours (i.e. less flexible, more rigid).

Circadian timing of attack onset

Migraineurs were asked to indicate on what time of the day attacks usually started, in 6 hour intervals (0.00-6.00 a.m.; 6.00-12.00 a.m.; 12.00-6.00 p.m.; 6.00-12.00 p.m.; or "can not indicate"). If 6-hour intervals were indicated, patients were asked to be more precise in 2-hour intervals, if possible.

Migraine characteristics, demographics, data on sleep quality and depression

Within the LUMINA cohort, data on migraine characteristics were available. Of both migraineurs and controls demographics, data on intoxications, sleep medication and sleep data (Pittsburgh Sleep Quality Index; range 0-21, with score >5 indicative for poor sleep quality [20]) were collected. For depression, data from the HADS questionnaire [21] (Hospital Anxiety and Depression Scale with Anxiety and Depression subscales; total range 0-42, with HADS-D score ≥8 indicative for depression), CES-D (the Center for Epidemiologic Studies Depression scale; total range 0-60, CES-D>16 indicative for depression [22]), and a combined life-time depression algorithm [8] (HADS-D≥8 or CES-D>16 or physician-made diagnosis of depression or use of antidepressants with indication of depression) were used [23].

Statistics

General characteristics were compared between migraineurs and controls using Student's t-tests for continuous variables, and Chi square tests for categorical data. To assess differences in chronotypes between patients and controls chi square tests and multinomial regression analyses were performed with chronotype as dependent variable (levels: early, normal, and late chronotype), adjusted for age and gender, and additionally for sleep quality and HADS depression score and shift work. Midsleep on free days corrected for sleep debt (MSFsc) was compared between patients and controls using a linear regression model, adjusted for age and gender. Continuous data on circadian rhythm's stability and amplitude were analysed using linear regression models (to identify determinants). The relationship between circadian timing of attack onset and chronotype was also assessed using Chi square tests and multinomial regression analyses. All data analyses were performed using SPSS 17.0 (SPSS inc., IBM, USA), with the statistical threshold at $p<0.05$.

Results

Study population

Questionnaires were sent to 2,875 migraineurs and 200 headache free controls. The total response was 2,578 (83.8%): 2,389 (83.1%) for migraineurs and 189 (94.5%) for controls. The characteristics are summarised in Table 1.

Confounders

Compared to controls, migraineurs more frequently were female, had a lower educational level, and had a slightly higher body mass index (BMI). They consumed less units of alcohol

per week, had higher total scores on the Pittsburgh Sleep Quality Index and Hospital Anxiety and Depression Scale -Depression subscale (HADS-D), as well as a higher prevalence of life-time depression.

Table 1. Baseline characteristics of study population. Baseline characteristics of migraineurs (n=2,389) and non-headache controls (n=189). *p*-values depicted in bold indicate significant differences ($p<0.05$), using independent-samples t-tests and χ^2 tests where appropriate. Y = years; F = female; BMI = body mass index; SD = standard deviation; PSQI = Pittsburgh Sleep Quality; HADS-D = Hospital Anxiety and Depression Scale, Depression sub-scale.

Variable	Total (n=2,578)	Migraineurs (n=2,389)	Controls (n=189)	*p*
Demographics				
Age y, mean (SD)	45.2 (11.9)	45.1 (11.7)	46.4 (14.2)	0.23
Gender F, n (%)	2,149 (83.4%)	2.047 (85.7%)	102 (54.0%)	**<0.001**
BMI kg/m², mean (SD)	24.5 (4.0)	24.6 (4.1)	24.1 (2.8)	**0.045**
Education level (%)				**0.022**
Low	163 (6.7%)	151 (6.7%)	12 (6.3%)	
Middle	838 (34.72%)	790 (35.0%)	48 (25.4%)	
High	1,447 (59.1%)	1,318 (58.3%)	129 (68.3%)	
Missing	130 (5.0%)	130 (7.6%)	0	
Intoxications				
Nicotine, packyears, mean (SD)	4.8 (9.1)	4.9 (9.2)	4.7 (8.3)	0.84
Alcohol; units/ week, mean (SD)	3.1 (4.4)	2.7 (3.8)	6.9 (7.5)	**<0.001**
Caffeine; units/ day, mean (SD)	5.9 (3.0)	5.9 (3.0)	5.6 (2.4)	0.18
Other				
PSQI total score, mean (SD)	6.3 (3.6)	6.5 (3.6)	4.2 (2.8)	**<0.001**
PSQI ≥6, %	1,330 (51.6%)	1,277 (53.5%)	53 (28.0%)	**<0.001**
HADS-D, score, mean (SD)	4.2 (3.6)	4.3 (3.6)	2.6 (3.0)	**<0.001**
Life-time depression	1,046 (40.6%)	1,017 (42.6%)	29 (15.3%)	**<0.001**
Shiftwork ever, n (%)	764 (29.6%)	716/2.383 (30.0%)	48/189 (25.4%)	0.19
Shiftwork last week, n (%)	186/756 (24.6%)	177/716 (24.9%)	11/48 (22.9%)	0.77
Shiftwork history, y, mean (SD)	10.7 (9.5)	10.8 (9.6)	8.5 (7.7)	0.06

Non-respondent data

Non-responder analysis in controls showed higher HADS-D score compared to responders due to one non-responder control who was an outlier with severe depression ($p=0.002$). In migraine patients, responders were slightly older ($p<0.001$), had a higher BMI ($p=0.03$), without differences in HADS-D scores ($p=0.09$) compared to non-responder migraineurs (data not shown).

Chronotypes in migraineurs and controls

Self-reported chronotypes

Chronotypes were distributed differently between groups (unadjusted proportions; $p<0.001$). Early chronotypes were more common in the migraine group (unadjusted: 1,167/2,387, 48.9% vs. 73/189, 38.6%), controls were more often normal chronotypes (44/189, 23.3% vs. 319/2.387, 13.4%). Late chronotypes did not differ (unadjusted: 901/2,387, 37.7%;

vs. 72/189, 38.1%). The adjusted odds ratio (OR) for a migraineur (vs. non-headache control) to have an early chronotype vs. a normal chronotype was 2.42 (95% C.I.: 1.58-3.69), and the OR for having a late vs. normal chronotype was 1.69 (95% C.I.: 1.10-2.61) (e-Table 1). In the overall model, there were significant effects of age (p<0.001), PSQI score (p=0.03) and HADS-D score (p=0.008), but not for gender (p=0.36) or shift work in the previous week (p=0.58). Sleep quality and HADS depression score were different between groups (p<0.05), but did not alter the difference between migraineurs and controls significantly.

Self-reported chronotypes in migraine subtypes

Earlier chronotypes were overrepresented in both migraine with aura and migraine without aura (χ2-square test; p=0.002; p=0.01) vs. the non headache controls. Multinomial regression showed that both migraine subgroups were more likely to have both early and late chronotypes vs. the non headache controls (e-Table 1).

Timing of mid sleep on free days corrected for sleep-debt (MSFc)

MSFsc was not different between migraineurs and controls (mean ± SD): 3:39±0:58 vs. 3:43±0:59, adjusted for age and gender p=0.33 (e-Table 2).

Amplitude and stability of the circadian rhythm

Amplitude of the circadian rhythm

Migraineurs were more languid (more tired after changes in sleep/wake pattern) compared to controls (mean ± SD): 48.9±0.6 vs. 46.1±1.3; p<0.001 (age and gender adjusted). Female gender, lower age and higher HADS-D score were correlated with greater languidnesss (higher scores) (Table 2). Higher attack frequency (p=0.009), but not migraine subtype (p=0.65), was associated with higher scores.

Table 2. Predictors of higher scores on languid-vigour (LV) and flexible-rigid (FR) subscales in migraineurs and non-headache controls. Higher score on LV scale reflects more languidness. On the FR scale, higher scores reflects more flexibility whilst lower scores reflect more rigidity. B = regression coefficient; SE = standard error; C.I. – confidence interval; MO = migraine without aura; F = female gender; HADS-D = Hospital Anxiety and Depression Scale, Depression subscale.

Variable	Languid-vigour				Flexible-rigid			
	B	SE	95% C.I.	*p*	B	SE	95% C.I.	*p*
Total group								
Migraine diagnosis	2.81	0.64	1.53 - 4.08	**<0.001**	-3.00	0.51	-4.00 - -2.00	**<0.001**
Age (year)	-0.22	0.01	-0.25 - -0.19	**<0.001**	-0.06	0.01	-0.09 - -0.04	**<0.001**
Gender (F)	2.43	0.46	1.53 - 3.33	**<0.001**	-3.38	0.36	-4.10 - -2.68	**<0.001**
BMI (kg/m^2)	0.03	0.04	-0.06 - 0.11	0.52	0.11	0.03	0.05 - 0.17	**0.001**
HADS-D score	0.59	0.05	0.50 - 0.68	**<0.001**	-0.36	0.04	-0.43 - -0.29	**<0.001**
Migraineurs								
MO subtype	-0.17	0.36	-0.87 - 0.54	0.65	0.46	0.28	-0.09 – 1.00	0.10
Attack frequency	0.46	0.17	0.12 - 0.80	**0.009**	-0.76	0.13	-1.02 - -0.50	**<0.001**

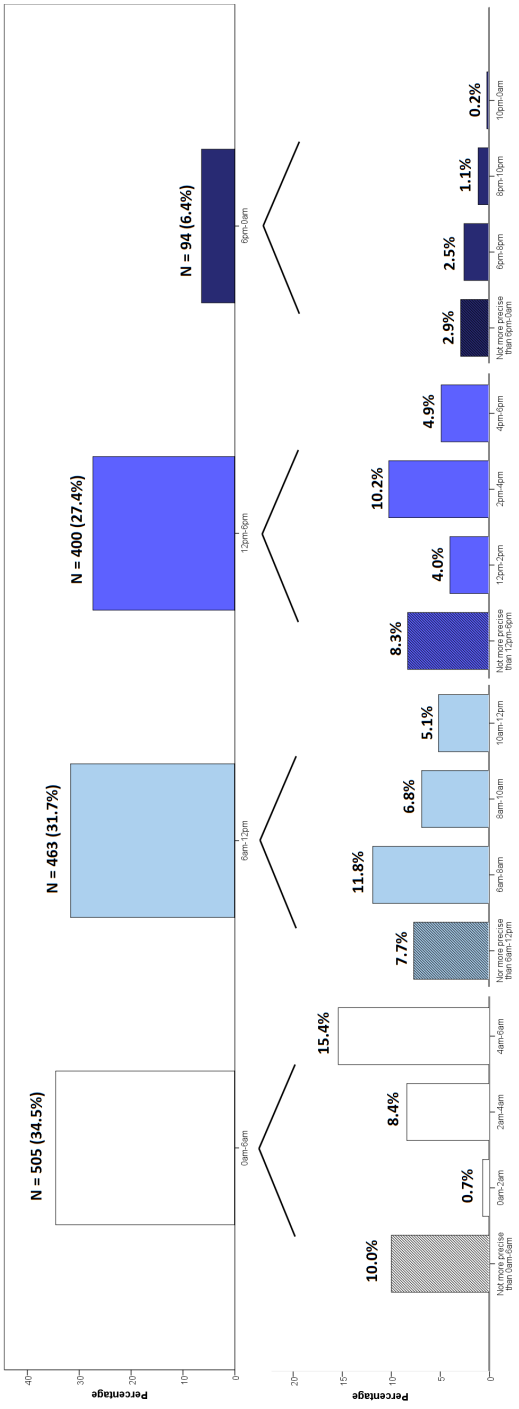

Figure 1. Distribution of circadian preference periodicity of migraine attack onset in migraine patients.
The upper panel depicts the timing of clinical onset of migraine attacks in 1,456/2,389 (61.0%) migraineurs who were able to specify the circadian timing of their attacks in 6h intervals. Attack most often began between 4 and 6 am (15.4% of total) or between 6 and 8 a.m (11.8% of total). In the lower panel, specifications into 2h intervals are depicted, with the bars accented in grey showing patients who could not further specify in 2h intervals. Percentages in the lower panel add up to 100%.

Stability of the circadian rhythm

Migraineurs were more rigid (less able to cope with changes in sleep/ wake pattern) than controls (reflected by lower scores): 48.0±0.6 vs. 51.0±1.0; p<0.001 (age and gender adjusted) (Table 2). Higher age, lower BMI, female gender and higher HADS-D score were associated with more rigidity (lower score). Higher attack frequency was correlated with lower scores (p<0.001).

Circadian timing of attack onset in migraineurs

In total, 1,462/2,389 (61.0%) of migraineurs indicated a specific circadian timing for their migraine attacks, most often between 0.00-6.00 am (505/1,462; 34.5%), and between 6.00-12.00 am (463/1,456; (31.7%)). Out of these, 1,050/1,462 (71.2%) were able to indicate the usual timing of the onset of their attacks in 2h segments: 2.00-4.00 am and 4.00-8.00 am were reported most frequently (together: 399/1,050; 38.0%) (Figure 1).

Patient chronotype linked to clinical migraine characteristics

Chronotypes were associated with attack time (χ2-test; overall model p=0.003 (Figure 2)). Early attacks (0-6am) were most often reported by migraineurs with early chronotypes (57.6%), whereas patients with late chronotypes reported later attacks (12-6pm) more often (post-hoc; p<0.001). In patients, earlier circadian attack onset was related to higher age (p<0.001) and migraine without aura subtype (p=0.008) (using multinomial regression; overall model significance p<0.001). Early chronotypes were associated with higher age (p<0.001) as well as lower BMI (p=0.011), lower HADS-D scores (p=0.003), and worse sleep quality (p=0.004), compared to late chronotypes. Attack frequency and migraine subtype were not associated with chronotype (e-Table 3).

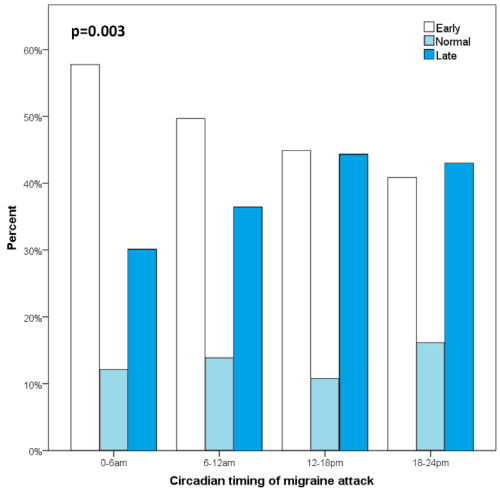

Figure 2. Chronotype in relation to migraine attack onset.

Early chronotypes are overrepresented among migraine patients with early attack onset. The proportion of migraine patients with early chronotype declines with advancing circadian attack onset time, whilst the proportion of late chronotypes increases. Normal chronotypes are evenly prevalent amongst subgroups with different attack onset times.

Discussion

We found that migraineurs are less prone to be of a normal chronotype compared to healthy controls, and that they are less flexible in adapting to changes in the sleep/wake cycle. Migraine attack onset peaks in the early morning and is related to early chronotype. These findings suggest a different setting of the circadian clock in migraineurs and that mechanisms which are involved in the initiation of migraine attacks are linked to chronobiological pathways.

In contrast to two other, smaller studies which reported contrasting results, our study found that both migraine with and without aura patients are less prone to be of a normal chronotype. Gori et al. also reported overrepresentation of both morning and evening type subjects in 100 migraine without aura patients vs. 30 healthy controls[2]. In 93 patients with menstrual migraine, Cevoli et al found no differences in chronotype distribution compared to 85 controls[12]. We found that over 60% of patients reported circadian periodicity of their attacks, and that those migraine attacks showed a predilection for the early morning, mostly in patients with early chronotype. This is in line with earlier smaller studies[1-3]. Fox et al reported that migraine attacks started most frequently between 4-8am, based on 3,598 migraine attacks in 1,698 patients[1]. In a 11-month prospective study with 58 female patients, Alstadhaug et al. found that migraine attacks tended to peak around the middle of the day[3]. Amplitude (languid-vigour) and stability (flexible-rigid) of the circadian rhythm have not been studied before in relation to migraine. Our data show that migraine patients are more languid, indicating that they have more difficulty to overcome the effects of reduced sleep. They also have a more rigid circadian rhythm, i.e. they prefer to sleep and be active at set hours. Both effects are most pronounced among patients with high attack frequency. The effect sizes, however, are small and the exact clinical relevance needs to be further studied.

Our study has several strengths. The study sample is very large and the patients are well characterised. Non-headache controls and patients were recruited in exactly the same way, minimising the risk of inclusion bias. The use of validated instruments for migraine diagnosis and chronotype[5, 14] assured large populations of well characterised migraine patients and healthy subjects and detailed evaluation of circadian rhythmicity. Thirdly, the web-based questionnaire was easy to fill out and send in, resulting in high response rates in both groups.

Some limitations of the study can be addressed. There were some differences between the migraine and control groups. Migraine patients were more often female, had lower education levels and used less alcohol. They showed lower sleep quality and higher depression scores. Ideally, the differences between the migraine and the control groups would have been smaller. To minimize potential bias, the primary analyses were adjusted for the effects of age and gender. As an additional check, additional corrections were performed for the effects of sleep quality and depression. These, however, did not affect the differences between migraineurs and controls. Furthermore, we were not able to include the specific MCTQ shift-work version[18] in this study, since it was published after our data collection period. However, in participants who indicated that they were doing shift-work at the time of filling out the questionnaire, additional questions for each of the possible

shifts separately were included. Unfortunate as the number participant with recent shift work (last week) was very small no useful separate analysis could be made.

Although the number of control subjects in our study was considerably smaller than the number of migraine patients in our study, and smaller than numbers from population based studies, the distribution of chronotypes in the control group is similar to the general population. We believe the smaller size of the control group has hardly affected the statistical power of the study. The number of cases (n = 2,389) was high and the number of controls (n = 189) was still considerable, resulting in post-hoc power of 0.93 to detect the 12% difference in proportion of early chronotypes between both study groups at alpha 0.05. With regard to circadian rhythm amplitude and stability scales, post-hoc power to detect the differences we found was 1.0 (See supplementary text). Increasing the number of controls would have involved disproportionately large and in fact unnecessary efforts leading to only moderate increase in study power.

The subjects within the LUMINA study have partly been self-selected, since registration and participation via the study website was obligatory. The population we invited for participation was highly motivated as was reflected by the high response rates (over 80%). Although we can not rule out a self-selection bias, since both patients and controls have been recruited similarly, we feel this potential bias has affected both groups in a similar way. Since, in the LUMINA cohort, only 4% of subjects were included from our dedicated headache outpatient clinic and 87% of participants have been diagnosed with migraine previously by a physician, we feel these patients are representative of the migraine population in our country.

Overrepresentation of early chronotypes, early circadian attack onset and high circadian rigidity suggest that migraineurs have a different setting of the endogenous pacemaker in the suprachiasmatic nucleus, the main circadian rhythm initiator. This nucleus has extensive projections to the hypothalamus and the pineal gland and is pivotal in regulating wakefulness, the sleep/wake cycle [24], and various other body rhythms. The suprachiasmatic nucleus has been suggested to play an important role in the pathophysiology of episodic brain disorders such as cluster headache and migraine. It is unknown where, how, and why migraine attacks begin and the hypothalamus might be the site of initiation. Several observations and arguments support this hypothesis. Anatomically, the hypothalamic A11 dopaminergic nucleus facilitates and modulates trigeminovascular nociception [25], the underlying mechanism for headache in migraine. Clinically, hypothalamic involvement is suggested by the nature of the premonitory symptoms which frequently occur several hours to even days before the headache and other features of the migraine attack begin; the circadian rhythmicity of migraine attacks [1,2]; the temporal relationship between fluctuations in female hormone levels (menarche, menstruation, pregnancy and menopause) and onset, recurrence and disappearance of migraine attacks in women, and changes in several other hormones [26]. Altered hypothalamic activation during spontaneous migraine attacks has also been detected in a positron emission tomography study [27]. More recently, a functional imaging study covering three untreated migraine attacks in one patient showed, in addition to hypothalamic activation, altered functional coupling to the trigeminal spinal nuclei prior

to an attack. Functional changes in the hypothalamo-brainstem coupling might be an important driver for attack initiation [28].

Early chronotypes are also overrepresented in depression and epilepsy [29]. These paroxysmal brain disorders show strong bidirectional comorbidity with migraine, suggesting overlapping pathophysiologic mechanism, possibly shared genetic factors [8]. These might predispose to early and late chronotypes and circadian rigidity. This hypothesis is further supported by observations in two rare genetic conditions, in which migraine is associated with marked changes in sleep pattern or biorhythm. First, in familial advanced sleep phase syndrome, a very rare, highly penetrant autosomal disorder caused by a mutation in the casein kinase 1-delta *(CK1-δ)* gene, patients suffer from extreme chronotype shifts leading to advanced sleep onset and offset. Patients in two of these families also have migraine with aura [30]. Second, transgenic mouse models expressing the R192Q *CACNA1A1* mutation that in humans causes familial hemiplegic migraine lack the physiological retardation in circadian adaptation to phase advance shifts (east-bound jetlag) [31].

In conclusion, most migraineurs are early birds and have difficulties in coping with (acute) changes in the sleep/wake cycle. Attack preferentially strike in the early morning. These observations underscore an important role for chronobiological mechanisms in migraine attack initiation.

Clinical Implications

- Migraine patients are less prone to be of a normal chronotype and they are more languid and more rigid when changes in circadian rhythm occur.
- 60% of migraine patients report diurnal periodicity of headache attacks, of which one third reports attack beginning between midnight and 6 am, and one third between 6 am and noon
- Taken together, these data suggest that chronobiological mechanisms play a role in migraine pathophysiology.

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Supplementary material

Supplementary text

Subject inclusion within the LUMINA study

Both migraine patients and controls were recruited via nationwide public announcement, advertising in lay press and via the research website, and were considered eligible after a two-step inclusion process using validated questionnaires via the especially designed LUMINA website. Additionally, patients from our outpatient headache clinic were invited to participate by a letter. On the website, patients were asked to fill out a screening questionnaire that has been validated previously. Firstly, if patients fulfilled the screening criteria, they were sent a web-based extended migraine questionnaire, based on the ICHD-II criteria [14, 15]. This questionnaire was validated before by performing a semi-structured telephone interview in 1,038 patients who had filled out the extended migraine questionnaire [14]. The specificity of the questionnaire was 0.95. We consider the cohort a well-defined web-based cohort, with 4% of subjects included from our dedicated headache outpatient clinic, 87% of the participants having been diagnosed as migraineurs previously by a medical doctor. In addition to questions that were necessary to diagnose migraine accurately, the extended questionnaire also included items on demographic factors, acute and prophylactic headache medication use, migraine attack frequency and allodynia. Participants without the needed internet skills were able to fill out the questionnaires on paper. Non-headache individuals willing to participate had to pass a screening questionnaire online via the research website. If this screening questionnaire did not show any indication for having migraine, cluster headache, chronic tension type headache or medication overuse headache, individuals were sent a subsequent in depth questionnaire. This second questionnaire again assessed possible headache complaints, together with demographic variables. Only individuals that fulfilled both the criteria of 'non-headache' in the screening and in depth questionnaire were considered eligible controls and were approached for this questionnaire study. Since recruitment of control subjects started at a later point in time and inclusion rate was slower, the number of migraine patients included exceeds the number of controls included in our study.

Respondents and non-respondents in this study

Eligible subjects (both migraine patients and non-headache controls) within the LUMINA study were sent an invitation to participate in this study into chronotype by e-mail. A reminder was sent twice. Subjects not having participated after two reminders were considered non-respondents. Baseline and demographic data of the non-respondents were available in the LUMINA study.

Sample size

The control group in our study was considerably smaller than the case group but, we believe, this has hardly affected the statistical power of the study. The number of cases (n = 2,389) was high and the number of controls (n = 189) was still considerable, resulting in a post-hoc power of 0.93 to detect the 13% difference in proportion of early chronotypes between both

study groups at alpha 0.05 (and a post-hoc power of 0.76 to detect a 10% difference). With regard to circadian rhythm amplitude stability, we have a post-hoc power of 100% to detect a difference of 2.0 (amplitude scale) and 3.0 (stability scale) between groups at alpha 0.05. Increasing the number of controls would have involved disproportionately large and in fact unnecessary efforts leading to only moderate increase in study power.

Supplementary tables

e-Table 1. Determinants for chronotype distribution in migraineurs and non-headache controls.

Variable	Early vs. normal chronotype			Late vs. normal chronotype		
	Odds ratio	95% C.I.	*p*	Odds ratio	95% C.I.	*p*
Migraine (vs. controls)	2.42	1.58-3.69	**<0.001**	1.69	1.10-2.61	**0.016**
Migraine without aura (vs. controls)	2.14	1.37-3.34	**0.001**	1.54	0.96-2.43	0.06
Migraine with aura (vs. controls)	2.76	1.71-4.43	**<0.001**	2.01	1.23-3.28	**0.005**
Age (year)*	1.01	1.00-1.02	0.11	0.98	0.96-0.99	**<0.001**
Gender (F)*	0.88	0.62-1.19	0.37	0.98	0.70-1.38	0.92
PSQI score*	0.96	0.92-0.99	0.01	0.98	0.94-1.01	0.19
HADS-Depression score*	0.99	0.95-1.02	0.17	1.02	0.99-1.07	0.19
Shift work last week*	0.88	0.55-1.41	0.59	1.05	0.65-1.01	0.84

F = female gender; C.I. = confidence interval. All analyses were adjusted for age, gender, PSQI and HADS score. Outcome of multinomial regression. * overall model significance was $p<0.001$ (age), $p=0.36$ (gender), $p=0.03$ (PSQI score); $p=0.008$ (HADS Depression score) and $p=0.58$ (shift work last week).

e-Table 2. Mid sleep and sleep duration in migraineurs and non-headache controls. Data are depicted for total group and for strata based on age and gender. MSFsc = Mid sleep on free days (clock time), corrected for accumulated sleep debt; SD = standard deviation. Higher age predisposed for earlier MSFsc (p<0.001), but gender was not associated (p=0.10).

	Controls				Migraineurs			
	n	*Work days* Ø ± SD	*Free days* Ø ± SD	*MSFsc* Ø ± SD	n	*Work days* Ø ± SD	*Free days* Ø ± SD	*MSFsc* Ø ± SD
Mid sleep time, h:min±SD								
< 21 years of age	189	3:04±42'	3:57±69'	3:43±59'	2.389	3:02±46'	3:51±68'	3:39±58'
21-30 years of age	4	3:31±23'	5:15±74'	4:48±54'	12	3:51±26'	5:25±58'	5:07±63'
> 30 years of age	32	3:12±38'	4:45±77'	4:18±76'	285	3:17±53'	4:40±60'	4:19±61'
Women	153	3:01±43'	3:46±62'	3:34±52'	2,092	2:59±44'	3:44±65'	3:33±55'
Men	102	3:04±37'	4:01±72'	3:49±63'	2,047	3:03±45'	3:51±67'	3:39±57'
	87	3:04±47'	3:53±67'	3:48±63'	342	2:57±50'	3:50±66'	3:39±63'
Sleep duration, h:min±SD								
< 21years of age	189	7:04±61'	7:41±83'		2.389	7:05±69'	7:36±92'	
21-30 years of age	4	8:14±55'	9:30±71'		12	7:48±52'	8:41±65'	
>30 years of age	32	7:20±47'	8:32±61'		285	7:31±67'	8:31±81'	
Women	153	6:57±63'	7:28±82'		2,092	7:01±69'	7:28±91'	
Men	102	7:18±64'	7:54±89'		2,047	7:07±69'	7:37±93'	
	87	6:48±55'	7:27±64'		342	6:56±71'	7:32±86'	

e-Table 3. Demographics and clinical characteristics in different chronotypes among migraineurs. BMI = Body Mass Index; MO = migraine without aura; AF = attack frequency; PSQI = Pittsburgh Sleep Quality Index; HADS-D = Hospital Anxiety and Depression Scale; Depression subscale.

Variable	Early	Normal	Late	*p*
Age y, mean (SD)	46.9 (11.0)	46.0 (12.1)	42.4 (11.9)	**<0.001**
BMI, mean (SD)	24.3 (3.9)	24.6 (3.9)	24.9 (4.4)	**0.011**
MO, n (%)	695/1.167 (59.6%)	203/319(63.6%)	561/901 (62.3%)	0.28
AF≤4 / months, n (%)	1.075/1.167 (92.1%)	287/319 (90.0%)	827/901 (91.6%)	0.47
PSQI, mean (SD)	6.3 (3.5)	7.0 (4.1)	6.6 (3.5)	**0.004**
HADS-D, mean (SD)	4.1 (3.4)	4.6 (3.8)	4.6 (3.7)	**0.003**

Chapter 4.

Restless legs syndrome in migraine patients: prevalence and severity

Van Oosterhout WPJ[1]
van Someren EJ[2,3]
Louter MA[1,4]
Schoonman GG[1,5]
Lammers GJ[1,6]
Rijsman RM[7]
Ferrari MD[1]
Terwindt GM[1]

[1]Department of Neurology, Leiden University Medical Center, Leiden, the Netherlands
[1]Department of Sleep and Cognition, Netherlands Institute for Neuroscience, an Institute of the Royal Netherlands Academy of Arts and Sciences, Amsterdam, the Netherlands
[3]Departemnts of Integrative Neurophysiology and Medical Psychology, Center for Neurogenomics and Cognitive Research (CNCR), VU University and Medical Center, Amsterdam, the Netherlands
[4]Department of Psychiatry, Leiden University Medical Center, Leiden, the Netherlands
[5]Department of Neurology, Elisabeth-Tweesteden Hospital Tilburg, Tilburg, the Netherlands
[6]Sleep Wake Center SEIN Heemstede, Heemstede, the Netherlands
[7]Centre for Sleep and Wake Disorders, Medical Centre Haaglanden, The Hague, The Netherlands.

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Abstract

Background
We aimed to study not only prevalence but more importantly severity and correlation between sleep quality and restless legs syndrome (RLS) in a large population of well-defined migraine patients as poor sleep presumably triggers migraine attacks.

Methods
In a large cross-sectional and observational study, data on migraine and RLS were collected from 2,385 migraine patients (according to ICHD-IIIb) and 332 non-headache controls. RLS severity (International RLS Study Group severity scale) and sleep quality (Pittsburgh Sleep Quality Index) were assessed. Risk factors for RLS and RLS severity were calculated using multivariable-adjusted regression models.

Results
RLS prevalence in migraine was higher than in controls (16.9% vs. 8.7%; multivariable-adjusted OR 1.83; 95% C.I. 1.18-2.86; p=0.008), and more severe (adjusted severity score: 14.5±0.5 vs. 12.0±1.1; p=0.036). Poor sleepers were overrepresented among migraineurs (50.1% vs. 25.6%; p<0.001). Poorer sleep quality was independently associated with RLS occurrence (OR 1.08; p<0.001) and RLS severity (p<0.001) in migraine patients.

Conclusion
Restless legs syndrome is not only twice as prevalent but also more severe in migraine patients, and associated with decreased sleep quality.

Introduction

Migraine is a disabling episodic headache disorder[1]. It is associated with a variety of both psychiatric and somatic comorbidities such as depression[2] and restless legs syndrome (RLS)[3-8]. RLS, also known as Ekbom's syndrome, is characterized by an urge to move, mostly associated by unpleasant leg sensations, occurring at rest, in a circadian pattern diminishing with motor activity[9,10].

Several studies have provided evidence for a positive bi-directional association between migraine and RLS in clinical cohorts[3-6]. RLS prevalence rates in migraine populations range from 11.4%-17.7%[3,6], and are about twice as high as prevalence in the general Western population of 5-10%[11,12]. Additionally, it is suggested migraine is very prevalent among RLS patients[6]. Recently, data from population-derived migraine cohorts have suggested a ~1.2 fold increased risk for RLS in both sexes[7,8]. In case-control studies, a four-fold increase in RLS prevalence among migraineurs was reported[13].

It is not known, however, if RLS is also more severe in migraine patients and whether it relates to poorer sleep quality in migraine patients, thereby possibly triggering new migraine attacks. So far, only a small study with not well-defined headache patients, and without adjustment for important confounders, studied severity of RLS[14].

The aim of this study was to investigate not only the prevalence but also severity of RLS in a large population of well-defined migraine patients, and investigate the association with sleep quality.

Material and methods

Subjects

Our study was conducted as a part of the LUMINA project[15]. Participants were Dutch adults aged 18 to 74 years of age, both migraine patients and healthy controls. Patients with migraine with and without aura fulfilled the International Classification of Headache Disorders (ICHD-IIIb) criteria. Controls did not suffer from migraine, cluster headache, chronic tension type headache, or medication overuse headache. Both migraine patients and controls were recruited via public announcement, advertising in lay press and via the research website, and were considered eligible after a two-step inclusion process using validated questionnaires (see Supplementary Text 1 for details). The study had been approved by the medical ethics committee of the LUMC. All subjects provided written informed consent prior to the procedure.

Study design

The design of the study was observational and on a cross-sectional base. In total, n=2,875 eligible migraine patients, fulfilling ICHD-IIIb migraine criteria[16] and n=347 healthy controls were sent an invitation to a digital questionnaire including questions on RLS and RLS severity. Questionnaires could be filled out between September 2010 and January 2014.

Subjects were reminded to participate twice per e-mail, and non-responders were defined as those who did not participate after the reminders.

Clinical characteristics

Within LUMINA, premonitory symptoms (symptoms <48 hours prior to headache onset), headache characteristics, and accompanying symptoms were assessed. Since no validated questionnaire on premonitory symptoms exists, a simple inventory was used. Premonitory symptoms were scored dichotomised and included n=17 items, of which n=5 can be considered 'dopaminergic' (yawning, craving, tiredness, depressive mood and hyperirritability) [17, 18]. The premonitory symptom score was calculated by summing individual items (*yes* = 1, *no* = 0 points; range 0-17). In all subjects (both migraine patients and controls), demographics, data on intoxications, sleep quality data and depression data were gathered. Medication overuse was defined as either i) ever use of simple analgesics on >15 days/month during >3 months; ii) ever use of ergotamines on >10 days/month during >3 months; iii) ever use of triptans on >10 days/month during >3 months, or any combination of the above.

RLS screening and severity questionnaires

As part of an extended questionnaire on sleep habits and sleeping problems, we included a screening questionnaire for RLS. This questionnaire comprised four "yes/ no" type questions based on the essential criteria proposed by the international RLS Study Group [19] and has been validated previously by a physician's diagnosis [20]. When all four criteria were fulfilled, RLS severity in the past week was measured using the International RLS Study Group severity rating scale [21], that consists of 10 items related to severity and frequency of RLS symptoms. Each question is a five-point Likert-scale, with a range from 0 (no RLS or no impact) to 4 (very severe RLS or very severe impact), so total score ranges from 0 to 40. Subjects with RLS were divided in groups with mild (0-10 points), moderate (11-20 points), severe (21-30 points), or very severe (31-40 points) RLS [22].

Depression

For depression, data from the self-administered Hospital Anxiety and Depression Scale (HADS) [23], Center for Epidemiologic Studies Depression Scale (CES-D) [24], and a combined life-time depression algorithm [25] (HADS-D≥8 or CES-D>16 or physician-made diagnosis of depression or use of antidepressants with indication of depression) were used.

Sleep quality and insomnia

The Pittsburgh Sleep Quality Index is designed to measure the quality and patterns of sleep in the past month and contains 19 self-rated questions from which seven component scores are calculated and summed into a global score. Higher scores denote a poor sleep quality: component score range from 0 to 3, and global scores range from 0 to 21. Poor sleep quality is defined with a PSQI score of ≥6 [26]. The Insomnia Sleep Index (ISI) is a self-administered questionnaire to assess insomnia and insomnia severity in the past week, with 7 self-rated questions using a 5 point Likert like scale (none/ mild/ moderate/ severe/ very severe; ranging from 0-4) [27]. The total score ranges from 0-28, with higher scores denoting more insomnia complaints, and dichotomisation into 'no insomnia'(≤14) and 'insomnia' (≥15) [28].

Statistics

General characteristics were compared between migraine patients and controls using a Student's t-test for continuous variables, and a Chi square test for categorical data. To assess whether migraine and RLS were associated (primary analysis), a binary logistic regression was performed with RLS status as dependent variable. In analysing determinants for RLS severity, we performed a linear regression analysis with continuous RLS severity score as outcome measure. The primary regression analysis was adjusted for age, gender, BMI, smoking (packyears), alcohol use and life time depression. All other analyses were adjusted for age and gender. Data analyses were performed using SPSS 17.0 (SPSS inc., IBM, USA). The statistical threshold was set to $p<0.05$.

Results

Study population

Questionnaires were sent to 2,875 migraine patients (1,755 migraine without aura, 1,120 migraine with aura) and to 347 controls, of which 2,385/2,875 (82.9%) and 332/347 (95.7%) responded respectively. Non-responder analysis showed that responders were older (44.9±12.1 vs. 41.2±12.9; $p<0.001$), had a higher BMI (24.5±4.1 vs. 23.9±3.8; $p=0.044$) and had a lower HADS score compared to non-responders (10.1±6.7 vs. 11.5±7.1; $p<0.001$). Gender, smoking, use of alcohol, use of caffeine and PSQI score did not differ. In the study population, migraineurs were more often female, lower educated, had lower alcohol and higher caffeine intake (Table 1).

RLS prevalence in migraine patients and controls

A total of 403/2,384 migraine patients (16.9%) fulfilled the essential criteria for definite RLS, compared to 31/332(9.3%) in controls($p<0.001$). RLS prevalence did not differ between migraine with aura and migraine without aura patients: 170/919 (18.5%) vs. 233/1,465 (15.9%); $p=0.100$. RLS prevalence was 19.7% in the subgroup of migraine patients aged 50 years and older (166/844). The multivariable-adjusted odds ratio (OR) for RLS in migraine vs. the control group was 1.83 (95% C.I. 1.18-2.86; $p=0.008$). ORs (95% C.I.) for RLS were 1.74 (1.08-2.79; $p=0.02$) in migraine without aura and 1.99 (1.24-3.20; $p=0.005$) in migraine with aura subgroups. Within the migraine group, migraine subtype was not a determinant for RLS (age and gender adjusted; OR 0.83; $p=0.10$), but medication overuse was (OR 1.54; $p<0.001$).

RLS severity

Overall, 146/434 (33.6%) respondents had mild RLS, 227/434 (52.3%) had moderate RLS, 58/434 (13.4%) had severe RLS, and 3/434 (0.7%) had very severe RLS. Severe to very severe RLS was more present among migraine patients than in controls: 60/403 vs. 1/31; $p=0.036$. The adjusted mean RLS severity score in migraine patients with definite RLS was higher compared to controls with definite RLS: 14.5±0.5 vs. 12.0±1.1; $p=0.036$ (adjusted for age and gender). In migraineurs with RLS, a higher number of dopaminergic premonitory symptoms was associated with higher RLS severity ($p=0.008$). Additionally, a history of acute migraine headache medication overuse ($p=0.026$), use of ergots ($p=0.045$), and prophylactics ($p=0.002$) were also linked to more severe RLS (Table 2).

Table 1; Baseline characteristics of study population. Baseline characteristics of migraine patients (n=2,385) and healthy controls (n=332). MO = migraine without aura; AF = attack frequency

Variable	Total n=2,717	Migraine patients n=2,385	Controls n=332	*p*[a]
Demographics				
Age y, mean (SD)	44.9 (12.1)	45.1 (11.6)	43.8 (15.2)	0.145
Gender F, n (%)	2,230 (82.1%)	2,045 (85.7%)	185 (55.7%)	<0.001
BMI kg/m^2, mean (SD)	24.5 (4.1)	24.6 (4.1)	24.2 (4.0)	0.154
Education level, n (%)[b]				0.008
Low	161 (6.3%)	144 (6.5%)	17 (5.1%)	
Middle	862 (34.0%)	771 (35.0%)	91 (27.4%)	
High	1,513 (59.7%)	1,289 (58.5%)	224 (67.5%)	
Migraine				
Subtype MO		1,466 (61.5%)		
AF 1-4/month		2,182 (91.5%)		
RLS				
Definite RLS, n (%)	434 (16.0%)	403 (16.9%)	31 (9.3%)	<0.001
RLS severity, mean (SD)[c]	14.1(0.4)	14.5 (0.5)	12.0 (1.1)	0.036
Anti-RLS medication[d]	13/220	13/204 (6.4%)	0/16 (0%)	0.298
Intoxications				
Nicotine, packyears, mean (SD)	4.6 (8.9)	4.5 (8.7)	5.4 (10.0)	0.088
Alcohol; units/ week, mean (SD)	3.1 (4.4)	2.7 (3.8)	6.3 (6.7)	<0.001
Caffeine; units/ day, mean (SD)	5.8 (3.0)	5.9 (3.0)	5.3 (2.6)	0.001
Other				
PSQI total score, mean (SD)	6.2 (3.6)	6.5 (3.6)	4.1 (2.7)	<0.001
PSQI ≥6, %	1,360 (50.1%)	1,275 (53.5%)	85 (25.6%)	<0.001
HADS, total score, mean (SD)[e]	10.1 (6.7)	10.6 (6.7)	6.4 (5.45)	<0.001
Anti-RLS medication	13/220 (5.9%)	13/204 (6.4%)	0/16 (0%)	0.298

MO, migraine without aura; AF, attack frequency. *P* values are uncorrected for multiple comparison. [a]In view of the significant gender disproportion between migraine patients and non-headache controls in combination with known higher RLS prevalence amongst females and higher alcohol consumption in males, *p*-values may reflect biased estimates and should therefore be interpreted with caution; [b]data available from n = 2204 migraine patients and n = 332 controls; [c]adjusted for age and gender; [d]additional data from n = 220/419 subjects with definite RLS; [e]data available from n = 2254 migraine patients and n = 316 controls.

Impact of RLS on sleep quality in migraine patients

Significantly more migraine patients with RLS (64.4%) compared to migraine patients without RLS (51.4%; $p<0.001$) had poor sleep quality, and severity of the global PSQI sleep quality score was higher (7.4±3.7 vs. 6.3±3.6; $p<0.001$). The different PSQI components are indicated in Table 3. Migraine patients with RLS also scored higher on the Insomnia Severity

Index than migraine patients without RLS (9.5±6.7 vs. 8.1±6.5; $p<0.001$). Clinical insomnia was more prevalent in the RLS subgroup: 97/403 (24.1%) vs. 361/1.979 (18.2%); $p=0.007$.

Migraine, RLS and depression

Mean HADS score was higher in the migraine group compared to healthy controls (10.6±6.7 vs. 6.0±5.4; $p<0.001$), and life-time depression was also more prevalent: 45.5% vs. 16.8%; $p<0.001$ (age and gender adjusted). In both groups, life-time depression was associated with RLS prevalence (overall odds ratio 1.60; $p<0.001$) and RLS severity (overall B=2.67; $p<0.001$), but this effect was strongest in the migraine group.

Table 2. Determinants for Restless Legs Syndrome severity. Multivariable-adjusted B's for RLS severity (according to IRLSSG-criteria). MO = migraine without aura; MA = migraine with aura; DPS = dopaminergic premonitory symptoms; PSQI = Pittsburgh Sleep Quality Index; n.a. = not applicable. Linear regression analyses were adjusted for age and gender.

Variable	Multivariable-adjusted determinants for RLS severity					
	Total n=434		Migraine patients n=403		Controls n=31	
	B	*p*	B	*p*	B	*p*
Demographics						
Age (y)	-0.002	0.937	0.011	0.704	-0.121	0.107
BMI (kg/m²)	0.149	0.033	0.144	0.047	0.187	0.457
Gender, F	-0.610	0.488	-0.739	0.466	-2.620	0.110
Migraine vs. controls						
Migraine vs. controls	2.475	0.036	n.a.	n.a.	n.a.	n.a.
MO vs. controls	2.175	0.085	n.a.	n.a.	n.a.	n.a.
MA vs. controls	2.914	0.015	n.a.	n.a.	n.a.	n.a.
Mig. characteristics						
MO subtype	n.a.	n.a.	0.058	0.926	n.a.	n.a.
>4 attacks/month	n.a.	n.a.	1.294	0.237	n.a.	n.a.
Medication overuse	n.a.	n.a.	1.440	0.026	n.a.	n.a.
Use of triptans	n.a.	n.a.	0.882	0.243	n.a.	n.a.
Use of ergots	n.a.	n.a.	2.993	0.045	n.a.	n.a.
Use of prophylactics	n.a.	n.a.	2.043	0.002	n.a.	n.a.
DPS (continue)	n.a.	n.a.	0.540	0.008	n.a.	n.a.
Intoxications						
Nicotine, packyears	0.054	0.095	0.063	0.068	0.005	0.943
Alcohol, units/ week	-0.138	0.063	-0.126	0.114	-0.095	0.620
Caffeine, units/ day	-0.025	0.814	-0.038	0.736	-0.002	0.996
Other						
PSQI total score	0.573	<0.001	0.579	<0.001	0.320	<0.001
PSQI ≥6	3.153	<0.001	3.229	<0.001	0.961	0.605
Life-time depression	2.666	<0.001	2.725	<0.001	0.398	0.811

p-values are uncorrected for multiple comparisons

Table 3. Pittsburgh Sleep Quality Index (PSQI) scores. Depicted are PSQI component and global scores (mean (SD)) of migraine patients with and without restless legs syndrome (RLS). Migraine patients with RLS have higher scores on almost all PSQI component scores, indicating worse functioning on these domains.

	Migraine with RLS (n=403)	Migraine non-RLS (n=1,981)	*p*
PSQI mean component score			
Subjective sleep quality	1.3 (0.7)	1.1 (0.7)	<0.001
Sleep latency	1.4 (1.0)	1.2 (1.0)	<0.001
Sleep duration*	0.7 (0.9)	0.6 (0.8)	0.002
Habitual sleep efficiency*	0.9 (1.0)	0.7 (0.9)	0.001
Sleep disturbance	1.6 (0.6)	1.4 (0.5)	<0.001
Sleep medications*	0.4 (0.9)	0.4 (0.8)	0.952
Daytime dysfunction	1.2 (0.8)	0.0 (0.7)	<0.001
PSQI mean global score	7.4 (3.7)	6.3 (3.6)	<0.001
Poor sleeper (PSQI≥6) (n,%)	259 (64.3%)	1,016 (51.3%)	<0.001

* Mann Whitney U-test

Discussion

In the present study, we found that prevalence of restless legs syndrome (RLS) is two times higher in a well-defined group of migraine patients than in non-headache controls. Most importantly, our study shows that RLS is more severe in migraine patients and is associated with poorer sleep quality, a known trigger factor in migraine.

The prevalence of RLS in our migraine and control groups were comparable to previously reported data in both clinic-and population-based cohorts of migraine patients and controls, ranging from 9.5-22.4%[3, 5-8, 29] and from 7.1-13.0%[7, 8, 12, 30-33]. Higher RLS severity in headache patients was recently suggested based on data from a small and less well characterised sample of headache patients[14]. Additionally, we found that RLS in migraine patients is more severe with increasing migraine severity, as reflected by use of prophylactics, or a history of medication overuse. The clinical relevance of this small difference is to be further determined since both reflect mild RLS severity. Unbalanced group sizes in our study could have affected the outcome. However, previous data reporting a higher RLS prevalence in chronic migraine vs. episodic migraine underlines our finding[34].

The strength of our study includes the large sample size, with data from over 2,300 well-defined migraine patients, representative for the population studied. Second, detailed validated questionnaires[15, 19] assured precise categorization, although RLS remains a clinical diagnosis. Third, the personalized web-based questionnaire facilitates filling out and sending in for participants, leading to a high participation rate[15]. Fourth, non-headache controls were recruited in exactly the same way as the migraine patients, minimizing inclusion bias. However, some limitations should also be addressed. First, the control group in our study was considerably smaller than the case group but, we believe, this has hardly

affected the statistical power of the study. The number of cases (n = 2,350) was high and the number of controls (n = 300) was still considerable, resulting in post-hoc power of 0.97 to detect a 8% difference in proportion of RLS prevalence between both study groups at alpha 0.05 (and a post-hoc power of 0.82 to detect a 6% difference). Increasing the number of controls would have involved disproportionately large and in fact unnecessary efforts leading to only moderate increase in study power. Secondly, some medication may affect symptoms of RLS[35]. In our study 18% of subjects with definite RLS used anti-RLS medication, of whom only five used dopaminergic medication. This small subgroup reported highest RLS severity (data not shown). Second, presence of nephrotic syndrome, iron deficiency or diabetes can contribute to RLS symptomatology[36, 37], and RLS mimicks could make classification more difficult[38]. These conditions can not fully be excluded based on the four RLS diagnostic criteria, as was shown before[39]. Furthermore, other co-morbidities could have affected both migraine and RLS. Preferably, analyses should be corrected for these possible confounders. However, these possible biases have affected both the non-headache and the headache group. Thirdly, there were some differences between the migraine and controls groups. Migraine patients were more often female, used less alcohol and more caffeine than controls. They showed lower sleep quality, as reflected by the PSQI score, and a larger proportion suffered from life-time depression. Since identical questionnaires for depression and sleep quality were used in both the migraine and the control group, they would have affected both groups in a similar way, and would therefore not have accounted for inter-group differences. By adjusting all the primary analyses for these factors we tried to minimize potential bias. Fourth, the majority (87%) of the migraine patients in our sample has been diagnosed with migraine by a physician. Therefore, we can not exclude that this group is enriched with more severe migraineurs compared to a genuine population-based sample since not all migraine patients consult a physician. This would then suggest that RLS is associated with more severe migraine.

RLS is associated with lower sleep quality and fragmented sleep, which are known triggers for migraine attacks. Therapeutic options for RLS include among others dopaminergic treatment[19]. Few studies have assessed the effect of anti-RLS therapy on alterations in co-morbid migraine. In a small (n=10) study of patients with concomitant migraine and RLS, dopaminergic treatment with immediate-release pramipexole improved both RLS symptoms in all patients, and headache frequency and severity in half over a period of five months[40]. Headache relieve was also reported in another case-study (n=40) in one-third of migraineurs when treating RLS, most often when using dopamine-3 receptor agonists such as gabapentin of pregabalin[14]. This could either be due to improving central dopaminergic dysfunction or by improving compromised sleep quality due to RLS. Effects of migraine treatment on concomitant RLS have never been studied.

In conclusion, the risk for RLS is doubled in migraine patients, but more importantly RLS is more severe and associated with poorer sleep quality which might trigger new attacks. Further studies are needed to investigate if treatment for RLS positively affects migraine attacks.

Clinical implications

- Migraine patients are less prone to be of a normal chronotype and they are more languid and more rigid when changes in circadian rhythm occur.
- 60% of migraine patients report diurnal periodicity of headache attacks, of which one-third reports attack beginning between midnight and 06:00, and one-third between 06:00 and noon.
- Taken together, these data suggest that chronobiological mechanisms play a role in migraine pathophysiology.

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Supplementary Material

Supplementary Text

Both migraine patients and controls were recruited via nationwide public announcement, advertising in lay press and via the research website, and were considered eligible after a two-step inclusion process using validated questionnaires via the especially designed LUMINA website. Additionally, patients from our outpatient headache clinic were invited to participate by a letter.

On the website, patients were asked to fill out a screening questionnaire that has been validated previously. Firstly, if patients fulfilled the screening criteria, they were sent a web-based extended migraine questionnaire, based on the ICHD-II criteria [15, 16]. This questionnaire was validated before by performing a semi-structured telephone interview in 1,038 patients who had filled out the extended migraine questionnaire [15]. The specificity of the questionnaire was 0.95. We consider the cohort a well-defined web-based cohort, with 4% of subjects included from our dedicated headache outpatient clinic, 87% of the participants having been diagnosed as migraineurs previously by a medical doctor. In addition to questions that were necessary to diagnose migraine accurately, the extended questionnaire also included items on demographic factors, acute and prophylactic headache medication use, migraine attack frequency and allodynia. Participants without the needed internet skills were able to fill out the questionnaires on paper.

Non-headache individuals willing to participate had to pass a screening questionnaire online via the research website. If this screening questionnaire did not show any indication for having migraine, cluster headache, chronic tension type headache or medication overuse headache, individuals were sent a subsequent in depth questionnaire. This second questionnaire again assessed possible headache complaints, together with demographic variables. Only individuals that fulfilled both the criteria of 'non-headache' in the screening and in depth questionnaire were considered eligible controls and were approached for this questionnaire study.

Since recruitment of control subjects started at a later point in time and inclusion rate was slower, the number of migraine patients included exceeds the number of controls included in our study.

Chapter 5.

Postdural puncture headache in migraineurs and nonheadache subjects: a prospective study

van Oosterhout WPJ[1]
van der Plas AA[1]
van Zwet EW[2]
Zielman R[1]
Ferrari MD[1]
Terwindt GM[1]

[1]Department of Neurology, Leiden University Medical Center, Leiden, the Netherlands
[2]Department of Medical Statistics, Leiden University Medical Center, Leiden, the Netherlands

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Abstract

Objectives

To prospectively assess (i) the incidence and duration of post-dural puncture headache (PDPH) in migraineurs and healthy subjects; (ii) the associated risk factors; (iii) the risk of getting a migraine attack shortly before or after lumbar puncture (LP).

Methods

As part of an extensive biochemical migraine research program, we assessed the occurrence, duration and characteristics of PDPH in a 160 migraineurs and 53 age- and gender matched healthy controls. In addition, we evaluated potential risk factors for PDPH as well as the risk of developing a migraine attack before or after LP.

Results

In total 64/199 (32.2%) subjects developed PDPH. Young age, low Body Mass Index, severe headache immediately after LP, and sitting sampling position, but not being a migraineur, increased the risk of PDPH (all $p<0.05$). Duration of PDPH was prolonged by history of depression, sitting sampling position, high perceived stress during the LP procedure, and multiple LP-efforts (all $p<0.05$). Migraine attacks were less likely to occur before or shortly after LP.

Conclusions

Migraineurs are not at increased risk of developing PDPH. PDPH duration is similar in migraineurs and age- and gender matched controls. Lumbar puncture does not trigger migraine attacks, and the stress of an upcoming LP might even have a protective effect against onset of migraine attacks.

Introduction

Postdural puncture headache (PDPH) occurs in 10-40% of subjects after lumbar puncture (LP), usually within five days[1, 2]. The headache typically comes on within 15 minutes after assuming an upright position and improves again within 15 minutes after lying down.[3] Frequently associated symptoms are neck stiffness, tinnitus, hyperacusis, photophobia, and nausea (Table e-1) [3-5]. Sometimes, patients may also experience blurred vision or diplopia[6]. In 95% of cases, the symptoms spontaneously resolve within a week[3].

Various pathophysiological mechanisms have been hypothesized for PDPH [7-11] and retrospective observational studies suggest young age [12-19], female gender [17, 19-21], low BMI [19, 22], previous PDPH [7, 18], and (migraine) headache prior to LP [6, 23-25] as risk factors. Prospective studies on the epidemiology of PDPH, however, are lacking.

As part of an extensive biochemical migraine research program, we were allowed by the Medical Ethical Committee to sample cerebrospinal fluid (CSF) from 160 well characterized migraineurs and 53 healthy controls. All subjects gave written informed consent and were followed for at least a week after LP. This gave unique opportunity to prospectively study the risk and course of PDPH in migraine and healthy subjects. For both populations, we assessed: (i) incidence and duration of PDPH; (ii) possible risk factors for PDPH; (iii) risk of migraine provocation by LP; and (iv) whether the prospect of undergoing LP could trigger or prevent migraine attacks in the preceding days. The results of our study may call for a re-evaluation of the ICHD-IIR criteria for PDPH.

Material and methods

Subjects

Migraineurs and healthy controls over 18 years of age were included as part of a study on biochemical changes in migraine. All subjects underwent full physical examination, including ophthalmoscopy. Exclusion criteria were: (i) use of anti-depressive or anti-coagulation medication; (ii) BMI>32; (iii) any oncological history; and (iv) any contra-indication for LP. Migraineurs were diagnosed according to the ICHD-IIR criteria[3] and could have no more than ten headache days per month. Healthy volunteers had to be free of any form of regular headaches. History of depression was scored by asking participants whether they ever were diagnosed with depression by a physician. Stress was assessed with a visual analogue scale, ranging from 0 (no stress at all) to 10 (most stressful event ever) regarding the upcoming LP. This score was assessed 10-15 minutes prior to the LP. None of the participants reported to have undergone an LP in the past in the setting of a scientific study. The exclusion criterion of any other neurologic disease has lead to the inclusion of LP naïve subjects, since use of the LP procedure is limited to a neurologic diagnostic setting. A very small proportion of subjects might have had epidural anaesthesia in the past, but we have no reason to believe this proportion would differ between migraineurs and controls. We therefore consider the study population LP naïve.

Standard protocol approvals, registrations, and patients consents

The study was approved by the Medical Ethical Committee. All subjects gave written informed consent and received a small financial compensation according to standard guidelines of the Center for Human Drug Research, Leiden, the Netherlands.

Study design

All LPs were performed between 09:00 a.m. and 01:00 p.m. by experienced neurology-residents and at least three days after a previous migraine. Participants were subjected to overnight fastening and were only allowed to drink water prior to the LP. With the patient in left lateral supine, a total of 14 mL of CSF was collected using a 0.9 mm traumatic Quincke needle (Mediplast, Sweden). If a successful puncture was not possible in the left lateral supine position, LP was performed with the subject in sitting position. Participants were mobilized after the procedure, since there is no evidence that prolonged recumbent position after LP may prevent PDPH[26, 27].

Follow up assessment

All participants were standard contacted three days after LP, but could contact the research team at any time earlier or later. The presence and severity of PDPH and migraine were evaluated in a semi-structured telephone interview using ICHD-IIR criteria [3] and, for PDPH, Lybecker's classification [6]. Conservative treatment with bed-rest and oral NSAIDs, fluids, anti-emetics, and caffeine, was recommended to all subjects with mild to moderate PDPH. Autologous epidural blood-patch was offered to all subjects with severe or PDPH not improving with conservative treatment.

Statistics

General characteristics were compared between the groups with and without PDPH using a Student's t-test for continuous variables, and a Chi square test for categorical data. To assess risk factors for PDPH, binary logistic regression was performed with migraine status as independent variable, adjusted for age, gender, and BMI. Additional risk factors were also analysed with the regression model, both uni- and multivariate. Multivariate analyses were performed in two ways: (i) adjusted for age, BMI and gender; and (ii) with significantly associated (p<0.05) variables from the univariate analysis. To identify factors for PDPH duration, a multiple linear regression (uni- and multivariate) was performed, adjusted for age, gender, and BMI. Variables were selected based on literature or because they were considered relevant. All data analyses were performed using SPSS 17.0 (SPSS inc., IBM, USA). *P* values less than 0.05 were considered significant.

To assess (i) whether LP could trigger migraine attacks or (ii) whether (the stress of) the prospect of undergoing LP, or the mere participation in the study, could trigger or actually prevent the occurrence of a migraine attack in the days preceding a *scheduled* LP, we conducted two separate analyses. For the first objective, we compared the proportion of migraineurs who developed an attack within the 3 days after LP with the proportion of migraineurs in a separate headache diary study, who had not had a migraine in the first three days of the diary study but who subsequently did develop an attack in the following three days. The diary study was previously carried out at our centre in 700 migraineurs who

kept a headache diary for a month. Of these, 41 later also participated in the present LP study. To test significance, a logistic regression model was fit with a dummy variable encoding LP (yes/ no) as the only covariate. A random-patient effect was also included to account for the fact that some subjects participated in both studies. In a secondary analysis we only included the 41 subjects who had participated in both studies, using McNemar's test.

To address the second question, we compared the proportion of migraineurs with an attack in the three days preceding a *scheduled* LP in the LP study with the proportion of migraineurs who had an attack in the first three days of the diary study. We fit a logistic regression model, including a random-patient effect. Again, we repeated the analysis also for the subgroup that had participated in both studies, using McNemar's test.

Results

Study population

Between January 2008 and September 2010, 160 migraineurs and 53 healthy controls (n = 213; 131 females) were included in the study (Figure e-1). LP was unsuccessful in 13/213 (6.1%) subjects (of which nine were migraineurs), leaving 200 subjects at risk for PDPH. After LP, one subject developed headache which could not reliably be attributed to either PDPH or episodic migraine. Retrospectively, this patient suffered from chronic migraine which started months prior to the study and should not have been included in our study. Therefore, the patient was excluded from the analysis. The baseline characteristics of the remaining 199 study participants (n=49 controls; n=84 with migraine with aura, of whom 19 with hemiplegic migraine; n=66 with migraine without aura) are described in table e-2. Migraineurs had a higher mean age (44.0±13.3 vs. 36.3±15.3, p=0.001), were more frequently female (98/150 [65.3%] vs. 24/49 [49.0%]; p=0.041), and were less likely to consume alcohol (44/150 [29.3%] vs. 42/49 [85.7%]; p=0.036) than controls.

PDPH incidence and characteristics

A total of 64/199 subjects (32.2%) developed PDPH, which was mild in 27/64 (42.2%), moderate in 18/64 (28.1%) and severe in 14/64 (21.9%). In 59/64 (92.2%) subjects the PDPH had started within two days and in 63/64 (98.4%) within three days. The median time to onset was 20.0 hours (range: 1.0 – 76.0; Figure 1). The overall median duration of PDPH was 5.1 days (range 1-18) and tended to be shorter in migraineurs (mean ± SD: 4.6 ± 2.4 days compared to controls (5.9 ± 3.6 days; p=0.098). In the 52 subjects with a spontaneous recovery, the median duration was 4.0 days (range 1-11). In the 12 subjects who were successfully treated with a blood patch, the median duration was 6.5 days (range 3-18; Figure 2. Information on associated symptoms was available for 62/64 (96.9%) subjects with PDPH of whom 55/62 (88.7%) reported the presence of such symptoms: vestibulo-cochlear symptoms (vertigo, dizziness, hypacusis and tinnitus) in 40/62 (64.5%); nausea and/ or vomiting in 40/62 (64.5%); cochlear symptoms (including hypacusis and tinnitus) in 19/62 (30.6%), ocular symptoms (diplopia) in 2/62 (3.2%) and musculoskeletal complaints (stiff neck or stiff shoulder region) in 23/62 (37.1%). Photophobia was reported by 3/62 (4.8%). PDPH was more prevalent in the subgroup that underwent LP in sitting position vs.

in lateral supine position: 13/20 (65.0%) vs. 51/179 (28.5%); *p*=0.001 (OR 3.5, 95%C.I. 1.2-10.1; *p*=0.022 when adjusted for number of LP efforts, age, gender, BMI).

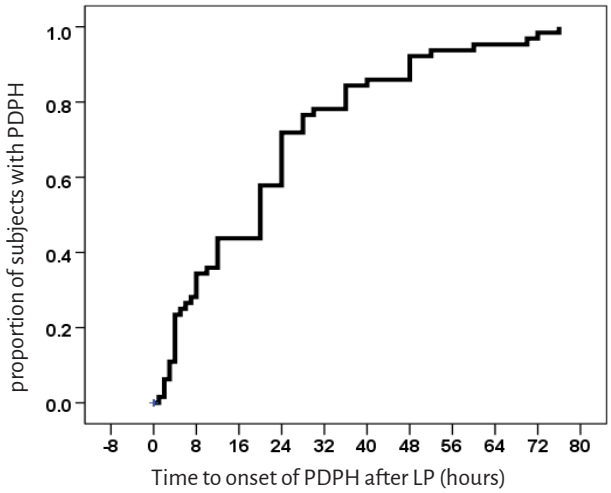

Figure 1. Cumulative onset of post-dural puncture headache in 64 subjects. 59/64 (92.2%) reported PDPH <48 hours and 63/64 (98.4%) <72 hours. Median time to onset was 20 hrs.

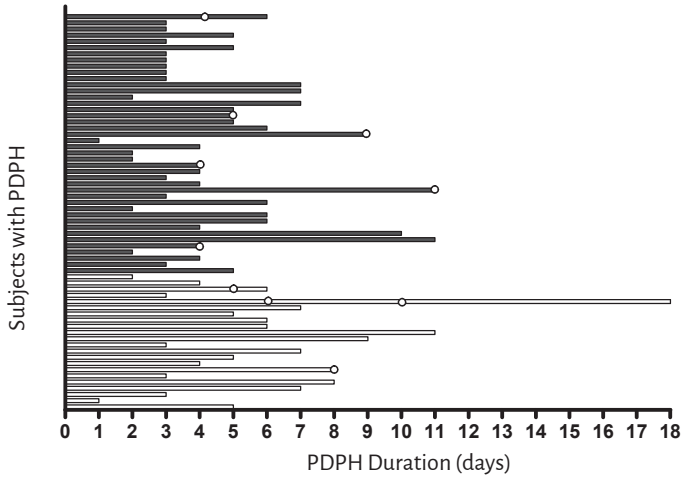

Figure 2. Duration (days) of PDPH in 64 subjects with PDPH. O = timepoint of autologous epidural blood patch; grey coloured bars = migraine patients; open bars = controls.

Differences between subjects with and without PDPH

Table 1 compares the baseline characteristics of the participants who developed PDPH and those who did not. Subjects with PDPH were younger, had a lower BMI, and reported higher scores on immediate post-LP headache severity. CSF opening pressure did not differ between subjects who developed PDPH (18.3±4.6 cmH_2O) vs. those who did not (17.4±4.2 cmH_2O; p=0.18), see Table 1. Fewer migraine subjects (42/150; 28.0%) reported PDPH than did controls (22/49; 44.9%; p=0.028). There was no difference in PDPH incidence between migraineurs with and without aura (data not shown). PDPH difference did also not differ between patients with hemiplegic migraine (2/19; 10.5%) vs. non-hemiplegic migraineurs (40/131; 30.5%; p=0.07). Excluding HM cases in a post-hoc analysis did not change the significant lower incidence of PDPH in the migraine group compared to controls.

Risk factors for occurrence and long duration of PDPH

Several risk factors were identified that significantly enhanced the risk of PDPH (Table 2). In a univariate analysis, young age and low BMI increased the risk of PDPH. The risk of PDPH was also increased in subjects who reported high headache severity immediately after LP and sitting sampling position. In a multivariate analysis (adjusted for age, gender and BMI) low age, low BMI, sitting position and high headache score after LP proved significant predictors. In a second multivariate analysis (using variables with p<0.05 association from univariate analysis) severe headache-after-LP and sitting position were confirmed as predicting factor. Table 3 indicates risk factors for longer PDPH duration. Both univariate and multivariate (adjusted for age, gender and BMI) analyses show that sitting sampling position, history of depression, multiple LP effort, and high perceived stress during the procedure were associated with a longer duration of PDPH. Lower level of education and absence of migraine family history showed a similar though non-significant trend. A second multivariate analysis (using variables with p<0.05) confirmed sitting position as an important risk factor.

Migraine after LP

In total 25/150 (12.0%) migraineurs experienced a migraine attack between 0 and 10 days after LP (median: 2 days), the majority (18/25; 72%) within 3 days. In the subgroup of migraineurs in whom the LP was performed on the first scheduled appointment, thus who did not had an attack in days -3 to 0 prior to the LP, the risk of experiencing a migraine attack within three days after LP was 13/94 (14%). This was substantially lower than the risk of experiencing a migraine attack on days +4 to +7 in the diary study for those migraineurs who had not suffered an attack on the first three/four days of the study (111/257; 43.2%; odds ratio = 0.74; p<0.0001). A similar trend for reduced risk of a migraine attack after LP was seen for the subgroup of 41 migraineurs who had participated in both the LP and diary study: 14/41 (34.1%) vs. 24/41 (58.5%; odds ratio = 0.25; p=0.37).

Perceived stress levels immediately before LP tended to be higher in migraineurs who did not experience an attack ≤ 3 days after LP (2.6 ± 3.0) than those in migraineurs who did experience an attack (1.6 ± 1.6; p=0.19). Stress levels during the LP procedure were very similar in migraineurs with (2.9 ± 2.4) and without an attack (3.2 ± 2.9; p=0.72). None of the controls experienced a migraine attack after LP.

Migraine preceding LP

Fewer migraineurs had an attack in the three days preceding LP (37/131; 28.2%) than in the first three days of the diary study (388/645; 60.2%; $p<0.0001$). This was also true for the subset of migraineurs who had participated in both the LP (21/41; 51.2%) and diary study (24/41; 58.5%; $p=0.04$).

Table 1; Baseline characteristics of study population. Baseline characteristics of subjects who developed PDPH (PDPH, n=64) versus subjects without PDPH (non-PDPH; n=135) of participants with successful LP (n=199). *p*-values depicted in bold indicate significant differences ($p<0.05$), using independent-samples t-tests and χ^2 tests appropriately. M = Migraine; LP = lumbar puncture; CSF = cerebrospinal fluid; VAS = visual analogous scale with range 1-10 (no stress/ pain – most stressful/ painful imaginable).

Variable	Total (n=199)	PDPH (n=64)	Non-PDPH (n=135)	*p*
Age; mean (SD)	42.1 (14.1)	37.9 (13.2)	44.1 (14.2)	**0.004**
Female (%)	122 (61.3%)	37 (57.8 %)	85 (63.0%)	0.486
Migraine (%)	150 (75.4%)	42 (65.6%)	108 (80.0%)	**0.028**
BMI	24.2 (3.0)	23.4 (3.0)	24.5 (2.9)	**0.022**
Education level (%)				
Low	17 (13.6%)	4 (8.9%)	13 (9.6%)	0.173
Middle	69 (34.6%)	22 (48.9%)	47 (34.8%)	
High	94 (47.2%)	19 (42.2%)	75 (55.6%)	
Comorbidity				
None	189 (95.0%)	61 (95.3%)	128 (94.8%)	0.952
History of depression	6 (3.0%)	2 (3.1%)	4 (3.0%)	
Epilepsy	4 (2.0%)	1 (1.6%)	3 (2.2%)	
Family History M.* (%)	108 (54.3%)	34 (53.1%)	74 (54.8%)	0.823
Intoxications				
Alcohol (%)	148 (74.4%)	49 (76.6%)	99 (73.3%)	0.626
Smoking (%)	37 (18.6%)	13 (20.3%)	24 (17.8%)	0.668
Drugs (%)	4 (2.0%)	3 (4.7%)	1 (<0.1%)	0.064
Caffeine (%)	188 (94.5%)	62 (96.9%)	126 (93.3%)	0.307
Hours sober; mean (SD)	11.3 (2.3)	11.8 (2.1)	11.1 (2.3)	0.052
LP effort; mean (SD)	1.4 (1.0)	1.7 (1.1)	1.3 (1.0)	**0.042**
Puncture traumatic (%)	16 (8.0%)	6 (9.3%)	10 (7.4)	0.655
CSF opening pressure; mean (SD)	17.7 (4.3)	18.3 (4.6)	17.4 (4.2)	0.184
Sitting position	23 (11.6%)	13 (20.3%)	10 (7.4%)	**0.008**
Pain during LP (VAS)	3.3 (2.4)	3.4 (2.5)	3.2 (2.4)	0.548
Stress prior to LP (VAS)	2.4 (2.7)	2.1 (3.1)	2.5 (2.5)	0.284
Stress during LP (VAS)	3.2 (2.8)	3.0 (2.7)	3.3 (2.9)	0.547
Headache directly after LP (VAS)	1.0 (1.6)	1.5 (2.0)	0.7 (1.4)	**0.009**

Table 2; Risk factors for PDPH onset. Odds Ratios (1: univariate; 2: multivariate, adjusted for age, gender and BMI; and 3: multivariate with variables $p<0.05$ in univariate analysis) for the risk of developing PDPH using a logistic model (n=199). *p*-values depicted in bold indicate significant differences ($p<0.05$).

Variable	1. Univariate OR [95% CI]	*p*	2. Multivariate OR [95% CI]	*p*	3. Multivariate OR [95% CI]	*p*
Age (years)	0.972 [0.951-0.993]	**0.010**	0.976 [0.955-0.998]	**0.033**	0.977 [0.954-1.000]	0.051
Gender (F)	0.787 [0.433-1.432]	0.433	0.701 [0.376-1.306]	0.263		
BMI (kg/m^2)	0.877 [0.793-0.971]	**0.011**	0.892 [0.803-0.990]	**0.032**	0.912 [0.816-1.019]	0.102
Migraine diagnosis	0.506 [0.264-0.969]	**0.040**	0.618 [0.306-1.248]	0.180	0.557 [0.274-1.134]	0.107
Education level						
Middle vs. low	2.531 [0.774-8.272]	0.124	2.143 [0.626-7.338]	0.225		
High vs. middle	1.398 [0.576-4.523]	0.576	0.812 [0.232-2.839]	0.744		
History of depression	0.760 [0.199-2.903]	0.688	0.740 [0.183-2.984]	0.672		
Family History Migraine	0.963 [0.535-1.734]	0.901	1.147 [0.614-2.143]	0.667		
Alcohol (yes/ no)	1.169 [0.590-2.317]	0.655	0.849 [0.407-1.770]	0.663		
Smoking (yes/ no)	1.046 [0.503-2.174]	0.904	1.096 [0.513-2.344]	0.813		
Drugs (yes/ no)	7.230 [0.737-70.87]	0.089	4.011 [0.384-41.92]	0.246		
Caffeine (yes/ no)	2.007 [0.421-9.563]	0.382	1.800 [0.371-8.730]	0.466		
Sober (hours)	1.136 [0.997-1.294]	0.056	1.138 [0.996-1.300]	0.057		
LP effort (1x vs. >1)	0.553 [0.281-1.090]	0.087	0.579 [0.286-1.172]	0.129		
Puncture traumatic (yes/ no)	1.407 [0.489-4.052]	0.527	1.591 [0.532-4.755]	0.406		
CSF opening pressure (cm H_2O)	1.054 [0.975-1.138]	0.184	1.057 [0.969-1.153]	0.209		
Sitting position (vs. supine)	3.186 [1.313-7.731]	**0.010**	3.321 [1.323-8.338]	**0.011**	3.210 [1.287-8.006]	**0.012**
Pain during LP (VAS 1-10)	1.005 [0.893-1.132]	0.931	0.990 [0.872-1.125]	0.883		
Stress prior to LP (VAS 1-10)	0.946 [0.840-1.066]	0.363	0.957 [0.845-1.083]	0.485		
Stress during LP (VAS 1-10)	0.960 [0.863-1.069]	0.458	0.948 [0.846-1.062]	0.356		
Headache after LP (VAS 1-10)	1.322 [1.103-1.583]	**0.002**	1.377 [1.139-1.664]	**0.001**	1.443 [1.169-1.782]	**0.001**

Table 3; Risk factors for longer PDPH duration. Regression coefficients (1: univariate; 2: multivariate, adjusted for age, gender and BMI; and 3: multivariate with variables $p<0.05$ in univariate analysis) for the duration of PDPH using a linear regression model (n=52 PDPH cases with spontaneous recovery). *p*-values depicted in bold indicate significant differences ($p<0.05$).

Variable	1. Univariate B [95% CI]	*p*	2. Multivariate B [95% CI]	*p*	3. Multivariate B [95% CI]	*p*
Age (years)	-0.016 [-0.067;0.034]	0.520	-0.017 [-0.075;0.041]	0.551		
Gender (F)	0.788 [-0.531;2.107]	0.236	0.807 [-0.600;2.214]	0.254		
BMI (kg/m^2)	-0.043 [-0.262;0.176]	0.692	0.031 [-0.231;0.292]	0.815		
Migraine diagnosis	-0.946 [-2.317;0.425]	0.172	-0.926 [-2.422;0.570]	0.219		
Education level						
Middle vs. low	2.667 [-0.159;5.492]	0.064	2.548 [-0.358;5.454]	0.084		
High vs. middle	2.667 [-0.942;4.799]	0.183	1.749 [-1.210;4.708]	0.240		
History of depression	6.578 [2.115;11.042]	**0.005**	7.598 [2.929;12.267]	**0.002**	-0.279 [-1.851;1.293]	0.722
Family History Migraine	-1.149 [-2.441;0.144]	0.080	-1.182 [-2.587;0.223]	0.097		
Alcohol (yes/ no)	0.945 [-0.528;2.419]	0.204	0.922 [-0.637;2.481]	0.240		
Smoking (yes/ no)	0.436 [-1.246;2.117]	0.605	0.738 [-1.029;2.505]	0.405		
Drugs (yes/ no)	1.187 [-1.643;4.017]	0.404	1.723 [-1.429;4.876]	0.277		
Caffeine (yes/ no)	1.090 [-2.352;4.532]	0.528	0.961 [-2.548;4.471]	0.584		
Sober (hours)	0.053 [-0.261;0.368]	0.735	0.060 [-0.263;0.382]	0.712		
LP effort (1x vs. >1)	-1.718 [-3.155;-0.281]	**0.020**	-1.629 [-3.173;-0.085]	**0.039**	-0.268 [-0.968;0.432]	0.444
Puncture traumatic (yes/ no)	0.721 [-1.524;2.966]	0.522	0.760 [-1.531;3.052]	0.508		
CSF opening pressure (cm H_2O)	-0.023 [-0.149;0.103]	0.718	-0.013 [-0.151;0.125]	0.852		
Sitting position (vs. supine)	2.750 [1.082-4.418]	**0.002**	3.003 [1.192-4.814]	**0.002**	2.534 [0.632-4.435]	**0.010**
Pain during LP (VAS 1-10)	0.161 [-0.111;0.433]	0.239	0.141 [-0.138;0.421]	0.314		
Stress prior to LP (VAS 1-10)	0.030 [-0.172;0.231]	0.770	0.057 [-0.153;0.268]	0.587		
Stress during LP (VAS 1-10)	0.369 [0.149;0.588]	**0.001**	0.356 [0.120;0.592]	**0.004**	0.345 [0.076-0.614]	**0.013**
Headache-after-LP (VAS 1-10)	0.189 [-0.152;0.531]	0.271	0.148 [-0.217;0.512]	0.419		

Discussion

This is the first prospective evaluation of the risk of PDHP and migraine after LP in migraineurs and healthy subjects. We carefully assessed and compared two large populations of well-characterized migraine patients with episodic migraine and age- and sex-matched healthy subjects, using in all subjects the same standardized LP procedure, pre- and post-LP detailed clinical characterization and one-week pro-active follow up.

Contrary to common belief [6, 28], but in line with an earlier small retrospective study [29], migraine patients did not have increased risk or longer duration of PDPH. In fact, we found a remarkably lower incidence of PDPH in migraineurs also when excluding the hemiplegic migraine cases. Notably, chronic migraineurs were not included in this study as well as migraineurs with active comorbid depression wherefore treatment was required. The finding that migraineurs were not at increased risk prompted us to further investigate and compare the underlying mechanisms for PDPH and migraine. In contrast to what is believed by some, LP did not trigger attacks in migraine patients. Interestingly, pre-LP stress levels tended to be higher in those participants who did not get an attack after LP, which would suggest a protective rather than a provoking effect of acute stress [30,31].

In our study we confirmed previously found risk factors for PDPH (young age and low BMI [18, 21, 32]) and identified headache immediately after LP and sitting sampling position as a previously unrecognized additional risk factor. Moreover, we showed that sitting sampling position, history of depression, multiple LP efforts, and higher perceived stress during the LP procedure were associated with a longer duration of PDPH. Sitting position seemed to have a larger effect than the number of LP efforts, although we feel these factors are strongly related. Higher levels of neuroticism or anxiety have been described before as risk factors for PDPH[33] but not for a longer duration of PDPH.

One might argue that using smaller sized a-traumatic needles [34, 35] and parallel bevel insertion rather than perpendicular [18, 36], could have potentially reduced the risk of PDPH. However, after careful consideration, we decided to use traditional procedures and needles, for several reasons. First, as the present study primarily was a "CSF biochemical profiling study", it was crucial to limit the sampling time to preclude *ex vivo* metabolisation [37]. Using larger sized needles and the simplest available procedure, allowed for higher CSF flow rates and briefer overall sampling times. An alternative could have been the use of 20 Gauge atraumatic needles [38]. However, a-traumatic needles are more difficult to use, often leading to higher failure rates [35], and that time there was no experience in our department with atraumatic needles. Furthermore, comparability with previously collected samples required us to use the same needles, since it is unknown what the effect of type of needle and consequent CSF sampling rate and flow velocity is on CSF metabolites. Finally, we wanted to compare our results with those obtained in previous studies using standard LP procedures. Indeed, our results for PDPH incidence [1,2], duration [17, 39], and lag time [6, 40], are all well in line with findings in previous, retrospective studies, supporting the external validity of our data. In future studies, however, the use of atraumatic needles should be considered.

Finally, despite clearly showing postural dependency of the headache, over one tenth of the subjects with PDPH did not have any of the associated symptoms as mandated by the ICHD-IIR classification criteria[3]. This finding would call for a re-evaluation of these criteria.

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Supplementary material

Table e-1; Diagnostic criteria for post-dural (post-lumbar) puncture headache (PDPH) as set by the Headache Classification Subcommittee of the International Headache Society[3].

Diagnostic criteria		
A.	Headache that worsens within 15 minutes after sitting or standing, and improves within 15 minutes after lying, with at least one of the following (see 1 to 5) and fulfilling criteria C and D:	1. Neck stiffness 2. Tinnitus 3. Hypacusia 4. Photophobia 5. Nausea
B.	Dural puncture has been performed	
C.	Headache develops within 5 days after dural puncture	
D.	Headache resolves either*:	1. Spontaneously within 1 week 2. Within 48 hours after effective treatment of the spinal fluid leak (usually by epidural blood patch).

* Note: in 95% of cases this is so. When headache persists, causation is in doubt.

Table e-2; Baseline characteristics of migraineurs (n=150) and healthy controls (n=49). *p*-values depicted in bold indicate significant differences ($p<0.05$), using independent-samples t-tests and χ^2 tests appropriately. M = Migraine; LP = lumbar puncture; CSF = cerebrospinal fluid.

Variable	Total (n=199)	Migraineurs (n=150)	Controls (n=49)	*P*
Age; mean (SD)	42.1 (14.1)	44.0 (13.3)	36.3 (15.3)	**0.001**
Female (%)	122 (61.3%)	98 (65.3%)	24 (49.0%)	**0.041**
BMI	24.2 (3.0)	24.3 (2.9)	24.1 (3.3)	0.746
Education level (%)				
Low	17 (13.6%)			
Middle	69 (34.6%)			
High	94 (47.2%)			
Comorbidity				
None	189 (95.0%)	140 (93.3%)	49 (100%)	0.179
Depressive	6 (3.0%)	6 (4.0%)	-	
Epilepsy	4 (2.0%)	4 (2.7%)	-	
Family History M.* (%)	108 (54.3%)	102 (68%)	6 (12.2%)	**<0.001**
Migraine subtype MO (%)	-	66 (44%)	-	
Intoxications				
Alcohol (%)	148 (74.4%)	44 (29.3%)	42 (85.7%)	**0.036**
Smoking (%)	37 (18.6%)	24 (16.0%)	13 (26.5%)	0.100
Drugs (%)	4 (2.0%)	1 (0.7%)	3 (6.1%)	**0.018**
Caffeine (%)	188 (94.5%)	142 (94.7%)	46 (93.9%)	0.834
Hours sober; mean (SD)	11.3 (2.3)	11.3 (2.1)	11.4 (2.8)	0.871
LP effort; mean (SD)	1.4 (1.0)	1.5 (1.1)	1.3 (0.8)	0.189
Puncture traumatic (%)	16 (8.0%)			
CSF pressure; mean (SD)	17.7 (4.3)	17.7 (4.5)	17.4 (3.8)	0.716
PDPH (%)	64 (32.2%)	42 (28.0%)	22 (44.8%)	**0.028**
Pain during LP	3.3 (2.4)	3.3 (2.3)	3.2 (2.6)	0.829
Stress prior to LP	2.4 (2.7)	2.4 (2.9)	2.1 (2.3)	0.544
Stress during LP	3.2 (2.8)	3.2 (2.8)	3.2 (2.9)	0.958
Headache immediately after LP	1.0 (1.6)	1.0 (1.6)	1.0 (1.6)	0.762

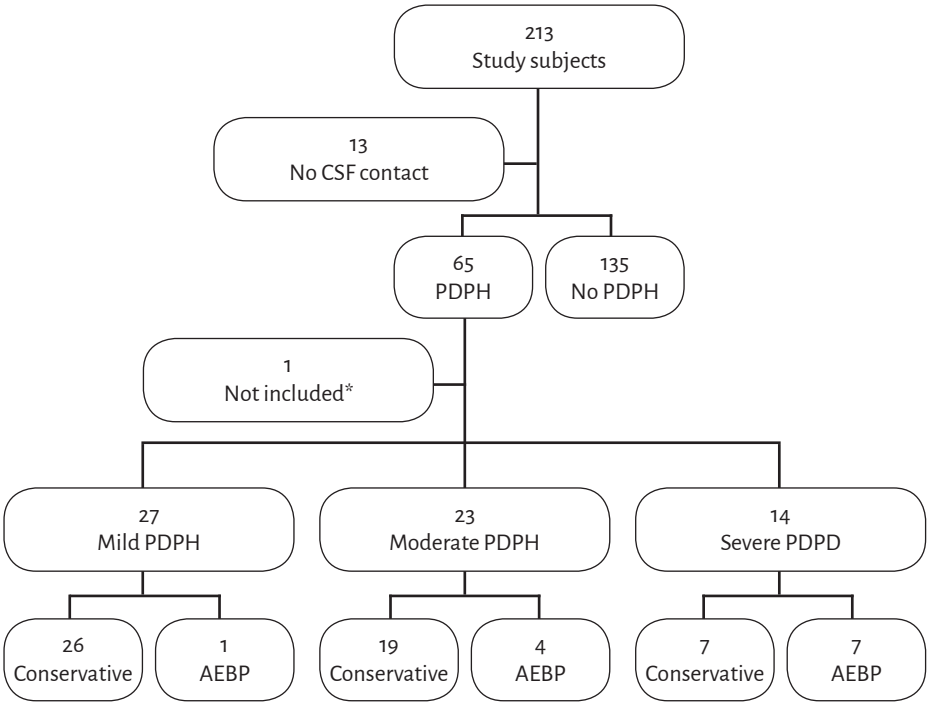

Figure e-1; Flowchart showing distribution of study subjects in relation to PDPH and selected treatment. AEBP = autologous epidural bloodpatch; Conservative = conservative treatment. *One female subject was excluded from the PDPH analysis due to headache symptoms that could be attributed to neither PDPH nor migraine, and were suggestive for chronification of migraine.

Chapter 6.

Abnormal cardiovascular response to nitroglycerin in migraine.

Van Oosterhout WPJ[1,2]
Schoonman GG[1,3]
Saal DP[1,4]
Thijs RD[1,5]
Ferrari MD[1]
van Dijk JG[1,6]

[1]Department of Neurology, Leiden University Medical Center, Leiden, the Netherlands
[2]Department of Neurology, OLVG Hospital, Amsterdam, the Netherlands
[3]Department of Neurology, Elisabeth-Tweesteden Hospital, Tilburg, the Netherlands
[4]Department of Neurology, Franciscus Gasthuis & Vlietland, Schiedam, the Netherlands
[5]Stichting Epilepsie Instellingen Nederland (SEIN), Heemstede, the Netherlands
[6]Section of Clinical Neurophysiology, Leiden University Medical Center, Leiden, the Netherlands

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Abstract

Introduction
Migraine and vasovagal syncope are comorbid conditions that may share part of their pathophysiology through autonomic control of the systemic circulation. Nitroglycerin can trigger both syncope and migraine attacks, suggesting enhanced systemic sensitivity in migraine. We aimed to determine the cardiovascular responses to nitroglycerin in migraine.

Methods
In sixteen women with migraine without aura and ten age and gender matched controls without headache intravenous nitroglycerin (0.5 μg·kg-1·min-1) was administered. Finger photoplethysmography continuously assessed cardiovascular parameters (mean arterial pressure, heart rate, cardiac output, stroke volume and total peripheral resistance) before, during and after nitroglycerin infusion.

Results
Nitroglycerin provoked a migraine-like attack in 13/16 (81.2%) migraineurs but not in controls (p=.0001). No syncope was provoked. Migraineurs who later developed a migraine-like attack showed different responses in all parameters vs. controls (all p<.001): the decreases in cardiac output and stroke volume were more rapid and longer lasting, heart rate increased, mean arterial pressure and total peripheral resistance were higher and decreased steeply after an initial increase.

Discussion
Migraineurs who developed a migraine-like attack in response to nitroglycerin showed stronger systemic cardiovascular responses compared to non-headache controls. The stronger systemic cardiovascular responses in migraine suggest increased systemic sensitivity to vasodilators, possibly due to insufficient autonomic compensatory mechanisms.

Introduction

Migraine is a common, paroxysmal headache disorder with a complex pathophysiology[1, 2]. The exact role of the vasculature is a matter of debate[3]. Some studies have suggested ictal [4] and interictal [5] functional changes in the systemic circulation in migraine. Interestingly, migraineurs also have an increased rate of vasovagal syncope (VVS) [6, 7] which partly explains the white matter abnormalities found in population based study[7].
Syncope is transient loss of consciousness due to cerebral hypoperfusion [8, 9] and also occurs often in the general population [8, 10]. In population-based studies, compared to non-migraine controls, more individuals with migraine had ever had syncope[11], frequent syncope (>5 attacks per lifetime) [6] and orthostatic intolerance [6] in between attacks. Syncope can also occur during migraine attacks[12]. One third of people with unspecified syncope have migrainous features during episodes of syncope[13]. Vasovagal syncope (VVS) is the most likely mechanism for syncope in migraineurs. VVS can be provoked with a tilt table test, which relies on a susceptibility to hypotension in the vertical position[14]. Administration of a systemic vasodilator such as nitroglycerin increases the incidence of tilt-induced VVS, likely due to increased venous pooling[14]. Nitroglycerin may also provoke migraine attacks, but only in migraineurs[15]. Nitroglycerin is de-nitrated to nitric oxide (NO) and vasoactive S-nitrosothiols. Both substances cause vasodilation through activation of cytoplasmic guanylate cyclase, which increases intracellular guanosine-3',5'-monophosphate (cGMP) and cytosolic calcium[15]. NO provokes migraine attacks either directly or indirectly through the NO-activated intracellular cascade[15].

Taken together, the systemic circulation of individuals with migraine seems abnormally susceptible to nitroglycerin. In this study, we assessed whether this response is indeed different in relation to a forthcoming migraine attack.

Methods

Participants

Participants were included as part of a prospective case-control study on cerebral changes in migraine. Sixteen women with migraine without aura (as defined by the ICHD-2 criteria (16) and in retrospect also fulfilling the ICHD-3 criteria [17]) were compared with ten control women without migraine, group-matched for age and body mass index (BMI). Eight (50%) migraineurs versus five (40%) controls had ever had at least one VVS (p=.62; Table 1). Migraine participants were recruited from the LUMINA database, described in detail elsewhere[18]. Controls were recruited via public announcement, and were not to have any primary headache disorder. None of the participants used any vasoactive or neuroactive medication.

Study procedure

After screening, eligible individuals were invited to the hospital. After overnight fastening, nitroglycerin was administered. Clinical features and cardiovascular and clinical parameters were monitored continuously immediately before (during 10 min), during (20

min) and immediately after (10 min) nitroglycerin-infusion. Headache severity was scored throughout the experiment using a visual rating scale, ranging from '0' (no headache) to '10' (most severe headache). Migraine participants were investigated at least three days after a previous migraine attack. All measurements were performed by a single investigator (WPJvO) in a quiet, temperature-controlled room with the participants lying comfortably in the supine position.

Administration of nitroglycerin

Nitroglycerin (0.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$ infusion for 20 minutes via cannulation of the antecubital vein in both the control and migraine group) was used to trigger migraine-like attacks in the migraine group[15]. This model is widely used and has good reproducibility and reliability [15, 19]. Infusion of nitroglycerin results in an immediate non-migraine headache in healthy individuals and migraineurs, followed by a migraine-like attack 4-6 hours after infusion in migraine patients but not in controls[19, 20].

Photoplethysmography

Continuous finger blood pressure measurements were performed with a Finometer Pro (Finapres Medical Systems BV, Amsterdam, The Netherlands; model 1; 2003.126). This is a reliable non-invasive beat-to-beat blood pressure measurement device using photoplethysmography, enabling analysis of blood pressure (mean arterial blood pressure; mmHg), cardiac output ($L \cdot min^{-1}$), total peripheral resistance ($mmHg \cdot (L \cdot min^{-1})^{-1}$), stroke volume (L), and heart rate ($beats \cdot min^{-1}$) [21, 22]. These parameters were obtained using the Beatscope software program (Finapres Medical Systems BV, Amsterdam, The Netherlands). The cuff was placed on the right index finger and the height correction system of the device was active during the whole measurement period, with participants keeping their arms immobilised in a sling with the hand above the heart. The participants were supine and at rest for 5-10 minutes before measurements started. We transformed beat-to-beat photoplethysmography data to averages per minute. The original continuous data were inspected for artefacts (WPJvO; JGvD), for instance due to talking, movements or coughing, in which case the results of that minute were treated as missing values.

Standard Protocol Approvals, Registrations, and Patient Consents

The study was conducted as part of the Leiden University Medical Center Neuro Analysis Programme (LUMINA)[18]. LUMINA and the present study were approved by the local medical ethics committee. All participants provided written informed consent prior to participation. Individual participants can not be recognised based on data published in this manuscript.

Statistics

Student's t-test was used to compare continuous, normally distributed variables, and the Fisher exact test for categorical data. Where appropriate, non-parametric tests were applied. Data were analysed in two ways: by ANCOVA (between-group comparison of pre-, during and post-infusion block averages; adjusted for age) and by a generalized estimating equations (GEE) model (between-group comparison of 1-minute averages during the nitroglycerin infusion period; repeated measurements). The primary analysis was the comparison between migraine participants in whom an attack developed after

nitroglycerin versus control participants. Group (migraine and control), time (the 1 minute average), and group*time interaction were included in the model. In a further explorative analysis, we assessed whether the cardiovascular response to nitroglycerin depended on the propensity of nitroglycerin to provoke a migraine attack in migraine subgroups. Analyses were performed using SPSS 23.0 (SPSS inc., IBM, USA). The statistical threshold was set to $p < .05$.

Data availability Statement

All data, methods and materials used to conduct this research are mentioned in this article. Research materials related to this paper can be accessed upon request to the authors.

Results

Clinical characteristics

Baseline characteristics of migraine and control participants did not differ (Table 1).

Table 1. Baseline characteristics of study population.

Baseline characteristics of migraine and non-headache control participants. Continuous variables are depicted as mean ± SD. *p*-values depicted in bold indicate significant differences ($p<.05$), using independent-samples t-tests and χ^2 tests appropriately. MO = migraine without aura. * = Fisher's exact probability test. N.c. = not calculable

Variable	Migraine patients N=16	Controls N=10	*P*
Demographics			
Age y	39.3 ± 9.7	37.1 ± 9.2	.57
BMI kg/m^2	23.0 ± 1.9	23.7 ± 1.8	.36
Gender F, n (%)	16 (100%)	10 (100%)	n.c.
Syncope			
Syncope ever, n (%)	8 (50.0%)	4 (40.0%)	.46 [a]
Syncope >1/yr, n (%)	2 (12.5%)	0 (0%)	.51 [a]
Headache			
Migraine subtype MO, n (%)	16 (100%)	-	-
Attack frequency per month	2.5 ± 1.0	-	-
Age at onset	18.8 ± 8.7	-	-

Baseline measurements

At baseline, mean cardiovascular parameters (total peripheral resistance; stroke volume; cardiac output; mean arterial pressure and heart rate) did not differ between individuals with migraine and controls (all $p>.05$; Table 2).

Table 2. Cardiovascular parameters in control participants and in migraineurs (in whom an attack developed), during and after nitroglycerin infusion.

Estimated means ± SE (ANCOVA, adjusted for age) are depicted. M = migraineurs; C = controls; MAP = mean arterial pressure (mmHg); HR = heart rate (beats·min^{-1}); CO = cardiac output (L·min^{-1}); TPR = total peripheral resistance (mmHg·(L·min^{-1})$^{-1}$); SV = stroke volume (ml beat^{-1}). Bold indicates p<.05.

	Baseline (10min)							NTG (20min)						
	M			C			p	M			C			p
TPR	0.82	±	0.04	0.77	±	0.07	.63	0.85	±	0.04	0.85	±	0.08	.96
SV	103.9	±	5.2	113.9	±	8.4	.33	85.9	±	3.1	98.7	±	6.0	.07
CO	6.6	±	0.3	6.8	±	0.5	.68	6.1	±	0.2	6.4	±	0.4	.54
MAP	88.7	±	2.1	92.0	±	3.5	.42	82.4	±	1.8	85.3	±	3.4	.47
HR	66.2	±	1.3	63.4	±	2.0	.24	72.4	±	1.0	65.1	±	1.8	**.001**

	Post-NTG (10min)						
	M			C			p
TPR	0.88	±	0.05	0.93	±	0.07	.55
SV	89.1	±	4.3	97.3	±	6.7	.31
CO	5.7	±	0.2	5.8	±	0.4	.93
MAP	81.5	±	2.5	83.7	±	3.9	.64
HR	66.3	±	1.2	60.4	±	1.8	**.01**

Effects of nitroglycerin on migraine attacks and syncope

Both migraine and control participants reported a mild to moderate headache during infusion of nitroglycerin (Figure 1), with a similar peak intensity (2.0 ± 2.5 points vs. 1.4 ± 1.8; p=.10) and no difference in time of onset of headache between groups (group*time interaction: p=.30). Thirteen of the sixteen (81.3%) migraineurs developed a migraine attack versus none of the controls (0%; Fisher's exact test p=.0001), with a mean ± SD of 314 ± 126 minutes (median 270 minutes; range 155-600 minutes) after NTG-administration. The six migraineurs in whom a migraine-like attack occurred within 270 minutes after infusion were labelled the 'early attack' group. The seven migraineurs who developed a migraine-like attack >270 minutes after infusion were labelled the 'late attack' group. No syncope was provoked.

Effects of nitroglycerin in migraineurs in whom an attack developed

Analyses of block averages showed that mean heart rate was higher in migraine participants (in whom an attack developed later) during infusion of nitroglycerin as compared to controls (p=.001; Table 2). The time-series analysis, using 1-minute averages, showed that stroke volume, cardiac output and mean arterial pressure decreased during infusion, whereas heart rate and total peripheral resistance increased (all p<.001) in both groups. For all parameters, but especially for total peripheral resistance, stroke volume and heart rate, nitroglycerin-induced changes were more pronounced in migraine participants compared to controls (p<.001 for group*time interaction in the analysis of each of the 5 outcome parameters). We observed a faster and more prolonged decrease in stroke volume and cardiac output in the group of migraineurs compared to the control group. Total peripheral resistance, after a slight increase, steeply decreased in the migraine group as compared to

a small and stable increase in the control group. Mean arterial pressure decreased in both groups, albeit after an initial small increase in the migraine group. Heart rate increased in both groups but more so in the migraine group (Figure 2).

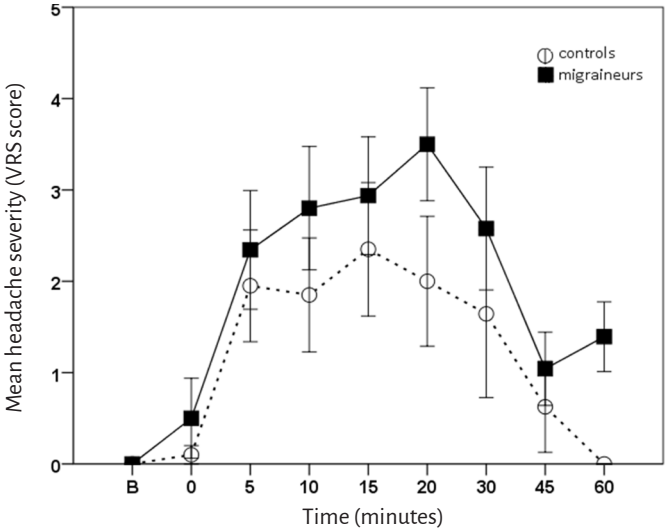
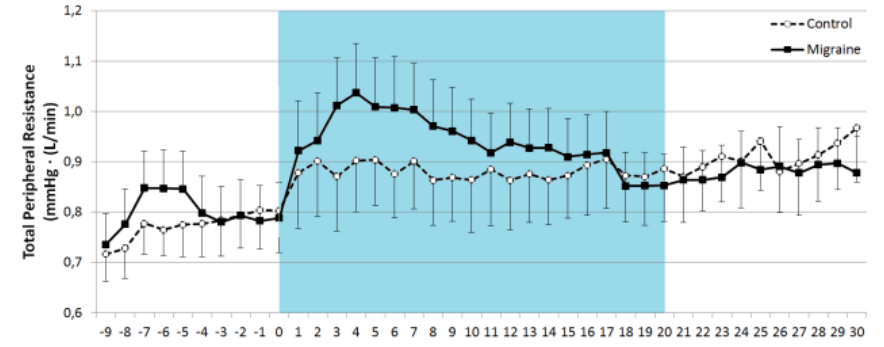


Figure 1. Headache severity in the nitroglycerin provocation experiment.
Both the migraine and the control group reported mild-moderate headache during nitroglycerin infusion (time 0 to 20min). Mean peak headache intensity did not differ between groups (student's t-test; 2.0 ± 2.5 points vs. 1.4 ± 1.8; p=.10) nor did time to onset (generalized estimating equations model for 0 – 20min period; group*time interaction: p=.30).

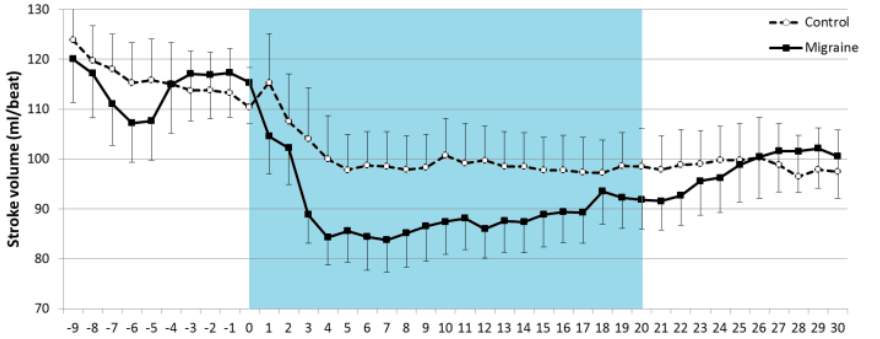
Table 3. Differences in baseline cardiovascular parameters between different migraine subgroups in relation to time-to-onset of migraine-like attack after NTG administration.
Means ± SD are depicted for unadjusted group means. MIG <4:30h = migraine patients with a migraine attack <4:30h after nitroglycerin; MIG >4:30h = migraine patients who developed a migraine attack >4:30h after nitroglycerin; No MIG = migraine patients who did not develop a migraine after nitroglycerin; TPR = total peripheral resistance (mmHg·(L·min^{-1})$^{-1}$); SV = stroke volume (ml·beat^{-1}); CO = cardiac output (L·min^{-1}); MAP = mean arterial pressure (mmHg); HR = heart rate (beats·min^{-1});* = Kruskal-Wallis test

	Baseline (10min)									
	MIG <4:30h N=6			MIG >4:30h N=7			No MIG N=3			p^a
TPR	0.86	±	0.26	0.87	±	0.35	0.63	±	0.15	.52
SV	106.0	±	33.7	111.4	±	39.7	134.9	±	17.7	.37
CO	6.5	±	1.9	7.2	±	2.6	8.5	±	1.7	.33
MAP	91.2	±	11.2	93.3	±	10.1	98.7	±	15.1	.79
HR	65.1	±	5.6	65.3	±	4.0	68.6	±	17.4	.81

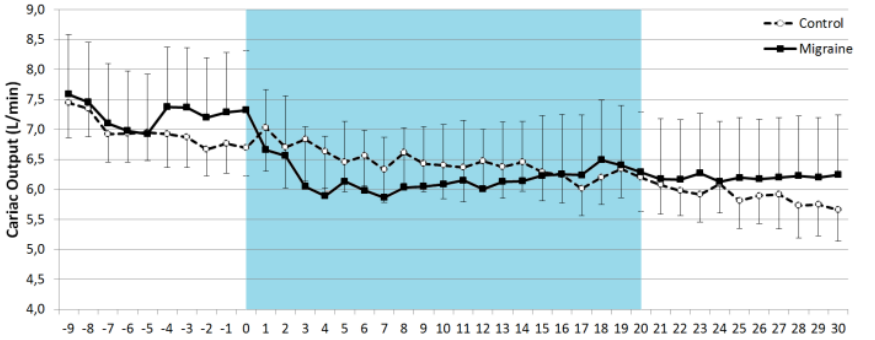
A. Total peripheral resistance



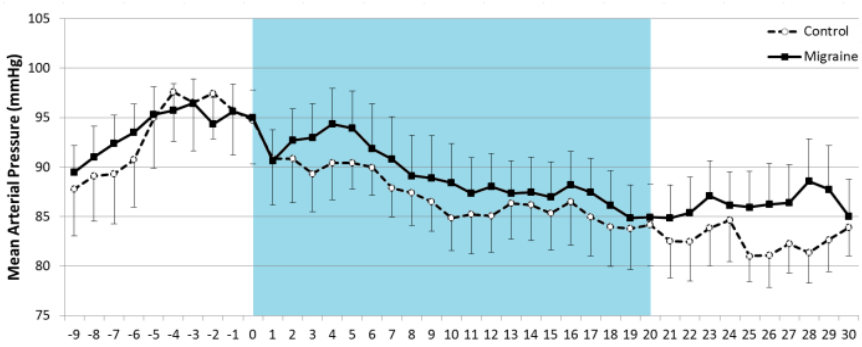
B. Stroke volume



C. Cardiac output



D. Mean arterial pressure



E. Heart rate

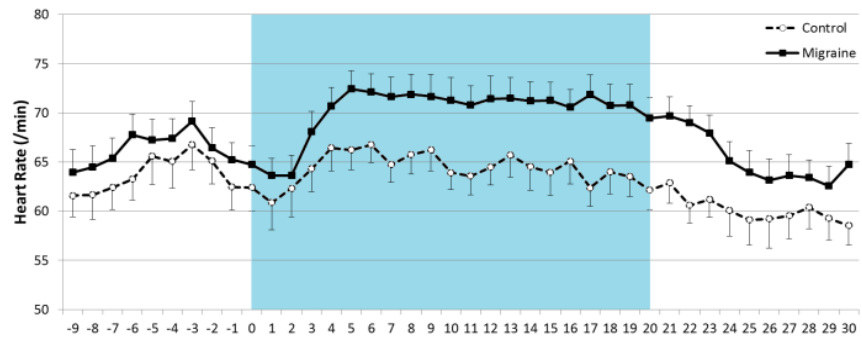
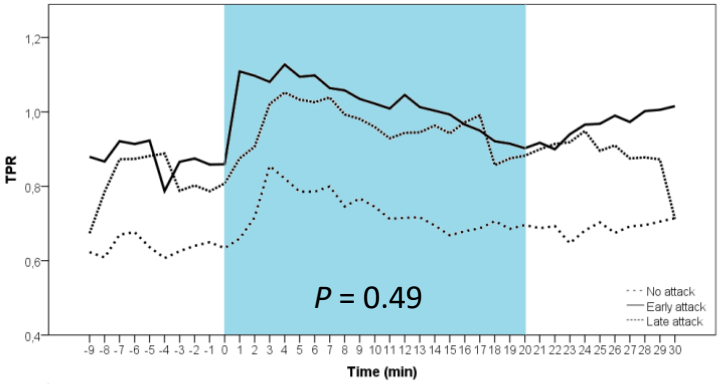


Figure 2. Cardiovascular responses during the course of nitroglycerin infusion in control participants and in migraineurs inn whom a migraine attack developed.
Time-series analysis showed that the minute-to-minute response to nitroglycerin differed between migraineurs and controls for several cardiovascular parameters. Graphs depict estimated means with 1 standard error as error bar.

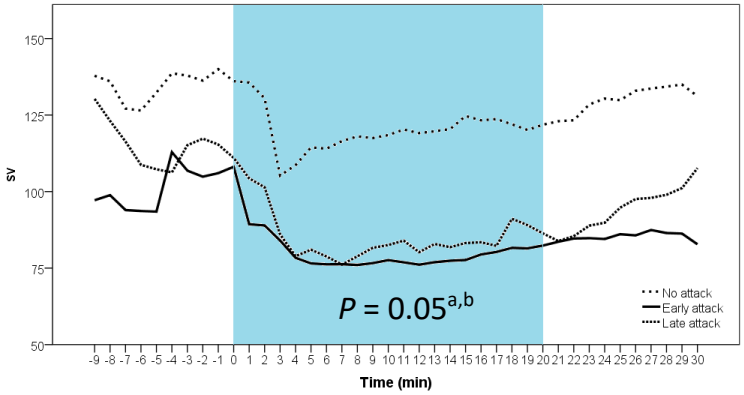
Effects of nitroglycerin in migraine attack onset subgroups

We compared the 'early attack' group with the 'late attack' and 'no attack' groups in an exploratory manner, due to the very small numbers per subgroup. The analyses suggested differences in cardiovascular responses between migraine participants who developed an attack versus those who did not (Table 3; Figure 3). These parameters reacted in the same general pattern during administration of nitroglycerin: those who later had an attack tended to have a lower baseline stroke volume (p=.051) and cardiac output (p=.048). However, our study was not powered to detect any differences. Post-hoc calculation shows that with a sample size of 6 per group stroke we only had a power of 0.39 and to detect a 50% difference in effect size.

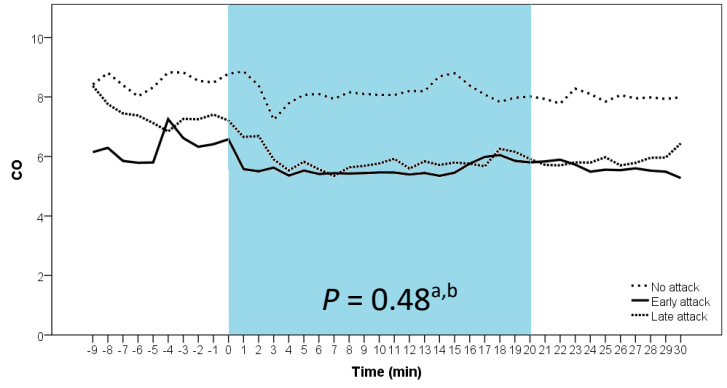
A. Total peripheral resistance



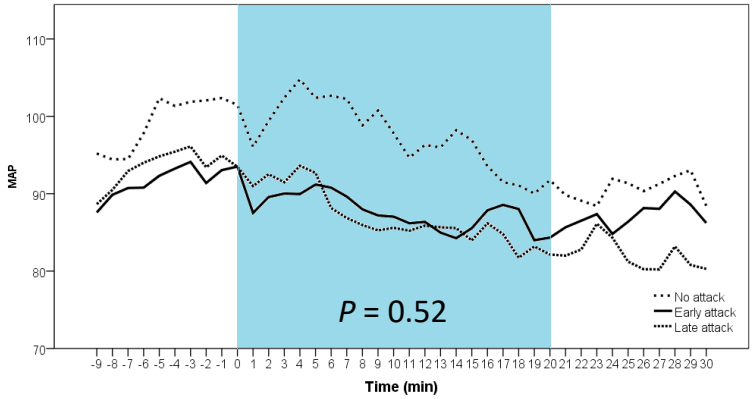
B. Stroke volume



C. Cardiac output



D. Mean arterial pressure



E. Heart rate

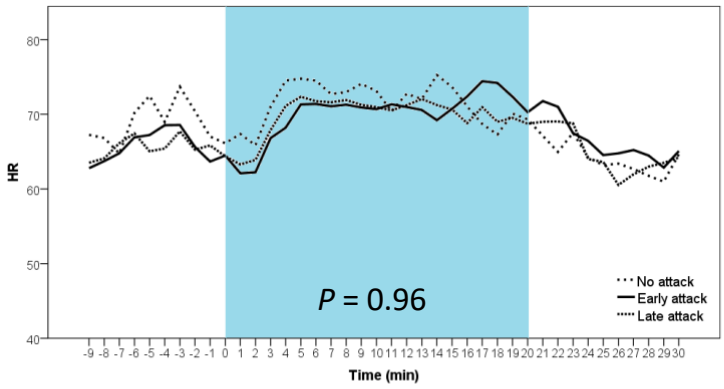


Figure 3. Cardiovascular response profiles during nitroglycerin infusion in migraine subgroups based on time-to-onset of the migraine attack.
Time-series analysis showed that the minute-to-minute response to nitroglycerin differed between migraineurs, classified in three groups based on time-to-onset of migraine attack. Graphs depict estimated means. Reported *p* values indicate overall level of significance (ANOVA-based). Superscript letters indicate post-hoc differences (LSD; $p < .05$) between early attack and no attack groups ([a]), and between late attack and no attack groups ([b]).

Discussion

We tested the hypothesis that the systemic responses to nitroglycerin differ between non-headache controls and migraineurs with a forthcoming attack. Using continuous photoplethysmography, we showed that intravenous nitroglycerin administration induced a faster and more prolonged decrease in stroke volume and cardiac output, a steep decrease in total peripheral resistance after an initial increase, a slightly higher mean arterial pressure, and a sustained increase in heart rate in migraine participants in whom an attack

developed later. The most pronounced differences occurred in those migraine patients in whom an early attack developed, whereas the changes were less marked in the three patients in whom no attack developed. The enhanced response of the systemic circulation to nitroglycerin in migraineurs suggest that the systemic vasculature is more susceptible to its (vasodilatory) effects.

Normal circulatory physiology consists of complex feedback mechanisms regulating blood pressure, maintaining adequate tissue perfusion throughout the body (for more details see Figure 4). Some of the differences in cardiovascular responses to nitroglycerin (the overall lower stroke volume, cardiac output, mean arterial pressure and steep decrease in total peripheral resistance after initial increase) that we have reported in this study suggest insufficient compensatory mechanisms to nitroglycerin-induced vasodilation in the migraine group. Interestingly, the mechanism described here in migraineurs is similar to the mechanism described in individuals with VVS who are exposed to nitroglycerin during head-up tilt [23] (Figure 4). Our data suggest that migraineurs have more venous pooling of blood and altered (arteriolar) total peripheral resistance after nitroglycerin in the supine position. Since some of the participants in both groups had had VVS in the past (year), we cannot exclude that the results might in part be due to VVS rather than to migraine itself. Subgroups in this study were too small to run separate analyses and further research is warranted to investigate this.

Theoretically the effects could be due to changes in only one circulatory compartment, e.g. the cerebral circulation. However, as cerebral blood flow comprises approximately 15-20% of the cardiac output and is tightly regulated to meet the brain's metabolic demands [24], our findings suggest involvement of the entire systemic circulation. It is speculative whether the change in systemic circulation might also cause a change in cerebral circulation. Photoplethysmography can unfortunately not distinguish between cerebral and non-cerebral parts of the peripheral vasculature. We can therefore only speculate to what extent our findings reflect changes in the cranial vessels. How much of the total peripheral resistance is based on the cranial vasculature is unknown. It is debated whether or not nitroglycerin may induce transient vasodilatation of extracranial vessels in migraine patients and non-headache individuals. If so, at least part of the decrease in total peripheral resistance could be due to cranial vasodilatation. Future studies should distinguish between these cerebral and systemic effects to further elucidate the underlying mechanisms.

Nitric oxide donors have a regulatory effect on vascular smooth muscle tone, but we cannot exclude a neuromodulatory effect of nitroglycerin within the central and peripheral nervous system [28]. It is therefore likely that its cardiovascular actions are not only confined to direct effects on blood vessels [29, 30].

The strengths of our study include the use of the standardised nitroglycerin provocation model [15, 19, 20], identical study protocols for patients and controls, and a homogenous study group. Furthermore, continuous monitoring of cardiovascular parameters before, during and after GTN infusion has not been reported before, according to our knowledge. Although

the inclusion criteria improve group homogeneity and interpretability of results, we are aware that they might limit generalization of our results to males or migraine with aura patients, as cardiovascular functioning and cardiovascular disease are gender dependent [31].

Only a few studies have investigated cardiovascular parameters in migraine [32-35]. Sublingual nitroglycerin induced increases in heart rate but no decreases in total peripheral resistance [35]. In a population-based study, higher diastolic and lower systolic blood pressures were found while mean arterial pressure was normal [32]. However, these and other cardiovascular parameters (cardiac output, heart rate, total peripheral resistance) did not differ in case-control studies [5, 33, 34], in which blood pressure was measured with upper arm cuffs, and only at baseline (no headache) conditions. The findings are nevertheless in agreement with our baseline data. Only one other experimental study reported on the cerebral vascular effects of nitroglycerin infusion in migraine, reporting a higher arterial sensitivity (reflected by higher middle cerebral artery blood flow velocities) to infusion as compared with healthy controls [36].

We can only speculate about underlying mechanisms explaining the differences in the magnitude of the cardiovascular responses to nitroglycerin. Hypothesising on underlying explanations, a stronger response of the systemic or cerebral vasculature might induce a migraine attack, but currently cerebral vasodilation is mostly regarded as an epiphenomenon in migraine [1]. Secondly, the systemic effect might occur independent of the migraine-inducing effects, both caused by one underlying mechanism. Sensitivity to nitric oxide (NO) may be enhanced, possibly due to higher perivascular concentrations of NO synthase [37]. NO is directly and further downstream implicated in neurogenic (central sensitisation; pain transmission) and vascular (vasodilation) mechanisms [15, 37]. Since or study was not powered to detect the possible differences between attack-onset subgroups, future studies could be developed to investigate these differences further.

There is a growing interest for the biological functions of the nitrate-nitrite-nitric oxide pathway, and specifically for the notion that the previously considered inert anions nitrate and nitrite can be recycled in vivo to form nitric oxide. It highlighted the possible therapeutic potential for this pathway, in particular in hypoxic states [38] and has already resulted in the first clinical studies in stroke [39]. We could postulate that migraine patients respond better to this effect, as we have shown that they have increased systemic cardiovascular sensitivity to nitric oxide.

Although we showed enhanced systemic cardiovascular responses to nitroglycerin in migraineurs, important remaining questions are whether this stronger response is due to migraine itself or to a concomitant susceptibility to VVS and what exactly is happening with the cerebral circulation during nitroglycerin triggering.

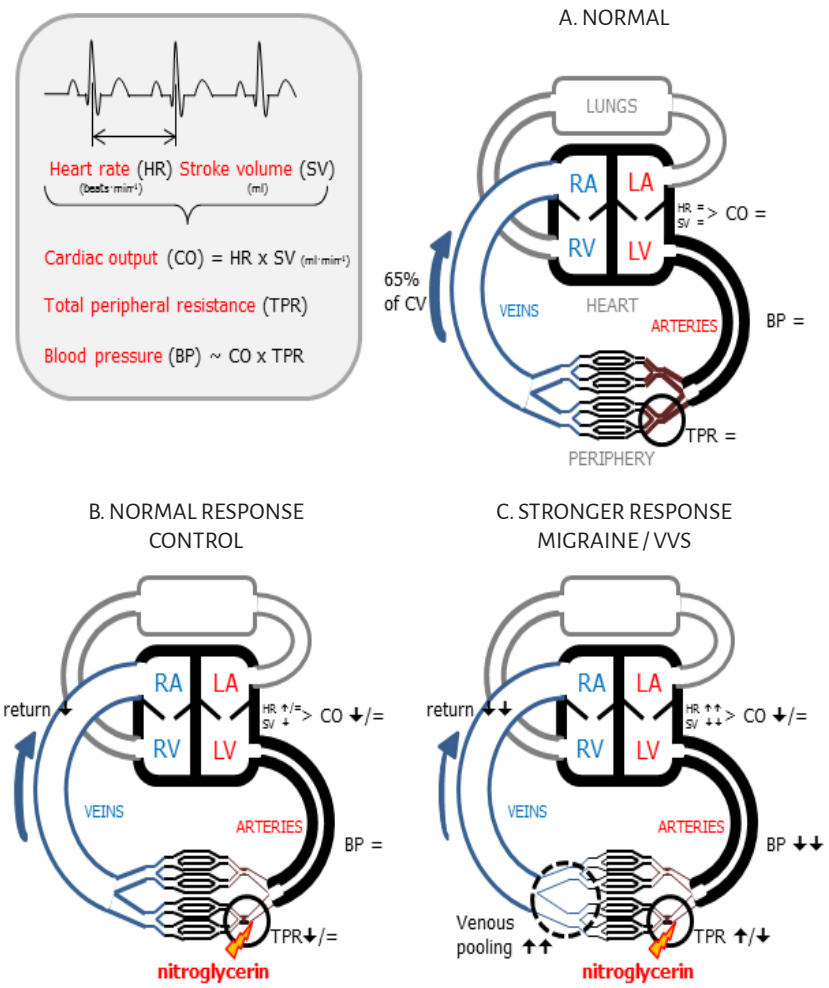


Figure 4. Normal circulatory physiology and cardiovascular responses to nitroglycerin in individuals with migraine and vasovagal syncope.

Blood pressure depends on cardiac output (CO) and on the total peripheral (arteriolar) resistance (TPR): blood pressure = CO x TPR (neglecting central venous pressure). Mean arterial pressure then is the average blood pressure during one cardiac cycle and is approximated by the sum of 2/3 x diastolic blood pressure and 1/3 of the systolic blood pressure. In turn, cardiac output (CO) is the product of the volume of blood pumped out by the heart per beat (stroke volume; SV) and heart rate (HR): CO = SV x HR. Blood pressure is therefore proportional to heart rate, stroke volume and total peripheral resistance. RA = right atrium; LA = left atrium; RV = right ventricle; LV = left ventricle, CV – circulating volume.

4A. Normally, the venous system contains 65% of the circulating volume and is responsible for the venous return to the heart. When blood pressure drops, autonomic control mechanisms should limit the decrease, aiming to maintain an adequate blood pressure. If, during a blood pressure decrease,

one or more of heart rate, stroke volume, or total peripheral resistance also decrease, the decreasing parameter must represent the cause of the blood pressure decrease. In contrast, if low blood pressure is accompanied by an increase in stroke volume, heart rate or total peripheral resistance, the increasing parameter represents an attempt at blood pressure restoration. For instance, a low blood pressure accompanied by a low stroke volume and high heart rate suggests that the heart rate attempts to compensate for the low stroke volume, and that the latter represents the primary problem.

4B. In individuals without migraine or vasovagal syncope nitroglycerin-induced vasodilation results in decreases in (arteriolar) total peripheral resistance, as well as in cardiac output and stroke volume via reduced venous return. These effects that have been described as results of nitroglycerin previously [30]. The cardiovascular system is largely able to compensate for these changes when a milder challenge is used (sublingual nitroglycerin spray). A stronger challenge, such as the intravenous infusion of nitroglycerin that was used in our study, can induce a decrease in blood pressure.

4C. The cardiovascular response to nitroglycerin is stronger in migraine and vasovagal syncope. In this study, the initial increases in total peripheral resistance and mean arterial pressure after nitroglycerin (in both the migraine and control group) suggested that venous and/or arterial compensatory mechanisms were evoked. The later decreases of mean arterial pressure and total peripheral resistance in the migraine group suggested that these mechanisms were insufficient leading to more pronounced responses. In vasovagal syncope, both spontaneous or nitroglycerin-induced, stroke volume and cardiac output decrease with total peripheral resistance either increasing (a; in cardio-inhibitory VVS [23]) or decreasing (b; in vasodepressive VVS [30, 40, 41]) reflecting autonomic compensatory mechanisms. Although the precise pathophysiology of VVS remains unclear, current theories hold that the fall in blood pressure is related to an impairment of venous return due to an inadequate venoconstrictive response; this may be accompanied by an inadequate arterial vasoconstriction during orthostatic stress [40].

Clinical implications

- Migraine and vasovagal syncope are comorbid conditions that may share part of their pathophysiology through autonomic control of the systemic circulation
- We found that intravenous nitroglycerin administration induced stronger systemic cardiovascular responses in migraine patients in comparison to controls
- The enhanced response of the systemic circulation to nitroglycerin in migraineurs suggest that the systemic vasculature is more susceptible to its (vasodilatory) effects

Acknowledgements

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Part II.

Imaging aspects

Chapter 7.

Hypothalamic functional MRI activity in the initiation phase of spontaneous and glyceryl trinitrate-induced migraine attacks

WPJ van Oosterhout[1,2]
AM van Opstal[3]
GG Schoonman[1,4]
JG van der Grond[3]
GM Terwindt[1]
MD Ferrari[1*]
MC Kruit[3*]
(* Both authors contributed equally)

[1]Department of Neurology, Leiden University Medical Center, Leiden, the Netherlands
[2]Department of Neurology, OLVG Hospital, Amsterdam, the Netherlands
[3]Department of Radiology, Leiden University Medical Center, Leiden, the Netherlands

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Abstract

Objectives

The hypothalamus has been suggested to be important in the initiation cascade of migraine attacks based on clinical and biochemical observations. The few imaging studies performed so far were all small, clinically heterogeneous, lacked non-migraine control groups and did not disentangle the changes due to the attack from those due the trigger compound. With a novel approach, we assessed hypothalamic neuronal activity in the early premonitory phase of glyceryl trinitrate (GTN)-induced and spontaneous migraine attacks.

Methods

We measured the hypothalamic blood oxygen level-dependent (BOLD) response to oral glucose ingestion with 3T functional MRI in 33 women, 19 with migraine without aura and 14 controls group-matched for age and body mass index on one day without prior GTN-administration, and on a second day, 90 minutes after GTN-administration to coincide with the premonitory phase of an induced attack. Interestingly, subgroups of patients with and without GTN-triggered attacks could be compared. Additionally, five migraineurs were investigated in a spontaneous premonitory phase. Linear mixed models were used to study between- and within-group effects.

Results

Without prior GTN-infusion, the BOLD-response to glucose was similar in migraine participants and controls ($p = 0.41$). After prior GTN-infusion, recovery occurred steeper and faster in migraine participants (versus day 1; $p < 0.0001$) and in those who developed an attack versus those who did not ($p < 0.0001$). Prior GTN-infusion did not alter the glucose-induced response in controls (versus baseline; $p = 0.71$). Just before spontaneous attacks, the BOLD-response recovery was also faster ($p < 0.0001$).

Conclusion

In this study, we found new and direct evidence of altered hypothalamic neuronal function in the immediate preclinical phase of both GTN-provoked and spontaneous migraine attacks.

Introduction

Migraine is a common, multiphasic, paroxysmal neurovascular brain disorder with recurring disabling attacks of headache, associated autonomic features and, in one third of patients, aura [1,2]. In up to 80% of patients, attacks may be preceded the 2-48 hours before by premonitory symptoms such as yawning, craving for specific food, fluid retention, tiredness, and mood changes [3-9]. When challenged with glyceryl trinitrate (GTN), most migraineurs (but not non-migraineurs), develop premonitory symptoms approximately 90 minutes after infusion and a migraine-like attack a few hours later [10].

The pathophysiology of migraine is complex. Although the underlying mechanisms for the aura, headache and associated symptoms are relatively well understood, how attacks begin is largely unknown [11]. The observation that attacks are usually preceded by premonitory symptoms strongly suggest that attacks actually begin well before the aura and headache phase. The episodic nature of attacks [12, 13], the clinical characteristics of premonitory symptoms [4], the observation that attacks may be triggered by acute changes in sleep pattern[14-18], and the finding that serum [19-24] and cerebrospinal fluid [21,22] levels of hypothalamic hormones are abnormal during [19, 21, 23, 24] and in-between [20, 22, 24] migraine attacks, all suggest a role for the hypothalamus in the initiation cascade of migraine attacks [11].

Studies using blood oxygen level-dependent functional MRI (BOLD fMRI) [25] or PET [26, 27] have indeed found hypothalamic cerebral blood flow (CBF) changes in GTN-induced [27] and a few spontaneous [25, 26] migraine attacks. These studies, however, were all small, clinically heterogeneous, or lacked non-migraine control groups. Moreover, the fMRI studies mainly investigated connectivity rather than hypothalamic function and no attempt was made to disentangle changes caused by GTN from changes due to the attack [27].

In the present study, we sought to better determine the role of the hypothalamus in the initiation of migraine attacks. As spontaneous migraine attacks are erratic, complicating studying the initiation phase of spontaneous attacks, we took advantage of the GTN migraine attack-provocation model [10]. We assessed and compared hypothalamic neuronal activity in women with (n=19) or without (n=14) migraine, with and without prior GTN infusion. Measurements with prior GTN infusion were done at 90 minutes after infusion to optimally coincide with possible premonitory symptoms. Five migraine patients could also be investigated during the premonitory symptoms phase of a spontaneous attack, enabling clinical validation of the findings in GTN-provoked attacks. Hypothalamic neuronal activity was measured as the fMRI BOLD response to glucose ingestion, which specifically activates glucose-sensitive neurons within the hypothalamus [28, 29]. The normal persisting drop in BOLD signal in response to glucose ingestion reflects reduced neuronal metabolic activity and is considered a signal of glucose satisfaction [28, 30]. The design we used enabled disentangling the effects due to the attack from those due to GTN.

Material and methods

Participants

We included 20 women with migraine without aura according to the ICHD-2 criteria [31] and 16 age- and body mass index (BMI) group-matched control women without a personal and 1st degree family history of migraine or any other regularly occurring headaches. Migraineurs also fulfilled the new ICHD-3 criteria [2]. All participants were recruited from the Leiden University Medical Centre Migraine Neuro Analysis (LUMINA) programme including individuals with migraine and non-headache controls from the Dutch population who all agreed to participate in migraine-related scientific research[32]. Participants with migraine were to have 1-6 migraine attacks and no more than 10 days of non-migraine headache per month. In addition they had to be free of migraine for at least 3 days before and 2 days after each study day, as was checked by a telephone call 7 days after each study day. Exclusion criteria for all participants included diabetes, premenstrual syndrome, hypertension, any psychiatric or neurologic disease, fever in the week prior and use of vasoactive, neuroactive or antibiotic medication in the two weeks prior to the measurement days.

Standard protocol approvals, registration, and patient consents

The study was approved by the local medical ethics committee and all subjects provided written informed consent prior to participation. The study was conducted according to the Declaration of Helsinki [33].

fMRI BOLD response to glucose ingestion

fMRI BOLD provides an indirect and non-invasive method to assess changes in neuronal activity in the brain by measuring changes in the BOLD signal. These changes occur due to changes in local concentrations of oxygenated and deoxygenated haemoglobin, local perfusion (blood flow and volume) and haematocrit, that result from changes in neuronal activity [34, 35]. Glucose-sensitive neurons within the lateral hypothalamus respond to glucose triggering after fastening. Physiologically, the hypothalamic BOLD response to oral glucose administration follows a typical pattern with an initial, relatively rapid and steep decrease of the signal becoming noticeable after about four minutes, and reaching its nadir after another four minutes. This is then followed by a slow recovery towards baseline levels over the next ten to twelve minutes [28, 29]. For glucose ingestion, a standard solution as used for the glucose tolerance test was made by mixing 300 mL tap water with 75 g glucose (Natufood, Natudis, Harderwijk, the Netherlands)[28].

GTN migraine provocation model

After cannulating an antecubital vein, GTN (0.5 micrograms·kg^{-1}·min^{-1}) is administered over 20 minutes with the study participant in supine position [10]. Immediately after infusion, all study participants (migraineurs and non-migraineurs alike) develop a brief, non-specific mild headache without associated features. In approximately 80% of migraineurs, but in none of non-migraineurs, this is followed, 3-6 hours later, by a migraine-like attack [10, 36-39]. In many migraineurs, GTN-provoked migraine-like attacks are preceded by premonitory symptoms, which typically start at around 90 minutes after GTN infusion [10, 36-39].

Study design

All participants were scanned on two separate days after overnight fasting and abstention of coffee, tea and alcohol; water intake was allowed. Prior to scanning all participants underwent a detailed standardised interview and full neurological examination. On the first (baseline) day, at around 9:00 am, this was followed by an fMRI scan which lasted for 21 minutes. About 7 minutes after beginning the fMRI scan, all participants ingested a standard glucose solution via a perioral tube, while remaining in supine position within the continuously recording MR scanner. On the second (= provocation) day, a 20 minute GTN infusion was started at 08:30 am and the post-GTN fMRI scan (with ingestion of glucose at 7 minutes after onset) was performed 90 minutes (mean ± SD: 91 ± 20) after start of the GTN infusion,. This timepoint was chosen to afford the highest likelihood of capturing possible premonitory symptoms of an ensuing GTN-provoked attack[10, 36-39].

Spontaneous attacks

Participants with migraine were also asked to come to the MRI as soon as they noticed an impending spontaneous migraine attack. They were then scanned during the premonitory phase of a spontaneous attack, using the same fMRI and glucose ingestion protocols.

Clinical parameters

We assessed premonitory symptoms, headache and migraine characteristics (according to the ICHD-2 [31] (also fulfilling the new ICHD-3 criteria [2]), and pain severity (numeric rating scale [NRS], ranging from 0 [no headache] to 10 [most severe headache possible]) before, every 5 minutes during, and every 30 minutes after GTN-infusion. Sociodemographic and clinical variables including migraine subtype, attack frequency and medication use, were recorded during a structured interview before the baseline study day

Data acquisition

MRI was performed on a 3.0 Tesla Achieva clinical scanner (Philips Healthcare, Best, the Netherlands) using a 32-channel phased array head coil. The same scan protocol was used for all MR sessions. It comprised of a whole brain high resolution 3D T1 sequence for imaging anatomical structures (TR 9.7 ms; TE 4.6 ms; flip angle 8°; FOV = 220x174x156 mm; 130 slices with a thickness of 1.2 mm and a voxel size of 0.86*0.86 mm), a structural hypothalamus scan (single slice scan, TR 550 ms, TE 10 ms, FOV = 208x208 mm, voxel size = 0.52x0.52x14 mm, scan time 1.14 min) and mid brain single slice fMRI scan (TR 120 ms, TE 30 ms, FOV 208x208 mm, voxel size = 0.81x0.81x14 mm, scan time 21.2 min, 500 dynamics). Anatomic images were screened for accidental findings by a neuroradiologist (MCK).

Data processing

Pre-processing and analysis of fMRI data was done using FSL version 5.03 [40]. Data was pre-processed as described in earlier studies [41]. Data were averaged for each set of 4 subsequent volumes, reducing the 500 dynamic scans to 125. The hypothalamus was segmented manually on the middle volume of the single slice MRI scan according to anatomical landmarks as previously described [41]. To correct for scanner drift, all hypothalamic BOLD values were corrected for the BOLD signal obtained in an internal reference ROI, drawn in grey matter, superior of the genu of the corpus callosum. To establish the post-ingestion

hypothalamic BOLD response to intervention, the mean pre-glucose signal (first 7 minutes of the 21 minute fMRI scan) was used for contrast. All data points (n=125) were divided by the mean baseline value and converted to percentages, yielding the percentage signal change relative to baseline, this percentage signal change was then averaged per minute.

Statistical analysis

General characteristics were compared using Mann-Whitney U tests for continuous variables, and Fisher exact tests for categorical data. Continuous data are presented as mean ± standard deviation, or as median with minimum-maximum. All fMRI results are reported as percentage BOLD change relative to the mean pre-glucose (reference) BOLD signal (0-7 minute pre-drinking). Data between minute 8 and 11 were omitted from the statistical analysis for artefacts in BOLD signal due to swallowing of the glucose solution. Data from minute 11 and up (11-21) were considered the post-glucose drinking BOLD response and were used for statistical analysis. Statistical analysis for comparison between groups (migraine; control), GTN (baseline; provocation) and/or migraine attack was performed by mixed model analysis as described earlier [41, 42]: group and GTN status were used as a fixed effect, time point as a variate and subject as a random factor. For within-group comparisons this model was applied to paired datasets.

Primary analysis was the difference in fMRI BOLD response to glucose at 90 minutes after GTN infusion versus the fMRI BOLD response to glucose without prior GTN infusion (i.e. baseline measurement) in participants with migraine, while focussing on those who developed an GTN-induced migraine-like attack. Secondary analyses included: i) baseline day differences between participants with migraine and controls; ii) effects of GTN on the fMRI BOLD response to glucose in participants and controls; iii) differences in fMRI BOLD response to glucose in GTN-induced versus spontaneous attacks; and iv) differences in post-GTN fMRI BOLD signal response to glucose in migraine participants in whom GTN did not provoke an attack versus controls . For further exploratory analyses, the fMRI BOLD response after glucose (average derived from 11 to 21 minutes post-glucose) was correlated with clinical migraine and demographic parameters using Pearson correlation coefficients. Uncorrected *p*-values of <0.05 were deemed significant for all tests. All statistical analyses were performed with the Statistical Package of Social Sciences (SPSS, version 23.0; SPSS, Chicago, III).

Data availability Statement

All data, methods and materials used to conduct this research are mentioned in this article.

Results

Participants

The study flow is depicted in Figure 1 (reasons for exclusion are given in the legends). Of the 20 migraine participants with a baseline scan two were excluded, leaving 18 participants eligible for the GTN scan. In two, post-GTN scans could not be performed. One post-GTN scan had to be excluded, leaving 15 post-GTN scans in migraine participants with also a baseline scan. Of the 16 migraine participants with a post-GTN scan, 13 (81%) developed

a migraine-like attack including the one of whom the scan had to be excluded due to movement artefacts. Paired data with both baseline and post-GTN scans were available for 15 migraine participants, 12 with and 3 without a provoked attack. In five migraine participants, scans could also be performed in the premonitory phase of a spontaneous attack. Of the 16 controls with a baseline scan, two were excluded and in four no post-GTN scan could be performed, leaving 11 controls with a post-GTN scan. Paired data with both baseline and post-GTN scan were available for 10 controls.

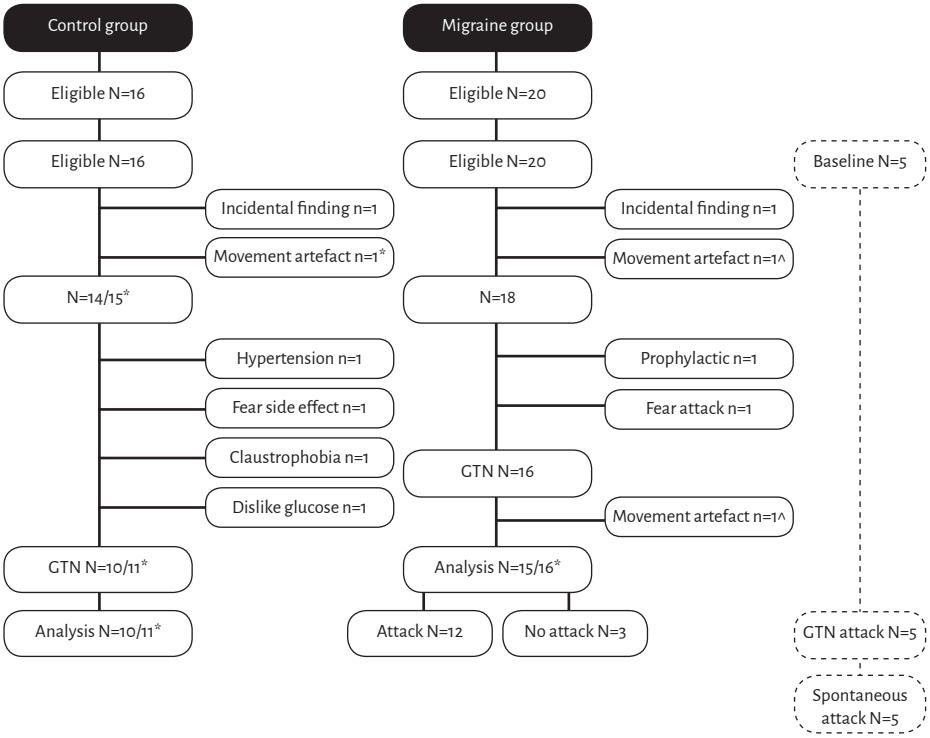

Fig. 1. Flowchart of study participants

Flowcharts depicting eligibility and exclusion of study participants at different stages of the study.
* = control participant in whom the baseline scan was excluded due to movement artefact, but GTN scan could be included in the unpaired analysis. ^ = migraine participant in whom both baseline and GTN scan were excluded due to movement artefacts.

Clinical and demographic characteristics

Clinical and demographic characteristics from all participants whose data were used in the analyses are summarized in Table 2. Demographic characteristics did not differ between the two groups. Participants with migraine had been free of migraine for 12.8 ± 8.7 (range 4-30) days before and >3 days after the attack-free measurement, and 10.3 ± 6.8 (range 4-30) days before and >3 days after the provocation day. There were no major baseline differences between the 13 migraine participants who developed a migraine attack after GTN and the 3 who did not.

Table 1. Description of clinical characteristics at baseline and after GTN-provocation in study participants.

Group	Age	Baseline	GTN-90	GTN effect	Headache onset delay (h:min)	Headache characteristics[a]	Headache intensity[c]	Associated symptoms[b]	Mimics usual migraine	Premonitory Symptoms
M	44	No HA	No HA	+	4:30	Left/throb/+	6	-/-/+/+	+	Yawning; nose feeling warm
M	40	No HA								
M	44	No HA								
M	46	No HA	No HA	-	n.a.	n.a.	n.a.	n.a.	n.a.	None
M	35	No HA	No HA	+	4:30	Right/throb/+	7	+/+/+/-	+	Yawning; nausea
M	50	No HA	No HA	+	5:20	Right/throb/+	9	+/+/+/+	+	Stiff neck; fatigue; problems concentrating
M	25	No HA	No HA	-	n.a.	n.a.	n.a.	n.a.	n.a.	None
M	50	No HA								
M	44	No HA	No HA	-	n.a.	n.a.	n.a.	n.a.	n.a.	None
M	48	No HA	No HA	+	3:35	Bil/pres/+	5	-/-/+/+	+	Stiff neck; osmophobia
M	50	No HA	Premonitory	+	2:50	Left/pres/+	8	+/+/+/+	+	Yawning; fatigue; feeling cold; polyuria
M	49	No HA	No HA	+	3:20	Right/pres/+	10	+/+/+/0	+	Fatigue; dry mouth
M	40	No HA	No HA	+	9:00	Right/throb/+	3	-/-/+/+	+	Feeling warm; fatigue; nausea
M	35	No HA	No HA	+	5:40	Left/pres/+	8	+/-/+/+	+	Craving; osmophobia; yawning; restless
M	51	No HA	No HA	+	6:20	Left/throb/-	5	+/-/+/-	+	Nausea; yawning;
M	28	No HA	Premonitory	+	3:40	Bil/pres/+	6	+/+/-/-	+	Fatigue; dry mouth; problems concentrating
M	32	No HA	No HA	+	4:10	Right/pres/+	6	+/+/+/+	+	Yawning; fatigue; heavy eyes; no appetite
M	24	No HA	No HA	+	7:40	Left/pres/+	6	+/-/+/-	+	Stiff neck; feeling cold
M	28	No HA	No HA	+	2:40	Bil/pres/+	9	+/-/+/+	+	Pressure feeling; light dizziness

table continues

Group	Age	Baseline	GTN-90	GTN effect	Headache onset delay (h:min)	Headache characteristics[a]	Headache intensity[c]	Associated symptoms[b]	Mimics usual migraine	Premonitory Symptoms
C	27	No HA	No HA	-						
C	25	No HA	No HA	-						
C	40	No HA	No HA	-						
C	46	No HA	No HA	-						
C	50	No HA	No HA	-						
C	44	No HA	No HA	-						
C	39	No HA	No HA	-						
C	44	No HA	No HA	-						
C	30	No HA	No HA	-						
C	45	No HA	No HA	-						
C	43	No HA	No HA	-						
C	46	No HA	No HA	-						
C	28	No HA	No HA	-						
C	29	No HA	No HA	-						
C	25	No HA	No HA	-						

For assessing the effect migraine attack phases, measurements were individually labelled as *no headache*, premonitory and *headache*. 'Premonitory symptoms' indicate symptoms that were reported by migraine patients >30min after GTN infusion, and that were recognised as their usual premonitory symptoms. M = Migraine patient; C = healthy control; GTN-90 = 90min after GTN infusion; + = provoked migraine attack after GTN infusion; - = no migraine attack provoked; HA = headache

[a] = lateralisation (left / right / bil = bilateral) / quality (throb = throbbing; pres = pressing) / aggravation

[b] = nausea / vomiting / photophobia / phonophobia

[c] = Numeric rating scale (NRS) score for maximum headache severity from 0 (no pain at all) to 10 (worst pain ever)

Table 2. Baseline characteristics of study population.

Variable	Migraine without aura n=19			Controls n=15		*p*
Socio-demographic						
Age, y [a]	44		24-51	40	25-50	0.34
BMI, kg/m² [a]	23.8		20.1-26.2	23.1	20.5-27.2	0.85
Righthandedness, n (%) [b]	17		(89.5%)	14	(93.3%)	0.69
Migraine specific						
Attack frequency / month ^	2	±	1-6			
Age at onset, y "	18.5	±	7.9			
Disease duration, y "	21.3		11.2			
Attack severity, NRS ^	8		5-10			

[a] = Mann-Whitney U test, depicted as median and minimum-maximum; [b] = Fisher exact test; ^ = depicted as median and minimum-maximum; " = depicted as mean ± standard deviation; Y = years; BMI = Body Mass Index; NRS = numeric rating scale

Clinical effects of GTN-infusion

GTN-infusion caused an immediate transient mild non-specific headache in all participants with migraine (mean numeric rating scale score = 3.8 ± 2.4) and controls (2.8 ± 2.0; $p = 0.25$). A delayed migraine-like attack developed in 13/16 (81%) participants with migraine at a mean of 4:54 ± 2:06 hours after start of the GTN infusion versus in 0/12 controls (0%; $p = 0.007$). All participants who developed a migraine-like attack reported one or more premonitory symptoms from 60 ± 54 minutes (median 180; range 30-240 minutes) after onset of the GTN-infusion and from 135 ± 116 minutes (median 112; range 30-460 minutes) before the headache started (Table 1). In contrast, none of those who did not develop a migraine-like attack, reported any premonitory symptom.

BOLD response to glucose: with versus without prior GTN-infusion

Without prior GTN infusion, the BOLD response to glucose was prototypical (steep decrease followed by a slow recovery) as reported previously [28, 29] and similar in the migraine and control group (Fig 2; $p = 0.41$). After prior GTN infusion, the BOLD response remained the same in the control group ($p = 0.71$; Fig 3A), but was clearly changed in the migraine group, with a much faster and steeper recovery phase after prior GTN infusion compared to the response without prior GTN infusion (total migraine group; intra-individual comparison: $p < 0.0001$; Fig 3B). A post-hoc analysis revealed that this response (after GTN-infusion) differed between patients with and without provoked migraine attack (between-group comparison; $p < 0.0001$; Fig 4B). The fast recovery had only occurred in the 13 migraine participants who later developed a migraine attack (intra-individual comparison versus own baseline: $p < 0.0001$). In those three who did not get an attack the recovery, in contrast, was slower (intra-individual comparison versus own baseline; $p < 0.004$).

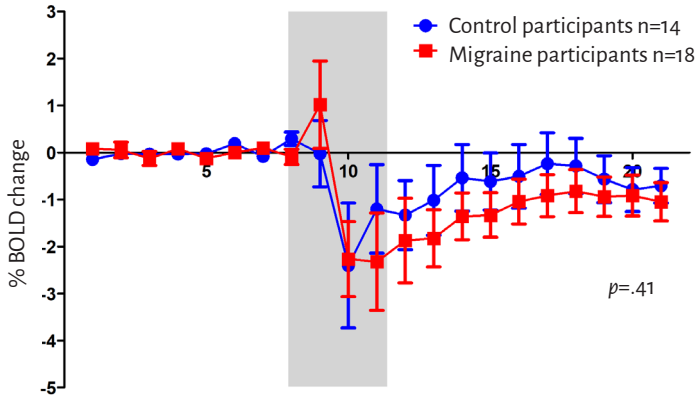

Fig. 2. Comparison of BOLD responses to glucose ingestion at baseline day in migraine and control participants

The BOLD response to glucose at a baseline day did not differ between migraine and control participants. Grey box indicates drinking period, of which data were omitted from the analysis since they are considered drinking artefacts. Error bars indicate 1 standard error.

Although the BOLD response to glucose after prior GTN infusion was visually different (faster and steeper recovery) in the total migraine group compared to controls, this difference did not reach statistical significance (between-group comparison: $p = 0.11$; Fig 3C), also not when only the 13 migraine participants who got an attack were included ($p = 0.12$).

Similar BOLD response in spontaneous and GTN-provoked attacks

Five migraine participants could also be studied in the premonitory phase of a spontaneous attack. In these patients the BOLD responses to glucose in spontaneous and GTN-provoked attacks were similar (intra-individual comparison; $p = 0.42$; Fig. 4C). Both responses differed visually from the attack-free measurement (Fig. 4D), but the response pattern in spontaneous attacks reached statistical significance at between-group level (versus baseline; $p < 0.0001$) but not at the intra-individual level likely due to small sample sizes ($p = 0.30$).

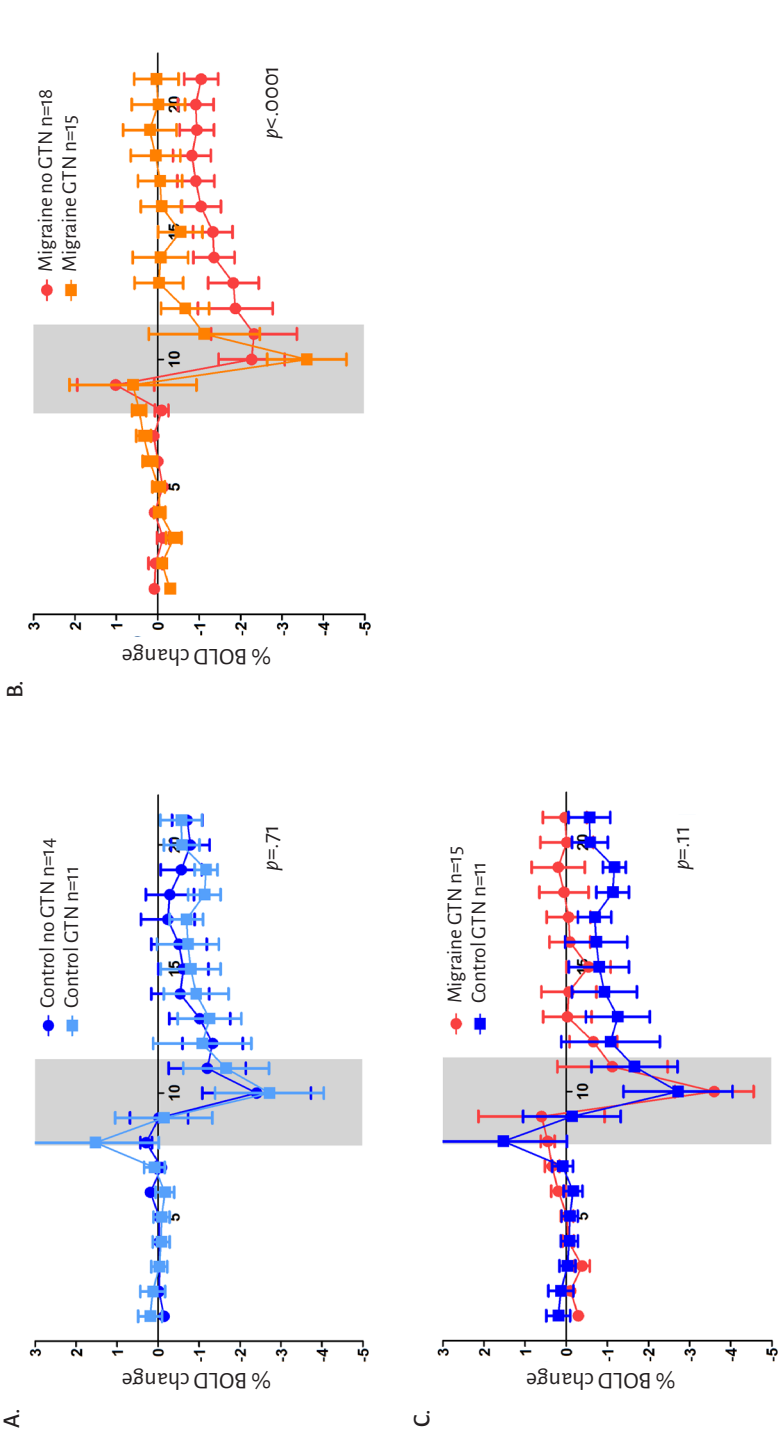

Fig. 3. Comparison of BOLD responses to glucose ingestion after GTN-provocation in migraine and control participants
The BOLD response to glucose after GTN did not differ from the BOLD response at baseline (without prior GTN-infusion) in controls (3A), but was higher in migraine participants (3B). The BOLD response to glucose after GTN is higher in migraine participants then in control participants, although not significant (3C). Grey box indicates drinking period, of which data were omitted from the analysis since they are considered drinking artefacts. Error bars indicate 1 standard error.

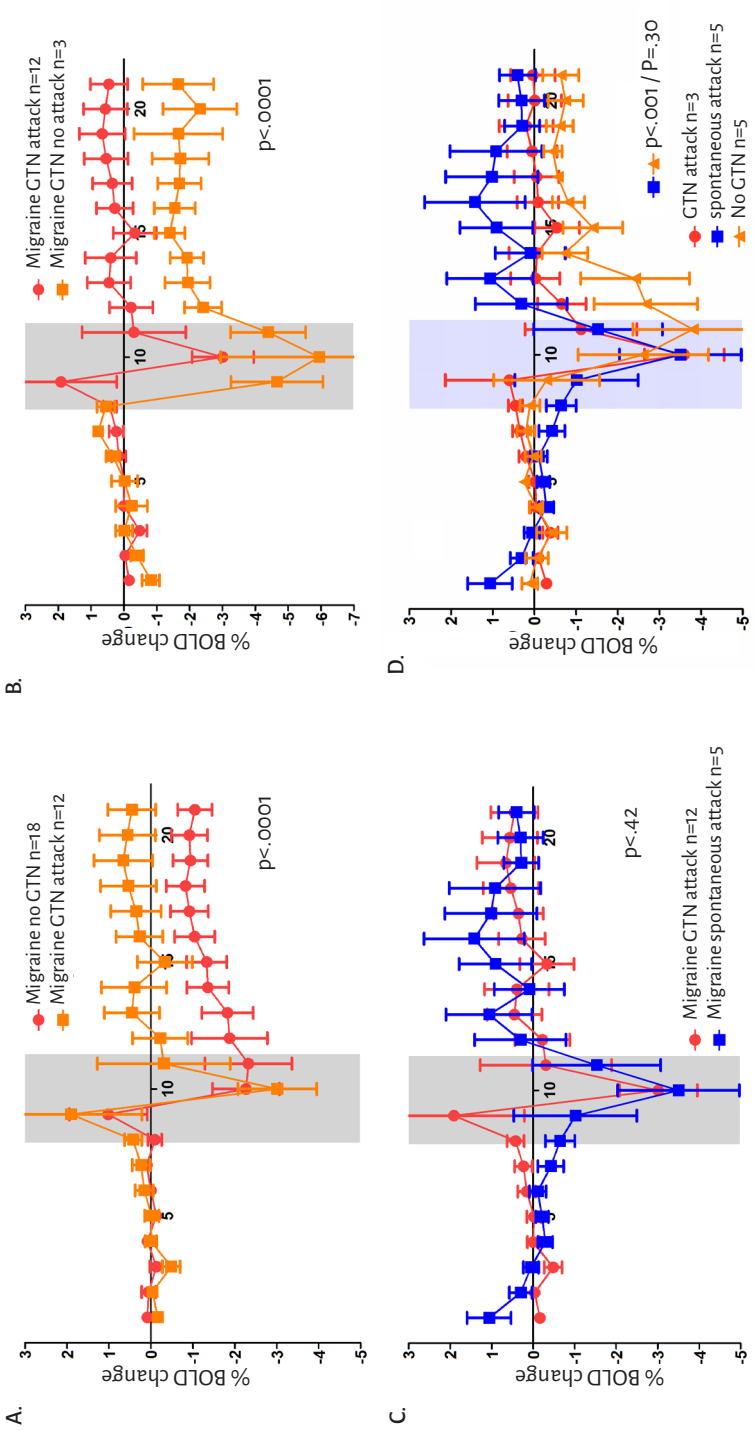

Fig 4. Comparison of BOLD responses to glucose ingestion in migraine participants in provoked and spontaneous attacks

In the pre-ictal phase of a GTN-induced migraine attack, the BOLD response to glucose is higher compared to the interictal response at baseline (paired analysis; 4A). The response pattern in spontaneous attack is similar to the pattern in GTN-induced attacks (4B). After GTN-infusion, this abnormal response >

(Continuation of fig 4 legend) pattern was only seen in the subgroup of migraine patients who developed a migraine-like attack, i.e. who were in the premonitory early phase of the attack. In the five migraine participants scanned during a spontaneous attack, the response pattern in the spontaneous attack was similar to that in a GTN-provoked attack (Fig 4C). Visually, the BOLD responses in both spontaneous attacks and GTN-provoked attacks differ from the response at baseline without GTN. The response pattern in spontaneous attacks reached statistical significance on between-group level ($p < 0.0001$) but not on intra-individual level due to small sample sizes ($p = 0.30$) (Fig. 4D). Grey box indicates drinking period, of which data were omitted from the analysis since they are considered drinking artefacts. Error bars indicate 1 standard error.

Discussion

To assess the role of the hypothalamus in the initiation of migraine attacks, we intra-individually compared hypothalamic neuronal activity in the premonitory symptom phase of 13 GTN-induced and 5 spontaneous migraine attacks with the activity outside an attack. Activity was also compared inter-individually with measurements with and without prior GTN infusion in 3 migraine participants and 11 controls who did not develop premonitory symptoms or an attack after GTN infusion. Hypothalamic neuronal activity was measured as the hypothalamic fMRI BOLD response to glucose ingestion, reflecting hypothalamic glucose-sensitive neuronal activity. Without prior GTN infusion, the hypothalamic BOLD response was similar in migraine participants and controls. However, while the response did not change after GTN infusion in controls, in the 13 migraine participants who developed a migraine attack, but not in the 3 who did not so, hypothalamic activity was different, with a faster and more abrupt recovery phase. Importantly, all hypothalamic responses and changes from baseline were similar in GTN-induced and spontaneous attacks, validating the findings in GTN-induced attacks as representative for what is occurring in spontaneous attacks.

In the early phases of provoked and spontaneous migraine attacks, migraine patients did not respond to glucose ingestion with a normal, persisting drop in the BOLD signal. Such a drop is considered to reflect reduced neuronal metabolic activity in the lateral hypothalamic area where glucosensitive neurons are located. This normal response is known across species[28] and is seen as a signal of glucose satisfaction, i.e. a normal 'satisfied' state after glucose ingestion[28,30]. The migraine patients rather showed an unresponsiveness to the glucose trigger during the early attack phase, implying a migraine-attack related disturbance of normal hypothalamic functioning; a disinhibited hypothalamic satisfaction. This abnormal response seems attack-specific, as it was not found in the control group, nor in the migraine group when there was no forthcoming attack. Although it is tempting to link this to the common premonitory symptom of craving, it would be oversimplifying to do so as the hypothalamic control of different homeostatic mechanisms is rather complex.

GTN influences cardiovascular parameters[43] and the changes we observed could theoretically have been due to GTN rather than related to the initiation cascade of an attack. However, one would then have expected similar changes to occur in the control

group. Moreover, we measured 90 minutes after GTN-infusion which significantly exceeds GTN $\tau_{1/2}$ (2.5-4 minutes).
Only two studies have previously investigated the role of the hypothalamus in the initiation phase of migraine attacks. The study by Maniyar et al showed activations in the posterolateral hypothalamus in 8 migraine with aura patients with premonitory symptoms after GTN infusion, using $H_2{}^{15}O$ PET cerebral blood flow as a marker for neuronal activity [27]. Although this suggests that the hypothalamus is pivotal in the early, premonitory phase of the migraine attack, a possible GTN effect cannot be excluded as there was no contemporaneous control group. Schulte et al [25] daily assessed the hypothalamic fMRI blood flow response to a trigeminal nociceptive stimulus (nasal administration of gaseous ammonia) for 30 consecutive days in a single migraine patient. They prospectively captured three migraine attacks and found an increased hypothalamic response in the 24 hours prior to onset of the migraine headache[25]. Although they did not include a control group to correct for possible diurnal, weekly or menstrual effects, the findings nonetheless suggest an important role for the hypothalamus in the early phases of the migraine attack. A third study found hypothalamic activation in migraine headache, but did not perform measurements during the premonitory phase. Collectively, these and our data suggest a pivotal role of the hypothalamus in the early phases of migraine attack initiation.

There is a growing interest in the hypothalamus as the site of initiation of a migraine attack initiator based on clinical and biochemical arguments [44-46]. In this study we have shown an increased hypothalamic BOLD response patterns to glucose after fasting in migraine patients. The ingestion of oral glucose induces a normal, transient silencing of the activity of glucosensitive neurons in the lateral hypothalamus. However, in the preictal phase of both spontaneous and GTN-triggered attacks this was followed by a much faster and steeper response than normally. The suppression of this hypothalamic state of hyperactivity was apparently temporarily and shorter than in non-migraine individuals and was suggestive of an impending migraine attack. We might draw an analogy between this 'overdrive' of 'craving' state of these neurons and the clinical symptom of craving experienced the hours before the migraine headache starts.

Our study has several strengths. The paired design in patients and controls enabled to disentangle the effects of GTN and the attack. Using a validated model to provoke premonitory symptoms and migraine-like attacks enabled us to capture the preclinical initiation phase of attacks [47] which is hardly possible for spontaneous attacks due to their erratic nature. Finally, we did manage to capture the presymptomatic phase of migraine attacks in 5 patients which allowed for a clinical validation of the findings in GTN provoked attacks. We included only female migraine without aura patients, limiting the overall generalizability. The result, however, was a homogeneous study group. Technically, the small region of interest made the data acquisition susceptible to e.g. drinking movement artefacts, leading to exclusion of three subjects from the analyses. We were not able to distinguish between the different hypothalamic nuclei. Possibly, different nuclei could be involved in different migraine attack phases, being hyperactive in one phase and hypoactive in the other. More sophisticated imaging techniques could perhaps be used in the future to disentangle these possible differential effects.

To conclude, this is the first study showing a disturbed function or reactivity of the hypothalamus during the earliest phases of both GTN-provoked and spontaneous migraine attacks. This emphasizes the role of the hypothalamus in the early phase of migraine attacks.

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Part III.

Biochemical aspects

Chapter 8.

Female sex hormones in men with migraine

WPJ van Oosterhout[1,2]
GG Schoonman[1,3]
EW van Zwet[4]
OM Dekkers[4]
GM Terwindt[1]
A MaassenVanDenBrink[5]
MD Ferrari[1]

[1]Department of Neurology, Leiden University Medical Center, Leiden, the Netherlands
[2]Department of Neurology, OLVG Hospital, Amsterdam, the Netherlands
[3]Department of Neurology, Elisabeth-Tweesteden Ziekenhuis, Tilburg, the Netherlands
[4]Department of Internal Medicine, Leiden University Medical Center, Leiden, the Netherlands
[5]Department of Internal Medicine, Division of Vascular Medicine and Pharmacology, Erasmus Medical Centre, Rotterdam, the Netherlands.

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Abstract

Objective
To assess the role of oestradiol and testosterone in men with migraine.

Methods
We measured 17ß-oestradiol (E2) and calculated free testosterone (T_f) in serum of 17 medication-free men with migraine and 22 men without migraine group-matched for age and body mass index, targeted at 20-28 kg/m^2. Blood was sampled on a single, for migraineurs interictal, day at 9am, 12am, 3pm and 6pm. Migraineurs were subsequently measured 3-4 times daily until an attack occurred. Clinical androgen deficiency was assessed using the Androgen Deficiency of Ageing Men questionnaire and the Aging Males' Symptoms scale. We analysed interictal data (mean ± standard error) with repeated measurement ANCOVA and longitudinal data by Generalized Estimated Equations models.

Results
Compared to controls, men with migraine had a lower interictal T_f/E2 ratio (3.9 ± 0.4 vs. 5.0 ± 0.3; p=.03) due to higher E2 (96.8 ± 6.1 pmol/L vs. 69.1 ± 5.6 pmol/L; p=.001) and similar T_f (357.5 ± 21.4 pmol vs. 332.6 ± 18.7 pmol/L; p=.35) levels. Pre-ictal T_f levels were increased in men with migraine reporting premonitory symptoms (p=.03). Men with migraine more frequently reported symptoms of androgen deficiency (11/18 [61.1%] vs. 6/22 [27.3%]; p=.031) which were also more frequently severe (p=.006); their age- and BMI-adjusted AMS scores were higher (27.0 ± 1.2 vs. 21.0 ± 1.0; p=.002).

Conclusions
In this study, non-obese men with migraine exhibited increased levels of the sex hormone oestradiol and show clinical evidence of relative androgen deficiency. The role of oestradiol in modulating migraine susceptibility and activity in men deserves further investigations.

Introduction

Migraine is a common, disabling, episodic brain disorder, typically characterised by recurrent attacks of severe headache, associated features and, in one third of patients, aura [1,2]. In up to two-thirds of migraineurs, attacks may be preceded by affective and physical premonitory symptoms [3,4]. Migraine prevalence and the frequency, duration and severity of migraine attacks are highly dependent on age, sex and, in women, events which are associated with marked fluctuations in female reproductive hormones [5-7]. In the fertile period, three times more women (24%) than men (8%) have active migraine and their attacks are on average more frequent, longer and more severe [5,7]. Furthermore, fluctuations in female sex hormones during puberty, menstruation, pregnancy, breast-feeding, menopause and post-menopause are associated with changes in attack frequency [5-7]. Additional evidence that sex hormones might modulate migraine risk and activity is coming from other observations [6,7]: migraine prevalence is higher in obese individuals with elevated oestrogen levels [8] possibly due to increased conversion from testosterone in adipocytes [9]; starting or stopping using oral contraceptives can be associated with either onset or disappearance of migraine attacks [10]; many male-to-female transsexuals develop migraine after starting oestrogen and anti-androgen therapy [11]; testosterone administration was associated with reduction in migraine frequency and severity in some women [12]; certain polymorphisms in sex hormone receptor genes were associated with increased risk of migraine [13]; and menstrual cycle-related changes in oestrogen levels influence the activity of the trigeminovascular system which is responsible for causing migraine headache [14,15]. Finally, sex-related differences and experimental manipulation of sex hormone levels modulated neurogenic vasodilatation and cortical spreading depression in animal migraine models [15,16], surrogate markers for migraine susceptibility. It is unknown whether sex hormones might modulate migraine risk and activity in men. Here we assessed interictal, pre-ictal and ictal levels of 17ß-oestradiol, free testosterone and the free testosterone to 17ß-oestradiol ratio in men with migraine, hypothesising that levels of 17ß-oestradiol and the ratio would be higher at baseline and would increase further towards the attack.

Material and Methods

Participants

We selected males between age 18 and 74 years, N = 18 with episodic migraine without aura according to the criteria of the International Classification of Headache Disorders (ICHD-IIIb)[17] and N = 24 controls without migraine themselves or in first degree relatives group-matched for age and body mass index (BMI). When we use the word controls, we refer to healthy controls without a history of recurrent headaches. Controls could in addition not have any other type of headache on ≥ 2 days per month. Migraineurs were excluded if: (i) they were unable to differentiate migraine from other headaches; (ii) had headache or were using acute headache medication on ≥10 days per month; and (iii) were daily using migraine prophylactic medication. Exclusion criteria for both migraineurs and controls were: (i) BMI <20 or >28; (ii) smoking during participation; (iii) frequent consumption of spicy foods; (iv) hypertension (defined as blood pressure >150/90mmHg or use of antihypertensive

medication; (v) intake of high-fat foods immediately before a measurement; (vi) history of hypogonadism; (vii) using any medication or supplements which could affect hormone levels; (viii) any liver or kidney condition; and (ix) coagulopathies such as haemophilia or a compromised immune system.

Standard Protocol Approvals, Registrations, and Patient Consents

The study was conducted as part of the Leiden University Medical Center Neuro Analysis Programme (see below) [18]. LUMINA and the present study were approved by the local medical ethics committee and all participants provided written informed consent prior to participation.

LUMINA programme

Both migraine patients and controls were recruited via nationwide public announcement, advertising in lay press and via the research website, and were considered eligible after a two-step inclusion process using validated questionnaires via the especially designed LUMINA website. Additionally, patients from our outpatient headache clinic were invited to participate by a letter. On the website, patients were asked to fill out a screening questionnaire that has been validated previously1. Firstly, if patients fulfilled the screening criteria, they were sent a web-based extended migraine questionnaire, based on the ICHD-II criteria(14, 15). This questionnaire was validated before by performing a semi-structured telephone interview in 1,038 patients who had filled out the extended migraine questionnaire[14]. The specificity of the questionnaire was 0.95. We consider the cohort a well-defined web-based cohort, with 4% of subjects included from our dedicated headache outpatient clinic, 87% of the participants having been diagnosed as migraineurs previously by a medical doctor. In addition to questions that were necessary to diagnose migraine accurately, the extended questionnaire also included items on demographic factors, acute and prophylactic headache medication use, migraine attack frequency and allodynia. Participants without the needed internet skills were able to fill out the questionnaires on paper. Non-headache individuals willing to participate had to pass a screening questionnaire online via the research website. If this screening questionnaire did not show any indication for having migraine, cluster headache, chronic tension type headache or medication overuse headache, individuals were sent a subsequent in depth questionnaire. This second questionnaire again assessed possible headache complaints, together with demographic variables. Only individuals that fulfilled both the criteria of 'non-headache' in the screening and in depth questionnaire were considered eligible controls and were approached for this questionnaire study.

Study Design

Blood samples were collected on a single (for migraineurs interictal) day at 9am, 12am, 3pm and 6pm. Participants with migraine were in addition measured three to four times daily on the following days until an attack occurred. The interictal (baseline) day was planned at least three days after the last migraine attack and within ten days from the next expected attack (based on the participant's historical attack frequency).

Hormone Level Assessment and Sample Storage

Levels of 17ß-oestradiol (E2; pmol/L), sex hormone-binding globulin (SHBG; nmol/L)

and albumin (g/L) were assessed using ECLIA immunoassays (Roche Diagnostics GmbH, Mannheim, Germany). Inter-assay coefficients of variability mean ± sd) were 5.3 ± 1.7 (SHB), 1.5 ± 0.5 (albumin), 2.1 ± 1.4 (oestradiol) and 5.1 ± 1.4 (total testosterone) respectively. The free testosterone fraction (T_f; pmol/L) was calculated using SHBG and albumin levels [19]. Previous studies have revealed that female sex hormone levels in men show only little inter- and intra-individual variability[20,21]. Serum total testosterone (T_t; nmol/L) was assessed by coat-a-count® radioimmunoassay (Siemens, Camberley, UK). Main outcome measure was the T_f/ E2 ratio. Prior to analysis, samples had been stored for a mean of 85.3 ± 38.5 days.

Migraine and Premonitory Symptoms

Using a standardised interview, we assessed at each measurement the presence and characteristics of headache and premonitory symptoms, including less-frequent micturition, ankle or wrist oedemas, changes in defecation, thirst, changes in appetite, craving for specific food, stiffness of limbs and/or face, stiff neck, difficulty with concentrating, mental agitation, physical agitation, fatigue, excessive yawning, hyperirritability, and mood changes such as depression [3]. The premonitory phase was defined as presence of ≥ 1 of the above symptoms which was then followed by migraine headache within 24 hours. Measurements were labelled afterwards as "baseline" (which in participants with migraine refers to interictal), "pre-ictal with or without premonitory symptoms", or "headache".

Items Relevant to the Reproductive System

We collected the following items that are relevant to the reproductive system and secondary sex characteristics: frequency of shaving of facial hair, age of dropping of voice in puberty, number of children, unwanted childlessness, delay in parenthood despite of attempts, help in fertilisation (in vitro fertilisation; sperm donation; surrogacy), and cryptorchidism.

Questionnaires on Androgen Deficiency

To assess clinical androgen deficiency, we used two validated questionnaires. The Androgen Deficiency of Ageing Men (ADAM) questionnaire contains 10 items regarding the most common symptoms observed with age-related decline in androgens (corresponding to bioavailable testosterone levels <70ng/dL) [22]. All questions are answered with *yes* or *no*. If question 1, 7, or any 3 other questions are answered positively, the results indicate an androgen-deficient state with a sensitivity of 0.88 and specificity of 0.60 [22]. The Aging Males' Symptoms (AMS) scale contains 17 items on ageing and clinical testosterone deficiency with per-item scores ranging from 1-5 (none to severe) [23]. Sum-scores range from to 17-85 with higher scores indicating lower health related quality of life and suggesting lower free testosterone levels. Scores can be categorized into no/few symptoms (17-26 points), mild (27-36 points), moderate (37-49 points), and severe symptoms (>50 points). Test-retest reliability is 0.8-0.9 for the total score [23]. Both questionnaires were filled out once prior to the first measurement.

Sample Size Calculations

The sample size was based on log [T_f/E2], since the ratio between these two hormones is a commonly used parameter in clinical studies. We based our sample size on intra-individual comparison, assuming a change of 25% to be clinically relevant [24]. We furthermore assumed

a 10% day-to-day variation in testosterone and 15% day-to-day variation in oestrogens [25]. With α set at 0.05 and β at 0.10, an intra-individual paired sample-size calculation resulted in 15 participants.

Statistics

General characteristics were compared between participants with migraine and controls using Student's t-tests for continuous variables and Chi square tests for categorical data. Baseline hormonal levels (both using the 9 am measurement and the average over the four baseline measurements of 9am, 12am, 3pm, 6pm) were compared between participants with migraine and controls using ANCOVA allowing for adjustment for age and BMI. Hormonal changes prior to an attack were assessed using a generalised estimated equations (GEE) model run with timing (baseline/ pre-ictal [<24 hours from beginning of attack] / headache) as dependent variable without additional correcting for age and sex [26]. Separate analyses were done for pre-ictal measurements with and without premonitory symptoms. Measurements up to 72 hours prior to the migraine headache were used in the analyses and measurements performed after acute migraine medication was taken were excluded. For research and financial reasons, we chose to only analyse migraine data from the baseline day, a prodromal day, and the headache phase. All hypothesis tests were 2-sided. Data analyses were performed using SPSS 17.0 (SPSS inc., IBM, USA). The statistical threshold was set at $p<.05$.

Data availability

All data, methods and materials used to conduct this research are mentioned in this article.

Results

Study Population and Measurements

We included 42 males, 18 with migraine without aura and 24 controls. Demographic characteristics were similar (Table 1). Data from two controls had to be excluded, one because he later turned out to have ever suffered from a migraine aura without headache and the other because of very low levels of LH suggestive of recreational use of androgen. Of one participant with migraine, no baseline interictal measurements were performed. Thus, interictal/baseline data were available for 17 participants with migraine and 22 controls. Follow-up data were available for 14 participants with migraine over a period of 2-11 days with 8-41 measurements per participant. In total 149 serum samples were analysed in 18 participants with migraine: interictal n=95; pre-ictal (<24h before onset headache) n=43 of which n=36 with premonitory symptoms; and during the headache phase n=18.

Female Sex Hormone Levels in Migraineurs

We assessed interictal/baseline levels in two ways: as a single measurement at 9 am and as mean of all 4 measurements on the interictal/baseline day. Interictal/baseline 17ß-oestradiol levels (9 am) were higher in participants with migraine (96.8 ± 6.1) compared to non-migraine controls (69.1 ± 5.6 pmol/L; p=.001) while free testosterone levels were similar (357.5 ± 21.4 vs. 332.6 ± 18.7 pmol/L; p=0.35; Figure 1). As a result the adjusted T_f/E2 ratio was lower in participants with migraine (3.9 ± 0.4) compared to controls (5.0 ± 0.3;

p=.03; Figure 1). Similar results were obtained for the means of the 4 measurements on the interictal/baseline day for 17ß-oestradiol (migraine: 92.5 ± 5.3 vs. controls: 67.6 ± 4.8 pmol/L; *p*=.002), free testosterone (migraine: 312.6 ± 17.2 vs. controls: 318.0 ± 15.5 pmol/L; *p*=.82), and adjusted T_f/E2 ratio (migraine: 3.6 ± 0.3 vs. controls: 4.9 ± 0.3; *p*=.007).

Table 1. Baseline Characteristics of the Study Population.

Variable	Men with Migraine (N=18)		Male Controls without Headache (N=22)	
Demographics				
Age, mean (SD), year	46.9	(16.4)	48.5	(17.2)
BMI, mean (SD), kg/m²	24.7	(4.1)	23.4	(1.8)
Clinical testosterone items				
Shaving frequency/week, mean (SD)	5.2	(2.1)	6.3	(2.4)
Age of dropping voice, mean (SD), year	13.5	(1.5)	13.8	(1.4)
Cryptorchidism, n (%)	1	(6)	1	(5)
Reproductive items				
Number of children, mean (SD)	0.9	(1.1)	1.3	(1.2)
Longer than expected time-to-conception, n (%)[a]	2	(20)	1	(7)
Unwanted childlessness, n (%)[b]	3	(30)	3	(19)
Aid in fertilisation, n (%)[b]	1	(10)	0	(0)
Migraine characteristics				
Migraine subtype MO, n (%)	11	(61)	-	
Attack frequency/month, mean (SD)	2.8	(1.6)	-	
Acute medication, n (%)	18	(100)	-	
Prophylactic medication, n (%)	0	(0)	-	

M = male; MO = migraine without aura; n.a. = not applicable. The section 'Reproductive items' only applies to those participants with a child wish, and therefore the total number is smaller than the entire group.

[a] In total n=24 participants had at least one child: n=14 controls and n=10 migraineurs

[b] In total n=26 participants (n=16 controls and n=10 migraineurs) had childwish

Pre-Ictal and Ictal Changes in Sex Hormone Levels

To analyse whether hormone levels change during or shortly before attacks compared to interictal/baseline we intra-individually compared the interictal/baseline, pre-ictal and ictal measurements in two ways. First, for all participants with migraine, irrespective of whether or not they had experienced premonitory symptoms in the pre-ictal phase, and second only for those who reported premonitory symptoms during the pre-ictal phase (Table 2; Figure 2). While there were no differences when analyzing all participants (Tf: *p*=.19; 17ß-oestradiol: *p*=.09; Tf/E2 ratio: *p*=.10), when only analyzing migraine participants with premonitory symptoms, Tf levels (*p*=.03) were different, but not the 17ß-oestradiol levels (*p*=.15) or Tf/E2 ratio (*p*=.08). Rerunning the prospective analyses using a linear mixed model approach yielded similar results. Post-hoc analysis revealed that pre-ictal T_f (358.6 ± 21.1 vs. 293.6 ±

23.0 nmol/L; *p*=.03) and 17ß-oestradiol (96.3 ± 7.7 vs. 73.2 ± 6.7; *p*<.001) levels were higher in migraine participants with premonitory symptoms compared to those without. Rerunning the analyses including only patients >50 years of age yielded similar results.

Clinical Evidence of Androgen Deficiency

The age and BMI adjusted mean ± SE AMS scores were higher in men with migraine (27.0 ± 1.2 vs. 21.0 ± 1.0; *p*=.002; pearson correlation with T_f levels: r=-0.05; *p*=.74), suggesting relative functional deficiency of T_f. Compared to controls, men with migraine did not report symptoms of androgen deficiency more frequently (ADAM questionnaire): 11/18 [61.1%] vs. 6/22 [27.3%]; *p*=.053, but they were more often mild/severe (AMS scale): 10/18 (55.6%) vs. 2/22 9.1%; *p*=.006 (Table 3).

Table 2. Hormone Levels during Different Phases of the Migraine Attack.

Hormone		Baseline		Pre-Ictal		Headache		*P* overall	*P* post-hoc pre-ictal
T_{free}, mean (SE), pmol/L	All	313.5	(18.5)	336.1	(17.0)	355.7	(32.3)	.19	
	PS+	313.2	(17.8)	354.1	(21.4)	354.8	(31.4)	.03	.03
	PS-	312.5	(18.1)	306.4	(24.8)	352.6	(32.7)	.41	
E2, mean (SE), pmol/L	All	94.2	(6.1)	90.3	(6.3)	98.6	(8.1)	.09	
	PS+	90.5	(6.1)	93.4	(7.1)	101.5	(8.4)	.15	<.001
	PS-	93.3	(5.7)	87.8	(6.5)	97.2	(7.3)	.08	
T_f/E ratio, mean (SE)	All	3.3	(0.2)	4.1	(0.4)	4.1	(0.5)	.10	
	PS+	3.3	(0.2)	4.2	(0.4)	4.1	(0.4)	.08	.98
	PS-	3.3	(0.2)	3.9	(0.4)	4.0	(0.4)	.25	
T_{total}, mean (SE) nmol/L	All	19.4	(1.3)	20.5	(0.9)	21.0	(1.5)	.59	
	PS+	19.4	(1.2)	21.8	(1.2)	21.2	(1.5)	.04	.005
	PS-	19.4	(1.3)	19.3	(1.0)	20.9	(1.6)	.45	

Variables are depicted as mean ± standard error (SE), derived from generalized estimated equation models. *P* values are not adjusted for multiple comparisons per endpoint / multiple endpoints.

Pre-ictal = 24 hours prior to begin of migraine headache.

PS+ = pre-ictal measurements with presence of premonitory symptoms.

PS- = pre-ictal measurements without presence of premonitory symptoms

T_{free} = Testosterone$_{free}$ (pmol/L)

T_{total} = Total testosterone, free and bound (nmol/L)

E2 = 17β-oestradiol (pmol/L)

P overall = comparison between baseline, pre-ictal, and headache phases

P pre-ictal = comparison between pre-ictal measurements with premonitory symptoms present vs pre-ictal measurements without premonitory symptoms

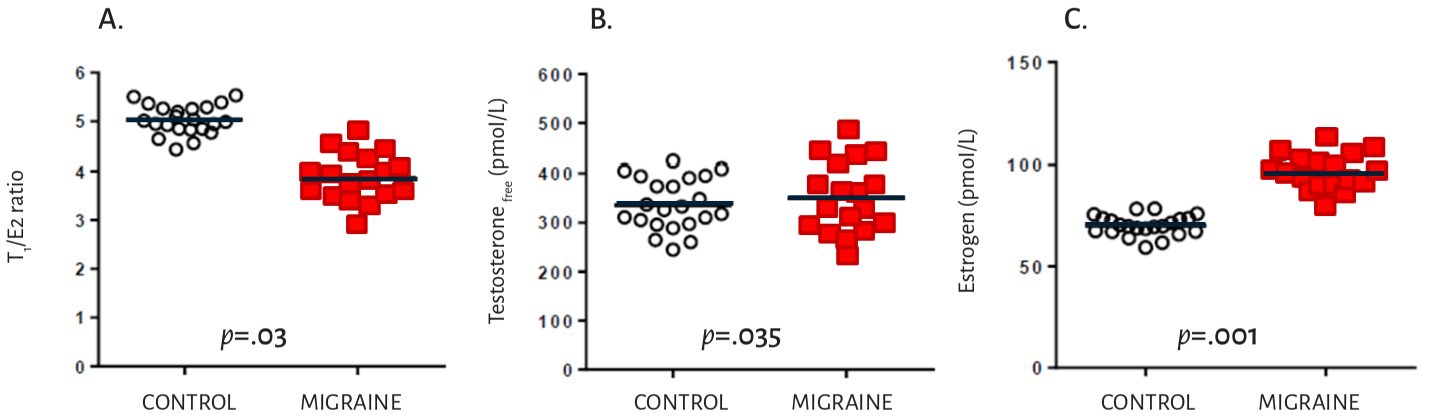

Figure 1. Tf/E2 Ratio and Levels of Free Testosterone and 17ß-Oestradiol in Men with Migraine and Non-Headache Controls at Baseline.
Depicted levels are adjusted for age and BMI. Tf:E2 ratio = free testosterone (pmol/L) / 17β-oestradiol (pmol/L) ratio

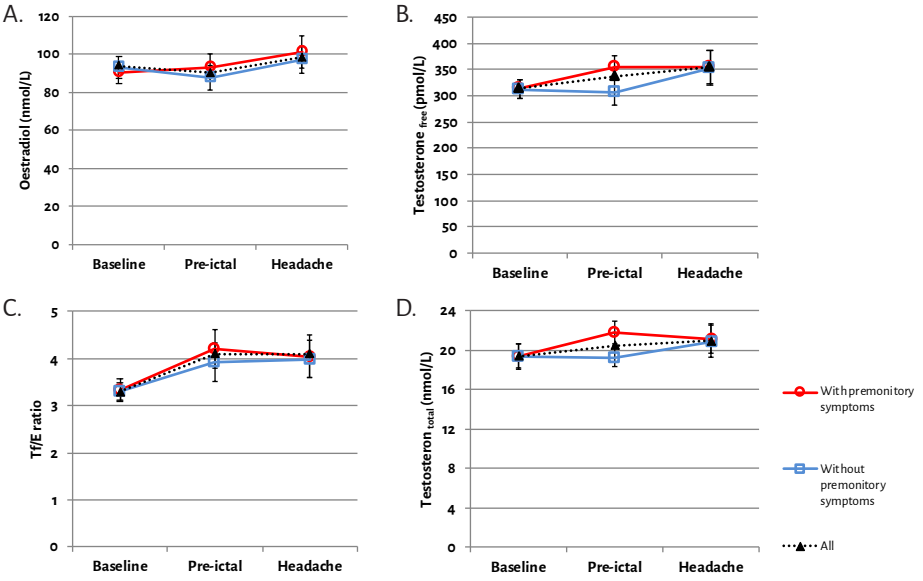

Figure 2. Hormone Levels during Different Phases of the Migraine Attack.
Depicted levels are means ± standard error of the mean. Tf:E2 ratio = free testosterone (pmol/L) / 17β-oestradiol (pmol/L) ratio

Table 3. Scores on Aging Males' Scale Score and on the Androgen Deficiency of Ageing Men questionnaire in male migraineurs and controls.

Variable	Men with Migraine (N=18)		Male Controls without Headache (N=22)		*p*
AMS score[a]					
Mean (SE)	27.0	(1.2)	21.0	(1.0)	0.001
AMS category[b]					0.006
No / few, n (%)	8	(44.4%)	20	(90.9%)	
Mild, n (%)	9	(50.0%)	2	(9.1%)	
Moderate, n (%)	0	(0%)	0	(0%)	
Severe, n (%)	1	(5.6%)	0	(0%)	
ADAM category[c]					0.053
Androgen deficiency	6	(23%)	7	(38.9%)	
No androgen deficiency	16	(72.7%)	11	(61.1%)	

[a] Adjusted for BMI and age
[b] no /few versus (mild / moderate / severe)
[c] Fisher's exact test
AMS = Aging Males's Scale
ADAM = Androgen Deficiency of Ageing Men questionnaire

Discussion

We prospectively assessed sex hormone levels in 17 well characterised non-obese men with migraine (with moderate attack frequency) and 24 controls without headache who were matched for age- and BMI on group level. Use of medications that could potentially affect hormone levels was carefully excluded. Men with migraine had increased oestrogen plasma levels, both absolute and relative to free testosterone, and reported higher scores on the ADAM and AMS scales reflecting functional androgen deficiency. Those who reported premonitory symptoms showed pre-ictal increase in testosterone and possibly also oestradiol.

While there is ample, though still circumstantial, clinical evidence that female sex hormones might modulate migraine susceptibility in women (see introduction and [5-7], little is known whether sex hormones have similar effects in males. We are aware of one study in which testosterone was measured in eight men with migraine during and outside attacks; in accordance with our study, no differences were found [27]. The pre-ictal rise in testosterone in our study might be related to a general stress-response anticipating the impending attack [28].

Unfortunately, our sample size is too small and the migraine characteristics are too homogeneous for additional analyses by headache frequency, intensity or duration. We can also not fully exclude that, possible due to self-selection, our results may only apply to patients with e.g. severe migraine. However, as the clinical characteristics of our study population seem rather typical for the average male migraineur (see Table 1), we believe such an alternative interpretation is unlikely.

There are several possible mechanisms through which changes in reproductive hormone levels might modulate migraine susceptibility. Women with menstrual-related migraine attacks seem to have a more rapid late-luteal phase drop in 17ß-oestradiol compared to women without menstrual-related migraines [7,29]. This might cause imbalance between long-lasting genomic effects of nuclear oestradiol receptors and short-lasting non-genomic effects via intra-membranous G-protein-coupled oestradiol receptors [30]. This imbalance might activate a cascade leading to neuronal sensitisation and ultimately triggering of migraine attacks [30]. Unfortunately, although in clinical trials with oestradiol-releasing skin creams [31-33] or oestrogen supplements [34], all aimed at preventing or delaying abrupt oestrogen-withdrawal, promising results were obtained, in clinical practice these measures are considered of only limited value[35].

In animal models, female and male sex hormones differentially affect two important basic mechanisms likely involved in migraine pathogenesis: susceptibility to cortical spreading depolarization (CSD), a putative surrogate marker of migraine susceptibility [15], and activation of nociceptive transmission within the trigeminovascular system [15,36]. The threshold for inducing CSD was lower and the velocity and frequency of CSD were higher in female compared to male transgenic mice carrying a human pathogenic *CACNA1A* familial hemiplegic migraine type 1 (FHM1) mutation[37]. Moreover, ovariectomy revoked

and orchiectomy enhanced these sex-related differences [37]. Female gonadal hormones enhanced CSD susceptibility, possibly by increasing cortical hyperexcitability, whereas male hormones had the opposite effect. These differential hormonal effects were only observed in mice carrying an FHM1 mutation and not in wild-type mice, supporting the hypothesis that female hormonal fluctuations affect migraine activity in genetically predisposed individuals [15,38]. In addition to their effects on CSD, sex hormones might also regulate sensitization of trigeminal neurons by modulating expression of nociceptive mediators such as calcitonin gene-related peptide (CGRP) and by affecting serotonin synthesis, dural mast cell density, and intracellular downstream signalling. Oestrogens seem to have a positive and androgens a suppressing effect on nociceptive transmission, with higher oestrogen levels reflecting higher activation states of these mechanisms [39]. Overall, the differential effects on CSD susceptibility and trigeminovascular activity might well explain, at least partly, why migraine is so much more prevalent among women, and why periods of major sex-hormonal fluctuations are so often associated with marked changes in migraine activity[6,7].

Taken together, in our current study non-obese men with migraine exhibited increased levels of the sex hormone oestradiol and show clinical evidence of relative androgen deficiency. Further studies in larger and additional populations are needed to validate these findings. What exactly the role is of oestradiol in men with migraine and whether fluctuations in oestradiol levels, like in women, might be associated with changes in migraine activity, deserves further intra-individual follow-up studies over multiple attack cycles.

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Chapter 9.

Reduced trigeminovascular cyclicity in patients with menstrually related migraine

K Ibrahimi[1]
WPJ van Oosterhout[2]
W van Dorp[3]
AHJ Danser[1]
IM Garrelds[1]
SA Kushner[4]
EMEH Lesaffre[5]
GM Terwindt[2]
MD Ferrari[2]
AH van den Meiracker[1]
A Maassenvandenbrink[1]

[1] Division of Pharmacology and Vascular Medicine, Department of Internal Medicine, Erasmus MC, Rotterdam, the Netherlands
[2]Department of Neurology, Leiden University Medical Center, Leiden, the Netherlands
[3]Department of Gynaecology, Erasmus MC, Rotterdam, the Netherlands
[4]Department of Psychiatry, Erasmus MC, Rotterdam, the Netherlands
[5]Department of Biostatistics, Erasmus MC, Rotterdam, the Netherlands

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Abstract

Objective
A case-control study to investigate the effect of the menstrual cycle on trigeminal nerve-induced vasodilation in healthy women and patients with menstrually-related migraine (MRM).

Methods
Using a laser-Doppler imager, we compared the vasodilator effects of capsaicin application and electrical stimulation (ES) on the forehead skin, a trigeminal nerve-innervated dermatome, in premenopausal MRM patients (n=22), healthy controls (n=20), and postmenopausal women without migraine (n=22). Blood samples were collected for female sex hormones measurements.

Results
Dermal blood flow (DBF) responses to capsaicin were higher in controls during days 1-2 than during days 19-21 of their menstruation cycle (Mean E_{max} ±SEM: 203±28 a.u. vs 156±27 a.u. (p=0.031) for 0.06 mg/ml capsaicin and 497±25 a.u. vs 456±24 a.u. (p=0.009) for 6.0 mg/ml capsaicin). In contrast, MRM patients demonstrated DBF responses without significant cycle-dependent variability (day 1-2 vs day 19-21: E_{max} 148±20 a.u. vs 154±20 a.u. (p=0.788) for 0.06 mg/ml capsaicin and 470±17 a.u. vs 465±20 a.u. (p=0.679) for 6.0 mg/ml capsaicin). DBF response to ES were not different between either MRM patients or controls, at either occasion. Estradiol levels on day 19-21 of the menstrual cycle were higher in healthy controls (Mean±SEM: 75±8 pg/ml) than in MRM patients (52±4 pg/ml, p=0.014). In postmenopausal women, DBF responses to capsaicin and ES, as well as estradiol levels at both visits, were all significantly reduced compared to MRM patients and controls (in all cases, $p<0.05$).

Conclusions
Our study provides evidence for a reduced menstrual cyclicity of both estradiol levels and the trigeminovascular vasodilator system in MRM patients.

Introduction

Migraine is twice as prevalent in women than in men [1]. Migraine incidence in women changes because of estrogen variations around menarche and menopause, but also during the menstrual cycle [2, 3]. Estrogen withdrawal increases migraine attack incidence migraine attacks;[4] a process that may be postponed by estradiol injections [5-7]. Migraine attacks associated with menstruation are generally perceived as more severe than attacks outside this period [8, 9].

The potent vasodilator calcitonin gene-related peptide (CGRP), a key mediator in migraine,[10] is released from primary afferents of the trigeminal ganglion, exerting its effects via the trigeminovascular system [11, 12]. Given the relationship between migraine attack incidence and hormonal fluctuations, an interaction between female sex hormones and CGRP seems likely [13, 14]. We have developed a human model to study trigeminal nerve-mediated vasodilatation, by applying capsaicin and electrical stimulation (ES) to the forehead skin, a trigeminal nerve-innervated dermatome [15]. Capsaicin activates the transient receptor potential vanilloid type 1 (TRPV1) channel, thereby enhancing CGRP release from trigeminal nerve terminals [15]. In contrast, ES appears to act directly on trigeminal nerve terminals, without the need for TRPV1 activation to evoke release of CGRP [15].

We investigated whether (i) varying levels of sex hormones during the menstrual cycle affect trigeminal nerve-mediated vasodilatation; and (ii) this pattern differs between menstrually-related migraine (MRM) patients and healthy controls. We hypothesized that (i) trigeminal nerve-mediated vasodilatory responses are increased preceding the menstruation, (ii) this increase is more pronounced in MRM patients than in controls, and (iii) trigeminal nerve-mediated vasodilatory responses are consistently low in postmenopausal women.

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

The Independent Ethics Committee of Erasmus MC, Rotterdam, The Netherlands, reviewed and approved the study protocol. All participants gave written informed consent after explanation of the study, which was conducted in accordance with local laws, the ethical principles of the Declaration of Helsinki, as well as the principles of Good Clinical Practice.

Design and procedures

For our case-control study design we compared MRM patients with age-matched healthy controls. We included postmenopausal women as a reference group (without menstrual cycle) for both the MRM patients and their age-matched healthy controls. MRM patients were recruited via a Dutch website inviting migraineurs to participate in research as part of the LUMINA project [16]. On the website, patients completed validated questionnaires based on the ICHD-III-b criteria to diagnose migraine and MRM [17]. MRM is classified as migraine without aura occurring on day 1±2 of the menstrual cycle with additional attacks of migraine at other times of the menstrual cycle [17]. Our MRM diagnosis was adapted from the ICHD-

III-b with a record of 3 menstrual cycles, of which 2 were prospective, and an interview. We recruited women without migraine via advertisement in local (Rotterdam, Netherlands) newspapers and flyers distributed in the Erasmus MC.

Data on medical history, medication, and information about the menstrual cycle or menopausal status were collected via an additional questionnaire. Information about headache frequency and severity of the MRM patients was acquired from the LUMINA database [16]. Only MRM patients not using migraine prophylactic treatment and consenting to refrain from the use of acute migraine therapy 48 hours prior to visits participated to prevent bias. MRM patients and healthy age-matched controls with a regular menstrual cycle, not using hormonal contraceptives, were eligible for inclusion.

Recruitment started March 2011 and continued until August 2012. Research was executed in the Internal Medicine Department of Erasmus MC. MRM patients did not have any medical condition besides migraine. Healthy age-matched controls and postmenopausal women were screened with a thorough interview checking for any (cardiovascular) disease or medication use. Non-smoking healthy women were included. For premenopausal women, one study visit was 19-21 days after the first day of menstruation and the second visit on day 1-2 of the subsequent menstruation (Figure 1). For postmenopausal women, two visits were scheduled 7-10 days apart. Weight, height and (supine) blood pressure were measured.

The first research visit was in July 2011 and the last one in September 2012. Migraine attack incidence was recorded from one month prior to the first research visit until two months later. Follow-up ended December 2012.

Forehead dermal blood flow (DBF) studies

Experiments were performed in a quiet, temperature-controlled room. Participants fasted for 3 hours before the measurements and both visits were during the same period of the day. After 15 min acclimatization, 3 electrodes containing a 0.5-ml reservoir were placed on the forehead (for details, see our model validation paper [15]). The electrodes were subsequently filled with three different types of solutions: normal saline, 0.06 mg/ml capsaicin, and 6.0 mg/ml capsaicin. A fourth electrode was placed in the neck region. An iontophoresis device (PeriIont 382b, Perimed, Sweden) was connected with the negative lead to the electrode containing saline and the positive lead to the electrode in the neck. DBF was measured with the PeriScan PIM-3 system (Perimed, Järfälla, Sweden).

DBF at the site of the electrodes on the forehead was continuously measured for 40 min. After 2 min baseline measurement, a current (0.2 mA) was applied for 1 min to the electrode containing saline. DBF was subsequently measured during 6 min. This process was repeated with increasing current intensities (0.4 mA, 0.6 mA, 0.8 mA), up to 1.0 mA.

Peripheral dermal blood flow response to ischemic stimulus

Post-occlusive reactive hyperemia (PORH) was measured at the volar site of the non-dominant forearm (area 1x1 cm). A blood pressure cuff was placed around the upper arm. After a 2-min baseline DBF measurement, the pressure in the cuff was quickly increased

to 200 mmHg, maintained for 5 min and subsequently released. PORH was continuously measured for 10 min.

CGRP measurements in saliva

Test subjects were given a cotton Salivette swab (Sarstedt AG & Co., Nümbrecht, Germany) to chew for 5 min. The swab was collected in the Salivette and stored at -80ºC. CGRP was determined by radioimmunoassay (Phoenix Pharmaceuticals, Inc, Burlingame, CA, USA), according to the manufacturer's instructions. Total protein content was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, Il, USA). Samples were measured in 2 blinded batches.

Estrogen and progesterone levels

Blood was collected via the cubital vein. Serum estradiol and progesterone levels were determined with the Coat-A-Count Estradiol and the Coat-A-Count Progesterone radioimmunoassay kits (Siemens Medical Solutions, Erlangen, Germany). Samples were measured in 2 blinded batches.

Statistical analysis

The number of cases and controls was based on the previously published validation study of our model[15]. Based on this study, a sample size of 20 is sufficient to be able to detect a 25% shift in the DBF response during the cycle and between the groups to 6 mg/ml capsaicin with an 80% power and 5% significance.

For each subject the maximal DBF (E_{max}) responses to capsaicin, and the ischemic stimulus were calculated. Similarly, for each subject the maximal DBF responses to each ES current intensity (0.2 mA-1.0 mA) was calculated. Individual data were analyzed in a blinded fashion. Group values are provided as mean values and standard error of the mean, except for the demographic data in Table 1, where standard deviations and ranges are presented as indicated. Differences between groups were analyzed by ANOVA and unpaired Student's t-test. Differences within groups were examined by Student's paired t-test. Repeated measurements ANOVA with multiple comparison tests were computed. Linear regression analysis was performed and Beta values with 95% confidence intervals are presented. The difference in DBF responses to capsaicin (Delta DBF) between groups and across the menstrual cycle were tested with the Mann-Whitney U test. A p-value <0.05 was considered to indicate statistical significance. No correction for multiple testing was applied.

Results

Subjects

Flow diagrams providing details on the recruitment are provided in Supplemental figure e-1. BMI, blood pressure, and heart rate were similar between groups (Table 1). Migraine attack incidence of the MRM patients was highest on day 1 of the menstrual cycle (Table 1).

Table 1. Demographics of the study population.

	Migraine patients	*Controls*	*Postmenopausal women*
Population, n	22	20	22
Age, years	37±7 (21-45)	33±9 (19-45)	60±5 (50-68)
BMI, kg/m²	22.9±3.9	24.0±1.6	23.8±2.3
BP, mmHg			
Systolic	105±17	109±9	118±9
Diastolic	66±7	64±6	71±6
HR , bpm	61±8	63±9	62±8
Age at migraine onset, years	18±7 (4-36)		
Disease duration, years	19±9 (6-41)		
Attack frequency, attacks per year			
1–2	1		
3–6	2		
7–12	5		
13–54	14		
Migraine incidence on day 1±2 of the menstrual cycle, No. of attacks			
Day 27	1		
Day 28	1		
Day 1	7		
Day 2	1		
Day 3	2		

BMI, body mass index. BP, blood pressure. HR, heart rate. Mean ± SD (Range)

Forehead DBF responses

In healthy controls, DBF responses to 0.06 mg/ml and 6.0 mg/ml capsaicin were significantly higher during day 1-2 of their menstrual cycle compared to day 19-21 (Figures 2A and 2B). In contrast, in MRM patients DBF responses to 0.06 mg/ml and 6.0 mg/ml capsaicin were similar throughout their menstrual cycle. The increase in DBF responses of healthy controls to 0.06 mg/ml capsaicin between visits (Delta: 47±20 a.u.) was significantly different from the slight decrease in DBF responses of the MRM patients (Delta: -6±24 a.u., p=0.040), whereas the slightly smaller increase in response to 6.0 mg/ml capsaicin (Delta: 41±14 a.u.) in healthy controls was not significantly different from that in MRM patients (Delta: 6 ±14 a.u., p =0.078). There was no association between DBF responses of MRM patients and the time to/since their migraine attacks. DBF responses in postmenopausal women were significantly lower than those in women with a menstrual cycle (controls and MRM patients) and were similar between visits. DBF responses to ES between visits did not differ for either of the groups (Figure 2C). Repeated measures ANOVA with a Greenhouse-Geisser correction revealed that mean DBF responses to ES differed significantly between stimulation intensities (0.2 mA-1 mA (F(2.017, 121.032): 414.878, p<0.0001). Repeated measures ANOVA revealed a group effect, with the lowest responses to ES in the postmenopausal women.

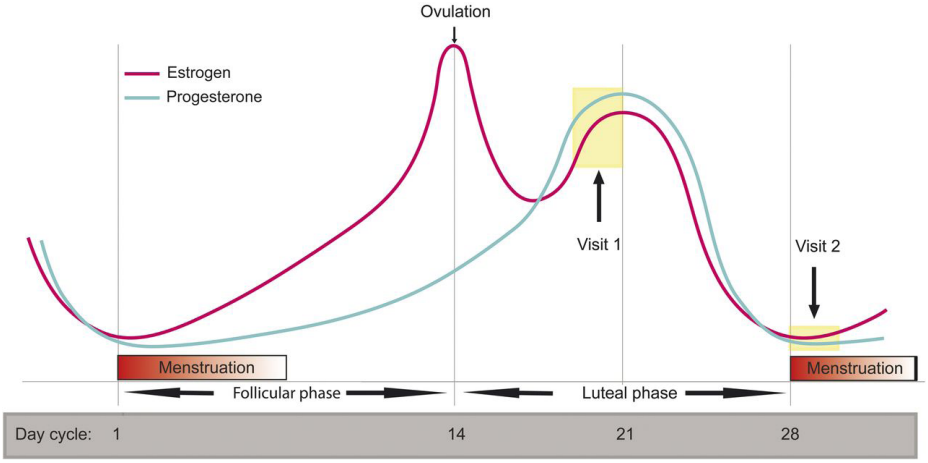

Figure 1. Estrogen and progesterone levels during menstrual cycle and time points of investigations
Yellow highlighted sections: time point of investigations for patients with menstrually related migraine and controls.

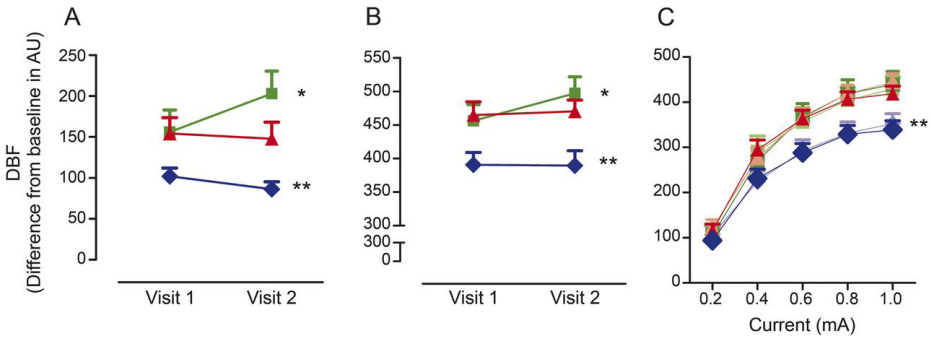

Figure 2. Maximal forehead DBF responses
DBF responses to 0.06 mg/mL capsaicin (A), 6.0 mg/mL capsaicin (B), and electrical stimulation (C). For controls (green square) and patients with MRM (red triangle): visit 1 = days 19–21 of the menstrual cycle and visit 2 = days 1–2 of menstruation. For postmenopausal women (blue diamond): visit 1 and visit 2 planned randomly with 7–10 days in between. (C) Visit 2 controls (light green square), visit 2 patients with menstrually related migraine (light red triangle) and visit 2 postmenopausal women (light blue diamond). *Significant difference in E_{max} between visit 1 and visit 2. **Significant difference in E_{max} during both visits compared to patients with MRM and controls. DBF = dermal blood flow; MRM = menstrually related migraine.

Hormone levels

Serum estradiol levels on day 19-21 of patients with MRM were significantly lower than those of the healthy controls (52±4 pg/ml vs. 75±8 pg/ml respectively), while no differences in estradiol levels between MRM patients and healthy controls (25±5 pg/ml vs. 21±4 pg/ml respectively) were present on day 1-2 of the cycle (Figure 3A). Serum progesterone levels in MRM patients and healthy controls at the two occasions were similar (Figure 3B). Estradiol

and progesterone levels during day 19-21 of the menstrual cycle were significantly higher than during day 1-2. Estradiol and progesterone levels of the postmenopausal women were low, consistent with their non-reproductive life stage.

Peripheral dermal blood flow response to ischemic stimulus

There were no significant differences in PORH responses between visits or between groups (Figure 4). Both age and the maximum PORH response were significant predictors for the E_{max} DBF response to 1 mA electrical stimulation (respectively: Beta:-2.455, 95% CI[-4.206; -0.705] and Beta: 0.813, 95% CI[0.165; 1.461]).

CGRP in saliva

No differences in salivary CGRP levels between visits or between groups were observed (Supplemental figure e-2). There was no correlation between saliva CGRP levels and the time to/since the last migraine attack of the MRM patients. The saliva volume collected from postmenopausal women was significantly lower than from healthy controls and patients with MRM on cycle day 19-21.

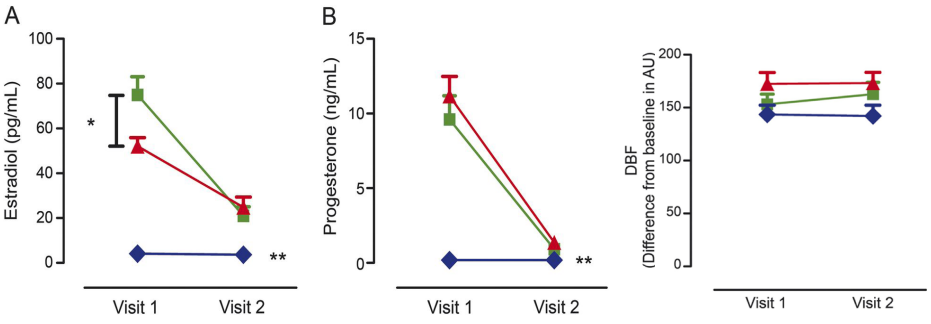

Figure 3. Serum estradiol and progesterone levels

Estradiol levels (A) and progesterone levels (B). For controls (green square) and patients with MRM (red triangle): visit 1 = days 19–21 of the menstrual cycle and visit 2 = days 1–2 of menstruation. For postmenopausal women (blue diamond): visit 1 and visit 2 planned randomly with 7–10 days in between. *Significant difference in estradiol levels between patients with MRM and controls. **Significantly lower levels in postmenopausal women during both visits compared to patients with MRM and controls. MRM = menstrually related migraine

Figure 4. Maximal postocclusive reactive hyperemia responses

For controls (green square) and patients with menstrually related migraine (red triangle): visit 1 = days 19–21 of the menstrual cycle and visit 2 = days 1–2 of menstruation. For postmenopausal women (blue diamond): visit 1 and visit 2 planned randomly with 7–10 days in between.

Discussion

We investigated the association between the menstrual cycling of female sex hormones on trigeminal nerve-mediated vasodilatation in healthy controls and MRM patients.

We clearly demonstrated a DBF response difference to capsaicin over the course of the menstrual cycle in healthy women, indicating that our model is suitable to detect cycle-dependent changes in trigeminovascular reactivity. In contrast, this cyclic DBF response difference was absent in MRM patients. Notably, DBF responses to electrical stimulation were similar throughout the menstrual cycle in both MRM patients and healthy controls. As expected because of their stable low female sex hormone levels, postmenopausal women showed no cyclicity in their DBF responses to either capsaicin or electrical stimulation between visits. Rather, when compared to the healthy controls and MRM patients, they responded significantly lower to both stimuli.

Another surprising finding of our study is the relatively low mean estradiol level detected during day 19-21 of the menstrual cycle of the MRM patients, which seems in agreement with their reduced cyclicity in DBF responses.

In accordance with our hypothesis, DBF responses to capsaicin during day 1-2 of the menstrual cycle were increased in healthy controls. The enhanced DBF response to capsaicin of healthy women around their menstruation is consistent with previously published data, where, in healthy women, sensory and vasomotor responses to intradermal capsaicin injections of the forehead were increased during the menstruation compared to responses during the luteal phase [18]. The higher responses were attributed to either the direct effect of estrogen withdrawal on trigeminal nerve innervated vasculature or to its effect on modulation of serum levels of ionized magnesium. Indeed, estrogen has been suggested to enhance neurogenic vasodilatation, primarily by CGRP, in rats [19, 20]. Since we did not detect cyclic responses to electric stimulation, the higher responses during menstruation in healthy controls may be attributed to differential activity of the TRPV1 channel. Alternatively, release of other neuropeptides in response to electrical stimulation might mask the CGRP-specific component of the DBF response. Finally, the lack of cyclic responses to electrical stimulation could be attributed to the stimulation time and intensity. With electrical stimulation, we applied a brief (1-min) stimulus with a 6-min recovery time. Therefore, this recovery time might have been sufficient for the replenishment of the readily-releasable pool of neuropeptide vesicles at the nerve terminals [21]. In contrast, with capsaicin application there is no recovery phase, but rather a constant stimulation during 40 min. This might lead to not only the exhaustion of the readily-releasable pool of vesicles, but also of the more slowly replenished neuropeptide vesicle reserve pool.

Contrary to our expectations, the DBF responses to capsaicin in MRM patients were unchanged throughout their menstrual cycle. Elevated CGRP levels in jugular blood during migraine attacks have been previously reported [22]. Consequently, we expected MRM patients to have higher DBF responses to capsaicin during day 1-2 of their menstrual cycle. Our results may be explained by activity-dependent transport of neuropeptide in the trigeminal

nerve. In particular, the higher release of CGRP from the dural fibres of the trigeminal nerve during the perimenstrual period might be due to enhanced anterograde transport from the cell soma, where CGRP is synthesized and packaged, to the nerve terminals from where it is synaptically released. Supporting such a potential mechanism is recent evidence from Drosophila that also suggests an activity-dependent mechanism of neuropeptide release [23]. Another possible explanation for the lack of cyclic responses in MRM patients could be an inhibition of TRPV1 channel function induced by the lower decline in estradiol levels in migraine patients as compared to healthy controls. Admittedly, the role of the TRPV1 channel in migraine is still ambiguous [24, 25].

We included salivary measurements of CGRP levels as these reflect the activation state of the trigeminovascular system. Although previous studies have detected elevated CGRP levels in saliva in migraine patients during the premonitory and headache phase of a migraine attack [26, 27], this finding was not replicated in a study with chronic migraine patients [28]. Notably, we observed similar levels of salivary CGRP in MRM patients and healthy controls. These data confirm our DBF measurements, as both the fibres of ophthalmic branch on the forehead (DBF response), as well as the fibres of the mandibular branch of the trigeminal nerve (saliva production), show similar responses in migraine patients independent from the timing in the menstrual cycle or the temporal relationship to a migraine attack. It is important to note that, contrary to the studies mentioned above, we did not collect saliva samples during the premonitory or headache phase of our migraine patients, since we primarily intended to investigate the relation between CGRP and the menstrual phase of healthy controls and migraine patients. Since this limits the conclusions that can be drawn from our salivary measurements, it would be interesting to study ictal salivary CGRP levels in MRM patients in future.

As hypothesized, the DBF responses to capsaicin application and electrical stimulation in postmenopausal women were consistently lower than in menstruating women. To verify whether these findings were caused by general, age-dependent decreases of microcirculatory function, we performed the PORH test. Notably, the PORH response between groups was not significantly different. The relatively low age and good health of the included postmenopausal women may explain the normal PORH responses. Though not significant, the PORH responses of the postmenopausal women tended to be lower than the PORH responses of the premenopausal groups (Figure 4). However, this slight difference in PORH responses is not of such magnitude to plausibly explain the significantly lower DBF responses of the postmenopausal women to capsaicin application and ES, which was confirmed by the regression analysis. Lower DBF responses to capsaicin application and electrical stimulation thus seem to be related to the low estradiol levels after the menopause, which specifically affect the trigeminovascular system and do not induce generalized microcirculatory dysfunction.

Our surprising finding of lower estradiol levels measured in migraine patients during day 19-21 of the menstrual cycle is not due to the mean age of our study groups. In fact, previous studies have reported higher estradiol levels in perimenopausal women [29]. In our study, the MRM patients had a slightly, but not significantly, higher mean age than the healthy

controls (Table 1), despite having lower estradiol levels during the luteal phase (day 19-21 of the cycle). A relationship between low luteal estradiol levels and MRM has never previously been reported. High estradiol levels preceding ovulation have been linked to migraine with, but not to migraine without aura [30]. Our observation is in accordance with previously published data [4], where the ratio between a urinary metabolite of estradiol measured during the luteal phase and during menses in menstrual migraine patients is the same as in our study (estradiol levels MRM patients: day 19-21:day 1-2 = 2:1). To replicate the data observed in our healthy controls, we used data from a previous study at our institution on healthy women with a regular menstrual cycle (n=9) [31]. Indeed, the ratio between serum estradiol during the luteal phase and menses was the same as in our current study (estradiol levels healthy controls: day 19-21:day 1-2 = 4:1). We conceive that the number of subjects in the current study and the replication group is relatively small to make definite statements about the hormone levels in MRM patients. Future studies should investigate these levels in a larger set of MRM patients.

Taken together, our data confirms the pre-existing theory that the premenstrual withdrawal of estradiol influences the trigeminovascular system. Moreover, our study provides evidence for a disturbed systemic as well as trigeminovascular cyclicity in patients with MRM, which may augment their susceptibility to migraine around menstruation.

Acknowledgements

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Chapter 10.

A human capsaicine model to quantitatively assess salivary CGRP secretion

WPJ van Oosterhout[1]
GG Schoonman[1]
IM Garrelds[2]
AHJ Danser[2]
KY Chan[2]
GM Terwindt[1]
MD Ferrari[1]
A Maassenvandenbrink[2]

[1]Department of Neurology, Leiden University Medical Center, Leiden, the Netherlands
[2]Division of Pharmacology and Vascular Medicine, Department of Internal Medicine, Erasmus MC, Rotterdam, the Netherlands

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Abstract

Background

Capsaicin induces the release of calcitonin gene-related peptide (CGRP) via the transient receptor potential channel V1 (TRPV1). The CGRP response after capsaicin application on the tongue might reflect the "activation state" of the trigeminal nerve, since trigeminal CGRP-containing vesicles are depleted on capsaicin application. We tested (i) the quantitative CGRP response after oral capsaicin application; (ii) the optimal concentration of red chili homogenate; and (iii) the day-to-day variability in this response.

Methods

Saliva was collected for two consecutive days after oral application of eight capsaicin dilutions (red chili homogenates) of increasing concentrations in 13 healthy individuals. Effects of homogenate concentration were assessed. Consecutively, saliva was sampled after application of vehicle and undiluted homogenates.

Results

CGRP secretion (pg/ml) increased dose-dependently with homogenate concentration (p<0.001). CGRP levels were highest after application of non-diluted homogenate (vs. baseline: 13.3 (5.0) vs. 9.7 (2.9); p=0.003, as was total CGRP secretion in five minutes (pg) with undiluted (vs. baseline): 89.2 (44.1) vs. 14.1 (2.8); p<0.001. The dose dependent response in CGRP was not affected by day (p=0.14) or day*concentration (p=0.60). Increase in CGRP (undiluted – baseline; pg/ml) did not differ between measurements on dose-finding (p¼0.67) and follow-up days (p=0.46).

Conclusion

Oral application of red chili homogenate is well tolerated and causes a dose-dependent CGRP release in saliva, without day-to-day effects in this response. This model could be used to noninvasively study the activation state of the trigeminal nerve innervating salivary glands.

Introduction

Capsaicin is derived from hot peppers of the *Capsicum* genus and binds to transient receptor potential channels, such as the TRPV1 (formerly known as vanilloid receptor type 1) [1]. Activation of the TRPV1 channels on trigeminal nerve terminals causes the release of calcitonin gene-related peptide (CGRP, a 37 amino-acid inflammatory neuropeptide present in the central, enteric and peripheral nervous system) as well as other inflammatory mediators [1;2], also from the major salivary glands and buccal mucosa [3-5]. The salivary glands are integrated into the neuroendocrine system through complex regulatory pathways, and some salivary peptides are also found in cerebrospinal fluid [6;7]. Clinically, non-stimulated elevated salivary CGRP levels were found during the premonitory or headache phase in two thirds of attacks [8;9], or at baseline [10]. The capsaicin-induced salivary CGRP response might thus reflect the "activation state" of the trigeminal nerve, innervating the salivary glands [10-13]. Activation of the sensory C fibres in the ophthalmic branch of the trigeminal nerve induces this salivary release of CGRP and other neuropeptides, blood vessel dilatation and increased glandular secretion [14;15]. Subcutaneous and intradermal application of capsaicin to the facial skin causes local flaring and pain sensation [16-19], and topical application of capsaicin was recently described to induce reproducible increases in forehead dermal blood flow as a marker of trigeminal nerve-mediated vasodilatation in humans [20]. However, quantification of CGRP release after dermal capsaicin application is difficult [17-19]. Application of capsaicin in the oral cavity also activates trigeminal nerve endings [16;21;22] and the subsequent release of CGRP in saliva can be quantified in animals [23]. In humans there are no good reference studies yet. This, however, is useful since it enables to study a variety of trigeminal conditions including headaches non-invasively and prospectively. The aim of this study is i) to quantify the CGRP response after capsaicin application; ii) to find the optimal concentration of red chili homogenate containing capsaicin for this response; and iii) to assess day-to-day variability.

Methods

Subjects

A total of 13 healthy controls (7 women; mean [SD] age 30.9 [5.9] years,) were included in the dose-finding pilot study using a cumulative dose-response design, aiming to find the (dilution of) red chili homogenate with highest salivary CGRP response, that was still well tolerated. Eleven healthy controls (partly overlapping with the first group; 6 women; mean [SD] age 30.9 [5.9] years, all with Western food habits) were subsequently included in a follow-up study, aiming to confirm the previous findings, in a design where a single dose was applied. All subjects provided written informed consent and the study was approved by the local ethics committee.

Study design and collection methods

In the dose-finding study, saliva was collected at two consecutive days after oral application of 8 capsaicin samples of inclining concentrations, from 10^6x dilution to pure, undiluted red chili homogenate. Per concentration, saliva was collected during 5 minutes (see Figure

1), and homogenates of increasing concentration were applied consecutively. All subjects prepared the mouth by expectorating, rinsing with tepid tap water twice, and discarding to the sink prior to the first measurement on every day. Subjects were given detailed verbal instructions (not to speak; not to move, not to swallow saliva) and were provided appropriate containers for saliva collection (50 mL conical tubes). Before the first sample, 2% citric acid solution was applied to the apex and sides of the tongue using a cotton-tipped applier, to stimulate salivary flow. On the second day, the whole procedure was repeated. Perceived heat sensation was measured during each homogenate concentration separately with a numeric rating scale (NRS) from 0 (no heat sensation at all) to 10 (highest heat sensation possible). The dose-finding study was non-randomized and non-blinded deliberately for several reasons. Firstly, the study was aimed at detecting the highest tolerable dose, enabling subjects to indicate tolerability step-by-step. Secondly, adequate blinding of capsaicin application is very difficult, since oral administration directly affects heat sensation and saliva production. Thirdly, with randomisation, and thereby possible early exposure to the undiluted homogenate, (de)sensitisation and depletion might largely affect the outcome parameters of subsequent measurements. In the follow-up study, we repeated salivary sampling in 11 subjects after application of only vehicle and undiluted red chili homogenates using the same protocol.

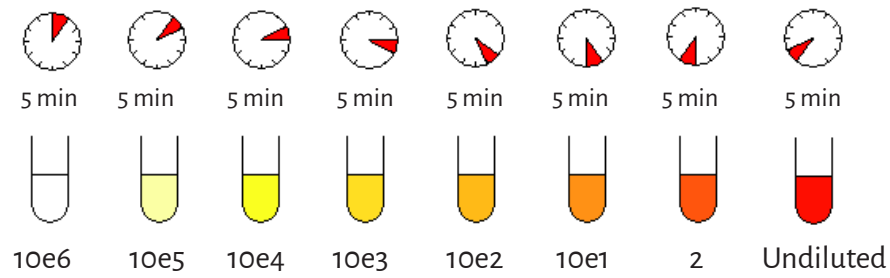

Figure 1. Study design.
Thirteen healthy volunteers were exposed on two consecutive days to 8 increasing concentrations of Madame Jeannet chili homogenates containing capsaicin. Exposure lasted 5 minutes for each of the homogenate dilutions, which ranged from 10^6 times diluted to pure homogenate. Secreted saliva was collected during each of these periods of 5 minutes.

Preparation of red chili homogenate

Red chili homogenates containing capsaicin were obtained from fresh Madame Jeanette chili peppers (*Capsicum chinense*). The green peduncles as well as the seeds were removed from the chili peppers. The remaining part was homogenized, without addition of extra fluids, and immediately frozen in aliquots at -80°C until use.

Saliva storage

Directly after sampling, saliva was placed on dry ice during the transfer to a freezer (-80˚C).

CGRP analysis and determination

CGRP was determined by radioimmunoassay (Phoenix Pharmaceuticals, Inc, Burlingame, CA, USA), according to the instructions of the manufacturer. Total protein was determined using the Pierve BCA Protein Assay Kit (Thermo Scientific, Rockford, Il, USA). Internal standards were used in every measurement. To assess potential cross-reactivity between the capsaicin in the red chili homogenates and the CGRP analysis kit, we also assayed homogenates themselves. Analysis of the undiluted homogenate indicated a mean [SD] CGRP concentration of 5.7 [0.4] pg / mL. These cross-reactivity effects were subtracted from outcome effects, and adjusted data were used for analysis.

Statistical analysis

CGRP responses after capsaicin application were quantified by using CGRP content (pg) and concentration (pg/ ml) in saliva as outcomes. CGRP in baseline samples was compared to levels after maximum capsaicin concentration using a paired student-t test. The optimum for CGRP response was defined by the capsaicin concentration (ranging from 10^6x diluted to pure, undiluted homogenate) leading to the highest CGRP content in salivary samples, whilst heat sensation was still acceptable. Day-to-day variability in CGRP response was assessed using a repeated measurement ANOVA with CGRP concentration and day as within-subject factors, and interaction between those variables. Correlation between heat sensation and capsaicin concentration (homogenate dilution) was assessed using linear regression. All data analyses were performed using SPSS 17.0 (SPSS inc., IBM, USA), and $p<0.05$ was considered to indicate significant differences.

Results

CGRP secretion at baseline and after capsaicin stimulation

The concentration of the homogenate significantly affected CGRP secretion ($p=0.001$). The highest CGRP levels were detected in samples after application of non-diluted chili homogenate in the dose-finding study (Table 1). Mean [SD] CGRP concentration (pg/ mL) was higher after undiluted homogenate compared to baseline (13.3 [5.0] vs. 9.7[2.9]; $p=0.003$; ratio 13.3/9.7=1.4/1), as was the total amount of CGRP (pg) secreted in 5 minutes (89.2[44.1] vs. 14.1 [2.8]; $p<0.001$; ratio 89.2/14.1=6.3/1). These findings were confirmed by data from the follow-up study: higher salivary CGRP concentration (pg/mL) using undiluted homogenate (15.6 [2.9] vs. 13.1 [2.0]; $p=0.038$), and higher totally secreted CGRP in 5 minutes (pg); 255.8 [85.1] vs. 98.3 [25.0]; $p<0.001$. Additionally, in the dose-finding study the ratio of salivary CGRP/ total protein (pg/ mg) was similar after stimulation of baseline vs. pure homogenates (19.3 [4.7] vs. 20.5 [5.8]; $p=0.45$), see Figure 3c.

Day-to-day reproducibility

Salivary CGRP concentration (pg/mL) increased in a dose-dependent manner after stimulation with capsaicin on day 1 ($p=0.001$) as well as on day 2 ($p<0.001$). Combining data from these two days also results in an overall significant positive correlation ($r=0.39$, n=200, $p<0.001$). Using ANCOVA, we found a significant effect of homogenate concentration ($p=0.001$) on CGRP response, but not for day and the interaction between concentration/day ($p=0.14$ and $p=0.60$ respectively).

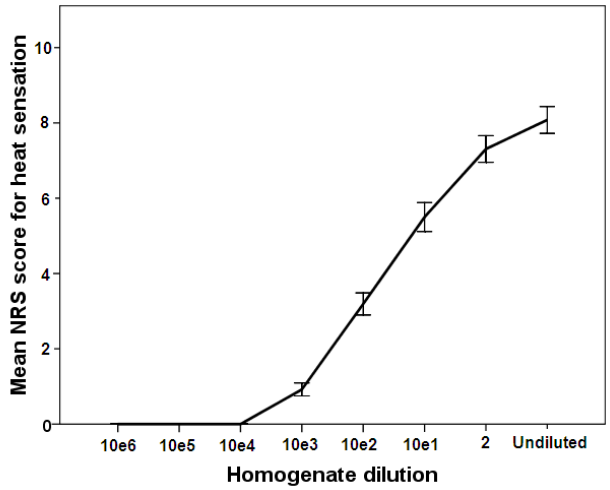

Figure 2. Subjective heat sensation.
Mean subjective heat sensation as reported by participants at increasing concentrations of orally applied capsaicin concentrations using a numeric rating scale (range 0-10). Participants perceived heat on from the sample that was diluted 1,000 (10^3 / 10e3) times. Error bars indicate +/- 1 standard error of the mean (SEM).

Table 1. CGRP concentration (pg/ mL saliva), amount of secreted CGRP (pg/ 5 minutes), and CGRP/ total protein ratio (pg/ mg) in samples at baseline and after application of undiluted homogenate (highest concentration) on day 1 and 2 (n=13) in dose-finding study.
UH = undiluted homogenate; min = minutes; TP = total protein.

			Day 1						Day 2	
			CGRP; pg/ 5min		[CGRP]; in pg / mL		CGRP /TP; pg/ mg		[CGRP]; in pg/ mL	
Subject	Gender	Age	Baseline	After UH	**Baseline**	**After UH**	Baseline	After UH	**Baseline**	**After UH**
1.	Male	39	41.0	*107.0	**11.7**	***16.5**	11.0	*14.5	**13.4**	**18.0**
2.	Male	32	67.7	131.5	**15.0**	**16.4**	16.7	16.1	**5.3**	**10.9**
3.	Male	26	52.7	38.2	**9.6**	**5.5**	8.2	6.7	**2.4**	**10.4**
4.	Female	32	38.3	64.5	**7.7**	**9.9**	13.3	12.1	**6.6**	**12.7**
5.	Female	28	50.5	64.8	**9.2**	**12.2**	11.4	16.1	**9.1**	**13.2**
6.	Female	29	64.4	102.9	**11.7**	**14.7**	10.7	16.6	**12.3**	**12.1**
7.	Female	26	47.3	150.2	**11.3**	**28.9**	10.6	36.6	**12.8**	**17.9**
8.	Male	26	44.7	53.1	**8.9**	**8.2**	9.9	7.7	**8.8**	**17.0**
9.	Female	42	35.0	81.3	**10.9**	**13.6**	12.6	11.4	**10.6**	**13.6**
10.	Female	39	45.0	55.5	**7.8**	**7.9**	10.4	12.4	**6.5**	**8.5**
11.	Male	32	28.2	*79.2	**11.3**	***15.8**	9.0	*22.0	**11.6**	**17.8**
12.	Female	23	35.1	48.8	**10.0**	**8.1**	17.7	13.0	**5.6**	**11.3**
13.	Male	28	27.6	241.6	**9.2**	**19.3**	10.7	13.6	**12.9**	**10.4**

*data not from UH tube, but from UH:2 tube.

The increase in salivary CGRP concentrations (pg/mL) between baseline and undiluted homogenate did not differ on the two consecutive days (mean [SD]: day 1 = 3.0 [6.0] and day 2 = 4.1 [3.3]; p=0.67), nor was it different from the follow-up study (2.5 [1.05]; ANOVA p=0.46).

Total saliva volume and protein content

The total volume of saliva and the level of secreted protein increased with increasing capsaicin concentration, but the ratio of CGRP / total protein remained stable after adjusting for cross-reactivity (Figure 3).

Heat sensation

Red chili homogenates were well tolerated by study participants. Participants reported increasing sensation of heat on from the 1:1,000 dilution on both days (Figure 2), and mean[SD] NRS score on pure capsaicin homogenate was 8.1 [1.8] on both day 1 and day 2. There was a strong, positive correlation between heat sensation and capsaicin containing homogenate dilution (r=0.87; p<0.001). None of the participants terminated their participation due to the experienced heat sensation. Dilution of ≥10^4x resulted in no heat sensation. Higher heat sensation was correlated with higher total amount of CGRP release (pg) in the sample (r=0.50, p<0.001).

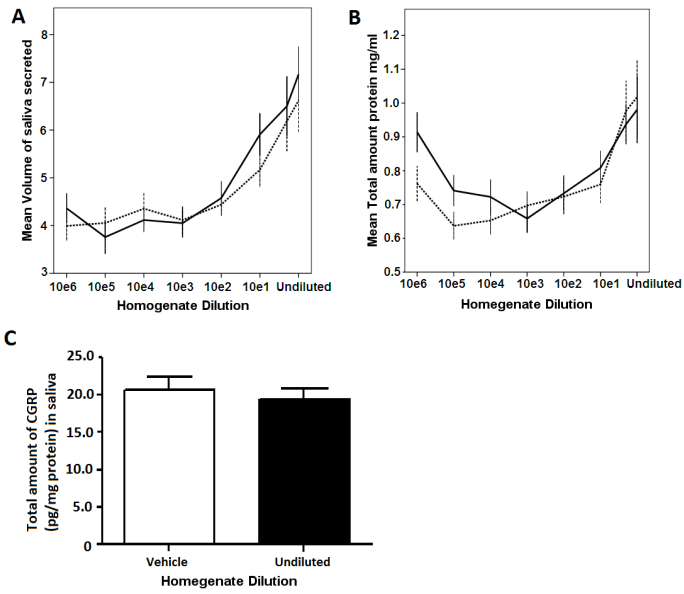

Figure 3. Total salivary volume and protein content with capsaicin stimulation.
3a Total secreted salivary volume (mL) depends on capsaicin concentration; **3b** Secreted protein content (mg/ mL saliva) depends on capsaicin concentration; **3c** Ratio of CGRP/ total protein (pg/ mg) does not depend on capsaicin concentration, after adjusting for cross-reactivity. In figures 3a and 3b, continuous lines depict day 1; dashed lines depict day 2.

Discussion

This study shows that oral application of red chili homogenate is well tolerated and causes a dose-dependent salivary CGRP release, without day-to-day effects in this response. Capsaicin (-containing homogenates) have been applied in several ways before in previous studies, including subcutaneously [18], intradermally [17;19], topically [16;20], and orally [24], but this is the first study to quantify the salivary CGRP release. Our data show that there are inter-individual differences in both baseline salivary CGRP content as well as after capsaicin application, indicating that CGRP levels are variable and that salivary CGRP levels increase in response to compounds activating the TRPV1 channel.

Capsaicin, being an irritant for mammals, produces a sensation of burning in any tissue with which it has contact, including buccal mucosa. From previous studies, we know oral application of capsaicin on the tongue and other orofacial tissues elicits burning pain, increases in blood flow and temperature, but no change in mechanical sensitivity in the glabrous lips or tongue [21]. In our study, we show that application of capsaicin concentrations leads to a higher saliva secretion, which is in accordance to previous research that additionally reported higher secretion rates [22]. This might be caused by the released CGRP itself, which is known to induce a delayed increase in rat salivary secretion [25]. Furthermore, our data suggest that, although total protein content may increase after capsaicin application, the salivary protein composition might not be altered, since at least the proportion of CGRP does not change.

In the follow-up study (with use of single dose homogenate), we found larger responses in CGRP secretion between vehicle and undiluted chili homogenates compared to the dose-finding study. The smaller CGRP response to the undiluted homogenate in the dose-finding study might be due to the cumulative application of inclining homogenate concentrations. This cumulative exposure to increasing concentration of chili homogenate is likely to have caused a partial depletion of CGRP-containing neurons innervating the salivary glands, and possibly to have induced early desensitization effects [25;26].

CGRP is expressed in subsets of peripheral and central nervous system neurons, including the trigeminal nerve branches that innervate the salivary glands. CGRP mediates the vasodilation part of neurogenic inflammation. Increased levels of CGRP reflect hyperactivation of the trigeminovascular system (TGVS) and subsequent signalling and processing of nociceptive stimuli in the brainstem [27]. Since sublingual and submandibular glands are partly innervated by sensory CGRP-containing trigeminal nerves [3-5], levels of salivary CGRP can be used as a biomarker for trigeminal nerve activation [10]. The trigeminal nerve is the major sensory nerve of the face with three major divisions (the ophthalmic, maxillary, and mandibular) that join together at the trigeminal ganglion. Although the three branches innervate different regions of the face, there is evidence in inflammatory disease that activation of one branch can cause activation of the other major branches through a process of sensitisation, leading to elevated neuronal activity [28;29]. It is, however, unknown, whether this is always the case, since a recent study showed that capsaicin induced activation of the ophthalmic branch does not have result in clinical headache [20]. Furthermore,

in migraine, referral pain to the lower branches of the trigeminal nerve is rarely reported. Overall, however, the CGRP present in saliva is most likely to originate from the trigeminal nerve endings[5]. This mechanism enables us to monitor salivary CGRP changes that reflect pathophysiological changes in other trigeminal branches [10]. Increases in salivary CGRP during trigeminal activation such is the case in migraine, have been reported before before [8, 9, 10] and suggest a direct or indirect causal pathway. Detailed insight in the effects of intracranial dural stimulation on salivary CGRP release would be very interesting. Unfortunately, results from such studies are not available.
Limitations of this study include a relatively small sample size. Furthermore, the age homogeneity of our study sample does not allow to generalize results to a younger or elder population. Although the mean increase in CGRP secretion using undiluted homogenate might be small (3.6 CGRP pg/mL saliva; ratio 13.3/9.7=1.4), the range in CGRP responses varied between subjects with CGRP concentration changes from -27% to +775%. These data indicate large inter-individual variability in CGRP response suggesting this secretion could be related to the (patho)physiological state of the subject.

Our study shows that it is possible to study trigeminal CGRP content and depletion with the use of the capsaicin model. Capsaicin stimulation is known to activate sensitive sensory neurons[30], leading to release of neuroactive biochemical substances including CGRP[1]. We speculate that the mechanism behind this effect might be the depletion of CGRP-containing vesicles within trigeminal nerve endings. Increased salivary CGRP levels might thus reflect a high activity status of the trigeminal system, i.e. many intraneuronal CGRP-containing vesicles. Capsaicin might also induce CGRP production in the nerve cells, and subsequent release of CGRP in target organs, in this case the salivary glands, leading to higher levels within the saliva. We could speculate that stimulation with capsaicin in this condition would result in a higher salivary volume and a higher CGRP content. The capsaicin model could be used to assess the CGRP loading and functionality of CGRP-containing trigeminal nerve endings innervating the salivary glands, thereby reflecting the activation status of other branches of the trigeminal system [10]. We hypothesise enhanced activity of the TGVS is reflected by enhanced CGRP depletion. Further development of analytic techniques may be of interest, especially for diseases in which dysfunction of the trigeminal system is known, for analysis of specific salivary compounds that are released into the saliva. In diseases such as (primary) headache syndromes including cluster headache[31] and migraine[10;31-36], but also in tension type headache[36], and other diseases, analytical techniques that help qualifying and quantifying salivary (neuro)peptides including CGRP could be useful in elucidating pathophysiologic mechanisms. Especially when combined with measurements that are able to assess functionality of the other branches of the trigeminal nerve (ophthalmic and maxillary) synchronously, salivary CGRP measurements may help in understanding involvement of the trigeminovascular system in a variety of conditions.

In summary, we found that non-diluted chili homogenate can be used as a well-tolerated and effective capsaicin provocation model to quantitatively measure salivary CGRP, with a good day-to-day reproducibility, which may be helpful in elucidating pathophysiologic mechanisms in diseases in which the trigeminal system is involved.

Clinical Implications

- Calcitonin gene-related peptide (CGRP) can be found in human saliva
- Salivary CGRP is released by salivary glands, partly innervated by efferents of the trigeminal nerve
- Oral application of undilated red chili homogenate causes a dose-dependent CGRP release
- The induced salivary CGRP response might reflect the activation state of the trigeminal nerve

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Part IV.

Summary

Chapter 11.

Conclusions, general discussion and future perspectives

Conclusions, general discussion and future perspectives

For this thesis, clinical investigations on the premonitory phase and early phase of both the spontaneous and triggered migraine attack were performed. In the first part, we studied clinical aspects and modulators of migraine attacks. In the second part, we used magnetic resonance imaging techniques to study metabolic and perfusion changes in the hypothalamus during the premonitory phase and early headache phase of both spontaneous and glyceryl trinitrate triggered migraine attacks. Lastly, several biochemical modulators and triggers of migraine attack onset were investigated.

Part I: Clinical aspects and modulators

Migraine is a clinically heterogeneous headache disorder characterized by recurrent, disabling attacks of severe and often unilateral headaches, accompanied by symptoms of photophobia, phonophobia, nausea and vomiting [1]. In one third of patients, transient neurological aura symptoms precede the headache [2]. It is pivotal for both epidemiological and clinical experimental studies to clearly distinguish between migraine with and without aura.

Diagnosing migraine aura reliably using a questionnaire

In epidemiological and genetic studies, but also in clinical experimental settings, it is important to be able to clearly distinguish between migraine patients with and without aura. These aura symptoms are most often visual, although sensory, motor or speech related symptoms can occur as well. Diagnosing patients can best be done using an interview. However, when the inclusion of large numbers of patients is necessary, accurate and reliable questionnaires are a well-desired tool. In **chapter 2** we assessed the validity of a self-administered, web-based migraine questionnaire in the setting of the well-defined Leiden University Migraine Neuro-Analysis (LUMINA) cohort. Computer-aided diagnoses were checked by semi-structured telephone-interviews in 1,067 patients. We compared a subset of seven questions with the full algorithm based on the ICHD-2 criteria [3, 4] and found that this subset provided a higher sensitivity (86% vs 45%), slightly lower specificity (75% vs 95%) and similar positive predictive value (86% vs 88%) in assessing aura. The study was the first to corroborate that an online questionnaire can accurately and reliably diagnose aura and enables detection of more aura cases with a low false-positive rate for the use in future epidemiological and genetic research.

Chronotypes and restless legs; underlining the link between migraine and the hypothalamus

Since migraine attacks also show seasonal and circadian periodicity [30-34], it is suggested that chronobiological, probably hypothalamic-mediated mechanisms play a role in the triggering and initiation of migraine attacks as well. In **chapter 3** migraine status was studied in relation to chronotype, i.e. the way an individual's endogenous circadian clock rhythm runs and synchronizes (entrains) to the 24-hour day. Off-center chronotypes, i.e. individuals who are really early birds or night owls, were overrepresented among migraine patients. Patients were more often early or late chronotypes as compared to the control group of non-headache subjects. As only few small studies had investigated this association before [32, 35], this study established the link between migraine (with and without aura) and off-center chronotypes. Interestingly, these data underlined that migraine attacks have a preponderance to strike in the early morning. In addition, for the first time, it was described that migraine patients can cope less well with alterations in circadian rhythm: they are less flexible and more rigid in adjusting the setting of their biological clock. These observations underscore an important role for chronobiological mechanisms in migraine attack initiation. Whether training or entrainment of the biological clock has an improving effect on migraine attack frequency or duration still needs to be established.

In **chapter 4** an increased prevalence of restless legs syndrome (RLS) in migraine patients

vs. controls was described, consistent with previous findings [5-11]. Interestingly, RLS was also found to be more severe among the migraine patients, and interfere more with sleep quality, thereby possibly moderating an additional triggering factor for migraine attacks. Migraine severity was correlated with RLS-severity. A number of hypotheses has been presented to explain the association between RLS and migraine [6]: dopaminergic dysfunction, dysfunctional iron metabolism, genetic linkage and sleep disturbances. RLS has since long been considered to be related to dopaminergic system dysfunction [12-19], and the A11 dopaminergic nucleus of the dorsal-posterior hypothalamus is hypothesized to be involved in both RLS [19-22] and migraine pathophysiology [23-25]. Although treatment of RLS with dopamine agonists leads to rapid and dramatic improvement in symptoms with dopamine agonists [26], the effect on migraine headache relief is not clear [27-29]. Whether treating migraine affects concomitant RLS symptoms also still needs to be established.

Post-dural puncture headache and experimental trigger factors for migraine attack initiation

In **chapter 5** it was described that migraineurs are not at increased risk of developing post-dural puncture headache (PDPH), in contrary to what was generally assumed before. The incidence and severity of PDPH were studied as part of an extensive biochemical migraine research programme in which cerebrospinal fluid sampling was performed using a defined protocol in 160 migraine patients and 53 age- and sex-matched healthy non-migraine control subjects. In total, 64 of 199 subjects (32.2%) with successful lumbar puncture developed PDPH. Young age, low body mass index, severe headache immediately after LP, and sitting sampling position, but not being a migraineur, increased the risk of PDPH. PDPH duration was prolonged by history of depression, sitting sampling position, high perceived stress during the procedure, and multiple lumbar puncture efforts. PDPH duration was found to be similar in migraineurs and control subjects. Furthermore, migraine attacks were less likely to occur before or shortly after lumbar puncture. This suggest that a lumbar puncture is not a trigger for a migraine attack, and that the stress of an upcoming lumbar puncture might even have a protective effect against onset of migraine attacks.

Based on the epidemiologic observation that migraine and vasovagal syncope are comorbid conditions and on the experimental observation that nitroglycerin can trigger both syncope and migraine attacks, in the study described in **chapter 6** the migraine-specific effects of intravenous nitroglycerin infusion on cardiovascular parameters were assessed. First, after nitroglycerin infusion, patients in whom an attack was provoked showed a stronger cardiovascular response compared to the non-migraine controls. Secondly, in patients who showed a stronger cardiovascular response in heart rate, stroke volume, and cardiac output during nitroglycerin infusion, a shorter time until the provoked migraine attack initiated was observed. Migraine patients in whom no attack was provoked had weaker cardiovascular responses, similar to the responses in the control subjects. The stronger systemic cardiovascular responses in migraine suggest increased systemic sensitivity to vasodilators, possibly due to insufficient autonomic compensatory mechanisms. As these autonomic mechanisms are partly controlled by hypothalamic signalling, a role for hypothalamic disfunctioning in attack susceptibility can be postulated.

Part II: Imaging aspects

Even though clinical evidence suggests hypothalamic involvement during the early phases of a migraine attack, very few imaging studies have provided evidence for this hypothesis. Only one functional Magnetic Resonance Imaging (fMRI) study focussing on the hypothalamus in migraine without aura attacks has been conducted. Three elaborate prospective studies have reported on activation of the hypothalamus during the migraine attack. Taken together, two out of these three studies provided insight in hypothalamic activity during the pre-ictal phase [36, 37], but control groups were lacking and generalizability was hampered by very small numbers [36-38]. Furthermore, they all used indirect measures for hypothalamic metabolic changes: either the Blood Oxygen Level Dependent (BOLD) signal or perfusion changes as measured with $H^2_{15}O$-PET as a proxy for neuronal activity.

Altered hypothalamic neuronal activity as measured with fMRI

In **chapter 7** an altered hypothalamic neuronal activity in response to an oral glucose load was reported in the early phases of nitroglycerin-triggered and spontaneous migraine attacks based on a study using functional Magnetic Resonance Spectroscopy (fMRI). Normally, the activity of the hypothalamus will remain low after ingestion of glucose, probably reflecting a 'satisfied' state of the glucosensitive neurons in the lateral hypothalamus [41, 42]. In the migraine group, the hypothalamus seemed not to respond to glucose during the premonitory phase, and activity did not show the normal, glucose-induced drop. The abnormal, prolonged increase we found in the migraine group might then reflect disinhibited satisfaction, which could link to the common premonitory symptom of craving. However, as the hypothalamus controls different homeostatic mechanisms, linking this abnormal response to one specific symptom would be an oversimplification. Our study is the first study showing an abnormal response of the fMRI-signal in the hypothalamus during the earliest phases of both nitroglycerin-provoked and spontaneous migraine attacks and corroborates the hypothesised role of the hypothalamus very early in the migraine attack, and additional studies are necessary to elucidate the precise role of the hypothalamus.

Part III: Biochemical aspects

In the third part several biochemical modulators of migraine attack onset in both male and female patients are described. Migraine prevalence and the frequency, duration and severity of migraine attacks are highly dependent on age, gender and, in women, events which are associated with marked fluctuations in the female reproductive hormones oestrogen and progesterone [43-45]. Testosterone suppletion had been suggested to modulate migraine frequency [46].

Blood hormone levels

Although there was considerable evidence from epidemiological, biochemical and experimental studies that female sex hormones might modulate migraine prevalence, frequency and severity in women, the role of oestradiol and testosterone in migraine

susceptibility and attack onset in male patients had not been studied. In **chapter 8** we described our prospective study into interictal, pre-ictal and ictal levels of 17ß-oestradiol and free testosterone in men with migraine and analysed the temporal relationship of changes in these levels with the occurrence of attacks. We found that interictally, male migraine patients have increased levels of 17β-oestradiol compared to non-headache controls, and that levels of free testosterone increase during the pre-ictal phase in patients who report clinical premonitory symptoms. Furthermore, we presented evidence for a relative androgen deficiency in the migraine group. Only one previous study had investigated testosterone levels in a total of eight male migraineurs. In accordance with our results, testosterone levels outside migraine attacks in that study did not differ from baseline [47]. We hypothesise that the pre-ictal rise in testosterone could reflect a general stress-response anticipating the impending attack [48].

Several mechanisms are proposed by which changes in the levels of reproductive hormones could modulate migraine susceptibility. The late-luteal phase drop in 17ß-oestradiol specific to patients with menstrual-related attacks [45, 49] is suggested to affect the balance between long-lasting genomic effects of nuclear oestradiol receptors and short-lasting non-genomic effect via intra-membranous G-protein-coupled oestradiol receptors [50]. This imbalance could induce neuronal sensitisation and ultimately triggering of attacks [50]. Secondly, female and male sex hormones differentially affect two important basic mechanisms likely involved in migraine pathogenesis as noticed in animal experiments. Female gonadal hormones lower the threshold for inducing cortical spreading depressions (CSDs). CSD is a putative surrogate marker of migraine susceptibility and is considered the biological correlate of the migraine aura [51]. Furthermore, female sex hormones activate the nociceptive transmission within the trigeminovascular system by modulating expression of nociceptive mediators such as calcitonin gene-related peptide (CGRP) and by affecting serotonin synthesis, dural mast cell density, and intracellular downstream signalling [51, 52]. Taken together, what exactly is the role of oestradiol in men with migraine and whether fluctuations in oestradiol levels, like in women, might be associated with changes in migraine activity, deserves further intra-individual follow-up studies over multiple attack cycles.

Trigeminal cyclicity and calcitonin gene-related peptide (CGRP) [ref paper]

Calcitonin gene-related peptide (CGRP) is a potent vasodilator and a key mediator in migraine [53], which is released from the primary afferents of the trigeminal ganglion exerting its effect via the trigeminovascular system [54]. In **chapter 9** a human model to quantitatively assess salivary CGRP secretion using capsaicin as a provocative agent is described. Capsaicin induces the release of CGRP via the transient receptor potential channel V1 (TRPV1). The CGRP response after capsaicin application on the tongue was considered to reflect the "activation state" of the trigeminal nerve, since trigeminal CGRP-containing vesicles were depleted on capsaicin application. The capsaicin in our study was derived from red chili homogenate, was well tolerated and caused a dose-dependent salivary CGRP release in our pilot study. Subsequently, we validated the optimal capsaicin concentration for provocation use in a second experimental set-up. Capsaicin (-containing homogenates) had been applied in several ways before in previous studies, including subcutaneously [55], intradermally [56, 57],

topically[58, 59] and orally[60], but we were the first study to quantify the salivary CGRP release. This model could be helpful in elucidating the pathophysiologic mechanisms in diseases in which the trigeminal system is involved.

Given the relationship between migraine attack incidence and fluctuations in sex hormones, in **chapter 10** the hypothesis was tested whether varying levels of sex hormones over the course of the menstrual cycle affect trigeminal nerve-mediated vasodilatation of the small vessels in the forehead, a dermatome innervated by the trigeminal nerve. In a prospective study, we compared dermal blood flow (as a proxy for vasodilation) during the mid-luteal phase and during menstruation phase after both topical capsaicin application and electrostimulation in patients with menstrual-related migraine, in age-matched healthy controls with a menstrual cycle, and in a reference group of post-menopausal women. In migraine we found a diminished cyclicity of dermal blood flow response to topical capsaicin application. The responses after electrostimulation and the salivary CGRP levels were similar throughout the menstrual cycle in all groups. Previous studies, however, have found elevated levels of salivary CGRP during the pre-ictal and ictal phases of the migraine attack [61, 62]. Since the time points in this study were not based on clinical migraine attack phase (but rather on menstrual cycle timing) no conclusions with regard to the salivary CGRP levels could be drawn. This study corroborated the hypothesis that the premenstrual decrease in oestradiol affects the trigeminovascular system, and underlined that patients with menstrual related migraine have both an altered systemic and trigeminovascular cyclicity. This might explain their augmented susceptibility to a migraine attack around the time of menstruation.

Overall resume and future perspectives

The premonitory phase and early phase of both spontaneous and nitroglycerin-triggered migraine attacks were explored in this thesis, in association with clinical modulators and trigger factors. Clinical research strategies, experimental designs, neuroimaging techniques and biochemical methods have revealed clinical risk factors, biochemical modulators and pharmacological triggers. Furthermore, newly discovered hypothalamus-specific alterations in metabolism and perfusion in the early phases of the migraine attack were described. Taken together, these results suggest that each migraine attack starts well before the initiation of the headache phase. The hypothalamus is postulated to have a pivotal role in the early phases of the migraine attack, and possibly affects attack susceptibility interictally as well.

Overall, the studies described in this thesis have improved our understanding that the migraine brain (and possibly the hypothalamus) differs from the non-migraine brain already at baseline. The studies described in this thesis also underline the importance of studying the entirety of the migraine attack: from interictal, via premonitory, to the headache phase. Longitudinal studies that collect data over these consecutive phases are of utmost necessity to further elucidate the complex biochemical alterations in the brains and bodies of migraine patients.

In depth profiling or defining of migraine patients, so-called phenotyping, either with very detailed questionnaires or face-to-face, should form the core of any future study in migraine, also when using large samples. Assessing the relationship between migraine and comorbidities or individual traits gives insight in underlying, shared mechanisms and can help identify important pathways or structures involved in migraine pathophysiology. Detailed neuro-imaging evaluation, using well-defined subjects and valid imaging techniques, can be used for studying brain structures, even those that are localized deep within the brain, such as the hypothalamus. S*tate of the art* imaging techniques are necessary since the presumed biochemical differences or changes are very small. The biochemical assessment of bodily fluids (cerebrospinal fluid, saliva, blood), provides an opportunity to study hormones, peptides or other molecules in a clinical context and with the possibility of repeated measurements over the course of a migraine attack. Therefore, performing such prospective studies in the future, although logistically very challenging, remains very important.

In conclusion, the studies in this thesis have improved our understanding of pathophysiological mechanisms in the early phase of migraine attacks and the role of the hypothalamus herein. The studies have shown that epidemiological, clinical, biochemical and imaging strategies are valid tools to attack onset mechanisms, and that longitudinal, repeated measurement study designs are well worth the logistical challenges. It remains pivotal to translate biochemical and imaging findings into novel treatment options for migraine patients.

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Chapter 12.

Nederlandse samenvatting

In dit proefschrift worden verschillende aspecten onderzocht die gerelateerd zijn aan de gevoeligheid voor het krijgen van migraineaanvallen in het algemeen danwel het uitlokken van een specifieke migraineaanval. Het onderzoek werd toegespitst op het begin van een migraineaanval en dan met name op de prodromale (in het Engels *premonitory)* fase[1]. Dat is de periode waarin een patiënt al wel aspecifieke klachten ervaart maar de hoofdpijnfase van de migraineaanval nog niet is gestart. De onderzoeken zijn ingedeeld naar drie onderzoeksstrategieën: 1) klinische verschillen en veranderingen, 2) (systemische) biochemische veranderingen en 3) lokale veranderingen gemeten met MRI in de hypothalamus, een diep in de hersenen gelegen structuur, die o.a. op basis van klinische verschijnselen bij migrainepatiënten al werd verondersteld een rol te spelen.

Inleiding over migraine (hoofdstuk 1)

In het eerste hoofdstuk worden de klinische verschijnselen van migraine besproken, evenals de onderliggende pathofysiologische mechanismen voor zover deze bekend zijn. Migraine is een neurologische aandoening waarbij hoofdpijn in aanvallen van 4-72 uur optreedt. De hoofdpijn is meestal eenzijdig, bonzend van karakter, neemt toe in ernst bij fysieke inspanning en belemmert de patiënt in zijn dagelijkse activiteiten. Als bijkomende verschijnselen kunnen misselijkheid, braken en overgevoeligheid voor licht, geluid en geuren optreden. De aanvalsfrequentie varieert van minder dan één aanval per jaar tot meerdere aanvallen per maand. Eén op de drie migrainepatiënten ervaart voorafgaand aan de hoofdpijn tijdelijk neurologische uitvals- of prikkelingsverschijnselen gedurende 5-60 minuten, die worden aangeduid met de term aura. Deze symptomen kunnen bestaan uit visuele symptomen, paresthesieën (onprettig gevoel zoals tintelingen of prikkelingen) aan een ledemaat en/of gelaat, moeite met spreken en, in uitzonderlijke gevallen, een verlamming van één zijde van het lichaam. Aanvullend rapporteren veel patiënten in de uren tot dagen voorafgaand aan de aura- en hoofdpijnfase aspecifieke klachten. Deze klachten bestaan uit onder andere vermoeidheid, concentratieproblemen, gapen, trek in specifieke voedingsmiddelen, het vasthouden van vocht (oedemen) en vaker/meer plassen. Deze symptomen worden prodromale verschijnselen genoemd. Er wordt gesuggereerd dat deze verschijnselen een weergave zijn van tijdelijk disfunctie van de hypothalamus, een diep in de hersenen gelegen kern die als belangrijkste functie heeft het in evenwicht houden van het milieu intérieur (de zogenaamde homeostasis).

Het is nog onduidelijk hoe een migraineaanval precies begint. Een huidige hypothese is dat de gevoeligheid voor het krijgen van een migraineaanval wordt bepaald door een combinatie van genetische factoren en modulerende factoren die de zogenaamde aanvalsdrempel kunnen verlagen. Modulerende factoren zijn mogelijk stress en hormoonconcentraties in het bloed. Daarnaast worden in de literatuur uitlokkende factoren beschreven, die het optreden van een nieuwe migraineaanval kunnen triggeren. Specifieke voedingsmiddelen,

1 De terminologie in het Nederlands wijkt enigszins af van de Engelse. Wanneer het Engels woord *premonitory* gebruikt wordt in de context van migraine, duidt dit de periode voorafgaand aan de hoofdpijnfase van een migraineaanval. Hierbij wordt dan in de terminologie geen onderscheid gemaakt tussen patiënten die dan wél al aspecifieke klachten hebben en patiënten die helemaal geen klachten hebben. In dit proefschrift heb ik ervoor gekozen om in het Nederlands het woord pre-ictaal (vóór de ictus, i.e. de hoofdpijnfase) te gebruiken om de *premonitory* periode aan te duiden. Het Nederlandse woord prodromaal gebruik ik vervolgens wanneer er in deze periode ook daadwerkelijk klachten of symptomen zijn.

weersveranderingen en ook stress worden vaak genoemd maar hard bewijs hiervoor ontbreekt tot nu toe. Meer is bekend over de onderliggende mechanismen van het migraineaura en de hoofdpijn. Op basis van dieronderzoek wordt gedacht dat een golf van depolarisatie van neuronen die zich langzaam over de hersenschors verspreidt (*cortical spreading depression*; CSD) het biologisch correlaat is van de aurasymptomen. Tijdens de hoofdpijnfase is het trigeminocervicale complex, de zenuw en hersenkern van de vijfde hersenzenuw (nervus trigeminus), geactiveerd. Verschillende andere zenuwbanen, zowel vanuit de hersenstam als vanuit de hersenschors, hebben hierop invloed.

Deel I: Klinische symptomen

Een nieuwe gevalideerde vragenlijst om de diagnose migraine in grote groepen individuen te stellen (hoofdstuk 2)

Om kwalitatief goed epidemiologisch en genetisch onderzoek te verrichten en daarmee de precieze pathofysiologische mechanismen bij migraine te doorgronden, zijn grote aantallen (tienduizenden) patiënten nodig. Het is derhalve om logistieke en financiële redenen belangrijk dat de diagnose migraine (en dan met name mét of zonder aura) redelijk betrouwbaar kan worden gesteld, gebruikmakend van een (online) vragenlijst als instrument. We hebben daarom in een populatie van 1.067 migrainepatiënten een nieuw ontwikkelde online vragenlijst gevalideerd, die gebaseerd was op de criteria van de internationale hoofdpijn vereniging (*International Classification of Headache Disorders* van de *International Headache Society*). De uitgebreide vragenlijst hebben we gevalideerd door bij deze groep eveneens een diagnose te stellen op basis van een semigestructureerd telefonisch interview. Het bleek dat de online vragenlijst nauwkeurig en precies de diagnose migraine met of zonder aura kon stellen in vergelijking met de interview diagnose als 'gouden standaard'. Tevens bleek dat het algoritme op basis van een selectie van 7 vragen uit deze lijst even goed was in diagnosestellen als het algoritme dat gebaseerd is op de complete internationale criteria. Deze vragenlijst wordt nu ook gebruikt bij het rekruteren van nieuwe migrainepatiënten in het migraineonderzoek van het LUMC.

Migrainepatiënten hebben een andere instelling van de biologische klok (hoofdstuk 3)

Bij veel patiënten hebben de migraineaanvallen een circadiaan (dag-nacht) of seizoensritme, wat suggereert dat de interne biologische klok, die een onderdeel vormt van de hypothalamus, anders afgesteld is dan bij individuen zonder migraine. Hoe de biologisch klok ingesteld is en synchroniseert met opgelegde veranderingen in het bioritme, zoals bijvoorbeeld door een jetlag of de dagelijkse aanpassing naar de 24-uurs dag, wordt chronotype genoemd. Chronotypes variëren van extreme ochtendmensen tot extreme avondmensen. De extreme chronotypen zijn geassocieerd met het hebben van epilepsie, een depressie of bipolaire stoornis. De chronotypeverdeling binnen een groep migrainepatiënten was nooit eerder goed onderzocht. Uit het vragenlijstonderzoek onder 2.875 migrainepatiënten en 200 individuen zonder hoofdpijn bleek dat patiënten minder vaak een gemiddeld chronotype hadden maar juist vaker een ochtend- (odds ratio 2,42) of avondmens (odds ratio 1,69) waren, zonder verschillen tussen migrainepatiënten mét en

zonder aurasymptomen. Verder toonden we aan dat de patiënten meer vermoeid waren na veranderingen in het slaap –waakpatroon en ook meer rigide waren in het omgaan met deze veranderingen, vooral in geval van een hogere aanvalsfrequentie. Migraineaanvallen leken het vaakst in de vroege ochtend te beginnen bij de ochtendmensen en juist vaker later op de dag bij de avondmensen. Deze bevindingen wijzen op een andere instelling van de biologische klok bij migrainepatiënten.

Het rusteloze benen syndroom komt vaker voor en is ernstiger bij migrainepatiënten (hoofdstuk 4)

Het rusteloze benen syndroom (*restless legs syndrome; RLS*) kenmerkt zich door een urgent gevoel de benen te moeten bewegen in rust, vaak in combinatie met pijn of krampen, waarbij de klachten zich volgens een circadiaan ritme voordoen en afnemen bij daadwerkelijk bewegen. Eerder epidemiologisch onderzoek toonde dat RLS ongeveer twee maal zo vaak voorkomt in populaties van migrainepatiënten (11,4-17,7%) in vergelijking met de gehele Westerse populatie (5-10%). In case-control onderzoek werd eerder een viervoudig verhoogde prevalentie van RLS onder migrainepatiënten gerapporteerd. Uit ons vragenlijstonderzoek onder 2,875 migrainepatiënten en 347 gezonde personen zonder hoofdpijn bleek dat migrainepatiënten een grotere kans hadden op het hebben van RLS (odds ratio 1,83; gecorrigeerd voor vertekenende variabelen), waarbij patiënten met migraine mét aura een grotere kans hadden (odds ratio 1,99) dan patiënten met migraine zonder aura (odds ratio 1,74). Voorts bleek dat de ernst van RLS in de migrainegroep groter was dan in de controlegroep. De ernst van de RLS in de migrainegroep was positief geassocieerd met het aantal prodromale symptomen dat wordt verondersteld samen te hangen met disbalans in dopamine. De slaapkwaliteit en depressieve symptomen bleken slechter bij zowel het hebben van migraine als het hebben van RLS. Pathofysiologisch wordt RLS geassocieerd met dopaminerge disfunctie, met mogelijk een rol voor de hypothalamus. Behandeling van RLS bestaat uit dopaminerge medicatie, waarbij er enig bewijs is dat de migraine daarmee ook in frequentie en ernst leef af te nemen, mogelijk indirect veroorzaakt door verbetering van de dopaminerge functie of van de slaapkwaliteit. Of andersom de behandeling van migraine ook een positief effect heeft op eventueel bijkomend RLS is onbekend. Aanvullend onderzoek zou zich hierop kunnen richten, aangezien het waarschijnlijk is dat eenzelfde mechanisme bij beide ziekten een rol spelen.

Migrainepatiënten zijn niet gevoeliger voor het krijgen van hoofdpijn na een ruggenprik (post-punctionele hoofdpijn), terwijl stress mogelijk een beschermende werking biedt (hoofdstuk 5)

Wanneer om diagnostische of therapeutische redenen een ruggenprik (lumbaalpunctie) wordt verricht, is de kans op het krijgen van hoofdpijn na een ruggenprik, de zogenaamde post-punctionele hoofdpijn, 10-40%. Deze hoofdpijn kenmerkt zich door toename bij overeind komen, en verbetering bij gaan liggen. Pathofysiologisch wordt de hoofdpijn veroorzaakt door lekkage van hersenvocht uit de nog niet gedichte opening in het harde hersenvlies. Niet iedereen krijgt post-punctionele hoofdpijn. Naast technische factoren (naalddiameter, vorm van de naaldpunt, manier van prikken) bepalen ook persoonskarakteristieken (leeftijd, BMI, geslacht) de kans hierop. In de literatuur werd gesuggereerd dat migrainepatiënten een grote kans hadden op deze hoofdpijn maar

duidelijke cijfers ontbraken. Uit ons onderzoek onder 160 migrainepatiënten en 53 gezonde controles bleek echter dat de incidentie van deze hoofdpijn niet verschilde tussen beide groepen. Voorts vonden we geen relatie tussen de ruggenprik en het ontstaan van een nieuwe migraineaanval. Als nevenbevinding toonden we aan dat de ervaren stress voorafgaand aan de ingeplande ruggenprik juist een beschermend effect tegen het optreden van migraineaanvallen leek te hebben.

Migrainepatiënten hebben een afwijkende cardiovasculaire respons op intraveneuze toediening van nitroglycerine (hoofdstuk 6)

Resultaten uit eerdere onderzoeken suggereren dat bij migrainepatiënten de cardiovasculaire respons (de reactie van het vaatbed en de hartfunctie) op een vaatverwijdende trigger afwijkend is vergeleken met gezonde controlepersonen. Migraine en vasovagale syncope ('flauwvallen') komen namelijk regelmatig voor binnen dezelfde patiënten (zogenaamde co-morbiditeit). Ook kunnen zowel migraineaanvallen als syncope worden uitgelokt bij gevoelige individuen door toediening van nitroglycerine. In ons onderzoek werd nitroglycerine intraveneus toegediend aan zestien migrainepatiënten en tien migraine-vrije controlepersonen. Hieruit bleek dat met name hartslag, slagvolume en cardiac output verhoogd waren tijdens infusie van nitroglycerine bij de patiënten. Opvallend was dat deze respons tijdens de infusie groter bleek, wanneer de tijd tot een door nitroglycerine uitgelokte migraineaanval daarna korter was. Deze sterke systemische respons bij migrainepatiënten suggereert een verhoogde systemische gevoeligheid op deze vaatverwijder. Mogelijk wordt dit veroorzaakt door insufficiënte compensatiemechanismen vanuit het autonome zenuwstelsel, die deels onder invloed staan van aansturing vanuit de hypothalamus.

Deel II: Neuro-imaging onderzoek

De hypothalamus bij migrainepatiënten lijkt onverzadigd tijdens de prodromale fase (hoofdstuk 7)

Met behulp van 3 Tesla functional Magnetic Resonance Imaging (fMRI) toonden we aan dat de hypothalamus in vroege, pre-ictale fase van zowel met nitroglycerine uitgelokte als spontane migraineaanvallen een afwijkende respons op een orale glucosetrigger liet zien. Bij de migrainepatiënten werd niet de normale, persisterende afname in perfusie als gevolg van de glucose-ingestie gezien (die wel werd aangetoond bij de controlegroep) maar juist een normalisatie. Dit zou kunnen passen bij een afwijkend setpoint van die hypothalame glucosensitieve cellen die het hongergevoel reguleren, waarbij deze blijven 'vragen' om glucose. Klinisch zou dit bijvoorbeeld kunnen passen bij de door patiënten genoemde trek in specifieke voedingsmiddelen tijdens de prodromale fase.

Deel III: Biochemische veranderingen

Mannen met migraine hebben hogere oestradiolspiegels in het bloed (hoofdstuk 8)

Bij vrouwen met migraine was reeds bekend dat de oestrogeenconcentraties een modulerende rol hebben op migrainegevoeligheid en het ontstaan van nieuwe migraineaanvallen. Bij mannen met migraine echter waren concentraties van geslachtshormonen nooit eerder onderzocht. Middels prospectief onderzoek onder 17 mannelijke migrainepatiënten en 22 mannen zonder migraine toonden we een hogere serumconcentratie oestrogeen aan in de patiëntengroep tussen de migraineaanvallen door. Voorts bleek de testosteronconcentratie licht te stijgen tijdens de vroege, prodromale fase van de daaropvolgende spontane migraineaanval. We beschreven verder dat er, op basis van gevalideerde vragenlijsten, aanwijzingen zijn voor klinisch verminderde androgeenfunctie bij de mannen met migraine. De verhoogde oestrogeenconcentratie die we vonden in ons onderzoek verklaart wellicht de migrainegevoeligheid van mannelijke migrainepatiënten. De eventuele rol van oestrogeen bij ontstaan van nieuwe aanvallen verdient verder onderzoek.

Bij vrouwen met menstruele migraine is sprake van een verminderd trigeminale cycliciteit gedurende de menstruatiecyclus (hoofdstuk 9)

Aangezien veranderingen in de concentraties van oestrogenen bij vrouwen met menstruele migraine samenhangen met het optreden van migraineaanvallen, veronderstelden we dat de activiteit van het trigeminovasculaire systeem eveneens varieerde gedurende de menstruele cyclus. We toonden in een prospectief experimenteel onderzoek aan dat de normale cycliciteit van de doorbloedingsrespons van de huid op lokale toediening van capsaïcine verminderd was bij patiënten met menstruele migraine in vergelijking met de groep vrouwen zonder migraine en de groep post-menopauzale vrouwen. Tevens waren de oestradiolspiegels bij de patiënten lager. De doorbloedingsrespons op elektrostimulatie en de CGRP-concentraties in het speeksel verschilden niet tussen de groepen. Deze bevindingen tonen dat oestradiol het trigeminovasculaire systeem beïnvloedt en dat patiënten met menstruele migraine zowel een verminderde systemische als trigeminovasculaire cyclische activiteit hebben.

Capsaïcine-applicatie is een valide en betrouwbaar provocatiemodel om CGRP-secretie in het speeksel te meten (hoofdstuk 10)

Middels een experimenteel onderzoek bij gezonde vrijwilligers toonden we aan dat de lokale applicatie van een capsaïcine homogenaat (mengsel) op de tong leidt tot CGRP-secretie in het speeksel op een dosisafhankelijke manier. Omdat de speekselklieren worden geïnnerveerd door de vijfde hersenzenuw, konden we met speekselonderzoek aantonen dat de CGRP-concentratie in het speeksel na depletie met capsaïcine de activiteit van het trigeminale systeem weerspiegelt. Met dit experimentele model kan voor het eerst ook kwantitatief de CGRP-respons worden gemeten. Dit model zou van waarde kunnen zijn om pathofysiologische mechanismen op te helderen bij ziekten waarbij het trigeminale systeem aangedaan is.

Conclusies en aanbevelingen voor de toekomst

In de algemene discussie (hoofdstuk 11) worden onze bevindingen in een breder persprectief geplaatst. Onze onderzoeken tonen dat reeds tijdens de pre-ictale of prodromale fase klinische en biochemische veranderingen meetbaar zijn, die suggereren dat de migraineaanval meetbaar start voor de hoofdpijnfase. De resultaten in dit proefschrift wijzen er verder op dat de hypothalamus niet alleen vroeg in de migraineaanval een belangrijke rol speelt maar wellicht ook al interictaal een abnormale setting heeft.

Er zullen waarschijnlijk nog vele epidemiologische en experimentele onderzoeken volgen om de onderliggende pathofysiologische mechanismen bij migraine op te helderen. Zowel accurate diagnosestelling en de behoefte aan een objectieve en sensitieve biomarker (een in of aan het lichaam meetbare indicator) worden daarom steeds relevanter. Om een beter begrip te krijgen van deze complexe aandoening is een alomvattende aanpak in toekomstig onderzoek belangrijk. Bij voorkeur worden meerdere migraineaanvallen, van de prodromale fase via de hoofdpijnfase tot de hier niet besproken herstelfase, nauwkeurig onderzocht met observationele en experimentele methoden. Gezien de klinische heterogeniteit is een steekproef van homogene migrainepatiënten daarbij van belang. Meer inzicht in de pathofysiologie en het vinden van zogenaamde biomarkers zullen dan in de toekomst behulpzaam kunnen zijn bij het ontwikkelen van specifieke anti-migraine medicatie

Addendum.

List of abbreviations.

^{1}H-MRS	^{1}H Magnetic Resonance Spectroscopy
^{31}P-MRS	^{31}P Magnetic Resonance Spectroscopty
ADAM	Androgen Deficiency of Ageing Men questionnaire
AEBP	Autologous epidural blood patch
AF	Attack frequency
AMD	Ageing Males' Symptoms questionnaire
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AUC	Area under the curve
BMI	Body mass index
BOLD-signal	Blood Oxygen Level-Dependent signal
BP	Blood pressure
c^{18}F-FDG-PET	c^{18}F fluorodeoxyglucose Positron Emission Tomography
CES-D	Center for Epidemiologic Studies Depression Scale
CGRP	Calcitonin gene-related peptide
CI	Confidence interval
CK1-δ	Casein kinase 1-delta gene
Cr	Creatinine
CRLB	Cramér-Rao lower bound
CSD	Cortical spreading depression
CSF	Cerebrospinal fluid
CTI	Circadian type inventory questionnaire
DBF	Dermal blood flow
DPS	Dopaminergic Premonitory symptoms
E2	17β-oestradiol
EPI	Echo-planar imaging
ES	Electrical stimulation
FHM	Familial hemiplegic migraine
fMRI	Functional Magnetic Resonance Imaging
FOV	Field of view
FSH	Follicle stimulating hormone
GABA	Gamma-aminobutyric acid
GEE-model	Generalized Estimating Equations model
GEM study	Genetic Epidemiology of Migraine study
GP	General Practitioner
GWAS	Genome Wide Association Studies
$H_2{}^{15}O$-PET	$H_2{}^{15}O$ Positron Emission Tomography
HA	Headache
HADS	Hospital Anxiety and Depression Scale questionnaire
HADS-A	HADS anxiety subscale
HADS-D	HADS depression subscale
HR	Heart rate
ICHD	International Classification of Headache Disorders
ICHD-2	ICHD version 2
ICHD-3beta	ICHD version 3beta
HIS	International Headache Society
IRLSSG	International Restless Legs Syndrome Study Group

IU	Institutional Units
Lac	Lactate
LH	Luteinizing hormone
LP	Lumbar puncture
LR	Likelihood ratio
LUMC	Leiden University Medical Center
LUMINA	Leiden University Medical Center Migraine Neuro Analysis
MA	Migraine with aura
MCTQ	Münich Chronotype Questionnaire
MO	Migraine without aura
MRI	Magnetic Resonance Imaging
MRM	Menstrually related migraine
MRS	Magnetic Resonance Spectroscopy
MSF	Time of midsleep on free days
MSFsc	Time of midsleep on free days, corrected for sleep debt
NAA	N-acetyl-aspartate
NAANAAG	N-acetyl-α-L-aspartyl-L-glutamate
NO	Nitric Oxide
NPV	Negative Predictive Value
NRS	Numeric Rating Scale
NSA	Number of Signal Averages
OR	Odds Ratio
PACAP	Pituitary adenylate cyclase-activating polypeptide
PAG	Peri Aqueductal Grey matter
PCh	Choline
PDPH	Post-Dural Puncture Headache
PET	Positron Emission Tomography
PORH	Post-occlusive reactive hyperaemia
PPV	Positive Predictive Value
PS	Premonitory symptoms
PSQI	Pittsburgh Sleep Quality Index
rCBF	regional Cerebral Blood Flow
RLS	Restless Legs Syndrome
ROC	Receiver Operator Curve
SD	Standard deviation
SE	Standard error
SHBG	Sex Hormone-Binding Globulin
SPSS	Statistical Package for the Social Sciences
TCC	Trigeminocervical complex
TE	Echo time
T_f	Free, unbound testosterone
TGVS	Trigeminovascular system
TR	Repetition time
TRPV1	Transient receptor potential cation channel subfamily V member 1
VAS	Visual Analogue Scale
VOI	Voxel of Interest

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Curriculum vitae.

Willebrordus Petrus Johannes van Oosterhout (given name: Ron) was born August 23, 1982 in Made en Drimmelen, the Netherlands. After graduating from the Sint-Oelbert grammar school in Oosterhout in 2000, he started medical school at the Leiden University Medical Center (LUMC). In 2002-2003 he was treasurer and board member of the faculty student board (Medische Faculteit der Leidse Studenten). From 2002 to 2005 he served as a board member and in 2005 president of the KNMG Studentenplatform (junior board within the Dutch Royal College of Physicians) and board member of the KNMG Federatiebestuur. During his medical studies, he performed a scientific internship on structural brain imaging in migraine and cluster headache in the department of Radiology. Furthermore, he participated in the LUMC Honours Class 'Cognition or how the Mind works' under supervision from prof.dr. M.A. van Buchem and prof.dr. E. Klasen in 2005. He obtained his medical degree in 2008 after finishing the clinical rotations.

From 2008 to 2012 he worked as a PhD candidate at the Department of Neurology at the LUMC, in collaboration with the Department of Radiology, supervised by prof.dr. M.D. Ferrari (neurologist), dr. M.C. Kruit (radiologist) and dr. G.G Schoonman (neurologist). From 2013 to 2017 he was a resident in Neurology at the LUMC supervised first by prof.dr. R.A.C. Roos and later by prof.dr. J.J. van Hilten. Additionally, he has been active within the International Headache Society as board member of the Trainees' and Residents' Special Interest Group, member of the programme committee of the 2017 International Headache Conference in Vancouver and the Newsletter Editorial Board. In 2016-2017 he participated in the national Residents' Talent Class Programme. In 2018 he started working as a qualified neurologist at the OLVG hospital in Amsterdam and the Zaans Medisch Centrum in Zaandam (ZMC) with a current staff position in the ZMC.