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Investigation of calcitonin-gene related peptide levels in tear fluid during nitroglycerin induced migraine attacks

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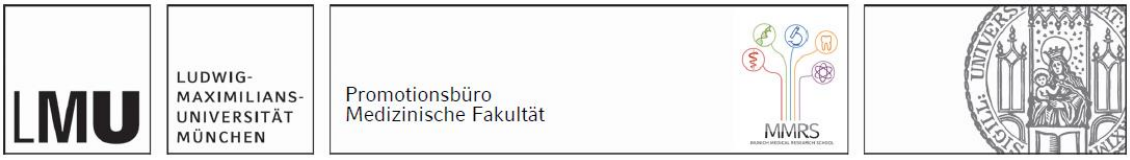

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Table of Contents

Abstract in German (Zusammenfassung)

Migräne ist eine primäre Kopfschmerzerkrankung, die sich durch wiederholte, häufig einseitige, schwere Kopfschmerzen über 4-72h äußert. Charakteristisch ist ein pochender Schmerz mit Photophobie, Phonophobie und/oder Übelkeit als Begleitsymptomatik. Trotz hoher Prävalenz in der Bevölkerung gibt es bisher keine in der Klinik etablierten diagnostischen Biomarker für Migräne. In der Pathophysiologie der Erkrankung spielt Calcitonin Gene-related Peptide (CGRP) eine entscheidende Rolle. CGRP ist ein multifunktionelles Neuropeptid, bestehend aus 37 Aminosäuren. Auch der Trigeminusnerv, welcher das Gesicht und die Meningen sensibel innerviert, spielt in der Entstehung von Migränekopfschmerzen eine wichtige Rolle. Während einer Migräneattacke wird CGRP von trigeminalen Nervenfasern freigesetzt, woraufhin es zu einer neurogenen Inflammationsreaktion und einer Aktivierung und Sensibilisierung nozizeptiver Trigeminusafferenzen kommt. Mehrere Studien haben bereits gezeigt, dass Plasma CGRP-Spiegel im Rahmen von Migräneattacken erhöht sind, jedoch ist die Datenlage hierzu uneinheitlich. CGRP wird während der Migräneattacke direkt von trigeminalen Fasern freigesetzt wird, welche auch die Kornea innervieren. Tatsächlich konnten Vorarbeiten bereits zeigen, dass das Neuropeptid in der Tränenflüssigkeit bei Migränepatient/-innen im Vergleich zu Kontrollproband/-innen interiktal und iktal erhöht ist.

In der hier vorliegenden Studie wurde die Freisetzung von CGRP in der Tränenflüssigkeit und im Blut von Migränepatient/-innen im Verlauf eines experimentell induzierten Kopfschmerzes untersucht. Die Induktion der Migränekopfschmerzen erfolgte mittels Glyceroltrinitrat (GTN), einem etablierten Modell zur Auslösung migränetypischer Kopfschmerzen. Außerdem wurden die CGRP-Konzentrationen in der Tränenflüssigkeit von interiktalen Migränepatient/-innen und gesunden Kontrollproband/-innen gemessen.

Ziel des Projektes war es, iktale und interiktale CGRP-Konzentrationen zu vergleichen und die CGRP-Konzentration im Verlauf einer experimentell induzierten Kopfschmerzattacke zu untersuchen.

Die CGRP-Konzentrationen in der Tränenflüssigkeit waren bei iktalen Migränepatient/-innen signifikant höher (n = 17; 1.88 ± 1.69 ng/ml) als bei gesunden Kontrollproband/-innen (n = 32; 0.90 ± 0.69 ng/mL). Bei Auftreten eines Kopfschmerzes nach GTN-Gabe, waren die CGRP-Konzentrationen in der Tränenflüssigkeit ebenfalls signifikant höher als interiktal. Es konnte ein Anstieg von 0.80 ± 0.77 ng/mL vor GTN-Gabe auf 1.42 ± 0.93 ng/mL bei Kopfschmerzen gezeigt werden. Nach Besserung der Kopfschmerzen zeigte sich ein signifikanter Abfall der CGRP-Konzentrationen in der Tränenflüssigkeit auf 1.10 ± 0.99 ng/mL. Im Blut konnten keine signifikanten Veränderungen der CGRP-Spiegel nachgewiesen werden.

Diese Ergebnisse können als weitere Validierung der Messung von CGRP in der Tränenflüssigkeit gewertet werden und unterstützen die Eignung von CGRP in der Tränenflüssigkeit als potenziellen Biomarker für Migräne.

Abstract

Migraine is a primary headache disorder. Its clinical presentation is characterised by recurring, unilateral headache attacks of moderate to severe intensity that last for 4-72h. The pain is usually described as pulsating or throbbing and is accompanied by photophobia, phonophobia and/or nausea. Despite its high prevalence, there are currently no biomarkers available for migraine diagnostics. Calcitonin Gene-related Peptide (CGRP) is one of the key players in migraine pathophysiology. CGRP is a multifunctional neuropeptide, consisting of 37 amino acids. Furthermore, the trigeminal nerve, which is responsible for the sensory innervation of the face and meninges, also plays an important role in migraine pathophysiology. During migraine attacks, CGRP is released by trigeminal nerve fibres and leads to neurogenic inflammation as well as activation and sensitisation of nociceptive trigeminal afferents. Many studies have already shown that plasma CGRP levels are elevated during acute migraine attacks. However, data on plasma CGRP levels in migraine patients is not congruent. During migraine attacks, CGRP is directly released from trigeminal fibres, which also innervate the cornea; previous work has shown that tear fluid CGRP levels in interictal and ictal migraineurs are higher than in healthy controls, suggesting that tear fluid CGRP levels directly reflect trigeminal activation.

In this study, we examined the release of CGRP into tear fluid and blood in migraineurs over the course of experimentally induced headache. For the induction of migraine attacks, glycerol-trinitrate (GTN) was used. Intravenous GTN administration is an established model for the induction of migraine-like headache in migraine patients. Furthermore, tear fluid CGRP levels were analysed in interictal migraineurs and healthy controls.

The goal of this study was to compare ictal and interictal tear fluid CGRP levels as well as to investigate CGRP levels in tear fluid over the course of GTN induced migraine headache.

Tear fluid CGRP levels were significantly higher in ictal migraineurs (n = 17; 1.88 ± 1.69 ng/ml) compared to healthy controls (m = 32; 0.90 ± 0.69 ng/mL). Tear fluid CGRP were also significantly higher during GTN induced headache than interictally; they rose from 0.80 ± 0.77 ng/mL at baseline to 1.42 ± 0.93 ng/mL during headache. After improvement of headache, tear fluid CGRP levels dropped significantly by 1.10 ± 0.99 ng/mL. Plasma CGRP levels showed no significant changes.

Our findings can be considered further validation of CGRP measurement in tear fluid and back CGRP in tear fluid as a potential biomarker in migraine.

1. Introduction

Migraine is a complex neurological and disabling primary headache disorder that affects over 1 billion people worldwide, causing estimated direct and indirect costs of around 100 billion euros per year in Europe alone. [1, 2] Clinically, it is characterised by recurring severe headaches accompanied by a variety of both neurological as well as systemic non-headache symptoms. [3] Often entailing a lack of participation in various areas of both personal and professional life, migraine can take a serious toll on the physical and mental health of people affected. [4] Even though the diagnostic criteria are clearly defined in the International Classification of Headache Disorders [3], the individual experience can be hard to convey, which is reflected by the following quote from the British author Virginia Woolf, who – like many others – is said to have been afflicted by migraine attacks [5]: “English, which can express the thoughts of Hamlet and the tragedy of Lear, has no words for the shiver and the headache […] let a sufferer try to describe a pain in his head to a doctor and language at once runs dry.” [6]

1.1 Diagnostic criteria

The International Classification of Headache Disorders (ICHD-3) classifies primary headache disorders – which, in contrast to secondary headache disorders, are not caused by an underlying condition – into four main groups: tension-type headache, migraine, trigeminal autonomic cephalalgias and other primary headache disorders. Migraine as a disorder can be subdivided into two main types: migraine without aura and migraine with aura. [3, 7]

Migraine is characterised by the presence of recurring headaches lasting between 4-72 hours in adults. Headaches must meet at least 2 of the following criteria: unilaterality, moderate or severe pain intensity, pulsating quality, and pain exacerbation through physical activity. Furthermore, headache must be accompanied by non-headache symptoms like nausea or photophobia and phonophobia (see Table 1, chapter 2.1.2). [3]

Migraine with typical aura is further characterised by a set of fully reversible symptoms occurring before or during the headache. Aura symptoms are numerous and can manifest through visual, sensory or speech disturbances, motor weakness or brainstem dysfunction. [3] Visual aura symptoms are by far most frequently reported, followed by sensory, aphasic and motor symptoms. [8] The most common manifestation of visual aura symptoms are bright dots or flashing lights, wavy lines, blind spots (scotoma) and tunnel vision. [9]

Migraine can also be categorised using headache frequency. Patients with episodic migraine experience < 14 headache days per month. Occurrence of headache on ≥ 15 days per month, with at least 8 days meeting ICHD-3 migraine criteria, is defined as chronic migraine. [3]

1.2 Epidemiology

Due to its high prevalence and because of the strain it puts on those affected, migraine is a major public health problem as well as a financial burden for countries all around the world and has been reported to be one of the leading causes of disability worldwide. [1, 4]

In 2015, the age-adjusted 3-month prevalence for migraine in the United States (US) was 15.3%, meaning that approximately 1 in 6 US Americans had experienced migraine or severe headache over the last 3 months. [10] Another review summarising data regarding migraine prevalence in Europe, showed that among 170.000 adult Europeans, the mean prevalence of migraine was 14.7% (17.6% in women, 8% in men). [11] In Germany, migraine prevalence in the general adult population in 2004 was 10.6%. [12] Migraine is most frequent in people aged 18 to 44, its prevalence decreasing with age and women are more often affected than men. [10]

Every year, roughly 2.5% of patients with episodic migraine develop chronic migraine. [13] Chronic migraine has an overall prevalence of 0.9% to 5.1% in the general population. [14]

Of all diseases and injuries analysed by the Global Burden of Disease (GBD) Study 2017, headache disorders had the second highest prevalence among both women and men. [15] Neurological disorders were ranked as the leading cause of disability-adjusted life years (DALYs), even though headache disorders have no direct impact on mortality. Migraine was ranked as the second leading cause of DALYs among neurological disorders. [16]

A German study found that, even though the one-year prevalence (in 2004) for headache (60.2%) was much higher than for migraine (10.6%), migraineurs more frequently reported headache-associated disability as well as more frequent medication use than patients with other types of headaches. However, less than half of migraine patients interviewed had consulted a physician for headaches in the past year and were using over-the-counter medication only. [12] Albeit migraine is very prevalent in the general population, these figures show that many people suffering from migraine do not seek medical help and thus might not get adequate treatment.

1.3 Clinical phenotype

The typical migraine attack can be divided into 4 stages: premonitory phase, aura, headache, and postdrome phase. [17]

Premonitory phase

Premonitory symptoms are symptoms that occur before the headache phase of a migraine attack (within 72h before) and are experienced by 7-88% of migraine patients. [17, 18] The most common symptoms are tiredness, concentration difficulties or neck

stiffness. By identifying their respective typical premonitory symptoms, some patients can even predict the onset of a migraine attack. [19]

Aura symptoms (as described in chapter 1.1) often occur before the onset of headache. An overlap of aura and headache phases is also commonly observed. [17, 20]

Headache phase

As previously described, the characteristics of migraine headache are defined by ICHD-3 (see Table 1). Headache quality is mostly described as throbbing (73.5%), aching (73.8%), pressing (75.4%) and stabbing (42.6%). [21] Headache pain is most often unilateral, unilaterality being most frequent in episodic migraine. It has also been established that headache pain during migraine attacks is most frequently located in eye or orbital (85.5%), frontal (79.8%), and temporal (78.7%) regions. [22] Non-headache symptoms, such as photophobia, phonophobia, osmophobia, and/or nausea, also occur during the headache phase. [3, 20]

Postdrome phase

Around 80% of migraine patients report non-headache symptoms after improvement of headache (postdrome). Symptoms observed in the postdrome phase are similar to those reported in the premonitory phase, the most common ones also being tiredness, concentration difficulties and neck stiffness. [23] It is therefore unclear if postdrome symptoms begin during or after headache or if they might be premonitory symptoms persisting past the headache phase. [17]

1.4 Migraine treatment

Migraine treatment is categorised into acute and preventive treatment. Acute medication is used to treat headache or nausea during a migraine attack. Preventive medications are drugs, most of which are not specific for migraine, that are taken regularly to reduce migraine frequency. [24] Alongside pharmacological treatment options, there are also different non-pharmacological approaches, such as different relaxation techniques (meditation or autogenic training), psychotherapy (cognitive-behavioural therapy) or biofeedback training which can help patients manage migraine frequency. [25]

1.4.1 Acute treatment

Acute migraine treatment can be classified into non-specific and specific drugs. Non-steroidal anti-inflammatory drugs (NSAIDs) and other analgesics like paracetamol or metamizole are considered non-specific, whereas triptans are disease-specific drugs in migraine treatment. [26] If monotherapy is insufficient, there are different options to combine different substances. [27]

For patients suffering from nausea during migraine attacks, the additional intake of prokinetics or anti-emetics can be effective. Oral metoclopramide and domperidone are recommended in the official migraine treatment guidelines. [24]

NSAIDs and analgesics

NSAIDs are suggested as a first line treatment option for a migraine attack. Paracetamol (acetaminophen) or metamizole are recommended as an alternative acute treatment if there are contraindications for NSAID intake. [24]

Triptans

Triptans are serotonin 5-$HT_{1B/1D}$ receptor agonists and the gold standard in treating acute migraine. They were initially thought to be effective in acute migraine treatment through their vasoconstrictive properties. However, more recent studies suggest that triptans act on both vascular and neuronal 5-HT receptors (B and D). [28] Thus, they do not only induce vasoconstriction but also lower calcitonin gene-related peptide (CGRP) levels and thereby alleviate migraine headaches (see chapter 1.6.3 for further explanations on the role of CGRP in migraine pathophysiology). [29]

Triptans are recommended if migraine attacks do not respond to NSAIDs and analgesics. [24] Triptans are highly effective regarding pain relief, more so than NSAIDs, acetylsalicylic acid (ASA) or acetaminophen. [30] Triptans can also be combined with NSAIDs (e.g. sumatriptan/naproxen) as a treatment option for recurrent headaches. [24]

1.4.2 Preventive treatment

The official treatment guidelines state that preventive treatment should be considered for patients suffering from more than 3 attacks per month that severely impact quality of life, last for more than 72 hours or do not respond to acute treatment options. It is also recommended for patients who experience side effects when using acute medication or whose intake of acute medication exceeds a frequency of 10 days per month. Preventive treatment is considered successful if attack frequency is reduced by at least 50%. [24]

Preventive treatment plays an important role in reducing the burden and disability imposed by high-frequency recurring headaches. A study from the US found that for more than 25% of migraineurs, preventive therapy could be considered. [31]

Recommended substances are beta blockers (propranolol, metoprolol), the calcium channel blocker flunarizine, anticonvulsive drugs (topiramate), antidepressants (amitriptyline) and CGRP antibodies (see chapter 1.5). Botulinum toxin type A injections can also be considered a preventive treatment in patients with chronic migraine. [24]

1.5 CGRP as a target for migraine treatment

For a long time, NSAIDs and triptans have been the main treatment options for acute migraine attacks and preventive treatments have been lacking disorder-specificity. Thus, there was a real need for the development of novel migraine-specific drugs. [32]

With the ever-growing body of evidence substantiating the importance of calcitonin gene-related peptide (CGRP) in migraine pathophysiology (see chapter 1.6), new drugs

targeting either the CGRP receptor or ligand with the goal of developing more specific migraine treatments.

CGRP ligand and receptor monoclonal antibodies (mABs)

The recent development of antibodies targeting either the CGRP receptor (Erenumab) or ligand (Galcanezumab, Framenezumab, Eptinezumab) has opened new doors in the preventive treatment of episodic and chronic migraine. [33] Large clinical trials have been conducted on all 4 available compounds – Erenumab [34-36], Galcanezumab [37], Framenezumab [38] and Eptinezumab [39] – and have shown a significant reduction in headache and acute medication use frequency. CGRP antibodies are considered safe and well tolerated by patients, as only minor adverse effects have been observed. [32]

As all other preventive migraine treatments were initially developed and approved for other diseases (see above), CGRP ligand and receptor mABs are the first preventive migraine treatment to be specifically developed to target a key pathway in migraine pathophysiology which might make them more effective than other migraine prevention therapeutics. [40]

CGRP receptor mABs are large molecules that cannot cross the blood-brain barrier (BBB) in a larger percentage. Thus, it is discussed that their site of action must be peripheral and that they most likely target receptors located in the trigeminal system. [41] Their effectiveness as preventive migraine treatments can be seen as evidence to support the important role of CGRP found in the trigeminal system in migraine pathophysiology.

Gepants

Gepants are small-molecule CGRP receptor antagonists that have been developed specifically for the treatment of acute migraine attacks. Gepants directly target CGRP receptors to relieve headache pain. [42] A total of 6 gepants has been developed and investigated in different pre-clinical and clinical trials: the efficacy of ubrogepant and rimegepant have been analysed in large clinical trials and were shown to both safely and effectively treat acute migraine attacks. [43-45] Ubrogepant was the first oral CGRP receptor antagonist to be approved by the US Food & Drug Administration (FDA) for use in acute migraine treatment. [46] Meanwhile, rimegepant can be used for both acute as well as prophylactic treatment of migraine. [45, 47]

1.6 Pathophysiology

Migraine is a complex neurological disorder with a genetic background, which can be triggered by different external factors, leading to activation of the hypothalamus and subsequently the trigeminal system, where CGRP is released by trigeminal fibres. [48, 49]

Numerous factors play a role in the initiation of migraine attacks. While there is a genetic component to the disease, different external triggering factors are also being discussed (such as fasting, stress or menstruation). [49-51] Migraineurs can show increased

cortical responsiveness to these external stimuli due to a disbalance between different excitatory and inhibitory neural circuits. This disbalance makes migraine patients more vulnerable to aforementioned triggering factors. [48, 49, 52]

Functional imaging studies have shown the involvement of the hypothalamus in the premonitory phase, suggesting its involvement in the early stages of migraine attacks and its amplifying role in pain transmission. [53, 54] Activation of the dorsal pons as well as the periaqueductal grey – which play an important part in regulating sensory stimuli as well as in modulation of nociception – has also been observed. [48, 55] Activation of these two regions could be the pathophysiological correlate of non-headache symptoms such as phono- or photophobia. It might also disinhibit trigeminal nociception. [48] Furthermore, functional coupling between the hypothalamus and spinal trigeminal nuclei is altered before and during the headache phase of migraine attacks. [54]

Whereas the prodromal symptoms are initiated centrally, the headache phase is induced by the activation of meningeal nociceptors and trigeminal pathways. [48, 56] The exact mechanisms that trigger this trigeminal activation and thereby the transition from premonitory to headache phase of the migraine attack remain unknown. [56] For example, cortical spreading depression (CSD) – a wave of neuronal depolarisation that propagates slowly across the cortex and is likely to be at the origin of visual aura symptoms – has been suggested as an initiator. [17, 57, 58] It has recently been shown that CSD can activate neurons in the trigeminovascular system (TVS), meaning that CSD may cause activation of meningeal nociceptors in the first place. [59]

1.6.1 The trigeminal system

The trigeminal system plays a key role in the development of migraine headache. [60]

The trigeminal nerve, consisting of three branches V1-V3 (ophthalmic, maxillary and mandibular) is responsible for the sensory innervation of the face and meninges. The mandibular branch (V3) also has a motor component, innervating the masticatory muscles. The soma of the sensory fibres of all three branches are found in the trigeminal ganglion. [61] These fibres can activate higher-level neurons located in the trigeminocervical complex (TCC), which consists of the trigeminal nucleus caudalis (TNC) and the cervical segments C1 and C2. Neurons from the TNC project to different thalamic, hypothalamic and brain stem nuclei, which in turn project to sensory cortices. [62]

The TVS consists of the trigeminal neurons, specifically C- and Aδ-fibres, which innervate the meningeal blood vessels and the cerebral arteries. Activation of these neurons is very likely to play a role in migraine headache as it leads to the secretion of different neuropeptides such as calcitonin gene related peptide (CGRP) and Substance P (SP), thereby inducing neurogenic inflammation. [63, 64]

1.6.2 Neurogenic inflammation

Neurogenic inflammation is an inflammatory process that consists of vasodilation, plasma protein extravasation (PPE), mast cell activation and degranulation, thereby releasing inflammatory mediators, activating meningeal nociceptors, and sensitising trigeminal afferents. [64, 65] This activation – as described above – is hypothesised to play a key role during the headache phase of a migraine attack. [66]

Edvinsson and colleagues have postulated that inflammation plays a key role in the peripheral nervous system, especially in the trigeminal ganglion, thereby possibly leading to peripheral sensitisation and chronification of headaches. [67]

Furthermore, sensitisation of peripheral trigeminal afferent sensory fibres might result in central sensitisation and repeated activation of downstream neurons in the trigeminal pain pathway, thereby potentially causing persistent headaches and the progression from episodic to chronic migraine. [48]

However, the preceding initiation of the neuroinflammatory process remains unclear. Recent studies have shown that evidence for PPE taking place during migraine attacks in humans is rather scarce [67], especially in relation to findings on the role of Substance P (see sub-chapter 1.6.5). This led to the assumption that neurogenic inflammation is probably not at the origin of migraine attacks and that there must be other triggers. However, as mentioned previously, CSD has been discussed as a potential activator of meningeal nociceptors in the first place. [17, 59]

As neuropeptides play an important role as inflammatory molecules during neurogenic inflammation and in the TVS, understanding the role of different **neurotransmitters and neuromodulators** has become a focus of migraine research. The research on calcitonin gene-related peptide (CGRP) has turned out particularly promising and the findings on its role in migraine pathophysiology have drawn a lot of attention.

1.6.3 CGRP and its role in migraine pathophysiology

Calcitonin gene-related peptide (CGRP) is a neuropeptide consisting of 37 amino acids and exists in two isoforms: αCGRP and βCGRP. αCGRP is derived from the calcitonin gene (CALC I gene) through alternative splicing and is mostly found in neurons of the central and peripheral nervous system. [68] βCGRP is derived from a second calcitonin gene (CALC II gene), located on chromosome 11, and is mainly found in the enteric nervous system. Their structure differs by only 3 amino acids. [69]

CGRP receptors are G protein-coupled receptors (GPCRs) formed by a calcitonin receptor-like receptor and a receptor activity-modifying protein (RAMP). [70]

Overall, CGRP has many different functions in the human body. For example, it is the most powerful microvascular vasodilator, which implicates its importance in the regulation of blood pressure. [69, 71] CGRP has also been suggested as a protective factor against hypertension and vascular disease. [69] CGRP induced vasodilation also leads to increased tissue blood flow, thereby facilitating wound healing. Enhanced blood

flow also plays an important role in neuroinflammation, which is one key mechanism of migraine pathophysiology. [66, 69]

Regarding the role of CGRP in migraine pathophysiology, it was already shown in 1984, that CGRP is produced in the trigeminal ganglia [72], where it is released by cell bodies of sensory C fibres. In the trigeminal ganglia, CGRP receptors are expressed by myelinated Aδ fibres and in satellite glial cells (SGCs). [73] CGRP receptors are also found in smooth muscle of blood vessels and on rodent mast cells. [74] The action of CGRP on Aδ fibres and SGCs is likely to cause migraine pain. CGRP directly activates the Aδ-fibres in the trigeminal ganglion. [75] Aδ fibres then project to the TCC, specifically the TNC and from there to different thalamic, hypothalamic and brain stem nuclei, which in turn project to sensory cortices. [62]

As the hypothalamus shows altered connections to trigeminal nuclei which are activated leading up to the migraine attack [54] and as trigeminal stimulation induces the release of CGRP [76], the following theory has been postulated: the TNC activates trigeminal ganglia, leading to the release of CGRP, which in turn stimulates Aδ fibres, thereby generating headache pain. [73]

Furthermore, CGRP seems to play a key role in the peripheral sensitisation taking place in trigeminal afferent neurons as mentioned above. [67] The continuous activation of Aδ fibres and SGCs through CGRP might lead to increased production of inflammatory cytokines in these cells and inflammation in the trigeminal ganglia. [67] A recent study showed that intra-ganglionic injection of CGRP in rats leads to increased neuronal and SGC activity, accompanied by heightened levels of cytokines. These findings suggest the implication of GCRP in the genesis of pain and at the same time highlight the role of SGCs. [77] This could lead to the conclusion that SGCs are stimulated by CGRP and as a result secrete inflammatory mediators. Thereby, they most likely activate neuronal afferent fibres. This theory can be seen as evidence for the theory of CGRP induced neuron-glia cross-excitation. [78]

Lastly, mast cell degranulation – as a part of neurogenic inflammation – has often been discussed as a potential cause of migraine attacks. Even though rodent mast cells express CGRP receptors, human mast cells do not. [74] Thus, CGRP induced mast cell degranulation probably does not play a role in migraine pathophysiology. The question of whether mast cell degranulation plays a different part in the development of a migraine attack remains unanswered. Normally, substance P is key to the induction of mast cell degranulation [79] but studies measuring plasma concentrations of substance P during acute migraine attacks have repeatedly shown negative results. [80]

1.6.4 Detection of CGRP in migraine patients

CGRP levels have been measured in different states in migraine patients. Firstly, CGRP was mostly analysed in the ictal state – meaning that patients had a migraine headache during time of detection. Consequently, CGRP levels have also been measured in the interictal state – meaning in a headache-free period in between migraine attacks. [81]

Ictal CGRP levels have been investigated in both spontaneous migraine attacks as well as during experimentally induced migraine attacks using glyceryl-trinitrate (GTN) (for the GTN model, see chapter 1.7). During spontaneous migraine attacks, elevated CGRP levels have been measured in both internal and external jugular blood in two independent studies. [80, 82] Furthermore, some studies have also found elevated plasma CGRP levels in peripheral blood during spontaneous and GTN induced acute migraine attacks. [83-86] However, these findings have been refuted by a recent study, which did not find a significant difference in ictal versus interictal CGRP levels (spontaneous attacks), neither in external jugular nor peripheral venous blood. [87] Goadsby et al. (who saw elevated CGRP levels in central venous blood) did not find a change in plasma CGRP levels from peripheral venous blood either. [80] Apart from measuring CGRP in plasma, levels have also been determined in saliva and were elevated during spontaneous migraine attacks. [88] Elevated CGRP levels were also observed in tear fluid of ictal migraineurs. [89] Overall, current data suggest that CGRP levels are elevated in different bodily fluids during the acute migraine attack.

On the other hand, the investigation of CGRP levels in **interictal** migraineurs has yielded heterogeneous results. Gallai et al., who observed elevated CGRP levels in peripheral venous blood from ictal migraineurs, found that interictal CGRP levels did not differ significantly from the control group. [84] In contrast, Ashina et al. detected elevated CGRP in peripheral blood in the interictal state. [90] Additionally, another study also found interictally elevated CGRP levels in blood samples drawn from the forearm. Results showed that interictal CGRP levels were higher in migraineurs than in the control group. [83] Furthermore, a study by Cernuda-Morollón and al. raised the question of whether plasma CGRP in interictal migraine patients could serve as a biomarker for chronic migraine. They found that CGRP levels were significantly higher in chronic migraine (74.90 pg/mL) than in patients with episodic migraine (46.37 pg/mL) and healthy controls (33.74 pg/mL). [91] Considering that samples were taken in the interictal state, these findings might further support the theory of CGRP playing a role in sensitisation and chronification. Lastly, interictally elevated CGRP levels have also been reported in tear fluid. [89]

The **effect of acute medication**, specifically triptans, on CGRP levels has also been investigated in different studies. In patients whose acute migraine attack was successfully treated with subcutaneous application of sumatriptan, a reduction of plasma CGRP levels from the external jugular vein has been observed. [29] The effect of triptans on CGRP levels in GTN induced migraine has also been studied. Juhasz et al. observed a significant drop in CGRP levels in blood drawn from the antecubital vein after successful treatment of migraine attacks with sumatriptan nasal spray. [86] Furthermore, Cady et al. not only found that salivary CGRP levels dropped after intake of rizatriptan, but elevated salivary CGRP levels could even predict the response to acute therapy with rizatriptan. [88]

1.6.5 Overview of other neuropeptides

Apart from CGRP, many other neuropeptides have been investigated in connection with the trigeminovascular system regarding migraine pathophysiology. Some examples are substance P (SP), vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP) and neuropeptide Y (NPY). [92, 93]

The following paragraphs will contain a short overview of the research done on SP, VIP and PACAP and their potential role as biomarkers for migraine.

Substance P (SP)

SP, like CGRP, is a sensory neuropeptide and plays an important role in nociceptive transmission. Upon noxious stimulation, SP is released from afferent trigeminal fibres [94, 95] and leads to neurogenic inflammation, which was thought to generate migraine attacks. As previously mentioned, a growing body of evidence has emerged which refutes this theory. For example, Goadsby et al. did not report any changes in SP levels during migraine attacks, neither in external jugular nor in cubital fossa venous blood. [80] Data is however controversial, as peripherally increased SP levels have been found in interictal migraineurs. [96]

Parasympathetic neuropeptides

VIP and PACAP are parasympathetic neuropeptides and functionally related. [97] They also act on G protein-coupled receptors (GPCRs): PAC1, VPAC1, and VPAC2. Whereas PAC1 is specific to PACAP, VPAC1 and VPAC2 receptors are highly affine to both VIP and PACAP. [98] Both have been investigated in relation to their role in migraine pathogenesis, which will be discussed further in the following paragraphs.

Vasoactive intestinal peptide

VIP is a peptide consisting of 28 amino acid residues and plays an important role as a parasympathetic neuropeptide. As described above, many migraine patients display parasympathetic symptoms like nausea or vomiting. Furthermore, cranial parasympathetic symptoms such as conjunctival injection, rhinorrhoea, nasal congestion, or lacrimation are also described. [99-101] This implicates the involvement of cranial parasympathetic fibres, which are found in the facial nerve and mostly derive from the sphenopalatine and the otic ganglia, in migraine pathophysiology. [93] VIP is widely found in both the sphenopalatine and otic ganglia, suggesting that VIP plays a key role in the cranial autonomic parasympathetic symptoms (CAPS) in migraine. [102] In line with this hypothesis, Goadsby et al. found that migraineurs presenting cranial autonomic symptoms showed elevated plasma VIP levels in external jugular blood. [80]

Pituitary adenylate cyclase-activating peptide

PACAP is a peptide existing in two isoforms (PACAP-27 and PACAP-38). [103] They are widely expressed throughout different tissues in the human body. PACAP-38 consists of 38 amino acids and is the predominant isoform in the nervous system. [97]

PACAP has been detected in trigeminal ganglia in rats [104] where it colocalises with CGRP and SP. [105] PACAP has also been detected in other migraine-related areas, such as the TNC or cerebral vessels. [106, 107] Furthermore, PACAP-38 has been shown to induce CGRP release in the TNC, but not in trigeminal ganglia. [107]

PACAP levels measured in both external jugular as well as peripheral venous blood of migraine patients were elevated during headache. [108, 109] Furthermore, it has been shown that the administration of intravenous PACAP can introduce migraine headaches in around 70% of migraineurs. [110]

1.7 GTN triggered migraine attack

To study the underlying pathophysiology of migraine attacks, different human experimental models to induce migraine headache have been developed. The infusion of glyceryl-trinitrate (GTN) has long been established as a robust experimental translational model for the induction and assessment of acute migraine attacks. The intravenous administration of GTN (0.5 µg/kg/min for 20 minutes) induces a mild immediate headache in both healthy controls and migraine patients. However, unlike healthy controls, up to 80% of migraineurs experience a delayed migraine-like headache that fulfils the IHS (International Headache Society) diagnostic criteria for migraine without aura [111, 112] and can be effectively treated with sumatriptan. [113]

GTN acts intracellularly in blood vessel walls by forming nitric oxide (NO). NO is released from endothelial cells and activates soluble guanylate cyclase (sGC) in smooth muscle cells of blood vessels, thus inducing an increase of intracellular cyclic guanosine monophosphate (cGMP). Via this pathway, NO causes vasodilation. [114] Initially, this mechanism was hypothesised to be the triggering factor of a GTN induced migraine because GTN acutely causes a mild headache and extracerebral vasodilation. [115] However, it was shown that the delayed migraine-like headache is not always accompanied by vasodilation [116], thereby underlining the fact that the vascular hypothesis is not sufficient to explain the pathogenesis of migraine headache.

Furthermore, Juhasz et al. have shown that CGRP levels increase significantly during GTN induced migraine attacks. CGRP concentrations also correlated with the intensity of the migraine attack and dropped after alleviation of headache. [85]

Considering that GTN only induces aura phenomena in less than 15% of patients suffering from migraine with aura, the model is mainly used to study the headache phase of a migraine attack. [112] It has therefore been suggested that GTN (via formation of NO) might only be involved in the headache phase of the migraine. [114]

As there is a latency of 4-6h [117] between GTN administration and the onset of migraine headache, NO is likely to trigger a slow pathway leading to a migraine-like headache, probably even on a gene transcription level. Activating the trigeminal system and inducing the transcription of several proinflammatory mediators, NO indirectly leads to

increased CGRP levels and thereby triggers migraine-like headaches by stimulating trigeminal Aδ fibres, SGCs and the TNC. [114]

However, there have also been studies questioning the reliability of GTN consistently inducing migraine. For example, the response rate to GTN varies from 50 to 80%. [112] One suggestion to explain this large variability in response rates is that there might be a relation between headache frequency and GTN induced migraine in migraineurs, but a significant correlation remains to be demonstrated. [118]

Nevertheless, most studies on this topic show that GTN can be used as a translational model to provoke migraine headache. [114] For our study, we needed a reliable translational model to induce migraine attacks, allowing us to measure neuropeptide concentrations at different stages of migraine headache. Considering that the literature on GTN induced migraine almost uniformly confirms the validity of this translational migraine model, we decided to use this approach in our study protocol.

1.8 CGRP in tear fluid - a potential biomarker for migraine?

There are currently no biomarkers for either migraine or any of the other primary headaches. A lot of research focuses on trying to find a reliable biomarker. As described in the above paragraphs, several neuropeptides have been analysed in detail and measured both interictally and during headache attacks. Results are often not unanimous and, in many cases, controversial. Hence, there is currently still a lack of evidence supporting the use of one of the mentioned neuropeptides as a valid clinical biomarker. Considering that studies on VIP and SP levels are not conclusive and the role of PACAP still being a fairly new area of research, the data available suggests that CGRP seems to be the most promising peptide to be considered as a potential biomarker for migraine. [119, 120]

As previously described, CGRP and the trigeminovascular system play an important role in the pathogenesis of migraine attacks. The trigeminal nerve has three branches (V1, V2, V3). V1 mainly innervates the dura mater and contains fibres innervating the dural vessels, where migraine pain is thought to originate through the release of CGRP from V1 fibres. [61, 121] Most importantly in relation to migraine, the first branch of the trigeminal nerve (ophthalmic nerve, V1) also innervates the temporal, frontal and orbital areas, where migraine pain is often located. [122]

A lot of studies have measured CGRP levels in the extracranial and peripheral venous blood circulation. There are, however, some drawbacks to these methods, like dilution through blood circulation. [123, 124] Furthermore, CGRP is not only released from trigeminal fibres but also has many other sources in the human body and has a very short half-life time of 7-9 minutes, which makes it hard to draw conclusions regarding CGRP in the trigeminal system from CGRP concentrations measured in peripheral blood. [123-125] CGRP has also been measured in saliva [126], with the hypothesis in mind that salivary CGRP levels might reflect activation of the third trigeminal branch.

Kamm et al., therefore, hypothesised that CGRP concentrations in tear fluid reflect trigeminal activation, particularly of the first branch. The basis of this theory is the fact that CGRP-positive fibres found in the cornea mainly stem from the trigeminal ganglion, where around half of the neurons express CGRP. [89, 127]

Based on this hypothesis, CGRP concentrations in tear fluid were measured in a previous study of our research group. It was shown that tear fluid CGRP levels were significantly higher in both interictal chronic and episodic migraine patients compared to healthy controls. CGRP levels were even higher in ictal migraineurs. These results support the hypothesis that measuring CGRP levels in tear fluid might be a more direct approach to trigeminal activation in migraineurs. Detecting CGRP in tear fluid being a non-invasive procedure, this method holds a lot of potential regarding the continuing search for a biomarker for migraine. [89]

With all the current data and evidence available on the role of CGRP in migraine, the potential of CGRP as a clinical biomarker is obvious. Measurement of CGRP in peripheral blood does however have the disadvantage of being diluted through blood circulation and that it cannot be differentiated between CGRP from trigeminal fibres versus other CGRP sources. [91, 128] Considering that the measurement of CGRP levels in peripheral blood is highly controversial because data is not congruent, plasma CGRP is currently not considered a valid biomarker for clinical practice. [128] The alternative of drawing blood from the external jugular vein is an invasive and unpleasant procedure and is not suitable for daily clinical practice. Thus, we believe that the investigation of CGRP in tear fluid as a potential biomarker for migraine is very promising.

As a target for new treatments, CGRP has already gained a lot of clinical importance in the field of migraine. If its use could be expanded from therapy to diagnostics, its relevance for everyday clinical practice would drastically increase. The development of a biomarker for migraine could open new doors regarding risk assessment (e.g., risk of chronification) as well as prediction of therapeutic response in primary headaches and might lead the way to more tailored treatment options for millions of migraine patients worldwide.

To further establish this method, we wanted to measure CGRP concentrations in tear fluid during migraine attacks. Thus, we decided to use the well-established model of GTN induced migraine to look at the changes in tear fluid CGRP levels throughout a migraine attack. We hypothesised that 1) tear fluid CGRP levels rise significantly during a migraine attack compared to baseline levels and 2) drop after improvement of headache or (successful) intake of acute migraine medication.

2. Methods

2.1 Participant recruitment

The study was approved by the ethics committee of the Ludwig-Maximilans-University (LMU) Munich (project number 18-827) in March 2019. Starting in August 2019, we recruited a total of 70 patients with episodic migraine and 48 healthy controls over a period of 15 months until October 2020. Participants were recruited through our outpatient headache centre and advertisements at different faculties of the LMU and at the University Hospital of the LMU.

All participants gave written informed consent prior to partaking in the study. For healthy controls, the participation consisted of a single appointment. Migraineurs were examined on two different days. On the first appointment, patients were examined in a (naturally occurring) interictal or ictal state. On the second appointment, participants were administered GTN to induce a migraine-like headache. Of the 70 patients with episodic migraine who were recruited for the first appointment, 37 were willing and suitable to participate in the second part of the study and were administered GTN to induce a migraine attack.

2.1.1 Inclusion criteria for migraineurs

Participants were included if they

- met the IHS (International Headache Society) criteria of The International Classification of Headache Disorders 3rd edition (ICHD-3) for migraine with or without aura (see Table 1) [3],
- had less than 15 headache days per month (i.e., episodic migraine),
- were over 18 years at time of participation,
- had sufficient command of the German language to give informed written consent for participation in the study.

Participants had to either present with an already existing diagnosis of episodic migraine or were examined by an expert clinician to verify whether the IHS migraine criteria were met.

2.1.2 Exclusion criteria for migraineurs

Participants were excluded if they

- had any severe pre-existing internal, neurological or psychiatric conditions. Certain medically adjusted conditions were not considered exclusion criteria (see results section below),
- had an intolerance to GTN,
- had regular issues with hypotension (meaning a systolic blood pressure lower than 90 mmHg),

- were taking phosphodiesterase inhibitors (danger of severe hypotension through interaction of with GTN) [129],
- were suffering from arterial hypertension as, due to the vasodilating effects of CGRP, its levels are likely elevated in patients with hypertension. [69] Blood pressure had to be below 140/90 mmHg on the first appointment. If the first measurement was over 140/90 mmHg, we measured a 2nd and a 3rd time. At least 2 of 3 measurements had to be under 140/90 mmHg. If blood pressure was higher, patients were asked to measure blood pressure at home twice a day (straight after waking up and before going to bed) for 3 consecutive days. Patients were only invited for the GTN appointment if the measurements at home were below 140/90 mmHg.
- had a prophylactic migraine treatment with CGRP antibodies within 4 weeks prior to sampling,
- had any pre-existing eye conditions (except corrected myopia or hyperopia),
- were wearing contact lenses on the day of sampling,
- were pregnant or breastfeeding at the time of study participation as plasma CGRP levels are elevated during pregnancy. [130]

Diagnostic criteria for migraine (ICHD-3)

A	At least 5 attacks meeting criteria B-D	
B	Headache duration (untreated or treated unsuccessfully):	4-72h
C	At least 2 of the following 4 characteristics:	1. headache location: unilateral 2. headache quality: pulsating 3. headache intensity: moderate / severe 4. aggravation through physical activity
D	Headache associated with at least one of the following accompanying symptoms:	1. nausea and / or vomiting 2. phonophobia and photophobia

Table 1 – Diagnostic criteria for migraine (ICHD-3). We used the ICHD-3 criteria to either confirm an existing diagnosis of episodic migraine or to make an accurate diagnosis during the first appointment. Participants also had to present < 15 headache days per month to meet the criteria of episodic migraine. Participants with ≥ 15 headache days per month were not included as the latter is defined as chronic migraine. For our study, we defined headache intensities as follows; light intensity: 1-3 on the NRS (numerical rating scale); moderate intensity: 4-6 on the NRS; severe intensity: ≥ 7 on the NRS. [3]

2.1.3 Inclusion criteria for healthy controls

Participants were included if they

- had no more than 2 days with mild headaches per month,
- did not have any history of headaches fulfilling any of the ICHD-3 criteria for migraine (see Table 1),
- were over 18 years old at the time of participation,
- had sufficient command of the German language to give informed written consent for participation in the study.

2.1.4 Exclusion criteria for healthy controls

Participants were excluded if they

- had any pre-existing severe internal, neurological or psychiatric conditions.
- were suffering from arterial hypertension. Blood pressure had to be below 140/90 mmHg on the examination day. If the first measurement was over 140/90 mmHg, we measured a second and a third time. At least 2 out of 3 measurements had to be under 140/90 mmHg,
- had taken any pain medication 48h hours prior to sampling,
- had any pre-existing eye conditions (except corrected myopia or hyperopia),
- were wearing contact lenses on the day of sampling,
- were pregnant or breastfeeding at the time of study participation.

2.1.5 Participant cohort

Because of initial recruitment problems, we initially included some patients and healthy controls not meeting one or more of the (non-critical) inclusion criteria. These participants could however be excluded before analysis.

Inclusion of migraineurs was considered separately for P1 and P2. Migraine patients had to meet the inclusion criteria mentioned above. We did, however (due to difficulties in participant recruitment) not exclude participants with some medically treated conditions without any symptoms (see results section). Patients were excluded if they had a diagnosis of arterial hypertension (even if adequately medically treated) or if they had elevated blood pressure during the appointment because the effects of arterial hypertension may influence CGRP levels. [131, 132] For P1, participants were included as ictal migraineurs if they indicated having had a headache within 48h prior to or past sampling. They were, however, excluded if they had taken pain-relieving medication within 48h before sampling. For P2, participants who indicated having had a headache within 48h prior to the appointment or at presentation were excluded. Participants who reported another headache surge within 48h after GTN administration were not excluded.

2.2 Study design

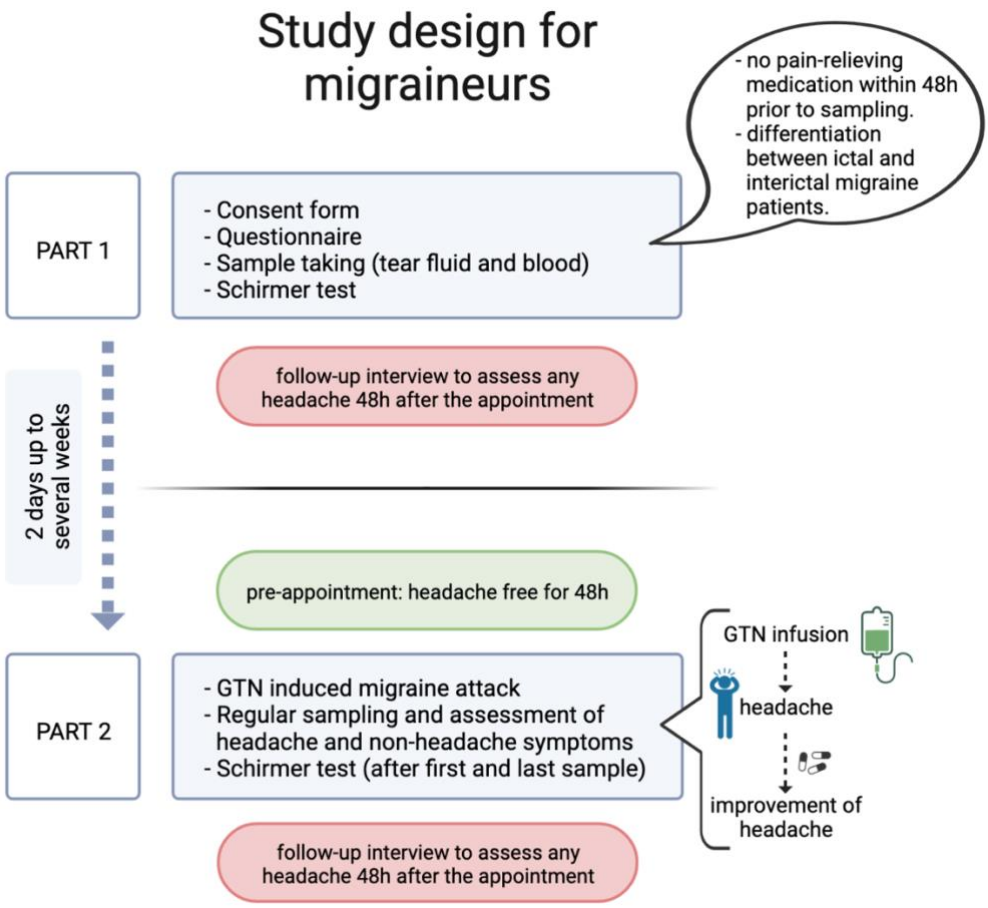

Figure 1 – Study design (migraine patients). For migraine patients, the study consisted of two consecutive appointments, part 1 and part 2. Both appointments had to be at least 2 days apart. **Part 1:** Headache history and epidemiological data was taken using a questionnaire and samples (blood and tear fluid) were collected. **Part 2:** participants were administered GTN i.v. to induce a migraine-like headache and regular samples (blood and tear fluid) were taken throughout the appointment. If a headache occurred, participants could take pain-relieving medication and samples were taken again after improvement of headache. A follow-up interview to assess any further headache was conducted after both appointments.

2.2.1 Part 1 – ictal and interictal migraine patients

For the **first appointment** (part 1, P1), the following information was acquired before taking samples: headache frequency, intensity, location and duration over the last 3 months, typical accompanying symptoms, triggering and alleviating factors for migraine headache, acute and prophylactic medication (present as well as previous), association with hormonal fluctuations in women, family history, presence of any other type of headache. Furthermore, we enquired general health, pre-existing conditions, medication use, allergies, alcohol consumption and smoking patterns. Additionally, we inquired about the presence of headache at the day of sampling, timing of the last headache and the latest use of pain medication.

After patients had been at rest in a seated position for at least 5 minutes, their arterial blood pressure was measured.

At the end of the interview, we proceeded to take the samples. Sampling was done in a non-fasted state between 9 a.m. and 6 p.m. To collect the tear fluid, plastic capillaries (plastic capillaries (ref. no. 100012), Sanguis, Nümbrecht, Germany) were used and held into the tear fluid at the lateral canthus of the eye. This method had already been established and described in the context of our previous study and has previously been used in other studies. [89, 133, 134] The collection of tear fluid is a non-invasive procedure and has no noteworthy risks. Much care was taken to avoid irritation of the eyes. The sampling process takes 1-2 minutes. Subsequently to collecting the tear fluid from both the right and left eye separately, both capillaries were immediately put in two 1.5ml tubes (pre-chilled), each containing 500 µl of tissue protein extractor solution (TPER; Pierce Rockford, IL).

Blood was then drawn from the antecubital vein into EDTA and serum tubes. For every blood sampling, we used 1 EDTA tube containing 250 µl of the protease inhibitor aprotinin (Trasylol 10 mg/ml, Sigma-Aldrich, Germany), 1 EDTA tube and one serum tube. All vials were pre-chilled.

Samples were processed straight after collection and kept cool at all times. Further processing of the samples will be described in chapter 2.4.

After sampling, we performed a Schirmer test on both eyes to quantify tear fluid production.

48h hours after the appointment, a follow-up interview was conducted to inquire about any headache or medication use after sampling.

2.2.2 Healthy controls

For healthy controls, the participation consisted of a single appointment (part 1, P1). Before sampling, the following information was acquired: general health, pre-existing conditions, medication use, allergies, alcohol consumption and smoking patterns. Furthermore, they were asked about any regular headache and the corresponding characteristics in order to exclude any persons suffering from recurring headaches meeting any of the migraine criteria. [3]

Subsequently, tear fluid and blood samples were taken. A Schirmer Test was performed to quantify tear secretion.

2.2.3 Part 2 – GTN induced migraine attack in migraineurs

The aim of the **second appointment** (part 2, P2) was to provoke a migraine attack in migraine patients through intravenous administration of GTN and to collect tear fluid and blood before (baseline) and at regular intervals after GTN administration. If patients reported a headache, the aim was to collect samples at a moderate to severe headache intensity. Patients were allowed to take their usual acute headache medication as

needed. Some patients reported a spontaneous improvement without taking any analgesics.

Patients arrived at the headache centre of the LMU (Klinikum Großhadern) between 8 a.m. and 9 a.m. and stayed between 6 up to 9 hours, depending on timing of headache onset after GTN administration. If patients did not report a headache after 7 hours after GTN administration, the appointment was finished.

When patients arrived, we enquired about the presence of any current headache, timing of the last headache and last intake of pain medication. Thus, we made sure patients had been headache free and had not taken any analgesics for the last 48h. Even though patients had already given informed consent to participation on the first appointment, we made sure to thoroughly explain the whole procedure a second time to put patients at ease.

After a short opening interview, blood pressure was taken after patients had been in a resting position for at least 5 minutes.

Consequently, we sampled tear fluid and blood for our baseline sample. The method of tear fluid sampling throughout day 2 was the same as described for sampling on day 1 in chapter 2.2.1. For the first blood withdrawal, a peripheral venous catheter (PVC) was introduced into the antecubital vein. This peripheral venous line was also used for the GTN administration and all further blood sampling to avoid the stress of repeated venipunctures as we took samples up to 9 times throughout the day.

A urine pregnancy test was conducted for all women of reproductive age before GTN administration to rule out the possibility of an existing pregnancy. All pregnancy tests we conducted were negative.

To induce the migraine attack, we administered intravenous GTN over 20 minutes. The respective dose was adjusted to the bodyweight (BW) of the participant (0.5 µl/kgBW/min) and GTN was diluted in a 0.5 litre sodium chloride solution 0.9%. Blood pressure was measured right before and every 5 minutes during administration. If blood pressure dropped significantly or if patients experienced symptoms of faintness or nausea, we paused the GTN administration. In that case, patients were given intravenous sodium chloride solution 0.9% to stabilise blood pressure. Upon clinical stabilisation, GTN administration was continued.

After GTN administration, participants had to stay in a seated position for 10 minutes and we measured blood pressure again to make sure they were hemodynamically stable.

The first sample would be taken 30 minutes after finishing GTN administration. Thereafter, the goal was to take samples approximately every 60 minutes, but in some patients, tear fluid and blood collection times had to be adapted due to dry or easily irritable eyes.

Headache characteristics (quality, localisation, intensity) and non-headache symptoms were assessed before every sampling. Blood pressure in a seated resting position was also always taken prior to every sampling.

Even though sampling was not possible at 60-minute intervals for all participants, the goal was to obtain samples from all participants at the following times:

(1) baseline sample (meaning before GTN administration),
(2) 30-60 minutes after GTN administration,
(3) at moderate intensity headache,
(4) after improvement of headache.

Participants were allowed to take their usual acute medication at any point but were asked – if possible – to wait until a headache of at least moderate intensity occurred.

Tear fluid and blood were taken right before and 60 minutes after acute medication intake and/or after a significant improvement of headache.

48h hours after the appointment, a follow-up interview was conducted with participants to enquire about any further headache or medication intake after sampling.

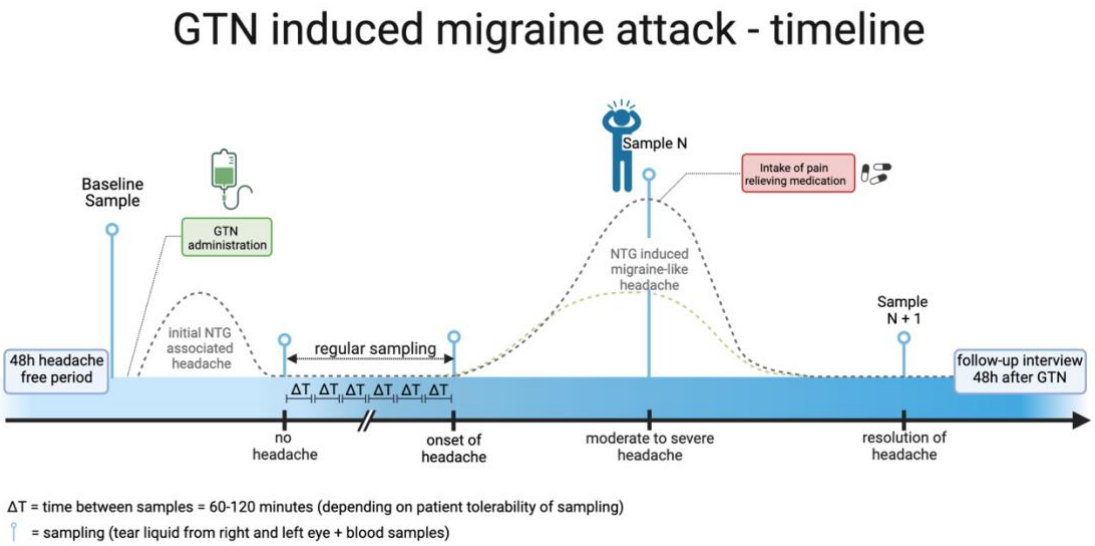

Figure 2 – **GTN induced migraine attack.** During the second appointment (part 2), migraineurs were given GTN intravenously over 20 minutes (0.5 µl/kgBW/min) to induce a migraine-like headache. Tear fluid (from both eyes) and blood samples were taken before, as well as 30-60 minutes after GTN. Samples were then taken at regular intervals, the frequency depending on the individual tolerance of tear fluid sampling. Headache quality, intensity (NRS), localisation as well as non-headache symptoms were assessed at regular intervals. Samples were taken after onset of a moderate to severe headache as well as after improvement of headache or at least 60 minutes after intake of pain-relieving medication.

2.3 Questionnaires

Part 1

For healthy controls, we had one questionnaire to assess epidemiological data as well as general health, weight, height, body-mass-index (BMI), pre-existing conditions, medication intake, allergies, as well as alcohol and nicotine intake. Also, a short

headache anamnesis was conducted to rule out that participants suffered from regular headaches.

Migraineurs were additionally further asked about headache frequency, intensity, location, and duration regarding the past 3 months. Typical non-headache attendant symptoms, triggering and alleviating factors, as well as acute and prophylactic medication history, role of hormonal fluctuation regarding headache frequency, family history and diagnosis of other existing headache disorders were inquired.

Part 2

In the second part of the study (P2), migraineurs were asked about current presence of headache, timing of last headache and last intake of pain-relieving medication. Beginning right after GTN administration, the presence of any headache was regularly enquired over the course of the day, always right before sampling. At every assessment, the following characteristics were also enquired: headache intensity, localisation, quality, aggravation by physical activity (e.g. walking) and accompanying non-headache symptoms. Over the course of the second appointment, headache intensity was assessed regularly using the NRS. The NRS is a numeric scale comprising 11 items from 0 to 10, with 0 corresponding to “no pain” and 10 corresponding to the “worst imaginable pain”. It is a reliable tool to quantify subjective perception of headache intensity. [135, 136] Items 1-3 corresponded to light pain, items 4-6 to moderate pain and 7-10 to severe pain.

All questionnaires used were developed specifically for this study.

2.4 Sample processing

2.4.1 Tear fluid samples

After tear fluid collection, capillaries were separately immersed in a 1.5 ml Eppendorf tubes and centrifuged for 5 minutes at 4000 revolutions per minute (rpm) at 4°C. After centrifugation, the capillary was taken out of the respective Eppendorf tube. We made sure to always keep samples cooled until storage at -80°C and measurement of peptide concentrations.

2.4.2 Blood samples

After taking blood samples, all 3 tubes were centrifuged for 10 minutes at 2000 rpm at 4°C, plasma was isolated into tubes and stored at -80°C. During the entire time, all samples were always stored using ice or cooling packs to keep them cool.

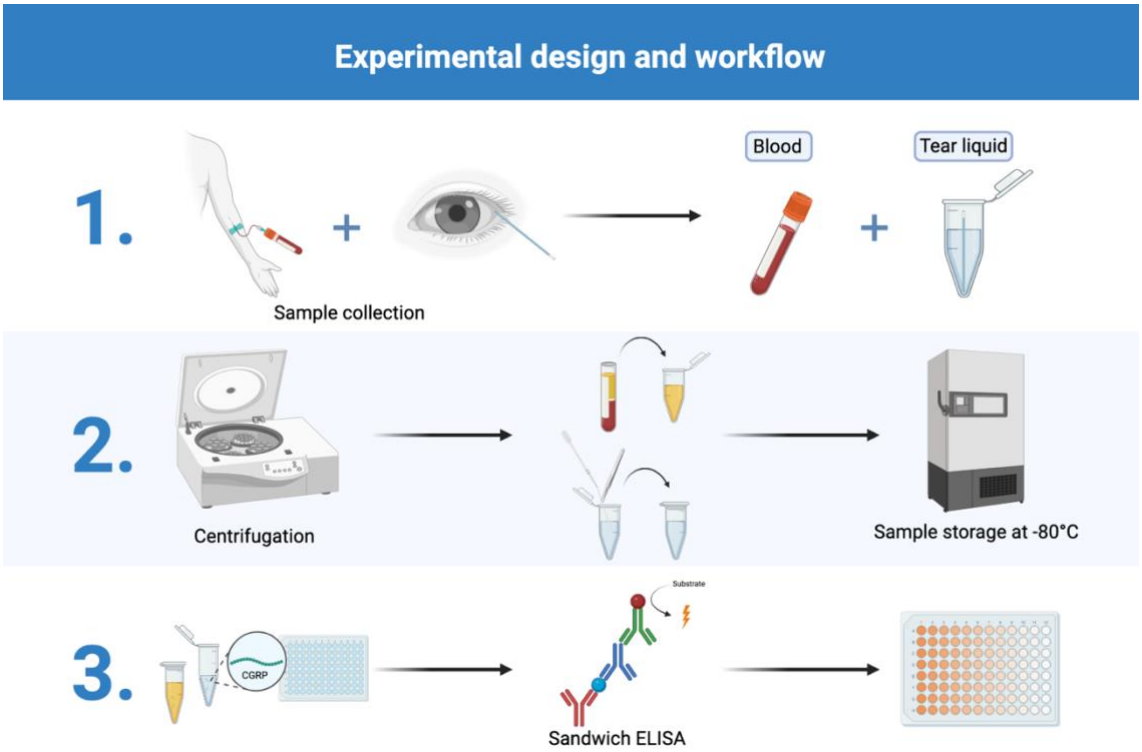

Figure 3 – Experimental design and workflow. 1. Blood and tear fluid samples were collected consecutively and stored coolly. **2.** Tear fluid was centrifuged for 5 minutes at 4000 rpm at 4°C. Blood samples were centrifuged for 10 minutes at 2000 rpm at 4°C and plasma was isolated and aliquoted into tubes. Samples were then stored at -80°C. **3.** CGRP levels were measured using a sandwich enzyme-linked immunosorbent assay (ELISA).

2.5 Schirmer Test

The Schirmer Test without anaesthesia was used to measure basal tear secretion and reflex tear secretion.

We always performed the Schirmer test on both eyes at the same time and removed the test strip from both eyes after 5 minutes to read the result.

The Schirmer Test was performed at the following times:

- Appointment 1: after tear liquid collection (migraine patients and healthy controls)
- Appointment 2: after the first and last tear fluid sampling (migraine patients only)

To avoid interference between sampling and Schirmer test results, we waited a minimum of 5 minutes after sampling before performing the Schirmer test. The Schirmer test was only performed in a part of participants as it was implemented into the study protocol after the study had already started.

2.6 Enzyme-linked immunosorbent assay

CGRP levels were measured in tear fluid of the right and the left eye respectively, as well as in plasma. A commercial sandwich enzyme-linked immunosorbent assay (ELISA)

from CUSABIO® (Wuhan, China) was used according to manufacturer's instructions. The indicated detection range lies between 1.56–100 pg/ml and the minimal detectable dose is 0.39 pg/ml. Intra-assay precision was indicated at < 8%, inter-assay precision at < 10%. For every sample, measurement was performed twice, and the average of both measurements was calculated. Absorption levels were determined by a BioRad spectrometer (BioRad Laboratories Inc., USA). Calibration curves with a 4PL curve fit (from arigoobio.com) were used to analyse the generated data and determine CGRP levels. This resulted in R^2 values over 0.99 for all measurements.

2.7 Statistics

The data collected through questionnaires during appointments and data generated through ELISAs was entered manually into a Microsoft Excel sheet (version 16.62, Microsoft Corp. 2016, Redmond, WA). After data collection was completed, the Excel sheet was transferred into SPSS Statistics 27.0 (IBM Corp. 2020, Armonk, NY).

Descriptive statistics are presented as mean values ± SDs. Kolmogorov-Smirnov and Shapiro-Wilk tests were performed to test normal distribution of data. To compare epidemiological data between groups, Mann-Whitney U Tests and T-Tests for independent samples (depending on distribution) as well as chi-square tests were used. To compare mean values of CGRP concentrations between migraineurs and healthy controls as well as between ictal and interictal migraineurs from the first appointment, Mann-Whitney U Test was used. For the comparison of CGRP from the first and second appointment, we used the Wilcoxon Signed Ranks Test. To detect differences in CGRP levels at different sampling points after GTN administration within one group of participants, we used the Friedman Test. To compare differences between different sampling points individually (baseline – headache; headache – post headache; baseline – post headache), we used the Wilcoxon Signed Ranks Test. We also compared CGRP levels between different categories at the three main sampling points using Mann-Whitney U Test. Statistical significance was assumed at p-values of $p < 0.05$. All analyses were carried out with SPSS 27.0.

Most figures were generated using SPSS Statistics 27.0. Figure 10 was created using Microsoft Excel. Figure 12, Figure 13 and Figure 14 were generated with seaborn version 0.11.2 [137] using python 3.7. Figure 1, Figure 2, Figure 3 and Figure 4 were created with BioRender.com.

3. Results

3.1 Participants

For this study, 118 participants were recruited in sum. A total of 48 healthy controls (35 female; 13 male) and 70 migraine patients (59 female; 11 male) took part in the first appointment. Of the 48 healthy controls, 16 had to be excluded, mainly because too little tear fluid could be obtained, elevated blood pressure levels or because they did not meet the headache criteria mentioned above (see chapter 2.1.2). 32 healthy controls were included. Of the migraine patients, 32 had to be excluded. The most frequent reasons were elevated blood pressure, lack of sampling material (tear fluid) or a pre-existing diagnosis of arterial hypertension. Other reasons are listed in the description of Figure 4.

Of the 70 migraineurs, a total of 37 patients (30 female; 7 male) took part in the second appointment and were administered GTN to induce a migraine attack. A total of 11 had to be excluded because they there was a lack of sampling material (tear fluid), they had elevated blood pressure, they presented at the clinic with a headache before GTN administration or they had an existing diagnosis of arterial hypertension.

Regarding the first appointment, data of 32 healthy controls (n = 32) and 38 participants with migraine (n = 38) were suitable for further analysis. Of the 37 participants who were administered GTN, data of 26 migraineurs (n = 26) was also suitable to be investigated further (see Figure 4).

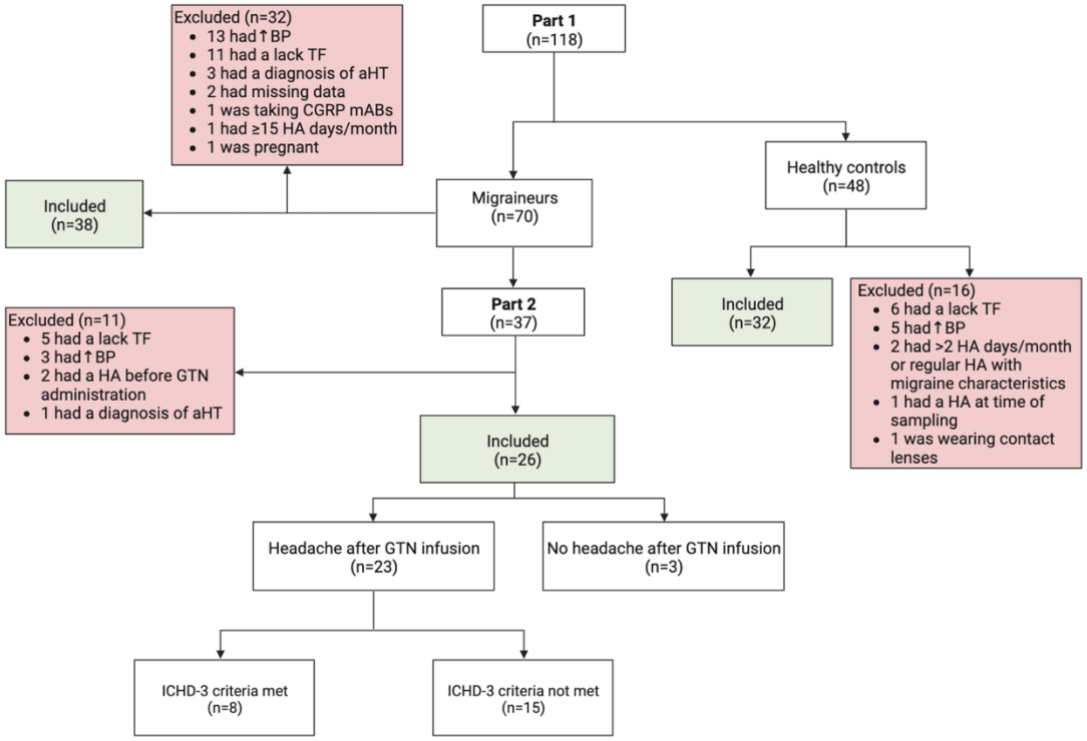

Figure 4 – Flowchart. BP = blood pressure; TF = tear fluid; HA = headache; aHT = arterial hypertension.

3.2 Part 1 (P1)

3.2.1 Epidemiological data

In part 1 of the study, the following two categories will be compared: migraineurs and healthy controls. Both groups were comparable in their age (26.2 ± 6.9 and 26.6 ± 7.8 years; *p = 0.934 Mann-Whitney U Test*) and body mass indexes (22.6 ± 3.3 and 22.1 ± 2.3 kg/m^2; *p = 0.682 Mann-Whitney U Test*). In both groups, most participants were female with no significant differences in gender between groups (χ^2 *[2] = 0.921, p = 0.337*). Migraineurs in our participant cohort had significantly more pre-existing medical conditions (χ^2 *[2] =4.924, p = 0.026*) than healthy controls. They also took more medication (migraine medication excluded) than healthy controls (χ^2 *[2] = 3.109, p = 0.078*), but with no statistically significant difference.

CLINICAL CHARACTERISTICS

	Migraineurs (n = 38)	Healthy controls (n = 32)	p-value
Female	32 (84.2%)	24 (75%)	p = 0.337
Age (y)	26.2 ± 6.9	26.6 ± 7.8	p = 0.934
BMI (kg/m^2)	22.58 ± 3.3	22.06 ± 2.3	p = 0.682
Migraine without aura	32 (84.2%)	--	--
Headache frequency (days/month)	7.8 ± 4.0	0.6 ± 0.7	< 0.001
Migraine frequency (days/month)	4.1 ± 3.1	--	--
Headache intensity (NRS)	7.9 ± 1.0	--	--
Duration (in h)	29.4 ± 20.6	--	--
Acute medication	37 (97.4%)	--	--
NSAIDs	30 (78.9%)	--	--
Triptanes	15 (39.5%)	--	--
Metamizole	3 (7.9%)	--	--
Migraine prophylaxis	6 (15.8%)	--	--
Betablocker	3 (7.9%)	--	--
Topiramate	1 (2.6%)	--	--
Amitriptyline	2 (5.3%)	--	--
Other medication*	8 (21.1%)	2 (6.3%)	p = 0.078
Other conditions**	10 (26.3%)	2 (6.3%)	p = 0.026

* Other medication: L-thyroxine, antidepressants, pantoprazole, insulin, antihistamines and oral contraceptives.

** Other conditions: Hypothyroidism, diabetes mellitus type 1, asthma, endometriosis, rheumatological illness, type A gastritis, coeliac disease and depression. The 2 healthy controls with a medically adjusted condition had hypothyroidism and endometriosis but no clinical symptoms.

Table 2 – Epidemiological data for appointment 1.

3.2.2 Measurement of CGRP levels

For all 70 participants (32 healthy controls and 38 migraineurs taken together), average tear fluid CGRP levels measured at 1.15 ± 1.60 ng/mL from the right eye and 1.09 ± 1.23 ng/mL from the left eye. There was no significant difference between tear fluid CGRP levels between both eyes (*p = 0.942, Wilcoxon Signed Ranks Test*). Therefore, CGRP levels in tear fluid will be indicated as the mean values between measurements from both eyes, as previously established by Kamm et al. [89] Average plasma CGRP levels were at 4.72 ± 3.83 pg/mL. Tear fluid levels were ~240 times higher than plasma levels.

3.2.3 CGRP levels in migraine patients versus healthy controls

Comparison between interictal and ictal migraineurs

To compare ictal and interictal CGRP levels in migraineurs, we could include 18 (f = 15, 83.3%) interictal participants and 17 (f = 15, 88.2%) ictal participants. Ictal participants were defined as migraineurs, who either presented with a headache at the clinic or had had a headache within 48h before or 48h after P1. Of the 17 ictal migraineurs, 4 indicated headaches during sampling, 11 indicated having had a headache within 48h after sampling (headache free at time of sampling) and 2 had headaches within 48h before sampling (headache free at time of sampling). A total of 3 participants had taken analgesics within 48h before presenting at the clinic; we did not include data of CGRP levels in further analysis as intake of acute medication has an influence on CGRP concentrations. [29, 86, 88] There was missing data for 2 plasma samples, which is why we only have plasma CGRP levels from 33 migraineurs (17 interictal and 16 ictal).

For interictal patients, the average CGRP level in tear fluid was 0.87 ng/ml SD ± 0.79 ng/mL. For ictal participants, the average CGRP tear fluid level was 1.88 ± 1.69 ng/mL). Respective plasma CGRP levels were at 5.16 ± 4.67 pg/mL in interictal and 3.84 ± 3.18 pg/mL in ictal migraineurs.

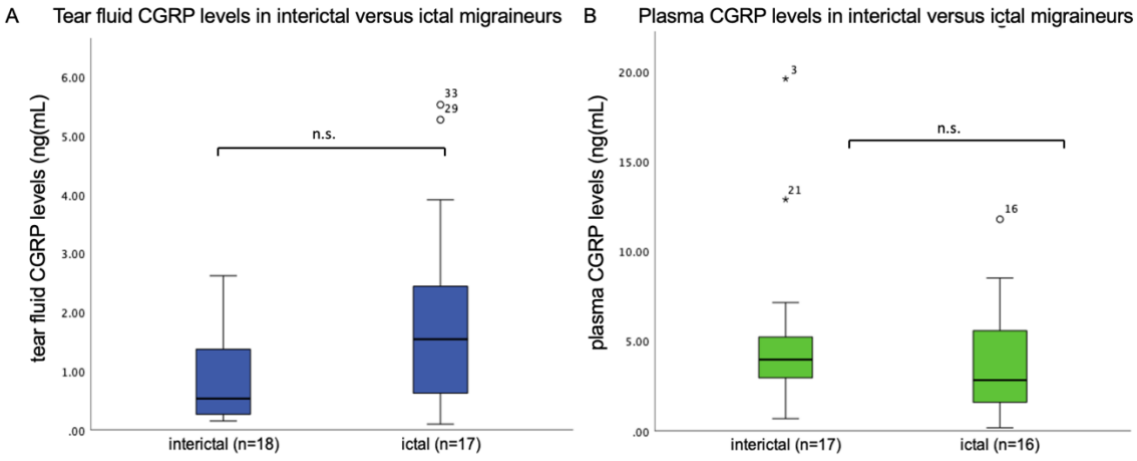

Figure 5 – CGRP levels in interictal and ictal migraine patients. (A) Tear fluid CGRP levels in both interictal and ictal migraine patients. Levels were higher in ictal migraine patients, but the difference was not statistically significant (p = 0.099 Mann-Whitney U Test). **(B)** Plasma CGRP

levels did not differ significantly between interictal and ictal migraineurs (p = 0.296 Mann-Whitney U Test).

Results show higher tear fluid CGRP levels in ictal compared to interictal migraine patients, while mean plasma CGRP levels were higher in interictal participants. Statistically, there was a trend for a significant difference between tear fluid CGRP levels in interictal and ictal migraineurs *(p = 0.099, Mann-Whitney U Test)*, and no significant difference in plasma CGRP levels *(p = 0.296, Mann-Whitney U Test)*.

Comparison between interictal migraineurs and healthy controls

The CGRP levels of 18 interictal migraineurs (15 females, 83.3%) were compared to those of 32 healthy controls (24 females, 75.0%). For interictal participants, the average CGRP level in tear fluid was 0.87 ± 0.79 ng/mL and in plasma, the CGRP concentration was 5.16 ± 4.67 pg/ml. For healthy controls, an average tear fluid CGRP level of 0.90 ± 0.69 pg/ml and a plasma CGRP level of 4.63 ± 3.43 pg/mL was measured.

In contrast to findings by Kamm et al. from the previous study [89], there was no statistically significant difference in tear fluid CGRP levels between interictal migraine patients and healthy controls (*p = 0.385 Mann-Whitney U Test*). There was no significant difference in plasma CGRP concentrations (*p = 0.817 Mann-Whitney U Test)* between interictal migraineurs and healthy controls either.

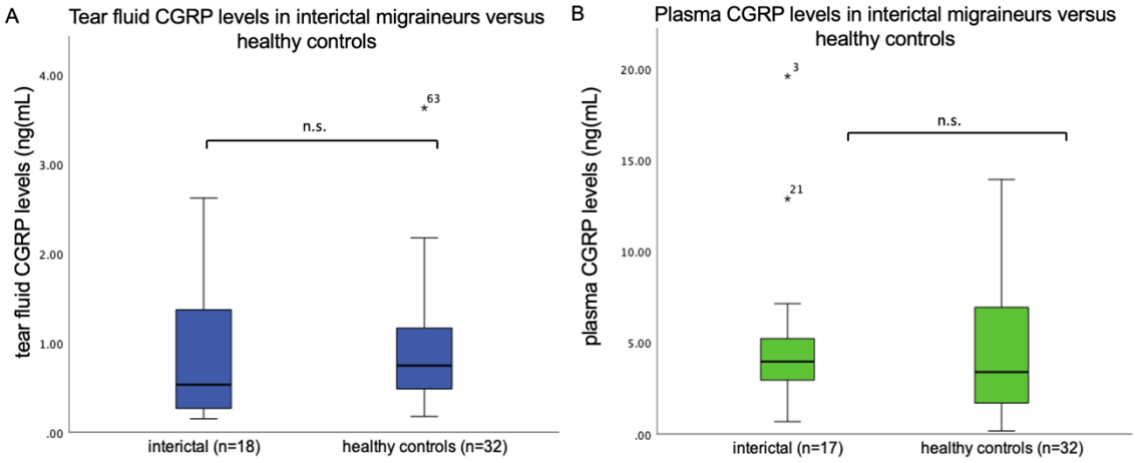

Figure 6 – CGRP levels in interictal migraine patients and healthy controls. (A) CGRP levels in tear fluid from interictal migraineurs and healthy controls were not significantly different (p = 0.385 Mann-Whitney U Test). **(B)** Plasma CGRP levels of interictal migraineurs and healthy controls did not differ significantly either (p = 0.817 Mann-Whitney U Test).

Comparison between ictal migraineurs and healthy controls

CGRP levels of 17 ictal migraineurs (f = 15, 88.2%) were compared to those of 32 healthy controls (f = 24, 75.0%). Tear fluid CGRP levels were measured at 1.88 ± 1.69 ng/ml for ictal migraineurs and at 0.90 ± 0.69 ng/mL for healthy controls, showing a statistically significant difference between both groups (*p = 0.034 Mann-Whitney U Test*). These results are in in line with previous findings from Kamm et al. [89] Plasma CGRP levels

were at 3.84 ± 3.18 pg/mL and 4.63 ± 3.43 pg/mL, meaning there was no statistically significant difference between the groups (*p = 0.382 Mann-Whitney U Test*).

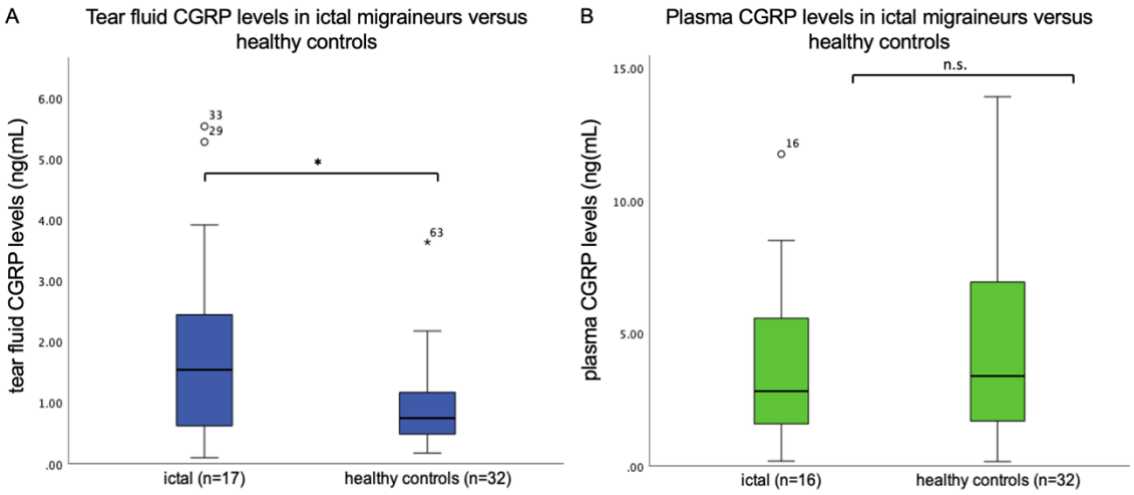

Figure 7 – CGRP levels in ictal migraine patients and healthy controls. (A) Tear fluid CGRP levels of ictal migraineurs and healthy controls. Levels were significantly higher in ictal migraineurs (p = 0.034 Mann-Whitney U Test). **(B)** Plasma CGRP levels in ictal migraine patients and healthy controls. There was no significant difference between both groups (p = 0.382 Mann-Whitney U Test).

3.3 Part 2 (P2)

3.3.1 GTN response – categories

As GTN has been shown to reliably provoke migraine attacks meeting ICHD-3 criteria in migraine patients, it was our method of choice for experimentally inducing headache (HA) in migraineurs. [111, 112] A total of 37 migraine patients were administered GTN (0.5 µg/kg/min for 20 minutes). A total of 26 participants met the inclusion criteria (see chapter 3.1).

We categorised the response to GTN administration according to the ICHD-3 criteria. 8 (30.8%) participants met the ICHD-3 criteria C and D (see Table 1, chapter 2.1.2) for migraine without aura (“HA, ICHD-3 met”). 15 (57.7%) participants developed a headache but did not meet ICHD-3 criteria C and D for migraine without aura (“HA, ICHD-3 not met”). 3 (11.5%) participants did not develop any headache (“no HA”) and were not analysed further.

With only 30.8% of participants developing a headache meeting IHS criteria for migraine after GTN administration, it is striking that the response rate to GTN was low in our study compared reports from literature. [114] Even though the liability of this model has been questioned as variable response rates from 50-80% have been reported, the response rate in this study (30.8%) is even lower than the indicated range. [112] For further elaboration on the potential causes of this discrepancy, see chapter 4.4.

In the following chapters, headache characteristics as well as non-headache symptoms will be described in further detail.

3.3.2 Epidemiological data

Participants from both categories did not differ significantly in either age (*p = 0.604, Mann-Whitney U Test*) or BMI (*p = 0.121, Mann-Whitney U Test*). In both categories, over 80% of participants were female and there were no significant differences in gender distribution (χ^2 *[2] = 0.003, p = 0.955*). Participants who developed a migraine attack had slightly more total headache and more migraine days per month, but there was no statistically relevant difference between both groups (*p = 0.074* and *p = 0.243, Mann-Whitney U Test*). Headache intensity during migraine attacks and average attack duration did not differ significantly either (*p = 0.066* and *p = 0.355, Mann-Whitney U Test*). In total, 4 patients took a regular migraine prophylaxis and there was no difference between groups (χ^2 *[2] = 0.494, p = 0.482*). None of our participants were taking CGRP antibodies as a migraine prophylaxis. A total of 5 patients had other pre-existing conditions and 6 were taking other non-migraine medication (see Table 3). There were no significant differences between groups regarding pre-existing conditions (χ^2 *[2] = 1.791, p = 0.181*) or intake of medication (χ^2 *[2] = 0.008, p = 0.931*)

CLINICAL CHARACTERISTICS

	GTN response			
	HA (n = 23)	**HA, ICHD-3 met** (n = 8)	**HA, ICHD-3 not met** (n = 15)	**p-value**
Female	20 (87.0%)	7 (87.5%)	13 (86.7%)	p = 0.955
Age (y)	26.30 ± 7.4	24.5 ± 4.2	27.3 ± 8.6	p = 0.604
BMI (kg/m^2)	23.00 ± 3.0	24.18 ± 3.1	22.37 ± 2.8	p = 0.121
Migraine without aura	20 (87.0%)	7 (87.5%)	13 (86.7%)	---
Migraine with aura	3 (13.0%)	1 (12.5%)	2 (13.3%)	---
Headache frequency (days/month)	7.5 ±3.7	9.9 ± 3.9	6.3 ± 2.9	p = 0.074
Migraine (days/month)	3.5 ± 2.7	4.4 ± 3.3	3.0 ± 2.3	p = 0.243
Headache intensity (NRS 0-10)	7.8 ± 0.9	8.4 ± 0.8	7.6 ± 0.9	p = 0.066
Duration (in h)	28.2 ± 20.5	33.3 ± 19.2	25.5 ± 21.3	p = 0.355
Acute medication	22 (95.7%)	8 (100%)	14 (93.3%)	p = 0.455
… NSAIDs	19 (82.6%)	7 (87.5%)	12 (80.0%)	---
… Triptanes	9 (39.1%)	4 (50.0%)	5 (33.3%)	---
… Metamizole	1 (4.3%)	1 (12.5%)	---	---
Migraine prophylaxis	4 (17.4%)	2 (25.0%)	2 (13.3%)	p = 0.482
… Betablocker	3 (13.0%)	1 (12.5%)	2 (13.3%)	---
… Amitriptyline	1 (4.3%)	1 (12.5%)	---	---
Other medication*	6 (26.1%)	2 (25.0%)	4 (26.7%)	p = 0.181
Other conditions**	5 (21.7%)	3 (37.5%)	2 (13.3%)	p = 0.931

* Other medication: L-thyroxine, pantoprazole, antihistamines and oral contraceptives.

** Other conditions: Hypothyroidism, endometriosis, rheumatological illness and depression.	

Table 3 – Epidemiological data for appointment 2 (GTN administration).

3.3.3 GTN administration

GTN was administered to a total of 37 patients, out of whom 26 patients could be included (see chapter 3.1). 3 were GTN non-responders (no GTN induced delayed headache) and 23 patients developed a delayed headache. Out of these, only 8 patients had a headache that met the ICHD-3 criteria for migraine. The average duration of GTN administration was 26 ± 4 minutes in all 26 patients. 24 patients developed an initial GTN-associated headache during GTN administration. Average maximum headache intensity during GTN administration across all 3 groups was 3.2 ± 1.3 on the NRS with no significant differences between the 3 groups (*p = 0.343, Kruskal Wallis Test*). GTN administration was well tolerated, and most patients experienced no side effects apart from headache. A total of 7 patients did however experience symptoms like nausea (n = 5) and/or dizziness (n = 3) during GTN administration. All 7 also had a delayed headache; 3 developed a headache meeting ICHD-3 criteria, 4 had a headache not meeting ICHD-3 criteria.

3.3.4 Delayed headache – characteristics and intensity

The maximum of the delayed GTN induced headache was at 253 minutes on average in all patients. In participants whose headache met the ICHD-3 criteria, the maximum headache intensity (of 5.4 ± 0.9 on the NRS) was indicated at 263 ± 117 minutes after GTN administration. Those who developed a headache not meeting ICHD-3 criteria indicated an average maximum of 4.0 ± 1.6 on the NRS at 248 ± 97 minutes after GTN administration. The onset of headache could not be determined as we only documented headache intensity at sampling points (approximately hourly) but not in between. Furthermore, in some participants, the delayed headache developed gradually without a headache free period between the initial GTN headache and the delayed headache Therefore the exact timing of delayed headache onset could not be determined.

	Maximum intensity (NRS)	Time until maximum intensity (min)
HA, ICHD-3 met (n = 8)	5.4 ± 0.9	263 ± 117
HA, ICHD-3 not met (n = 15)	3.3 ± 1.5	248 ± 97
HA (n = 23)	4.0 ± 1.6	253 ± 102

Table 4 – Maximum headache intensity (NRS) and time until maximum intensity was reached.

The intensity of the induced headache varied between 1 and 6 on the numerical rating scale (NRS). A total of 16 participants indicated a maximum headache intensity of at least 4 on the NRS (at least medium headache intensity). Figure 5 shows the maximum headache intensity that all 23 participants indicated over the course of GTN induced headache/migraine attack. The figure also shows that participants whose headache met the criteria of a migraine attack indicated maximum headache intensities between 4 to 6

on the NRS. 46.7% of participants (7 out of 15) who developed a headache that did not meet migraine criteria experienced mild headaches (< 4 on the NRS). 53.3% of participants (8 out of 15) experienced headache intensities of 4 to 6 on the NRS but did not meet the ICHD-3 criteria.

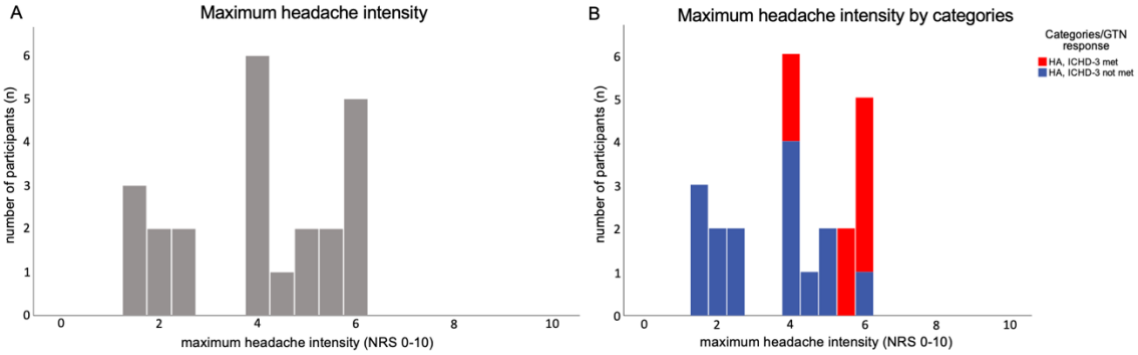

Figure 8 – Maximum headache intensity. (A) shows maximum headache intensities indicated by all participants with a GTN induced headache (n = 23). **(B)** Here, we compare maximum headache intensities in patients with GTN induced migraine meeting ICHD-3 criteria (n = 8) and headache not meeting ICHD-3 criteria (n = 15).

A total of 14 (60.9%) patients had a headache free period between the initial GTN induced headache (headache resolved completely between in under 60 minutes after the end of GTN administration) and the delayed headache response; 4 of them developed a headache meeting ICHD-3 criteria and 10 experienced a headache that did not meet ICHD-3 criteria. Another group of 9 (39.1%) patients did not report a headache free period; 4 of them had a GTN induced migraine attack (headache meeting ICHD-3 criteria) and 5 had a headache not meeting ICHD-3 criteria. Patients without a headache free period after the initial GTN headache experienced a gradual intensification of the immediate headache and 5 of them developed a headache of at least medium intensity.

	Headache quality				**Headache localisation**		
	Pulsating	Stabbing	Pressing	Pulling	Right	Left	Bilateral
HA, ICHD-3 met (n)	6	1	1	0	5	1	2
HA, ICHD-3 not met (n)	2	4	8	1	5	6	4

Table 5 – Headache quality and localisation. This table shows descriptive statistics of headache quality and headache localisation. It is noticeable that participants who developed a migraine attack mostly described their headache as pulsating and unilateral (right), whereas those who did not had mostly pressing headaches with no one localisation being particularly frequent.

3.3.5 Ictal non-headache symptoms

To meet the ICHD-3 criteria for a migraine attack, participants had to present either nausea or phonophobia and photophobia in addition to a headache meeting certain criteria like moderate intensity, pulsating quality, unilaterality, or worsening with physical activity (see Table 1). A total of 23 participants experienced some form of headache and 20 of them experienced attendant symptoms. 8 of the 23 (35%) also met the ICHD-3

criteria. The most frequently experienced migraine typical non-headache symptom was photophobia (n = 9). Phonophobia and nausea incurred less frequently (n = 6 for both).

In total, 12 of the participants from both categories also experienced other non-headache symptoms. The following attendant symptoms were indicated by participants: dizziness, unwellness, difficulties concentrating, yawning, petulance, lacrimation, diarrhoea, or aura symptoms (e.g., disturbed vision or paraesthesia).

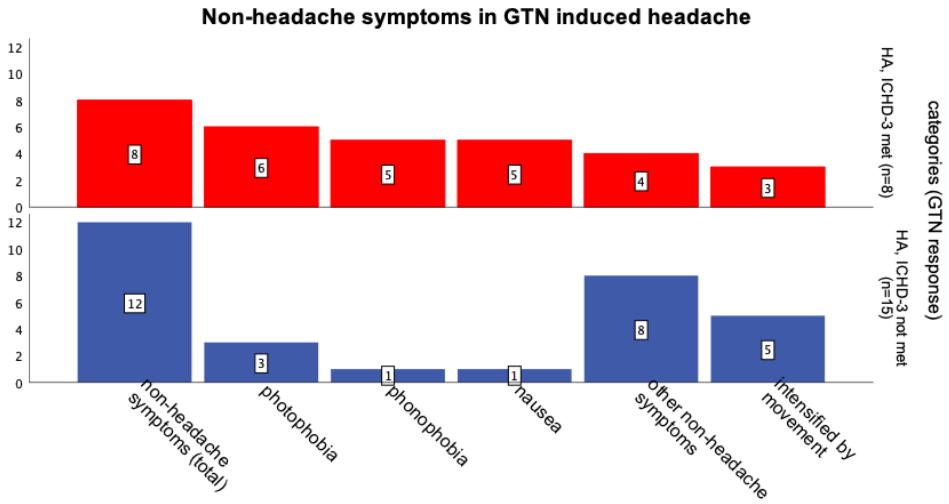

Figure 9 – Non-headache symptoms, totals. This graph shows the total number of participants, categorised after GTN response, who experienced attendant symptoms and which symptoms were most frequent. It also shows how many participants indicated that their headache worsened with physical activity.

In Figure 10, the temporal occurrence of the three migraine-typical accompanying symptoms is illustrated. It was observed that most attendant symptoms occurred between 4-5 hours after GTN administration. The time until occurrence of the highest indicated headache intensity lay at 264 minutes (4.4h) after GTN administration, meaning that there is an overlap between occurrence of attendant symptoms and severe headache. However, this analysis is limited as we only have data regarding the presence of attendant symptoms at sampling times, meaning that for some participants, data is missing for certain periods of time because samples were taken less frequently due to lower tolerance to tear fluid sampling. Therefore, we also illustrated attending symptoms at time of most severe headache in Figure 11.

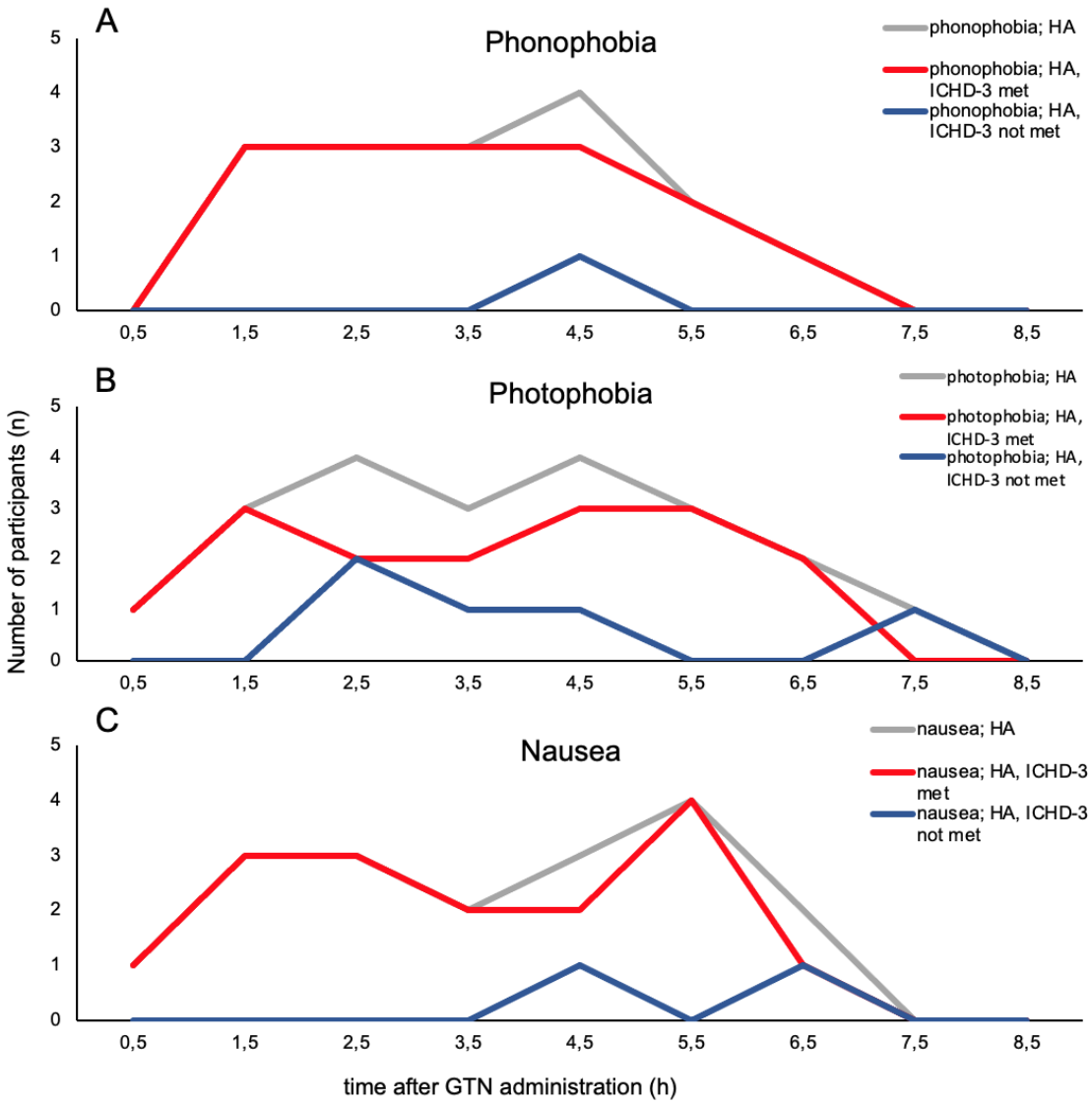

Figure 10 – Phonophobia, photophobia and nausea over time. This graph shows the distribution of the three migraine-typical attendant symptoms in our participant cohort over time. Data is missing for certain periods of time because samples were taken less frequently due to lower tolerance to tear fluid sampling. Therefore, we binned data from <1h at 0.5h, data from ≥1h to <2h at 1.5h, etc. **(A)** shows the trend of participant numbers experiencing phonophobia after GTN administration. **(B)** shows the number of participants who experienced photophobia as a non-headache symptom. **(C)** shows the number of patients who felt nauseous after GTN attribution.

Figure 11 shows the number of participants experiencing the three main non-headache symptoms during most intense headache: 8 people from both categories (ICHD-3 criteria met and not met) experienced photophobia (9 in total), 5 experienced phonophobia (6 in total) and 3 felt nauseous (6 in total). This shows that most participants who experienced these symptoms, suffered them parallel to headache.

In Table 6, we summarised the most frequent non-headache symptoms during spontaneous migraine attacks (non GTN induced) in all participants who developed a headache after GTN administration. These were inquired using the questionnaire in the first appointment of the study. In those who developed a migraine attack after GTN

administration (n = 8), 87.5%, 100% and 62.5% regularly experience phonophobia, photophobia and nausea respectively during spontaneous migraine attacks. During GTN induced migraine, in the same group of patients, 62.5% experienced phonophobia, 75% photophobia and 62.5% nausea (see Figure 9). Regarding non-headache symptoms, these numbers suggest a similarity between spontaneous and GTN induced migraine attacks. Considering patients who developed a headache that did not meet ICHD-3 criteria after GTN administration (n=15), 86.7%, 80% and 80% regularly experience phonophobia, photophobia and nausea respectively during spontaneous migraine attacks. After GTN administration, 6.7% of them experienced phonophobia, 20% photophobia and 6.7% nausea. These numbers suggest that non-headache symptoms (namely phonophobia, photophobia and nausea) are less frequent in GTN induced headache not meeting ICHD-3 criteria than during spontaneous migraine attacks.

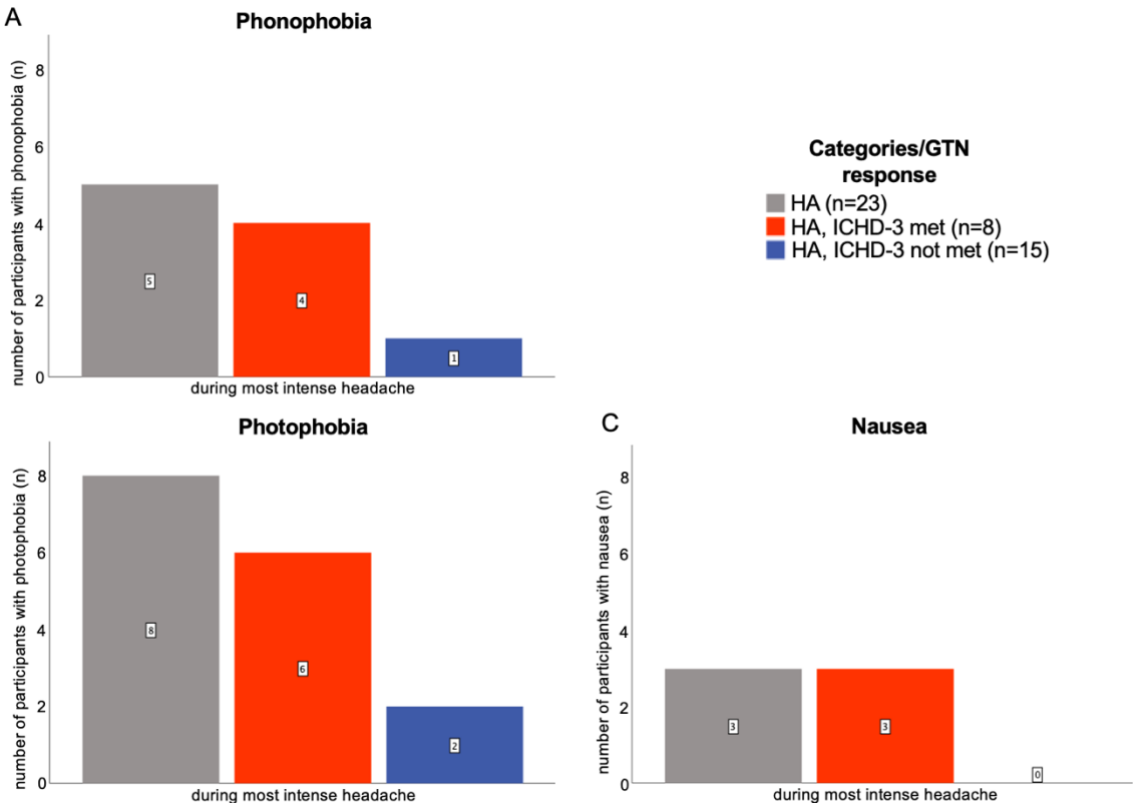

Figure 11 – Phonophobia, photophobia and nausea during most intense headache. (A) Number of participants who experienced phonophobia after GTN administration at the sampling point with most intense headache. **(B)** Number of patients with photophobia at the same point in time. **(C)** Number of patients feeling nauseous at the sampling point with most intense headache.

		Response to GTN					
		HA (n = 23)		HA, ICHD-3 met (n = 8)		HA, ICHD-3 not met (n = 15)	
		regularly	rarely	regularly	rarely	regularly	rarely
Symptoms described in spontaneous migraine attacks	Phonophobia	20 (87.0%)	2 (8.7%)	7 (87.5%)	1 (12.5%)	13 (86.7%)	1 (6.7%)
	Photophobia	20 (87.0%)	2 (8.7%)	8 (100.0%)	--	12 (80.0%)	2 (13.3%)
	Concentration difficulties	19 (82.6%)	--	7 (87.5%)	--	12 (80.0%)	--
	Nausea	17 (73.9%)	5 (21.7%)	5 (62.5%)	2 (25.0%)	12 (80.0%)	3 (20.0%)
	Dizziness	6 (26.1%)	4 (17.4%)	2 (25.0%)	1 (12.5%)	4 (26.7%)	3 (20.0%)
	Osmophobia	6 (26.1%)	1 (4.3%)	2 (25.0%)	1 (12.5%)	4 (26.7%)	--

Table 6 – Attendant symptoms participants experience during spontaneous (non GTN induced) migraine attacks.

3.3.6 Intake of acute medication

After GTN administration, patients were allowed to take their usual acute medication at any point but were asked if possible to wait until a headache of at least moderate intensity occurred. They could use the same acute medication that would normally take in spontaneous migraine attacks. Considering all 23 participants who developed a delayed headache after GTN administration, a total of 13 participants (56.5%) took pain relieving-medication, 6 from the group of patients with a headache meeting ICHD-3 criteria and 7 from the group of patients with a headache not meeting ICHD-3 criteria. 10 participants (43.5%) did not take any painkillers, either because they only developed a light headache or because their headache resolved spontaneously. Of the 13 participants who took pain-relieving medication, all had developed a headache of at least moderate intensity. Participants took either NSAIDs, triptans or metamizole with most participants opting for NSAIDs (see Table 7). On average, the intake of medication took place at 308 ± 100 minutes after GTN administration. All participants experienced a relief of headache intensity after medication intake. The average duration until improvement of headache after medication intake was 60 ± 20 minutes. In 3 participants, headache resolved completely. 20 participants had a remaining mild headache at the end of the session but all with an intensity of NRS ≤ 2. On average, headache improved by 3.4 ± 1.5 points on the NRS in all 23 patients. In patients who had taken acute medication (n = 13), headache improved by 4.3 ± 0.9 points on the NRS.

	Response to GTN	
	HA, ICHD-3 met (n = 8)	HA, ICHD-3 not met (n = 15)
Intake of pain-relieving medication	6 (75.0%)	7 (46.7%)
NSAIDs	4 (50.0%)	4 (26.7%)
Triptan	2 (25.0%)	2 (13.3%)
Metamizole	1 (12.5%)	1 (6.7%)

Table 7 – Intake of pain-relieving medication after GTN administration.

3.3.7 Follow-up interview

We conducted a follow-up interview with all participants at least 48 hours after GTN administration. From the participants who developed a GTN induced migraine attack (ICHD-3 criteria met), 6 (75%) reported headaches within 48h hours after the appointment. 3 out of those 6 reported having had another migraine attack. In those who developed a GTN induced headache not meeting ICHD-3 criteria, 8 (53.3%) participants reported a headache in the follow-up interview and 3 reported having had a migraine attack within 48 hours after the appointment. 7 participants from both categories had to take analgesics within 48 hours after the appointment.

		Reported within 48h after GTN administration		
		Headache	Migraine	Pain-relieving medication
Response to GTN	HA, ICHD-3 met (n=8)	6 (75.0%)	3 (37.5%)	2 (25%)
	HA, ICHD-3 not met (n=15)	8 (53.3%)	3 (20.0%)	5 (33.3%)

Table 8 – Follow-up interview 48h after GTN administration. This table shows the number of participants from categories 1 and 2 who, within 48h after the GTN appointment, indicated having experienced headache or even a migraine attack as well as the number of participants who took pain-relieving medication within 48h after the appointment.

3.3.8 CGRP levels during GTN induced headache

To test our hypothesis that tear fluid CGRP levels directly reflect trigeminal activation [89], we measured tear fluid CGRP levels during GTN induced headache. As elevated plasma CGRP levels have been measured during GTN induced migraine attacks, we also determined the corresponding plasma CGRP levels. [85, 86]

Before getting into the analysis of CGRP levels in GTN induced headache, we compared CGRP tear fluid and plasma levels from appointment 1 and baseline samples from appointment 2. We only compared individuals who had been headache free for 48h before and during sampling for both P1 (n = 29) and baseline P2 (n = 23). As expected, there were no significant differences in either tear fluid CGRP levels (p = 0.689, *Mann Whitney U Test*) or plasma CGRP levels (p = 0.368, *Mann Whitney U Test*). For a visual representation, see Figure 16 (Appendix A: Figures).

The following sub-chapters show CGRP levels trends in both tear fluid and plasma in GTN induced headache. Tear fluid CGRP levels are always indicated as the mean value

between levels from the right and left eye (see chapter 3.2.2). Measurements were carried out for the following samples:

(1) baseline (before GTN administration),
(2) most severe headache indicated,
(3) after (improvement of) headache.

CGRP levels from these 3 key sampling points of 23 participants were compared. Based on response to GTN, we differentiated between migraine headache meeting ICHD-3 criteria and headache not meeting ICHD-3 criteria.

CGRP levels in GTN induced headache

In a first instance, we compared CGRP levels for all participants who developed a delayed headache, regardless of whether the latter met ICHD-3 criteria for migraine. Data from 23 participants (f = 20, 87.0%) was analysed. For 1 patient who developed a headache not meeting ICHD-3 criteria, there was missing data for plasma CGRP levels. Hence, we only have data on plasma CGRP levels for 22 patients.

Average tear fluid CGRP levels were higher during headache than at baseline and dropped after improvement of headache:

(1) Baseline: 0.80 ± 0.77 ng/mL,
(2) Most severe headache: 1.42 ± 0.93 ng/mL,
(3) After headache: 1.10 ± 0.99 ng/mL.

There was a significant difference in CGRP tear fluid levels at the 3 points of sampling *($p < 0.001$ Friedman)*. However, changes in plasma CGRP were not significant *($p = 0.485$ Friedman).*

We also separately compared CGRP levels between the different sampling points using the Wilcoxon Signed Ranks Test. We found that tear fluid CGRP levels differed significantly between all 3 sampling points. There was no significant difference between plasma CGRP level measurements.

Figure 12 shows tear fluid and plasma CGRP levels as well as the trend of measurement from all individuals (n = 23). In tear fluid, most individual trends show a rise of CGRP levels from baseline to headache and a drop after improvement of headache. In plasma, no clear CGRP level trends become apparent. For a more in detail visualisation of CGRP levels in individuals with both GTN induced headache meeting and not meeting ICHD-3 criteria, see Figure 13 and Figure 14.

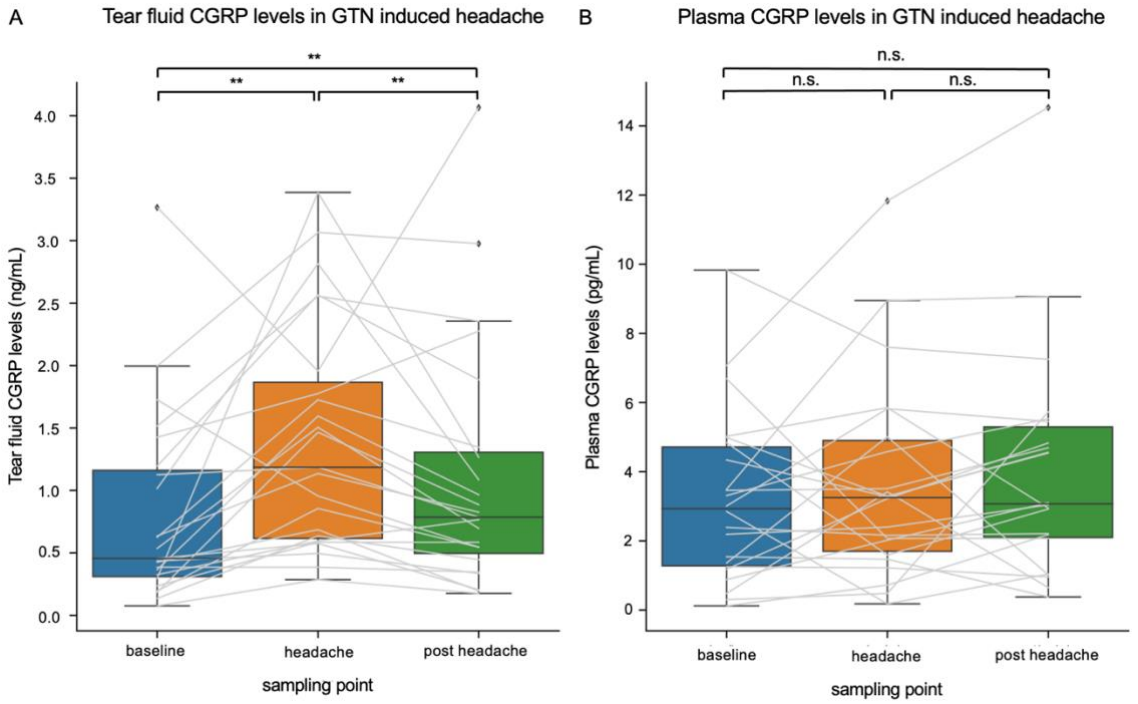

Figure 12 – CGRP levels in all patients GTN induced headache. (A) shows CGRP tear fluid levels in all patients who developed a delayed headache after GTN administration (n = 23). Tear fluid levels differed significantly between the 3 key sampling points (baseline – headache: p = 0.001; headache – post headache: p=0.006; baseline – post headache: p = 0.009; Wilcoxon Signed Ranks Tests). The grey lines show trends of tear fluid CGRP levels from all 23 individuals. **(B)** shows plasma CGRP levels in the same group. Levels did not differ significantly between the 3 key sampling points (baseline – headache: p = 0.355; headache – post headache: p = 0.205; baseline – post headache: p = 0.306; Wilcoxon Signed Ranks Tests). The grey lines show trends of plasma CGRP levels from all 23 individuals.

CGRP levels in GTN induced migraine attack (ICHD-3 criteria met)

Mean tear fluid CGRP levels from participants who developed a migraine like headache (meeting ICHD-3 criteria) after GTN administration were as follows:

(1) Baseline: 0.63 ± 0.44 ng/mL,
(2) Most severe headache: 1.45 ± 0.89 ng/mL,
(3) After headache: 1.02 ± 0.62 ng/mL.

There was a significant difference in CGRP tear fluid levels between the 3 points of sampling *(p = 0.002 Friedman)* but no significant differences in plasma CGRP levels *(p = 0.687 Friedman)*. Tear fluid CGRP levels rose significantly from baseline to headache and dropped significantly after improvement of headache. Post headache, CGRP levels were still significantly higher than at baseline. When comparing individual trends (see Figure 13), it becomes apparent that most trends show a rise after GTN administration as well as a drop after improvement of headache. In one individual, CGRP levels remained at a similar level throughout all 3 sampling points. One trend also shows a continuous rise, even after improvement of headache.

For plasma levels, no significant differences between the 3 sampling points could be detected. There were no clear trends in plasma CGRP levels either. In 2 patients, the

same pattern as in tear fluid becomes apparent: a rise after GTN administration and a drop after improvement of headache. The other trends are, however, very heterogeneous and show no clear patterns. One patient (M GTN 23) had a baseline CGRP level of 9.83 pg/mL and showed a continuous drop throughout measurements. In tear fluid, they showed a baseline level of 1.01 ng/mL, a headache level of 2.81 ng/mL and a post headache level of 1.08 ng/mL (close to baseline). After GTN administration, they indicated a maximum headache intensity of 6 on the NRS and experienced nausea, photophobia and phonophobia. They took rizatriptan at 290 minutes after GTN administration which led to an improvement of headache (NRS 1). Interestingly, this patient indicated having experienced persisting headaches for more than 48 hours after the appointment and indicated having taken 3 separate doses of triptans within 48 hours after the appointment. This persisting headache might be a possible explanation for the overall relatively high CGRP levels.

Overall, Figure 13 shows that tear fluid CGRP levels show a clear trend over the course of a migraine attack and peak during headache, whereas in plasma, no clear trends become apparent. This suggests superiority of tear fluid to plasma for measuring CGRP secreted during migraine and that tear fluid CGRP levels directly reflect trigeminal activation during migraine headache as already hypothesised by Kamm et al. [89]

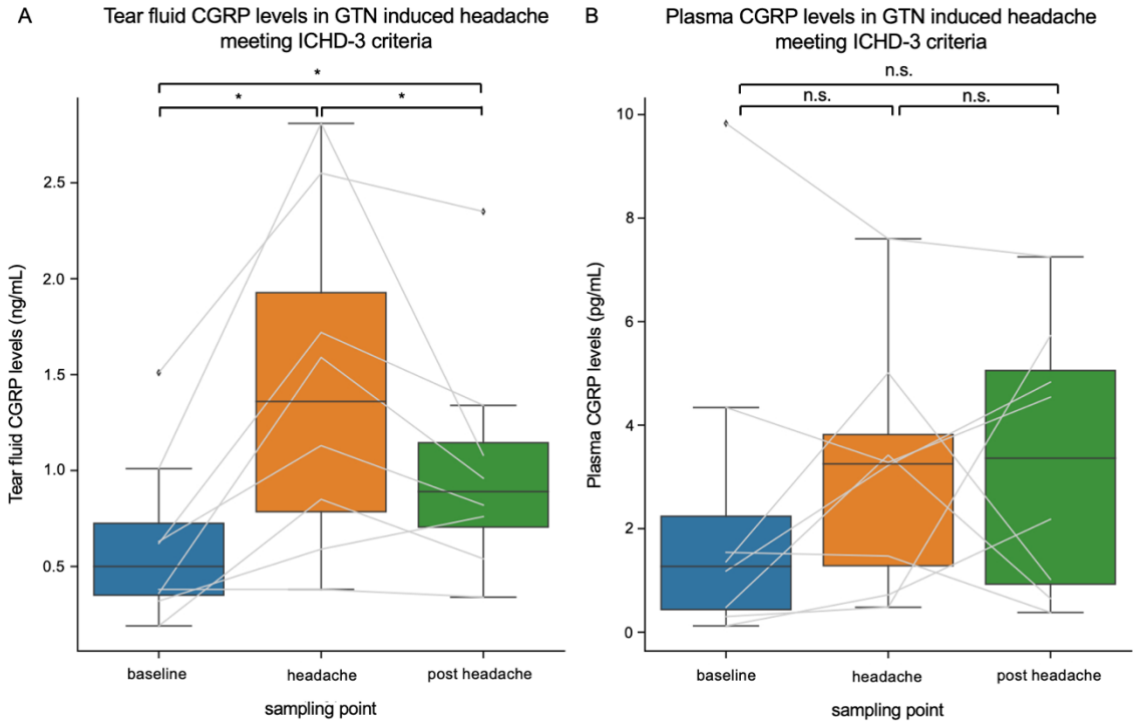

Figure 13 – CGRP levels in patients with GTN induced migraine attack. Both graphs show CGRP levels from patients who developed a migraine attack after GTN administration (n = 8). **(A)** Tear fluid CGRP levels differed significantly at the 3 key sampling points (baseline – headache: p = 0.012; headache – post headache: p = 0.025; baseline – post headache: p = 0.017; Wilcoxon Signed Ranks Tests). The grey lines show trends of tear fluid CGRP levels from all 8 individuals. **(B)** shows plasma CGRP levels, which did not differ significantly (baseline – headache: p = 0.327; headache – post headache: p = 0.779; baseline – post headache: p = 0.484; Wilcoxon Signed Ranks Tests). The grey lines show trends of plasma CGRP levels from all 8 individuals.

CGRP levels in GTN induced headache not meeting ICHD-3 criteria

15 participants developed a headache after GTN administration that did not meet migraine criteria. In those participants, a significant change of tear fluid CGRP levels after GTN administration could also be observed between the 3 sampling points *(p = 0.002 Friedman)*. Plasma CGRP levels did however show no significant changes in this group either.

CGRP tear fluid levels in participants with headache not meeting ICHD-3 criteria:

(1) Baseline: 0.89 ± 0.90 ng/mL
(2) Most severe headache: 1.40 ± 0.97 ng/mL
(3) After headache: 1.15 ± 1.15 ng/mL.

Comparing the 3 sampling points separately, we found a significant rise of CGRP in tear fluid after GTN administration *(p = 0.031, Wilcoxon Signed Ranks Test)*. The drop of CGRP levels after improvement of headache was, however, not significant (*p=0.056, Wilcoxon Signed Ranks Test*), neither were levels significantly higher post headache than at baseline (*p = 0.112, Wilcoxon Signed Ranks Test*). The comparison of individual tear fluid CGRP levels in patients with GTN induced headache not meeting ICHD-3 criteria (Figure 14) shows a similar trend as in patients with GTN induced migraine attacks (Figure 13). However, overall trends are more heterogeneous. Most individuals show a rise after onset of headache and a drop after improvement of headache. However, some trends also show either a continuous rise or drop throughout. Furthermore, there was one outlier (M GTN 52), with particularly high baseline and post high headache CGRP levels. We further looked into data of this individual. Besides suffering from episodic migraine, this individual had no other conditions and took no medication on a regular basis. They indicated not having had a headache for over 4 weeks preceding GTN administration. After our appointment, they indicated having had a continuous headache for 48 hours (headache intensity of 3 on the NRS).

There were no significant differences between plasma levels between sampling points. There were no apparent trends in individual plasma CGRP levels either, which were very heterogeneous in this group.

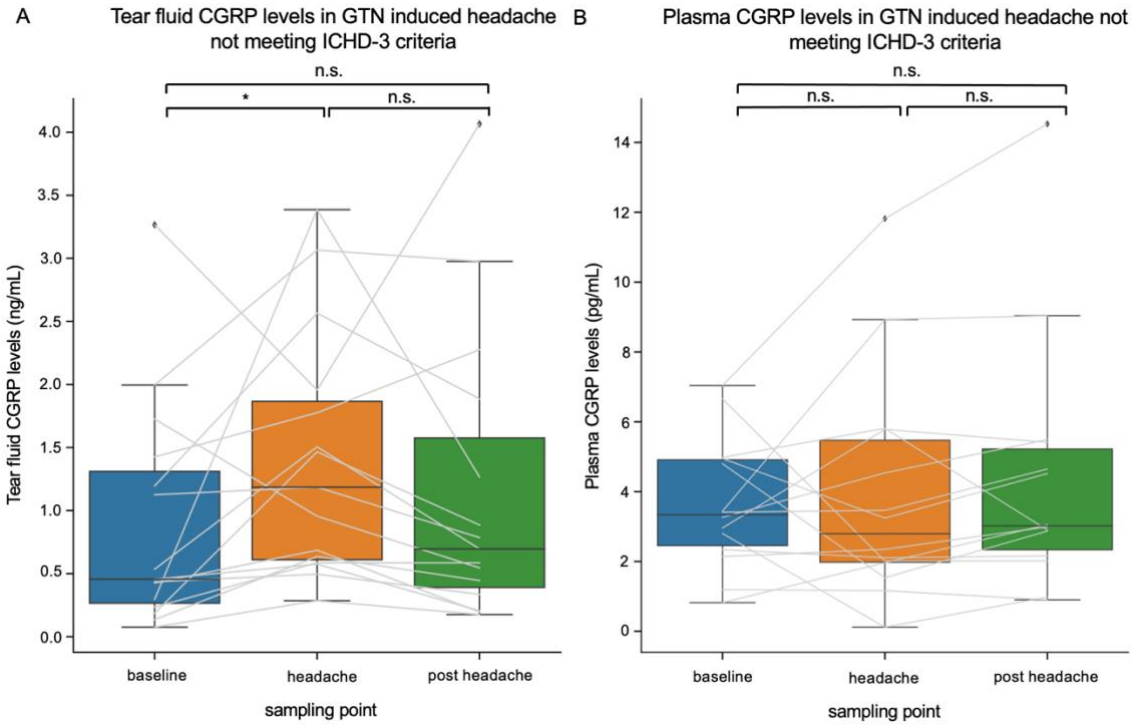

Figure 14 – CGRP levels in patients with GTN induced headache (ICHD-3 criteria not met). In this figure, we show CGRP levels in patients who developed a headache that did not meet the ICHD-3 migraine criteria after GTN administration (n = 15). **(A)** shows tear fluid levels before, during and after headache. Difference between baseline and headache sample was significant (p = 0.031, Wilcoxon Signed Ranks Test). Differences between headache and post headache as well as baseline and post headache were not significant (p = 0.056 and p = 0.112, Wilcoxon Signed Ranks Test). The grey lines show trends of tear fluid CGRP levels from all 15 individuals. **(B)** shows the corresponding plasma CGRP levels, which did not differ significantly (baseline – headache: p = 0.638; headache – post headache: p = 0.075; baseline – post headache: p = 0.397; Wilcoxon Signed Ranks Tests). The grey lines show trends of plasma CGRP levels from all 15 individuals.

CGRP levels in GTN induced headache – ICHD-3 criteria met versus not met

We also compared CGRP tear fluid levels at the 3 key sampling points between participants depending on their response to GTN.

Before GTN administration, baseline CGRP tear fluid levels were 0.63 ± 0.44 ng/ml in patients who developed a migraine attack and 0.89 ± 0.90 ng/ml in patients who developed a headache not meeting ICHD-3 criteria. There was no significant difference between the two groups (*p = 0.846 Mann-Whitney U Test*). Plasma CGRP levels did not differ significantly either (*p = 0.056 Mann-Whitney U Test*).

Regarding the point of most severe headache, tear fluid CGRP levels in patients with migraine were 1.45 ± 0.89 ng/ml. In comparison levels in patients whose headache did not meet ICHD-3 criteria were 1.40 ± 0.97 ng/ml. There was no significant difference between both groups (*p = 0.846 Mann-Whitney U Test*). Plasma CGRP levels did not differ either (*p = 0.585 Mann-Whitney U Test*).

After improvement of headache, tear fluid CGRP levels dropped to 1.02 ± 0.62 ng/ml in those with migraine and 1.15 ± 1.15 ng/ml in headache not meeting ICHD-3 criteria (*p = 0.519 Mann-Whitney U Test*). Plasma levels were not significantly different between both groups either (*p = 0.699 Mann-Whitney U Test*).

To conclude, neither tear fluid nor plasma CGRP levels at the 3 key sampling points did not differ significantly between participants who developed a migraine-like headache and those with a headache not meeting ICHD-3 criteria. In both groups, tear fluid CGRP levels rose significantly from baseline to headache. The drop of tear fluid CGRP levels after improvement of headache was only significant in participants with a GTN induced headache meeting ICHD-3 criteria.

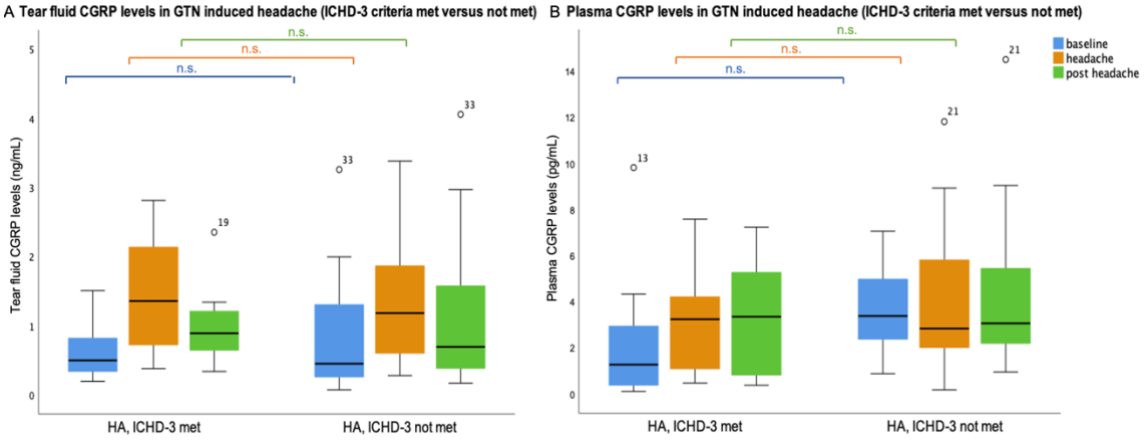

Figure 15 – CGRP levels in GTN induced headache (ICHD-3 criteria met versus not met). Here we compare tear fluid CGRP levels in patients who developed a GTN induced migraine attack versus those who developed a headache that did not meet ICHD-3 criteria for migraine. **(A)** shows tear fluid CGRP levels. There were no significant differences between groups. Comparison of tear fluid CGRP levels; baseline: p = 0.846 Mann-Whitney U Test; headache: p = 0.846 Mann-Whitney U Test; post headache: p = 0.519 Mann-Whitney U Test. **(B)** shows plasma CGRP levels. There were no significant differences between groups. Comparison of plasma CGRP levels; baseline: p = 0.056 Mann-Whitney U Test; headache: p = 0.585 Mann-Whitney U Test; post headache: p = 0.699 Mann-Whitney U Test).

3.4 Schirmer Test

The Schirmer test was implemented into our study protocol during the course of the study. Hence, we have data for 17 migraineurs and 9 healthy controls from the first appointment. Descriptive statistics of Schirmer tests are shown in Table 9. We compared Schirmer test results from the right and left eye separately.

For data of the first appointment, we compared Schirmer test results between interictal migraineurs, ictal migraineurs and healthy controls. We considered both eyes separately. There were no significant differences between any of the groups in neither the right nor the left eye.

At the second appointment (GTN administration), we conducted two Schirmer tests, one at the beginning (test 1) and one at the end of the appointment (test 2). Results from

Schirmer test 1 and Schirmer test 2 (mean values of n = 16 patients) of the right eye showed no significant difference (*p = 0.239, Wilcoxon Signed Ranks Test*). There was no significant difference regarding the left eye either (*p = 0.124, Wilcoxon Signed Ranks Test*). Therefore, we can conclude that there was no significant difference in reflex tear fluid production between the beginning and the end of the second appointment.

However, the limited availability of Schirmer test data must be considered. The Schirmer test can therefore not be used as a representative tool to quantify reflex tear fluid production of all participants in this study.

		Schirmer Test		
		Right eye; mean ± SD (mm)	Left eye; mean ± SD (mm)	Average of both eyes; mean ± SD (mm)
1st appointment	Interictal (n=8)	18 ± 12	19 ± 10	18 ± 11
	Ictal (n=9)	23 ± 12	21 ± 12	22 ± 11
	Healthy controls (n=9)	15 ± 11	14 ± 8	15 ± 8
2nd appointment				
Schirmer test nr. 1	HA, ICHD-3 met (n=4)	32 ± 4	28 ± 11	32 ± 4
	HA, ICHD-3 not met (n=12)	26 ± 8	22 ± 8	26 ± 8
Schirmer test nr. 2	HA, ICHD-3 met (n=4)	30 ± 8	22 ± 14	26 ± 11
	HA, ICHD-3 not met (n=12)	22 ± 12	19 ± 10	21 ± 10

Table 9 – Results from Schirmer tests from the first and second appointment. For the 1st appointment, we differentiated between interictal and ictal migraineurs as well as healthy controls. For the 2nd appointment, we performed a Schirmer test before taking the first sample and after taking the last sample. They are labelled Schirmer 1 and Schirmer 2 in this table.

4. Discussion

4.1 Key findings

Based on the hypothesis that tear fluid CGRP levels directly reflect trigeminal activation in migraine patients [89], the aim of this study was to measure CGRP levels in tear fluid and plasma in interictal and ictal migraineurs compared to healthy controls as well as analyse tear fluid CGRP levels over the course of a GTN induced migraine attack. To the best of our knowledge, this is the first study to investigate CGRP levels in tear fluid over the course of an experimentally induced headache.

Part 1 (P1)

Data from the first part of our study showed significantly higher tear fluid CGRP levels in ictal migraineurs compared to healthy controls. We also observed a trend of higher CGRP levels in tear fluid of ictal compared to interictal migraine patients; there was however no statistically significant difference between both groups. Tear fluid CGRP levels of interictal migraineurs and healthy controls did not differ significantly. Regarding plasma CGRP levels, there were no significant differences between groups.

Part 2 (P2)

We categorised participants depending on their GTN response. The first category included participants who developed a migraine-like headache attack. The second category comprised participants who developed a delayed headache that did not meet ICHD-3 criteria for a migraine attack. We then investigated CGRP levels at 3 key sampling points:

(1) Baseline sample, taken before GTN administration and in an interictal state,
(2) Sample taken at most severe indicated headache,
(3) Sample taken after improvement of headache.

We then compared CGRP levels at these 3 sampling points. In participants with GTN induced migraine, tear fluid CGRP levels were significantly higher during maximum headache compared to baseline levels and dropped significantly after resolution/improvement of headache. Post headache levels were also significantly higher than baseline levels. In patients with GTN induced headache that did not meet ICHD-3 criteria, tear fluid CGRP levels rose significantly from baseline to headache sample but the difference between the headache and post headache as well as the baseline and post headache samples were not significant. There were no significant changes in plasma CGRP levels either group. This suggests superiority of tear fluid to plasma for measuring CGRP secreted during experimentally induced migraine, supporting the hypothesis that tear fluid CGRP levels directly reflect trigeminal activation during migraine headache. [89] Lastly, CGRP levels at the 3 key sampling points after GTN administration did not differ significantly between patients with GTN induced migraine and headache not meeting ICHD-3 criteria. In both groups, tear fluid CGRP

levels rose significantly from baseline to headache. However, the drop of tear fluid CGRP levels after improvement of headache was only significant in participants with a GTN induced headache meeting ICHD-3 criteria for a migraine attack.

4.2 Tear fluid CGRP levels

As shown before, we also found higher CGRP levels in tear fluid compared to plasma CGRP levels (~170 up to 490 times higher), highlighting the advantage of measuring tear fluid CGRP concentrations. Tear fluid CGRP concentrations were also consistent with levels found by Kamm et al. [89] Furthermore, we found significantly higher tear fluid CGRP levels in unmedicated ictal migraineurs compared to healthy controls. CGRP levels in ictal migraineurs were higher than in interictal migraine patients, but not significantly. We did, however, not see higher CGRP levels in tear fluid of interictal migraineurs compared to healthy controls controls, in contrast to our previous study. Total headache frequency was higher in the previous study (14.7 ± 9.8 days/month in episodic and chronic migraineurs combined) compared to our study (7.8 ± 4.0 days/month; only episodic migraineurs). However, Kamm et al. reported no significant differences between tear fluid CGRP levels between episodic and chronic migraineurs as well as no significant correlation between headache frequency and CGRP levels. [89] Therefore, headache frequency most likely did not cause the different findings in our study. In the predecessor study, average tear fluid CGRP levels were indicated at 1.09 ± 1.47 ng/ml (n = 30) in interictal episodic migraine patients. We measured average tear fluid CGRP levels of 0.87 ± 0.79 ng/ml (n = 17). This shows that we measured lower average CGRP levels in tear fluid and had a smaller cohort of interictal migraine patients, which could be considered as an explanation. We also had a smaller cohort of healthy controls (n = 32 versus n = 48). Another possible explanation is that – compared to the previous study by Kamm et al. – we conducted follow-up interviews 48h after the first appointment. In our study, participants who had a headache within 48h after sampling were categorised as ictal migraine patients. Headaches occurring after sampling were, however, not documented in the previous study. [89] Sample processing was the same in both studies and is therefore unlikely to have caused the different findings. Furthermore, we used pre-chilled vials, reduced processing times to the best of our abilities, and always stored samples on ice as these aspects are important to avoid false low values when measuring CGRP. [81, 123, 138] In summary, the difference in results compared to the previous study could be due to a stricter definition of ictal versus interictal through the implementation of a post 48h interview (11 out of 17 participants were headache free during sampling but indicated having experienced a headache within 48h after sampling) and a smaller patient cohort.

In line with the hypothesis that CGRP concentrations in tear fluid reflect activation of the first trigeminal branch, we found that CGRP tear fluid concentration rose significantly over the course of GTN induced migraine and dropped significantly after headache improvement in patients with GTN induced headache (n = 23). In patients with GTN induced migraine (n = 8), this trend was even more clear when comparing trends of tear

fluid CGRP levels in individual participants. These findings are also in line with findings from early studies showing elevated jugular vein CGRP levels as well as elevated salivary CGRP levels in the acute migraine attack. [80, 82, 88] A recent study has shown that salivary CGRP can be used to monitor different phases of a migraine attack in 80% of patients (CGRP dependent attack vs 20% having developed a CGRP independent attack). [139] Our findings suggest that measuring tear fluid CGRP could also be used for monitoring of the acute migraine attack. This proposition is further backed by CGRP concentrations being higher in tear fluid than in peripheral blood, thereby allowing to detect very subtle changes. [81]

We also found that after improvement of migraine headache, CGRP levels did not return to baseline but remained significantly higher than at baseline. This is surprising as plasma CGRP levels have been shown to decrease parallel to headache intensity in migraine attacks (after treatment with sumatriptan). [86] It has also been shown that triptans can normalise CGRP levels. [88] Hence, the normalisation of elevated CGRP levels in migraine attacks that have been observed in other studies might be attributed to intake of triptans. In our study, only 13 patients (56.5%) took acute medication, and 4 patients (17.4%) took triptans. This might be the explanation for elevated CGRP levels even after clinical improvement of headache. Another possible explanation might be that trigeminal activation lasts well past the headache phase and into the prodromal phase. [53] Furthermore, we know from the conducted follow-up interviews that 14 (60.8%) patients reported suffering (migraine and headache not meeting ICHD-3 criteria) headaches and 7 (30.4%) had taken acute migraine medication within 48 hours after the appointment. This could also explain why CGRP levels in tear fluid were higher after improvement of GTN induced migraine compared to baseline samples.

Whereas in patients with GTN induced migraine (n = 8), CGRP levels differed significantly between all three sampling points (baseline – headache; headache – post headache; baseline – post headache), this was not the case for patients with GTN induced headache not meeting ICHD-3 criteria (n = 15). In this group, we also detected a significant rise in tear fluid CGRP levels from baseline to headache samples. A possible explanation for this might be the fact that the delayed headache – although not meeting ICHD-3 criteria – might have been a milder form of a migraine headache not reaching the full extent of a migraine attack or that acute pain medication was taken before a migraine attack could fully develop. Furthermore, the rise of tear fluid CGRP levels in migraineurs who developed a headache not meeting ICHD-3 criteria for migraine might explain why tension-type headache in migraine patients responds to treatment with triptans. [140] Levels did, however, not differ between headache and post headache or baseline and post headache samples. This raises the question of whether tear fluid CGRP levels might be used to differentiate between migraine and headache not meeting ICHD-3 criteria. However, more data and specific studies are necessary to investigate this question further.

Kamm et al. were the first to measure tear fluid CGRP levels in migraine patients. [89] Tear fluid CGRP levels measured in this study were consistent with levels measured by

Kamm et al. in the previous study. [89] Furthermore, as hypothesised, we found a significant change in tear fluid CGRP levels over the course of a GTN induced migraine attack. These results contribute to the validation of our method and further back the hypothesis that CGRP in tear fluid reflects trigeminal activation in migraineurs.

4.3 Plasma CGRP levels

Measuring CGRP levels in peripheral venous blood has shown mixed results in different studies. Some studies found significant differences between healthy controls and migraineurs. Others only detected a significant difference between ictal migraineurs and healthy controls. [80, 83, 89] We found no significant differences in plasma CGRP levels between migraineurs and healthy controls. We did not observe any significant changes in plasma CGRP levels after GTN induced headache. Our negative results for plasma CGRP levels undermined the body of evidence refuting CGRP levels in peripheral plasma as a useful tool to qualify CGRP secretion in migraine. The varying results found in different studies are most likely caused by differences in methods used as well as a lack of homogeneity of study groups. [81, 138] Other important factors are probably the dilution of CGRP in the bloodstream, contamination from other CGRP sources than the trigeminal fibres and the short half-life of CGRP (7-9 minutes). [123-125] One theory is that the fast degradation of CGRP in blood circulation might be the reason for negative results in studies with longer processing times. [123] We always drew blood straight after collecting tear fluid. Blood samples were stored on ice during the 5-minute centrifugation period of tear fluid. Plasma samples were then centrifuged for 10 minutes, plasma was pipetted into separate tubes and stored in a freezer at -80°C. Processing times were reduced to the best of our abilities; however, it cannot be excluded that they might have influenced our study results regarding plasma CGRP levels. Furthermore, the usage of pre-chilled vials and peptidase inhibitors, as well as storage on ice immediately after sampling play a key role. [81, 123, 138] However, this was carefully put into practice during the sampling process in our study and is therefore most likely not the cause of negative results. In any case, the currently available data on plasma CGRP levels further accentuate the need for an easy and accessible way of quantifying trigeminal CGRP released during migraine attacks.

4.4 GTN induced migraine attack

As previously mentioned, studies have shown that up to 80% of migraineurs experience a delayed more severe headache that meets the ICHD-3 diagnostic criteria for migraine without aura. [111, 112, 141] However, the published response rate of GTN induced migraine varies from 50 up to 80%. [112] Considering participants who developed a headache fulfilling the IHS diagnostic criteria as GTN responders, we observed a response rate of 30.8% (8 out of 26 participants) in our study, which does not match what is described in literature. However, 61.5% (16 out of 26 migraine patients) developed a headache of at least medium intensity (NRS ≥ 4). Furthermore, 30.8% of patients (8 out

of 26 participants) reported a headache that was described as pulsating/throbbing and 65.4% (17 out of 26) reported a unilateral headache; both characteristics are featured in the HIS migraine criteria. [3] So even though only 8 patients had a proper migraine attack, headache characteristics that are typical for migraine attacks were more frequently reported. In the group of patients who developed a GTN induced headache (n=23), a total of 20 patients (87.0%) reported non-headache symptoms. The most important non-headache symptoms in migraine, namely photophobia, phonophobia and nausea [3], were experienced by 9 (39.1%), 6 (26.1%) and 6 (26.1%) patients respectively. To compare these numbers of GTN induced headache to spontaneous migraine attacks in the same 23 patients: 20 (87.0%) regularly suffer from photophobia, 20 (87.0%) from phonophobia and 17 (73.9%) from nausea during spontaneous migraine attacks. This shows a discrepancy incurrence of photophobia, phonophobia and nausea in spontaneous migraine versus GTN induced headache in our study cohort.

In those who developed a migraine attack (n = 8), the average latency between GTN administration and the most intense headache was 255 minutes (4.3h). This latency is in line with previous literature (4-6h). [117]

The discrepancy between response rate in our study compared to other studies might be due to previous studies categorising participants with a GTN induced headache described as similar to their usual migraine attacks as GTN responders, which we did not do. [112, 141] Thomsen et al., who only categorised patients with a GTN induced headache meeting ICHD-II criteria as GTN responders, did however also observe a higher response rate (80%) than we did. [111] Another possible explanation might be a potential relation between GTN induced migraine and headache frequency. Christiansen et al. showed that GTN induced headache tends to be more intense in migraineurs with higher migraine frequency. However, there was no statistically significant difference between groups. [118] It must also be noted that they defined rare attacks as ≤ 4 attacks/year. Our participant cohort had an average migraine attack frequency of 3.5 ± 2.7 days per month, which is comparable to the group with frequent attacks in aforementioned study. Therefore, our data cannot accurately be compared to the study by Christiansen et al.

Furthermore, there was no significant difference between duration of GTN administration in GTN responders and non-responders as well as no difference in intensity of the initial GTN-associated headache during administration between both groups. We followed the established application protocol of intravenous GTN administration of 0.5 µg/kg/min over 20 min. The time of application varied a little as some patients had a clinically manifest drop of blood pressure during the administration and we had to pause the administration. We also calculated the response rate including data from those who had to be excluded (n = 35) – except for the 2 patients who presented with a headache already before GTN administration – and the response rate we found was similarly low (25.7%).

4.5 Clinical implications

There are currently no biomarkers available that can be used in the diagnosis of migraine. As measuring CGRP in peripheral blood has yielded heterogeneous results and measuring CGRP in central venous blood is an invasive method, the development of an alternative method to measuring CGRP levels in plasma would be worthwhile. The measurement of CGRP in tear fluid needs further validation but it is likely to directly reflect trigeminal activation, thereby possibly being an eligible candidate for a diagnostic biomarker. In general, the usage of CGRP as a clinical diagnostic biomarker to objectify disease severity is rather controversial. [142] It might, however, in the future be possible to use tear fluid CGRP as a marker to objectify as well as predict patients' response rates to different migraine medications. [142] For example, it has already been shown that higher levels of salivary CGRP are significantly associated with a better response to prophylactic treatment with the CGRP antibody Erenumab in episodic migraine patients. [143] Furthermore, a study investigating the measurement of CGRP in blood plasma found that CGRP plasma concentrations cannot be used to compare pathologies between different individuals. It may, however, be suitable to track CGRP changes in one individual to assess trends over the course of an illness. [138] Even though our findings do not support this hypothesis for plasma CGRP as we saw no patterns at all, it might also be an option to use tear fluid CGRP to track individual courses of disease.

As proposed by Alpuente et al., there might be a difference between CGRP dependent and CGRP independent migraine attacks which have been differentiated through salivary CGRP measurement. [139] This hypothesis could also be tested through measuring CGRP in tear fluid as this would allow to further classify migraine attacks based on the underlying pathophysiology and in a next step compare efficiency of different acute migraine medication in CGRP dependent versus CGRP independent migraine attacks. As a perspective, it might also be possible to further classify episodic migraine depending on interictal CGRP tear fluid levels, thereby predicting the response rate to prophylactic treatments targeting CGRP (CGRP antibodies).

As previously mentioned, in patients with GTN induced headache not meeting ICHD-3 criteria (n = 15), we only detected a significant rise of tear fluid CGRP levels from baseline to headache. Levels did, however, not differ between headache and post headache or baseline and post headache. These results stand in contrast to our findings in patients who developed a migraine attack after GTN administration (n = 8). In the latter, tear fluid CGRP levels differed significantly between all 3 sampling points. This implicates that different kind of headaches elicit different CGRP level trend patterns. For example, it would be interesting to analyse differences in CGRP levels in tension-type versus migraine headache. However, more data is needed to further investigate this question.

4.6 Limitations

Even though sampling of tear fluid is a non-invasive method that is tolerated by most people, we still observed that in some individuals, sampling is limited by low tolerability of touch of the eye which can make sampling difficult. Sampling is also complicated in people with dry eyes, in whom it is sometimes not possible to collect enough tear fluid for quantification of CGRP, which is why we took fewer samples in these participants. Some people also have very high reflex tear production, which might cause dilution and consequently false low values of CGRP in those individuals. Therefore, despite tear fluid sampling being non-invasive and easy in most people, the method is sometimes not as straightforward due to the afore mentioned difficulties, which can be a disadvantage compared to taking blood samples.

To better assess headache characteristics and attendant symptoms after GTN administration, it would have been better to give participants a headache questionnaire evaluating intensity (NRS), headache quality and location as well as attendant symptoms every hour, regardless of sampling times. In our study, these were only documented at sampling points. As mentioned above, we took fewer samples in patients with dry eyes or low tolerance to tear fluid sampling and therefore also have fewer data on headache characteristics and attendant symptoms. This was a limiting factor in analysing headache patterns after GTN administration.

Furthermore, the Schirmer test was only introduced into the study protocol halfway through the study after external recommendation to do so. For further studies, it would be of benefit to perform a Schirmer test on all participants as this might be a helpful tool to quantify reflex tear fluid production caused by the irritation of touch of the cornea. This would allow to investigate a potential correlation between the quantity of reflex tearing to tear fluid CGRP levels.

Moreover, if both healthy controls and migraineurs had been given GTN, it would have been interesting to compare respective tear fluid CGRP levels after GTN administration.

Lastly, it is widely known that CGRP can be elevated under other circumstances than migraine, such as during pregnancy, dialysis, osteoarthritis, and different flushing syndromes. [69, 130, 144, 145] In our study, recruitment of migraineurs willing to participate in GTN administration and experimental induction of headache turned out difficult. We therefore included patients with certain medically adjusted conditions as the number of included participants would otherwise have been too low (see chapters 3.2.1 and 3.3.2). We did, however, strictly exclude all patients with a diagnosis of arterial hypertension or who presented elevated blood pressure levels at our clinic as it is widely accepted that CGRP plays an important role in arterial hypertension. [69] In the vascular system, CGRP is mainly found in perivascular nerve endings. [146] Due to its vasodilating effects, CGRP secretion is likely to be activated in patients with hypertension. However its exact role in hypertension is still not fully understood. [69] By excluding all patients with elevated blood pressure or a diagnosis of arterial hypertension from our study, we avoided the latter as a confounding factor in CGRP levels.

4.7 Prospect

In this study, we measured CGRP levels over the course of a GTN induced migraine attack. We did, however, observe a lower response rate to GTN than described in previous studies and only 34.8% of participants had a headache meeting ICHD-3 criteria for a migraine attack. It will therefore be interesting to compare results from this study to CGRP tear fluid levels measured during spontaneously occurring (non GTN induced) migraine attacks.

Furthermore, it would be worth comparing CGRP levels in right and left eye tear fluid and see if there is a correlation between localisation of migraine headache. If tear fluid CGRP levels reflect trigeminal activation, as we hypothesised, CGRP levels might be significantly higher on the side where the headache manifests.

We found a significant drop of tear fluid CGRP after intake of acute medication. It has already been shown that tear fluid CGRP levels are significantly lower after use of pain-relieving medication compared to interictal migraineurs. [89] Reduced plasma CGRP levels have also been shown after sumatriptan intake in migraineurs with GTN induced migraine. A drop in CGRP levels was only observed in patients whose headache improved after intake of medication. [86] Cady et al. even found that elevated salivary CGRP levels can predict the response to acute medication with rizatriptan. [88] As salivary CGRP levels and tear fluid CGRP levels most likely reflect CGRP secretion from the third (V3) and first (V1) branches of the trigeminal nerve respectively, it would be interesting to investigate if tear fluid CGRP levels could be used as a predictor for response rate to triptans but also to other acute migraine medications like NSAIDs. So far, there are no biochemical markers to predict therapeutic response of migraine patients to different medications. The establishment of a such a marker would be a substantial step forward in migraine diagnostics and might facilitate the choice of an appropriate and patient-tailored acute migraine medication.

Moreover, Alpuente et al. found that baseline salivary CGRP levels can predict treatment response to the CGRP antibody erenumab in episodic migraineurs. [143] Another study showed that plasma CGRP levels can predict response to preventive treatment with Onabotulinumtoxin type A in chronic migraine patients. [147] These findings might be another big step forwards for precision medicine in migraine. Setting up a similar study measuring tear fluid CGRP before and after treatment with different prophylactic migraine treatments would be an interesting way to further test the suitability of tear fluid CGRP as a biomarker in migraine for the prediction of treatment response.

As CGRP-independent and CGRP-dependent migraine attacks have been proposed based on measurements of salivary CGRP, this assumption could also be tested measuring CGRP in tear fluid. [139] As both tear fluid and saliva CGRP concentrations are hypothesised to reflect trigeminal activation of the first and third branches respectively, it might be interesting to set up a study comparing CGRP levels in both fluids in a homogenous patient cohort. This would be an opportunity to compare both

methods directly and to investigate which fluid is better suited for monitoring of migraine attacks or objectively testing therapeutic response based on CGRP levels.

In summary, our findings can be considered further validation of the method of measuring CGRP in tear fluid and suggest that tear fluid is superior to blood for measuring CGRP levels during migraine attacks. Further research is needed to test the suitability of tear fluid CGRP levels as a clinical biomarker regarding different diagnostic questions (e.g., prediction of treatment response to acute medication or prophylactic treatments). Nevertheless, our findings strongly suggest that further investigation of tear fluid CGRP levels could open doors to more patient-tailored treatment options in migraine as tear fluid CGRP concentrations seem to be a promising candidate in the quest for a clinical biomarker in migraine.

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List of Figures

List of Tables

List of Abbreviations

aHT	arterial hypertension
ASA	acetylsalicylic acid
BMI	body-mass-index
BP	blood pressure
BW	bodyweight
CAPS	cranial autonomic parasympathetic symptoms
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene-related Peptide
DALY	disability-adjusted life years
ELISA	enzyme-linked immunosorbent assay
GBD	global burden of disease
GPCR	G protein-coupled receptor
GTN	glyceryl trinitrate
HA	headache
ICHD	international classification of headache disorders
IHS	international headache society
mABs	monoclonal antibodies
NO	nitric oxide
NPY	neuropeptide Y
NRS	numerical rating scale
NSAID	non-steroidal anti-inflammatory drug
PACAP	pituitary adenylate cyclase activating peptide
sGC	soluble guanylate cyclase
SGC	satellite glial cell
SP	substance P
TCC	trigemino-cervical complex
TF	tear fluid
TG	trigeminal ganglion
TNC	trigeminal nucleus caudalis
TVS	trigeminovascular system
VIP	vasoactive intestinal peptide

Appendix A: Figures

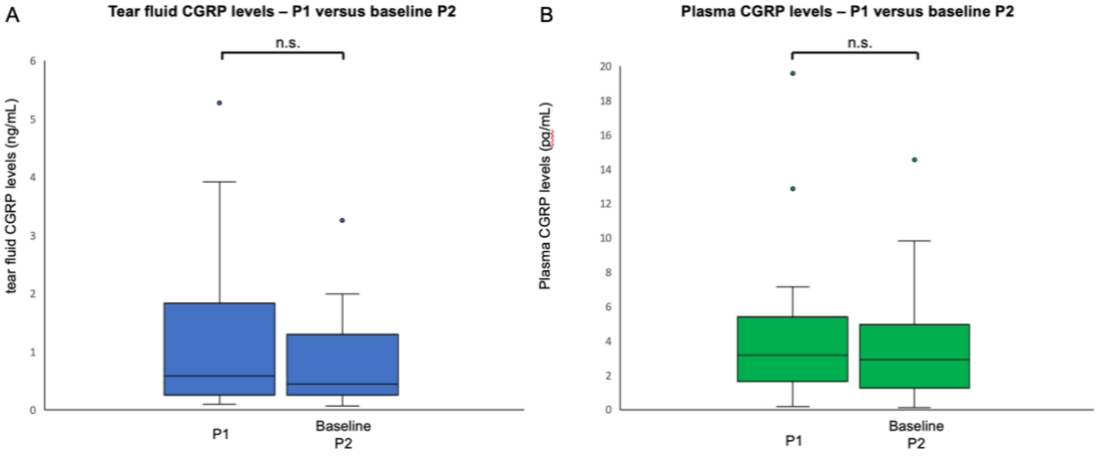

Figure 16 – comparison of CGRP levels between appointment 1 and 2. This figure shows CGRP tear fluid and plasma levels from appointment 1 (P1) and baseline samples of appointment 2 (P2). We only compared individuals who had been headache free for 48h before and during sampling for both P1 (n = 29) and baseline P2 (n = 23). **(A)** Tear fluid CGRP levels were measured at 1.15 ± 1.21 ng/mL at P1 and 0.80 ± 0.74 ng/mL at P2 (baseline). There was no significant difference between tear fluid CGRP levels (p = 0.689, *Mann Whitney U Test*). **(B)** Plasma CGRP levels were measured at 4.32 ± 3.99 pg/mL at P1 and 3.64 ± 3.25 pg/mL at P2 (baseline). There was no significant difference between plasma CGRP levels (p = *0.368, Mann Whitney U Test).*

Appendix B: Questionnaire for healthy controls

Fragebogen für gesunde Kontrollprobanden

Rechtes Auge:_____ Linkes Auge:_____

Aktuell Schmerzen?_____

Datum:_____

Name:_____ Geburtsdatum:_____

Gewicht:_____ Größe:_____

Geschlecht: ☐weiblich ☐männlich

1. Was machen Sie beruflich:

☐selbständig ☐Angestellter ☐Beamter ☐Ausbildung
☐Hausfrau/-mann ☐arbeitslos ☐Rentner/-in, seit wann:_____

2. Arbeiten Sie im Schichtdienst? ☐nein ☐ja

3. Familienstand: ☐ledig ☐verheiratet ☐geschieden
☐verwitwe(r)t

4. Haben Sie Kinder? ☐nein ☐ja, wie viele?_____

Allgemeine Erkrankungen

5. Wie würden Sie Ihre allgemeine Gesundheit im letzten Monat beschreiben?

☐sehr gut ☐gut ☐mittel ☐schlecht

6. Leiden Sie an neurologischen Erkrankungen?

☐nein ☐ja. Welche:_____

7. Hatten Sie schon einmal eine Migräne- oder Cluster-Kopfschmerz-Attacke?

☐nein ☐ja, wann und wie häufig:_____

8. Wurde bei Ihnen jemals eine andere Erkrankung diagnostiziert (Art, Hypertonie, Asthma, Herzbeschwerden, Magenulzera)?

☐nein ☐ja. Welche:_____

9. Nehmen Sie regelmäßig Medikamente ein?

☐nein ☐ja. Welche:_____

10. Nehmen Sie zusätzlich andere Medikamente ein?

☐nein ☐ja. Welche:_____

11. Sind psychiatrische Erkrankungen bei Ihnen bekannt?

☐nein ☐ja. Welche:_____

12. Sind psychiatrische Erkrankungen in Ihrer Familie bekannt?

☐nein ☐ja. Welche:_____

13. Hatten Sie eine Operation in den letzten 6 Monaten?

☐nein ☐ja. Welche:_____

14. Leiden Sie an Allergien?

☐nein ☐ja. Welche:_____

15. Rauchen Sie Zigaretten?

☐nein nie ☐Ex-Raucher, aufgehört:_____

☐ja. wieviel/Tag:_____ ☐Gelegenheitsraucher

16. Trinken Sie regelmäßig Alkohol?

☐nein nie ☐ja, bis zu 2-3x/ Woche ☐ja, mind. 2-3x/Woche

☐gelegentlich

wieviel/Tag:_____ was?:_____

Kopfschmerz-Anamnese

17. Wie viele gewöhnliche Kopfschmerzen haben Sie im Monat?_____

18. Wie lange dauern diese Kopfschmerzen gewöhnlich an?

☐ 30min- 2h ☐ 3-4h ☐ 5-12h ☐ 12-24h ☐ mehrere Tage
☐ 7 Tage

19. Wie würden Sie diese Kopfschmerzen als drückend, ziehend oder dumpf beschreiben?

☐ja ☐nein, eher als:_____

20. Treten diese Kopfschmerzen beidseitig auf?

☐ja ☐nein, nur:_____

21. Werden diese Kopfschmerzen von einem der folgenden Symptome begleitet:

☐Übelkeit ☐Erbrechen ☐Lichtempfindlichkeit
☐Lärmempfindlichkeit ☐andere:_____

22. Seit wann leiden Sie an diesen Kopfschmerzen?_____

23. Hatten Sie jemals eine Kopf- oder Nackenverletzung, die eine medizinische Behandlung bedarft hat?

☐nein ☐ja. Bitte näher beschreiben:_____

Appendix C: Questionnaire for migraine patients (parts 1 and 2)

Fragebogen für Migränepatienten – Untersuchungstag 1

Probandennr.:

µl Rechtes Auge: µl Linkes Auge:

Dauer Abnahme re Auge: Dauer Abnahme li Auge:

Aktuell Schmerzen?

Wann zuletzt Kopfschmerzen?

Wann zuletzt Einnahme von Akutmedikation?

Blutdruckmessung aktuell?

Interview 48h post: Kopfschmerzen? Akutmedikation?

Datum: Uhrzeit:

Gewicht: Größe:

Geschlecht: ☐weiblich ☐männlich

1. Was machen Sie beruflich:

☐selbständig ☐Angestellter ☐Beamter ☐Ausbildung
☐Hausfrau/-mann ☐arbeitslos ☐Rentner/-in, seit wann:

2. Arbeiten Sie im Schichtdienst? ☐nein ☐ja

3. Familienstand: ☐ledig ☐verheiratet ☐geschieden ☐verwitwe(r)t

4. Haben Sie Kinder? ☐nein ☐ja, wie viele?

Migräne-Anamnese

5. Um welche Migräne-Kopfschmerzen handelt es sich bei Ihnen?
☐Migräne ohne Aura
☐Migräne mit Aura
☐episodisch
☐chronisch

6. An wie vielen Tagen im Monat leiden Sie durchschnittlich an einer Migräneattacke?

7. Wie lange dauert eine Migräneattacke unbehandelt in der Regel an?

☐ nicht mehr als 2h ☐ 3-4h ☐ 5-12h ☐ 12-24h ☐ mehrere Tage
☐ 1 Woche oder länger

8. Auf einer Skala von 0-10, wobei 0 kein Schmerz und 10 der schlimmste vorstellbare Schmerz bedeutet, wie schmerzhaft würden Sie Ihre Migräneattacken einschätzen?

9. Wo sind die Schmerzen normalerweise lokalisiert?

☐hinter dem rechten Auge ☐hinter dem linken Auge ☐hinter beiden Augen
☐über der rechten Schläfe ☐über der linken Schläfe ☐über beiden Schläfen
☐über der rechten Augenbraue ☐über der linken Augenbraue ☐über beiden Augenbrauen
☐am Hinterkopf, rechtsseitig ☐am Hinterkopf, linksseitig ☐am Hinterkopf, beidseitig

10. Wie ist die Schmerzqualität der Migräne-Schmerzen?

☐pulsierend/pochend ☐schmerzend/drückend ☐stechend
☐bohrend ☐wie ein enges Band um den Kopf
☐dumpfer Schmerz ☐wie glühende Nadeln ☐anders:

11. Welche Begleitsymptome treten bei Ihnen auf?

Begleitsymptome	regelmäßig	selten
Lärmempfindlichkeit		
Lichtempfindlichkeit		
Geruchsüberempfindlichkeit		
Sehstörungen		
Schwindel		
Übelkeit		
Erbrechen		
Durchfall		
Kältegefühl/ Frieren		
Lakrimation		
Gerötetes Auge		
Naselaufen/ Nasenverstopfung		
Berührungsempfindlichkeit im Gesicht ipsilateral zum Schmerz während der Attacke		
Sprachschwierigkeiten		
Konzentrationsschwierigkeiten		
Bewusstseinsverlust		

12. Triggert oder verschlimmert einer der folgenden Punkte Ihre Migräne? Bitte alle benennen.

☐Stress (Ärger, Sorgen) ☐starker Sonnenschein ☐Wetterumschwung
☐Ruhigwerden nach Stress ☐lautes Geräusch ☐schweres Heben
☐Fliegen ☐sportl. Aktivität ☐bestimmte Gerüche/ Perfum
☐ausgelassene Mahlzeiten ☐sexuelle Aktivität ☐Husten, Anstrengung, Beugen
☐best. Nahrungsmittel (Schokolade, Käse, Bier, Glutamat) ☐Müdigkeit
☐andere:_____

13. Verbessert eine der folgenden Aktivitäten die Migräneschmerzen?

☐Ausruhen ☐Sport ☐Ruhe und Dunkelheit
☐heiße/ kalte Kompresse ☐Massage ☐warme Dusche
☐Druck über dem Migräneschmerz ☐andere:_____

14. Nur für Frauen: Ändert sich die Migräne durch folgende Punkte?

☐Menstruation ☐orale Kontrazeption ☐Schwangerschaft
☐andere hormonelle Medikamente/ Behandlungen

15. Beeinträchtigen diese Migräne-Schmerzen Ihre übliche Tagesaktivität erheblich?

☐nein ☐ja, inwiefern:

16. Werden Sie nachts von der Migräne geweckt?

☐nie ☐gelegentlich ☐oft

17. Haben andere Familienangehörige Migräneattacken?

☐nein ☐ja. Verwandtschaftsverhältnis:_____

18. Seit wann leiden Sie an Migräne-Kopfschmerzen?_____

19. Wann und durch wen wurde die Diagnose gestellt?_____

20. Wie stark beeinträchtigt Migräne Ihre Lebensqualität?

☐stark ☐mittel ☐wenig ☐gar nicht

Therapie

21. Welche Behandlungen sind bei Ihnen erfolgt?

1)_____

2)_____
3)_____

22. Welche Medikamente wurden bei Ihnen zur Attackenbehandlung eingesetzt?

☐Analgetika _____
☐Triptane _____
☐Opiate _____
☐Antirheumatika _____
☐Ergotamintartrat _____
☐andere:_____

23. Welche davon waren wirksam?

1)_____
2)_____
3)_____

24. Was hat diese Therapie Ihrem Gefühl nach beeinflusst?

☐Frequenz der Migräneattacken
☐Dauer der einzelnen Attacke
☐Intensität der einzelnen Attacke

25. Welche Prophylaktika haben Sie eingenommen? von:_____bis:_____

Prophylaktika	Einnahme		Erfolg			
	von	bis	ja	gering	nein	weiss nicht
	…….	…….	☐	☐	☐	☐
	…….	…….	☐	☐	☐	☐
	…….	…….	☐	☐	☐	☐
	…….	…….	☐	☐	☐	☐
	…….	…….	☐	☐	☐	☐
	…….	…….	☐	☐	☐	☐
	…….	…….	☐	☐	☐	☐
	…….	…….	☐	☐	☐	☐

26. Sind bei Ihnen nichtmedikamentöse Maßnahmen durchgeführt worden?

Maßnahmen	Dauer		Erfolg			
	von	bis	ja	gering	nein	weiß nicht
Akupunktur	…….	…….	☐	☐	☐	☐
Homöopathie	…….	…….	☐	☐	☐	☐
Psychotherapie	…….	…….	☐	☐	☐	☐
Verhaltenstherapie	…….	…….	☐	☐	☐	☐

Physikalische Medizin	…….	…….	☐	☐	☐	☐
Andere:	…….	…….	☐	☐	☐	☐
……..	…….	…….	☐	☐	☐	☐
……..	…….	…….	☐	☐	☐	☐

27. Leiden Sie neben den Migräne-Kopfschmerzen noch unter anderen Kopfschmerzen?

☐ Cluster-Kopfschmerzen
☐Spannungskopfschmerzen
☐andere:_____

Kopfschmerz-Anamnese

28. Wie viele gewöhnliche Kopfschmerzen haben Sie im Monat?_____

29. Wie lange dauern diese Kopfschmerzen gewöhnlich an?

☐ 30min- 2h ☐ 3-4h ☐ 5-12h ☐ 12-24h ☐ mehrere Tage
☐ 7 Tage

30. Wie würden Sie diese Kopfschmerzen als drückend, ziehend oder dumpf beschreiben?

☐ja ☐nein, eher als:_____

31. Treten diese Kopfschmerzen beidseitig auf?

☐ja ☐nein, nur:_____

32. Werden diese Kopfschmerzen von einem der folgenden Symptome begleitet:

☐Übelkeit ☐Erbrechen ☐Lichtempfindlichkeit
☐Lärmempfindlichkeit ☐andere:_____

33. Seit wann leiden Sie an diesen Kopfschmerzen?_____

34. Hatten Sie jemals eine Kopf- oder Nackenverletzung, die eine medizinische Behandlung bedarft hat?

☐nein ☐ja. Bitte näher beschreiben:_____

Allgemeine Erkrankungen

35. Wie würden Sie Ihre allgemeine Gesundheit im letzten Monat beschreiben?

☐sehr gut ☐gut ☐mittel ☐schlecht

36. Wurde bei Ihnen jemals eine andere Erkrankung diagnostiziert (Art, Hypertonie, Asthma, Herzbeschwerden, Magenulzera)?

☐nein ☐ja. Welche:_____

37. Nehmen Sie regelmäßig Medikamente ein?

☐nein ☐ja. Welche:_____

38. Nehmen Sie zusätzlich andere Medikamente ein?

☐nein ☐ja. Welche:_____

39. Sind psychiatrische Erkrankungen bei Ihnen bekannt?

☐nein ☐ja. Welche:_____

40. Hatten Sie eine Operation in den letzten 6 Monaten?

☐nein ☐ja. Welche:_____

41. Leiden Sie an Allergien?

☐nein ☐ja. Welche:_____

42. Rauchen Sie Zigaretten?

☐nein nie ☐Ex-Raucher, aufgehört:_____

☐ja. wieviel/Tag:_____ ☐Gelegenheitsraucher

43. Trinken Sie Alkohol?

☐nein nie ☐ja, bis zu 2-3x/ Woche ☐ja, mind. 2-3x/Woche

☐gelegentlich

wieviel/Tag:_____ was?:_____

44. Nehmen oder nahmen Sie jemals illegale Drogen ein?

☐nein nie

☐ja

Welche?_____

Welcher Zeitraum?_____

Wie häufig?_____

Fragebogen für Migränepatienten – Untersuchungstag 2

Probandennr.: _____

Datum: _____ Uhrzeit: _____

Proband/in weiterhin mit Teilnahme einverstanden? _____

Aktuell Schmerzen? _____

Wann zuletzt Kopfschmerzen? _____

Wann zuletzt Einnahme von Akutmedikation? _____

Blutdruckmessung aktuell? _____

Interview 48h post: Kopfschmerzen? Akutmedikation? _____

_____

1. Interiktale Abnahme

µl Rechtes Auge: _____ µl Linkes Auge: _____

Dauer Abnahme re Auge: _____ Dauer Abnahme li Auge: _____

2. Gabe von Glyceroltrinitrat

Gewicht: _____

Dosierung: _____

Uhrzeit Infusionsbeginn und -ende: _____

VP vor Infusionsbeginn:

Blutdruck: _____ Herzfrequenz: _____

VP post 5min:

Blutdruck: _____ Herzfrequenz: _____

VP post 10min:

Blutdruck: _____ Herzfrequenz: _____

VP post 15min:

Blutdruck: _____ Herzfrequenz: _____

VP Infusionsende:

Blutdruck: _____ Herzfrequenz: _____

Adverse Events während Infusion? _____

_____

Kopfschmerzen während Infusion? _____

3. Abnahme von TF nach 60min

Uhrzeit: _____

Aktuell Schmerzen? _____

KS-Seite? _____

Blutdruckmessung aktuell? _____

µl Rechtes Auge: _____ µl Linkes Auge: _____

Dauer Abnahme re Auge: _____ Dauer Abnahme li Auge: _____

4. Abnahme von TF nach *60min* bei mittelstarken KS

Uhrzeit: _____

Aktuell Schmerzen? _____

KS-Seite? _____

Blutdruckmessung aktuell? _____

µl Rechtes Auge: _____ µl Linkes Auge: _____

Dauer Abnahme re Auge: _____ Dauer Abnahme li Auge: _____

5. Abnahme von TF nach *120min* bei mittelstarken KS

Uhrzeit:

Aktuell Schmerzen?

KS-Seite?

Blutdruckmessung aktuell?

µl Rechtes Auge: µl Linkes Auge:

Dauer Abnahme re Auge: Dauer Abnahme li Auge:

6. Abnahme von TF nach 60min nach Einnahme von Akutmedikation

Uhrzeit:

Aktuell Schmerzen?

KS-Seite?

Welche Akutmedikation?

Blutdruckmessung aktuell?

µl Rechtes Auge: µl Linkes Auge:

Dauer Abnahme re Auge: Dauer Abnahme li Auge:

7. Abnahme von TF nach deutl. Besserung des Kopfschmerzes

Uhrzeit:

Aktuell Schmerzen?

KS-Seite?

Blutdruckmessung aktuell?

µl Rechtes Auge: µl Linkes Auge:

Dauer Abnahme re Auge: Dauer Abnahme li Auge:

8. Zusätzliche Abnahme I

Uhrzeit:

Aktuell Schmerzen?

KS-Seite?

Blutdruckmessung aktuell?

µl Rechtes Auge: µl Linkes Auge:

Dauer Abnahme re Auge: Dauer Abnahme li Auge:

9. Zusätzliche Abnahme II

Uhrzeit:

Aktuell Schmerzen?

KS-Seite?

Blutdruckmessung aktuell?

µl Rechtes Auge: µl Linkes Auge:

Dauer Abnahme re Auge: Dauer Abnahme li Auge:

GTN-induzierter Kopfschmerz; Tag 2; Probandennr.:_____

	GTN-Ende	+15min	+30min	+45min	+60min	+75min	+90min	+105min	+120min
Uhrzeit									
KS-Intensität (VRS 0-10)									
KS-Qualität									
KS-Seite									
Begleitsymptome									
Blutdruckmessung			X		X				
Abnahme von TF					X				
Blutentnahme					X				
Akutmedikation									

	+135min	+150min	+165min	+180min	+195min	+210min	+225min	+240min	+255min
Uhrzeit									
KS-Intensität (VRS 0-10)									
KS-Qualität									
KS-Seite									
Begleitsymptome									
Blutdruckmessung									
Abnahme von TF									
Blutentnahme									
Akutmedikation									

	+270min	+285min	+300min	+315min	+330min	+345min	+360min	+375min	+390min
Uhrzeit									
KS-Intensität (VRS 0-10)									
KS-Qualität									
KS-Seite									
Begleitsymptome									
Blutdruckmessung									
Abnahme von TF									
Blutentnahme									
Akutmedikation									

	+405min	+420min	+435min	+450min	+465min	+480min	+495min	+510min	+525min
Uhrzeit									
KS-Intensität (VRS 0-10)									
KS-Qualität									
KS-Seite									
Begleitsymptome									
Blutdruckmessung									
Abnahme von TF									
Blutentnahme									
Akutmedikation									

Appendix D: Ethics committee approval

LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

ETHIKKOMMISSION BEI DER LMU MÜNCHEN

Ethikkommission · Pettenkoferstr. 8 · 80336 München
Katharina Kamm
Klinikum der Universität München
Klinik u. Poliklinik f. Neurologie
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Anschrift:
Pettenkoferstr. 8a
D-80336 München
18.03.2019 Hb/lh

Projekt Nr: **18-827** (bitte bei Schriftwechsel angeben)

Beratung nach Fakultätsrecht

Studientitel: Nachweis von Calcitonin Gene-related Peptide (CGRP) und anderen Neuropeptiden in der Tränenflüssigkeit im Verlauf von spontanen und GTN-induzierten Kopfschmerzattacken in Patienten mit Migräne im Vergleich zu gesunden Kontrollprobanden
Antragsteller: Katharina Kamm, Klinikum der Universität München, Klinik u. Poliklinik f. Neurologie, Klinikum Großhadern, 81377 München

Sehr geehrte Frau Kamm,

besten Dank für Ihr Schreiben vom 11.03.2019 mit der Beantwortung unserer Fragen bzw. Erfüllung der Auflagen und den noch ausstehenden bzw. überarbeiteten Unterlagen.

Die Ethikkommission (EK) kann Ihrer Studie nun die ethisch-rechtliche Unbedenklichkeit zuerkennen.

Vorsorglich möchte ich darauf hinweisen, dass auch bei einer positiven Beurteilung des Vorhabens durch die EK die ärztliche und juristische Verantwortung für die Durchführung des Projektes uneingeschränkt bei Ihnen und Ihren Mitarbeitern verbleibt.

Allgemeine Hinweise:

- Änderungen im Verlauf der Studie sind der EK zur erneuten Prüfung vorzulegen.
- Schwerwiegende unerwartete studienabhängige Ereignisse sind der EK mitzuteilen (trifft nur für interventionelle Projekte zu).
- Das Ende der Studie ist anzuzeigen und das Ergebnis vorzulegen.
- Die ärztliche und juristische Verantwortung bei der Durchführung der Studie verbleibt uneingeschränkt bei Ihnen und Ihren Mitarbeitern. Bitte berücksichtigen Sie, dass diese Bewertung die ggf. erforderliche Konsultation des behördlichen Datenschutzbeauftragten nach Art. 26 BayDSG nicht ersetzt.
- Die Ethikkommission erklärt, dass an der Bewertung des vorliegenden Antrags niemand beteiligt war, der gemäß Bayerischem Verwaltungsverfahrensgesetz (BayVwVfG) Art. 20 als befangen anzusehen ist.

Für Ihre Studie wünsche ich Ihnen viel Erfolg.

Mit freundlichen Grüßen

[redacted]

Prof. Dr. W. Eisenmenger
Vorsitzender der Ethikkommission

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I wish to dedicate this thesis to Boma Suzette and Oma Marlene: *Ich wünschte ich könnte diesen Moment mit euch teilen und weiß gleichzeitig, dass ihr unglaublich stolz wäret.*