

The role of genetics on migraine induction triggered by CGRP and PACAP38

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THE 4 ORIGINAL PAPERS ARE

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4. Guo S, Christensen AF, Liu ML, Janjoo BN, Olesen J, Ashina M. Calcitonin gene-related peptide induced migraine attacks in patients with and without familial aggregation of migraine. *Cephalalgia*. 2016 Mar 18.

INTRODUCTION

Migraine is a disabling and very prevalent neurological disorder with a strong genetic component [1,2], manifesting primarily as attacks of severe headache accompanied by symptoms such as nausea, vomiting, photo- and phonophobia. In addition, many migraine patients experience premonitory symptoms (PS), such as fatigue, neck stiffness, yawning, mood swings or hunger, that precede and forewarn their migraine attacks [1]. The most common form of migraine is migraine without aura (MO) but a third of patients have migraine with aura (MA), which is commonly presented as visual disturbances before the onset of their migraine headache.

Migraine is ranked by the World Health Organization (WHO) as one of the 20 most invalidating diseases of the world [2], and in Europe alone, over 100 million people suffer from migraine [3]

causing huge economic costs for the society [4]. In spite of this, the mechanisms of migraine are yet not fully clarified. Thus, there is an urgent need to explore and understand the initiating mechanisms of migraine further. One way to do this is by conducting experimental human studies using endogenous substances that can trigger migraine attacks e.g. calcitonin gene-related peptide (CGRP) and pituitary adenylate cyclase-activating peptide-38 (PACAP38).

The overall aim of the present PhD thesis was to investigate the role of genetics to induction of migraine attacks by neuropeptides CGRP and PACAP38. Additionally, we also investigated biochemical changes in the blood after PACAP38 and whether CGRP and PACAP38 could induce premonitory symptoms.

Human models of migraine

Human provocation models of migraine have generated important data on mechanisms underlying migraine pathophysiology by CGRP and PACAP38 [5–7]. Both CGRP and PACAP38 are strong vasodilators and have in recent years gained considerable interest in the migraine field [8]. CGRP induces migraine attacks in 65% of MO patients [9], but not in normal subjects [10], and the development of CGRP-antagonists has proven effective in the treatment of migraine [11–13]. PACAP38 induces migraine in 65–75% of MO patients [14,15] and its receptor (PAC1) has emerged as another possible target for novel migraine treatment [8]. Furthermore, studies have shown that plasma levels of CGRP and PACAP38 are elevated during spontaneous migraine attacks [16–20].

Interestingly, both CGRP and PACAP38 activate adenylate cyclase by transmembrane receptors leading to increased formation of intracellular cAMP in vascular smooth muscle cells of cerebral arteries [21,22]. This indicates that cAMP-dependent pathways play a central role in migraine [9,23] and is supported by the fact that cilostazol, a drug which causes intracellular accumulation of cAMP, is one of the most powerful migraine-inducing compounds [24]. Yet, the exact pathophysiological mechanisms behind CGRP and PACAP38-induced migraine attacks are still unclear.

Provocation models of migraine can also be used to study premonitory symptoms (PS). Intravenous infusion of another migraine trigger, glyceryl trinitrate (GTN) - a nitric oxide donor (NO), that activates the cyclic guanosine monophosphate (cGMP) signaling pathway, was reported to induce PS in migraine patients with activation of the hypothalamus [25,26]. Since PS is considered as the first sign of a migraine attack, a better understanding of its underlying mechanisms is crucial to elucidate how a migraine attack begins. Developing a model to trigger PS of migraine reliably is therefore important. Whether CGRP and PACAP38 can induce PS and be used a PS model is unknown.

Heritability of migraine, family and twin studies

Heritability is the proportion of phenotypic variation between individuals that is due to genetic variation and is mostly calculated from twin studies [27]. A meta-analysis of migraine twin studies in adults found a heritability of 45% [28], meaning that almost half the risk of developing migraine can be attributed to genetic factors, whereas the other half may be attributed to factors such as the environment. This study also showed that monozygotic twins had a higher correlation of migraine than dizygotic twins [28]. Thus, migraine has a clear tendency to run in families. The risk of having MO doubles if you have a first-degree relative with MO [29]. Accordingly, aggregation of MO in first-degree relatives of probands with migraine implies enrichment of migraine susceptibility genes [30,31]. However, having a first-degree relative with MA gives an almost four-fold increased risk of MA, but no increased risk of MO, indicating that the inheritance of MO and MA is different [32].

Familial hemiplegic migraine

Familial hemiplegic migraine (FHM) is a rare subtype of MA characterized by transient hemiparesis during the aura phase. The current understanding in molecular genetics of migraine pathophysiology predominantly comes from studies of FHM, which is inherited in a monogenic autosomal dominant fashion [32–34]. So far three FHM mutation genes have been identified [35–37] all encoding ion transporters suggesting that disturbances in ion and neurotransmitter balances in the brain are responsible for the FHM. However, the FHM genes showed no association to MO or MA, suggesting that ion transporter genes play no major role in the common forms of migraine. Yet, others believe that FHM is an extreme entity on the migraine spectrum, and therefore a possible disease model for the study of genetic mechanisms in migraine in general [32].

Genetics of migraine without aura

In the last decades, a lot of effort and approaches have been used to identify causative genes for MO, such as the classical linkage analysis and candidate gene approach. However, specific genetic variants have for the first time been robustly identified with MO in the last few years by the approach of genome-wide association studies (GWAS). In a GWAS, hundreds of thousands of gene variants, the so-called single nucleotide polymorphisms (SNPs), that are distributed over the genome are tested in a hypothesis-free manner and compared between cases and controls for disease association. An SNP is a relatively common variation at a single position in the DNA among individuals that may confer risk of a disease.

GWAS have become the most used approach to identify common gene variants that confer susceptibility to complex disorders. This is partly because of the "common disease, common variant" hypothesis, which suggests that the genetic liability to common complex diseases such as migraine to a large part results from the accumulated effect of a high number of common gene variants, each contributing with a small to moderate effect. An argument for this is that if rare variants of high effect size were involved in MO, they should already have been detected by linkage studies. The success of GWAS studies in identifying a high number of risk variants for MO supports this view. Recently, 12 SNPs conferring risk of MO have been identified in a large GWAS meta-analysis including a total of 23,285 MO patients and 95,425 population-matched controls [38]. Interestingly, one of the identified genetic variants is localized within the *MEF2D* gene, which regulates the expression of PACAP38 [39].

However, the functional consequences of these SNPs and their biological actions are yet unclear [38].

Provocation model and migraine genetics

Pharmacological migraine provocation may be a novel approach to explore the contribution of genetics to migraine susceptibility [40]. CGRP and GTN have previously been used to investigate the functional consequences of genetics in FHM patients. Both studies showed that FHM patients do not show hypersensitivity to CGRP or GTN as seen in MO patients [41,42], indicating that FHM and the common form of migraine share different pathophysiological pathways. Furthermore, a previous study indicated that susceptibility of migraine-like headache to pharmacological provocation with GTN is associated with familial aggregation of migraine (family load) [43]. However, in this study provocation experiments were conducted in healthy volunteers and the International Headache Society (IHS) criteria for migraine were not used.

Combining the provocation model and genetics for migraine without aura (MO) has never been done before. Whether the hypersensitivity to CGRP- or PACAP38-induced migraine response experienced by two-thirds of MO patients may be explained by genetics e.g. familial predisposition, a high number of risk-conferring SNPs or maybe a specific SNP is unknown.

SPECIFIC AIMS

The specific aims of this thesis were:

- To investigate if family history of migraine (family load) or a high number of risk conferring SNPs contributes to susceptibility of CGRP-induced migraine attacks.
- To investigate if family load or the PACAP38-associated SNP contributes to susceptibility of PACAP38-induced migraine attacks.
- To investigate whether PACAP38 infusion causes changes in endogenous production of PACAP38, VIP, CGRP, TNF α and S100B.
- To investigate whether CGRP and PACAP38 can induce PS and if they are associated with family load.

METHODS

Volunteers

MO patients were recruited from a cohort of 1010 unrelated patients from the Danish Headache Center who were previously genotyped for the 12 single nucleotide polymorphisms (SNPs) associated with MO [38]. All patients strictly fulfilled the Headache Classification Committee of the International Headache Society's criteria for MO [33]. Exclusion criteria were any other type of headache; intake of any preventive medication and serious somatic or psychiatric diseases. Female participants in the reproductive age used safe contraceptive methods.

Healthy volunteers were recruited via announcement on a Danish website for recruitment of participants for experimental research projects: www.forsogsperson.dk. Exclusion criteria were any type of headache, daily intake of any medication (except oral contraceptives); serious somatic or psychiatric diseases.

All participants gave written informed consent prior to inclusion in the studies that was approved by the Ethics Committee of Copenhagen (H-2-2011-141 and H-2-2013-033) and conducted in accordance with the updated Helsinki declarations.

Experimental design of the studies

Study I and II were conducted using a double-blinded design. The data of study III were collected during study II, and the data of study IV were collected during study I and II.

In study I, all patients received a continuous infusion of 1.5 µg/min human α-CGRP for 20 min, whereas in study II, they received an infusion of 10 pmol/kg/min PACAP38. In study III, additional 6 healthy controls received intravenous infusion of saline for 20 min.

Infusion was given after baseline measurements. Headache characteristics, non-headache symptoms, adverse events and vital signs were recorded every 10 min until 120 min from the beginning of infusion in study I and 90 min in study II. Moreover, in study II, blood samples were collected at baseline and 20, 30, 40, 60 and 90 min. Next, the patients were discharged from the hospital and were carefully instructed to continue recording their headache and non-headache symptoms by a self-administered questionnaire every hour until 12 h after the start of infusion or until they went to bed. The patients were allowed to take their usual acute migraine medication at any time, but were instructed to take the medication when the headache and associated symptoms mimicked their usual migraine attacks.

Headache and migraine

Headache intensity was recorded repeatedly on a verbal rating scale (VRS) from 0 to 10 [44]. Headache localization, characteristics and associated symptoms were also recorded to determine the type of headache according to the International Headache Society (IHS) criteria [33]. In addition, we recorded whether the induced headache mimicked the spontaneous migraine attacks of the patients.

We used the previously described definition for an induced migraine attack (Box 1) [15,23,24].

BOX 1

The following definition was used for an induced migraine attack:

Migraine attack fulfilling either (1) or (2):

- (1) Headache fulfilling criteria C and D for migraine without aura according to the IHS criteria [33].
 - C. Headache has at least two of the following characteristics:
 - Unilateral location
 - Pulsating quality
 - Moderate or severe pain intensity (moderate to severe pain intensity is considered ≥ 4 on VRS)
 - Aggravation by cough (in-hospital phase) or causing avoidance of routine physical activity (out-hospital phase).
 - D. During headache at least one of the following:
 - Nausea and/or vomiting
 - Photophobia and phonophobia.
- (2) Headache described as mimicking the patient's usual migraine attack and treated with acute migraine medication (rescue medication).

SNPs

As the SNPs are bi-allelic, each patient has 0, 1 or 2 risk alleles for each SNP; hence the total numbers of at-risk-alleles can theoretically range from none to 24. In study I, we recruited the 20 patients with the highest and lowest number of risk alleles, respectively. High and low SNP load definition was based on the number of risk alleles of the patients we ultimately included in the two groups. Thus, we assured the most possible difference between the groups. In the present study, high SNP load was defined as patients identified with ≥ 14 risk alleles, whereas low SNP load was defined as patients identified with ≤ 9 risk alleles. In study II, we recruited 16 patients with double risk alleles of rs2274316 (*MEF2D*), which is associated with PACAP38 expression, and 16 MO-patients without the allele. Subsequently, we obtained information of family predisposition of the patients (the proband) that completed the study. This approach allowed us to stratify patients into two groups based on family and SNP load. Moreover, all participants and investigators were blinded in respect to family and SNP load on the day of experiment.

Family history of migraine

The history of migraine of the patient's first-degree relatives (parents, siblings and children) was obtained via a telephone interview based on a validated semi-structured questionnaire [45,46]. Migraine (MO or MA) were diagnosed according to the latest IHS criteria [33]. Patients identified with ≥ 2 first-degree relatives with migraine were defined as having a high family load, whereas patients identified with ≤ 1 first-degree relatives with migraine were defined as having a low family load.

Blood samples

In study II, blood samples were collected, before and after PACAP38 infusion, to determine the plasma or serum levels of PACAP38; CGRP; vasoactive intestinal peptide (VIP) as markers for release from parasympathetic and sensory perivascular nerve fibers [47]; the inflammatory cytokine tumor necrosis factor alpha (TNFα) as a marker for mast cell degranulation [48]; S100 calcium binding protein B (S100B) as marker for glial cell activation or leakage of the blood brain barrier (BBB) [49,50].

Premonitory and non-headache symptoms

The following non-headache symptoms were recorded using a questionnaire: Unusual tiredness, yawning, stiff neck, hunger, poor concentration, mood swings, nausea, photophobia and phonophobia. These symptoms were chosen because a prospective electronic diary study showed that they were the most common PS [1]. Based on a previous provocation study of PS using GTN [25], we defined PS as "non-symptoms before the onset of pain in migraine". Accordingly, we defined three phases: 1) Premonitory phase as before the onset of headache; 2) headache phase as during headache including migraine; and 3) postdrome phase as after the end of headache.

In addition, we also applied the strict International Headache Society (IHS) definition for a PS as the following: "Symptoms preceding and forewarning of a migraine attack by 2–48 h, occurring before the onset of pain in migraine without aura" [33].

Vital signs and adverse events

Heart rate (HR) and mean arterial blood pressure (MAP) were measured using an auto-inflatable cuff. During the in-hospital phase the subjects were questioned for the presence of any adverse events by the investigator every 10 min.

Statistical analysis

All data were presented as mean values \pm standard deviation (SD), except headache scores, which were presented as median values.

Differences between two groups for e.g. incidence of migraine attacks, premonitory symptoms and adverse events, were analyzed as categorical data with Fisher's exact test. Differences in AUC for headache intensity scores were tested using the non-parametric Mann-Whitney U-test. Logistic regression analyses were performed with incidence of migraine attacks as the outcome variable and number of risk alleles as predictor variable.

The difference between two groups over time was assessed by repeated-measurements ANOVA (RM-ANOVA) with the interaction between time and group (time * group) being the term of interest. Post hoc unpaired t-tests for comparing changes from baseline at each time point were performed only when RM-ANOVA revealed significant results.

All analyses were performed with SPSS Statistics version 19 for Windows (Chicago, IL, USA). $P < 0.05$ was considered the level of significance.

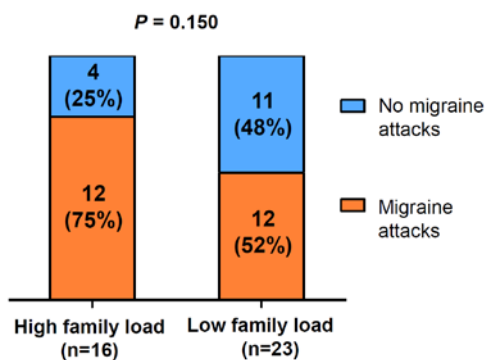
RESULTS

Study I: Role of genetics on CGRP-induced migraine attacks

Forty genotyped MO-patients completed this study [36 F, 4 M, mean age 45 years [range 19 - 65]], and we assessed family history of migraine of all patients except one, who was adopted with unknown relatives and therefore excluded in the analysis for family load. Sixteen patients had a high family load of migraine, whereas 23 had a low family load. In total, CGRP induced a migraine attack in 63% (15 out of 40) of patients.

CGRP infusion induced a migraine attack in 75% (12 out of 16; 95% CI: 48–93%) of patients with high family load compared to 52% (12 out of 23; 95% CI: 31–73%) with low family load ($P=0.150$) (Fig 1). Median time to onset of migraine attacks in patients with high family load was 1.92 h (range 0.33-12 h) and in patients with low family load was 3.5 h (range 0.16-9 h)($P=0.977$).

Figure 1:

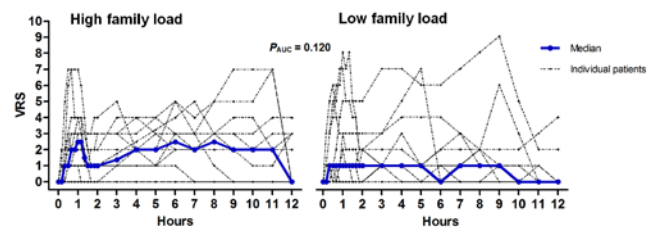


Family load of the participating migraine patients ($n=39$) and whether they got a migraine attack or not after CGRP infusion. High family load was defined as ≥ 2 first-degree relatives with migraine, whereas low family load was defined as ≤ 1 first-degree relatives with migraine. There was no significant difference in number of CGRP induced migraine attacks between MO-patients with high and low family load. Data are shown as n (%).

Seven out of 16 (44%) patients with high family load took rescue medication compared to 10 out of 23 (43%) with low family load ($P=0.751$). Both high family load and low family load patients responded well to their rescue medication and had a significant reduction in headache intensity 2 h after treatment ($P=0.021$ and $P=0.022$).

We found no statistical difference between the two groups in the incidence of any head pain or in the AUC for headache intensity over the 12 h observation period (Fig. 2).

Figure 2:



Median (thick blue line) and individual (thin lines) headache intensity on a 0-10 VRS for 16 MO-patients with high family load and 23 MO-patients with low family load. There was no significant difference in the AUC between high and low family load (AUC_{0-12h} : $P=0.120$).

Thirteen out of 20 patients (65%) with high SNP load developed a migraine attack after CGRP compared to 12 out of 20 patients (60%) with low SNP load ($P=1.000$). The median number of risk alleles in patients with high SNP load was 14 (range 14-19) compared to 8 (range 6-9) in patients with low SNP load. We found no significant difference between the two groups in the AUC for headache intensity over the 12 h observation period ($P=0.947$).

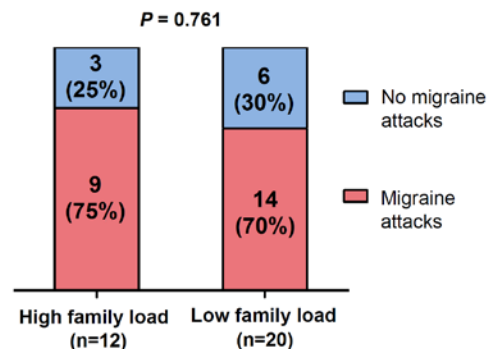
Logistic regression analyses showed no association between number of risk alleles and incidence of migraine attacks. Likewise, incidence of migraine attacks showed no association with any particular SNP ($P>0.05$).

Study II: Role of genetics on PACAP38-induced migraine attacks

Thirty-two genotyped MO-patients completed study II and family history of migraine was assessed for all patients. Twelve patients had a high family load of migraine, whereas 20 had a low family load. In total, PACAP38 induced a migraine attack in 72% (23 out of 32) of patients.

PACAP38 induced a migraine attack in 75% (9 out of 12) of patients with high family load compared to 70% (14 out of 20) with low family load ($P=0.761$) (Fig. 3). The median time to onset of delayed migraine attacks in patients with high family load was 6 h (range 0.33-10 h) and 5 h (range 2-9 h) in patients with low family load.

Figure 3:

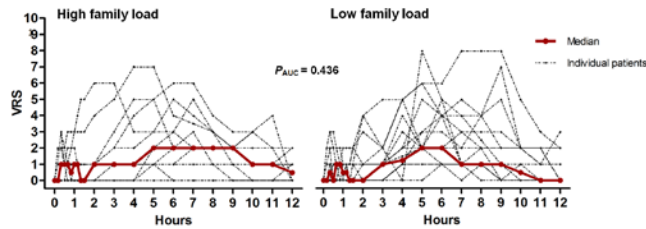


Family load of the participating migraine patients ($n=32$) and whether they reported a migraine attack after PACAP38 infusion. High family load was defined as ≥ 2 first-degree relatives with migraine, whereas low family load was defined as ≤ 1 first-degree relatives with migraine. There was no significant difference in incidence of PACAP38-induced migraine attacks between MO-patients with high and low family load. Data are shown as n (%).

Seven out of 12 (58%) patients with high family load took rescue medication compared to 15 out of 20 (75%) with low family load ($P=0.325$). Both high and low family load patients responded well to their rescue medication ($P=0.002$ and $P=0.002$).

There was no significant difference between the two groups in the incidence of any head pain or in the AUC for headache intensity over the 12 h observation period (Fig. 4).

Figure 4:



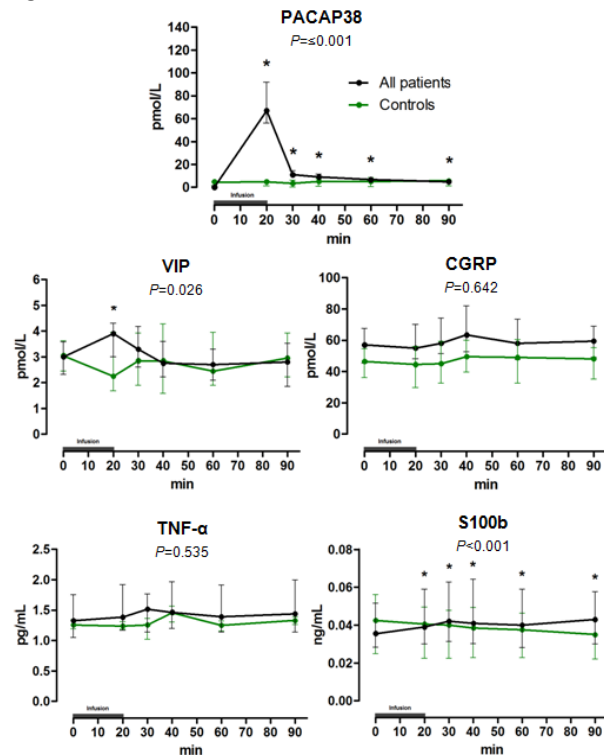
Median (thick red line) and individual (thin lines) headache intensity on a 0-10 VRS for 12 MO-patients with high family load and 20 patients with low family load. There was no difference in the AUC between high and low family load ($AUC_{0-12\text{h}}$: $P=0.436$).

Sixteen patients carried double risk alleles of the *MEF2D* gene variant and 16 were non-carriers. Eleven patients (69%) with the risk allele developed a migraine attack after PACAP38 compared to 12 patients (75%) without risk allele ($P=1.000$).

Study III: Biochemical changes after PACAP38

Blood samples were collected from the 32 MO-patients who received PACAP38 and 6 healthy volunteers who received saline. We found significant differences in plasma concentrations for PACAP38, VIP and S100B between migraine patients who received PACAP38 infusion compared to controls (Fig. 5), but not for CGRP and TNF-alpha.

Figure 5:



Effects of PACAP38 on plasma concentrations of measured biochemical variables in migraine patients compared to saline infusion in healthy volunteers. Data are shown as median with interquartile range (IQR). Migraine patients (black) who received PACAP38 infusion ($n=32$), healthy volunteers (green) who received saline infusion ($n=6$). P -value: RM ANOVA. *Post hoc unpaired t -test of changes from baseline: P -value <0.05 .

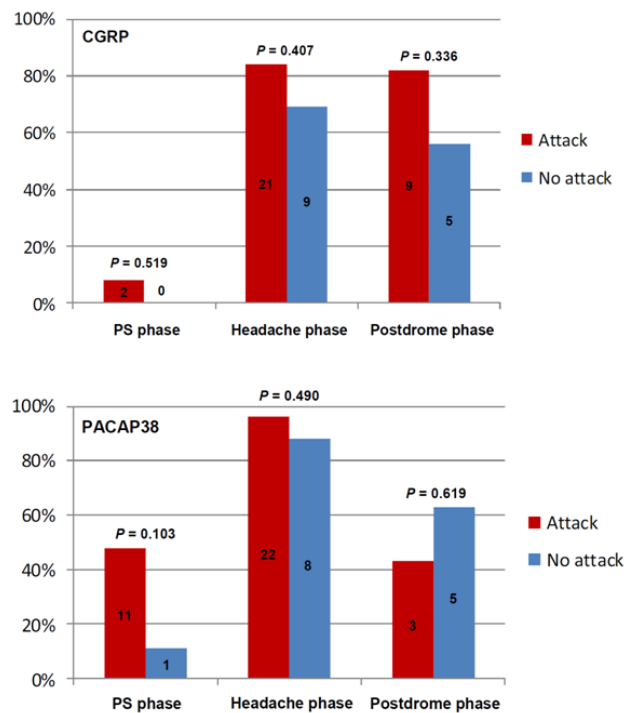
We found no significant difference in the biochemical variables between patients who developed ($n=23$) and those who did not develop ($n=9$) migraine attacks after PACAP38 (P -values >0.05). Additionally, we found no significant difference in the biochemical variables over time (0-90 min) between the patients with ($n=16$) and without ($n=16$) the *MEF2D* gene variant (P -values >0.05).

Study III: Premonitory symptoms induced by CGRP and PACAP38

As previously mentioned, 25 out of 40 (63%) patients developed a migraine attack after CGRP and 23 out of 32 (72%) patients developed attack after PACAP38.

In the premonitory phase, only 2 out of 25 (9%) reported non-headache symptoms before CGRP-induced migraine attacks, which fulfilled our criteria of a PS, whereas 11 out of 23 patients (48%) reported PS symptoms before PACAP38-induced migraine attacks (Fig. 6). However, we found no significant difference in PS during the premonitory, headache or postdrome phase, between the two groups of patients who *did* and *did not* develop a migraine attack after CGRP or PACAP38. In addition, none of the patients after CGRP infusion reported a PS that fulfilled the strict IHS definition [6], whereas 2 out of 23 (9%) patients after PACAP38 infusion fulfilled the IHS definition of PS [6].

Figure 6:



Incidence (%) of any non-headache symptoms during the premonitory (PS), headache and postdrome phase after CGRP (attack: $n=25$, no attack: $n=13$) or PACAP38 (attack: $n=23$, no attack: $n=9$). Patients who did not develop a headache nor had a postdrome phase after CGRP or PACAP38 were not included in the figure. Migraine associated non-headache symptoms (nausea, photo- and phonophobia) were excluded during the headache phase. Number of patients is shown in the columns.

Retrospective assessment of PS showed that 21 out of 28 (75%) patients with high family load reported to have PS prior their spontaneous migraine attacks, whereas 24 out of 43 (56%) with

low family load reported to have PS ($P=0.101$). In addition, we found no significant difference in the incidence of PS symptoms induced by CGRP or PACAP38 between patients with high and patients with low family load.

DISCUSSION

Genetics and CGRP- or PACAP38-induced migraine attacks (Study I and II)

Study I and II are the first functional studies investigating a relation between genetics of MO and migraine provocation. We found no association between familial aggregation of migraine and hypersensitivity to CGRP or PACAP38 infusion in MO-patients. In addition, we found that specific SNPs or a high number of SNPs could not explain the susceptibility to migraine attacks after these migraine triggers.

Family load

A central question in the interpretation of our results is whether family load is a good marker for genetic load and how to define high family load.

The family load is probably the best indicator we have at the moment for genetic enrichment in MO-patients. No genes have so far been discovered to be associated with MO and all the currently identified SNPs associated with migraine have low effect sizes. Possible explanations of the missing heritability could be due to undetected genes, epistasis and epigenetics, which all could be reflected in familial predisposition.

Familial predisposition is a risk factor for a majority of common chronic diseases (diabetes, cardiovascular disease, asthma and several cancers) and greater increase in risk is associated with an increasing number of affected first-degree relatives [51–55]. We based our definition of high family load (≥ 2 first-degree relatives with migraine) on studies of other diseases showing that two first-degree relatives significantly increased the risk of disorder [52,54]. In addition, since migraine affects roughly 15% of the population [3,56], having one first-degree relative with migraine is relatively likely to occur by chance.

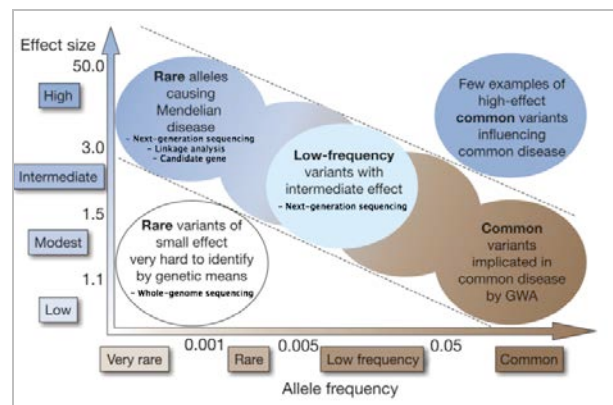
The strengths of the present studies of family load include a well-characterized patient group and the use of direct telephone interview to diagnose first-degree relatives [45,46] according to the latest IHS criteria [33]. Direct interview with each relative is required to obtain accurate information on migraine in families, because proband report is not sufficiently sensitive [57,58]. Moreover, we had participants and investigators blinded in respect to family load and genotype.

SNPs

The clinical contribution of SNPs is questionable because they explain only a tiny fraction of the genetic risk of migraine and their exact biological actions are unknown [38]. GWAS studies typically identify variants that contribute only modestly to disease risk (effect odds ratio ≤ 2) (Fig. 7). In addition, it is very likely, that only a small fraction of relevant risk variants has been discovered. The main goal of these SNPs is therefore not prediction of disease, but rather to identify new biological mechanisms, and possibly new drug targets. For instance, the PACAP38-associated risk allele has an odds ratio of 1.07 and is localized intronically within the *MEF2D* gene [39]. The *MEF2D* protein is a transcription factor that is highly expressed in brain [59], and a transcriptional study using microarray found evidence that *MEF2D* regulates PACAP38 expression [39]. However, the causality between this risk allele and the *MEF2D* gene is yet to be established and it is also unknown how the gene variant may affect the expression or sensi-

tivity of PACAP38. The biological relevance of these SNPs may therefore be questioned. However, some studies have indicated that the low effect sizes do not necessarily imply low biological importance. For example, it has been shown that an SNP with small effect size on disease can have a large clinical effect e.g. efficacy of statins on high levels of blood cholesterol [60–62]. Additionally, a GWAS of prostate cancer showed that the 1% of the population with the highest number of genetic risk variants had a 50% absolute risk of developing prostate cancer. The use of these genetic markers could also improve prediction when added on top of family history [63]. Thus, we performed explorative analysis on the migraine-associated SNPs, because we hypothesized that a high number or specific risk alleles (*MEF2D* gene variant) had a stronger effect on the susceptibility to CGRP or PACAP38-induced migraine attacks despite its small effect size on migraine risk. However, the explorative analysis failed to support our theory.

Figure 7:



The spectrum of potential genetic effects illustrated by effect size and allele frequency. These two key components determine which approach is most suitable in detecting associations with disease phenotype. Most interest has been on the variants within the dotted lines. (Modified from [40])

Biochemical changes after PACAP38 (Study III)

It has been suggested that the migraine-inducing and vasodilatory properties of PACAP38 may be caused by activation of the: 1) parasympathetic nervous system [23], 2) perivascular sensory nerve endings [8], 3) mast cell degranulation [64–66] or 4) central brain structures [67]. We showed that PACAP38 infusion caused increased plasma levels of VIP and S100B compared to controls, but *not* CGRP and TNF-alpha. In addition, we showed that biochemical variables were not different in patients who developed a delayed migraine attack compared to those who did not, and that the PACAP38-associated *MEF2D* gene variant had no influence on plasma levels of PACAP38.

PACAP38 effect on VIP, CGRP, TNF α and S100B

VIP is a parasympathetic neurotransmitter that is structurally related to PACAP38 [68]. A previous study reported ictal increase of VIP levels in a subgroup of migraineurs with autonomic symptoms [16], and recently a study showed elevated levels of VIP in chronic and episodic migraine patients interictally [69]. We demonstrated that infusion of PACAP38 lead to an immediate increase in plasma levels of VIP, which normalized after discontinuance of infusion. This may indicate release of VIP from parasympathetic nerve endings [47,70].

CGRP is expressed in sensory nerve fibers of the cranial vasculature [71,72] and plasma levels of CGRP has been reported to be elevated during spontaneous attacks in MO patients [16–19].

To our knowledge, no studies have investigated plasma levels of CGRP after infusion of PACAP38. Although our study showed that PACAP38 infusion did not cause changes in CGRP levels, including in patients who later reported migraine attacks, we cannot exclude possible changes in plasma CGRP during PACAP38-induced delayed migraine attacks.

TNF α is an inflammatory cytokine released upon mast cell degranulation [73]. Mast cells are located perivascularly in the dura [74] and have been suggested to be involved in migraine pathophysiology [65]. Plasma levels of TNF α is reported to be elevated during spontaneous migraine attacks [75]. The present study used TNF α as a marker for mast cell degranulation and failed to demonstrate any changes, including in patients who reported delayed attacks. Collectively, these data question the role of mast cells in PACAP38 responses, but our findings do not rule out delayed ictal changes in plasma levels of TNF α .

S100B is a calcium-binding protein, produced and released mostly by glial cells in the CNS [76]. It has been suggested that isolated S100B increase may be an early marker of blood brain barrier (BBB) opening and is not necessarily related to neuronal damage [50]. A small study causing iatrogenic BBB disruption with mannitol showed that serum S100B increased (~50%) significantly [50]. If PACAP38 causes leakage of the BBB, it would enable the passage of molecules that normally do not enter the brain. Present findings of increased serum levels of S100B (10-20%) over the observation period (0-90 min) suggest that infusion of PACAP38 may alter the BBB permeability. However, we cannot rule out that elevated S100B levels reflect release from the peripheral nervous system [77] in response to inflammation induced by PACAP38.

Premonitory symptoms induced by CGRP and PACAP38 (Study IV)

We found that CGRP did not induce PS, whereas PACAP38 induced PS in 48% of patients. However, CGRP and PACAP38 did not induce more PS in patients who developed an attack compared to those who did *not* develop an attack. In addition, we found that patients with a familial predisposition of migraine were not more susceptible of having PS, and they did not report more PS induced by CGRP or PACAP38. The strength of this study is the large sample size of provoked patients (attack versus *no* attack group were compared) and the use of a detailed questionnaire for recording the PS.

Our findings, in particular the lack of PS symptoms after CGRP infusion, suggest peripheral mechanisms of CGRP-induced migraine attacks. CGRP has shown to pass the BBB very poorly [78] and CGRP-antibodies, which also do not cross the BBB [79], have shown efficacy as preventive treatment for migraine [13,80]. Nonetheless, PACAP38 showed a clear tendency to induce PS in at least some patients, although it also crosses the BBB poorly [81]. This could be because PACAP38 is able to cross the BBB modestly by a specific saturable transporter [82] and thus exerting some central effects [67].

Provocation study by GTN has showed to induce PS in 36% (12 out of 33) of migraine patients [25]. However, no studies have ever compared non-headache symptoms in patients who reported and did *not* report attacks. In contrast to CGRP and PACAP38, GTN is a lipophilic compound that easily crosses the BBB and hence may activate brain structures [26,83]. GTN studies defined PS as symptoms before the onset of the triggered migraine headache [25,26], which is different from the definition of IHS [33]. In the present study, we used the same definition for PS as in GTN studies because pharmacologically induced migraine attacks

usually develop within hours after start of infusion [9,15,23,84], and the induced PS may therefore develop only shortly before or simultaneously with the onset of headache. Prospective data on PS showed no 2-hour gap between the end of premonitory symptoms and the beginning of pain [1]. Thus, the strict and arbitrary IHS definition for a spontaneous PS, stating that the symptom must begin 2-48 h prior the headache or aura in migraine patients [33], may *not* be applicable in migraine attacks induced by pharmacological triggers. We support the view [85] that the IHS definition of PS should be regarded as “symptoms preceding and forewarning the migraine attack prior to the onset of head pain”, and encourage the International Headache Society Classification Committee to reconsider their definition.

Limitations

We acknowledge that we were not able to get in touch with all first-degree relatives by phone. Accordingly, migraine diagnosis in 18 out of 98 (18%) relatives was based on report from the proband or parents. Furthermore, we did not account for the number of siblings in our calculation of high and low family load. Another factor that might influence our results is the use of different preventive medication among the patients which could reduce the incidence of migraine attacks or PS. Moreover, in regard to induction of PS we did not have a control group of healthy volunteers or placebo treated patients.

Our sample size could also be a limitation in regard to genetics, but a single provocation study with more than 100 patients would be difficult to execute. Larger sample size may be achievable by genotyping all patients included in provocation studies, possibly in multicenter fashion, over for example 10 years and subsequently stratification.

One might argue whether CGRP- and PACAP38-induced migraine attacks are different from spontaneous attacks. The pain in the infusion model is in general milder compared with spontaneous migraine attacks [86], but the reason could be that patients treat these induced attacks relatively early before head pain becomes severe and develops into severe migraine attack. We believe CGRP and PACAP38 induce genuine migraine attacks because the induced attacks mimic the usual spontaneous attacks of the patients, and it responds effectively to their usual migraine medication. In addition, migraine is known to have many triggers e.g. alcohol, stress and menstrual cycle, and we see no arguments against why CGRP and PACAP38-triggered attacks should be any different. Yet, we acknowledge that it may be more correct to refer to these induced attacks as migraine-like attacks.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In the present thesis we have investigated 1) the role of genetics in CGRP- and PACAP38- induced migraine attacks, 2) biochemical changes after PACAP38 and 3) whether CGRP or PACAP38 may induce PS. We demonstrated that:

- PACAP38 and CGRP induce migraine attacks in 63% and 72% of MO patients, respectively.
- Familial predisposition has no effect on migraine response or PS induced by CGRP or PACAP38.
- SNPs have no effect on CGRP- or PACAP38-induced migraine attacks.
- PACAP38 infusion causes changes in plasma concentrations for VIP and S100B, but not CGRP and TNF-alpha, suggesting activation of parasympathetic nerve endings rather than sensory nerve endings or mast cell degranulation.
- CGRP does not induce PS in MO patients.

- PACAP38 induces PS in 48% of patients, but does not induce more PS in patients who develop an attack compared to those who do *not* develop an attack.

Both CGRP and PACAP38 activate cAMP-dependent pathways [9,23]. However, our findings suggest that family load and SNPs do not influence this pathway. Nonetheless, many aspects should be further investigated. For example, we could investigate more distinct groups of patients in regard to family load, such as patients with no familial predisposition versus patients with ≥ 3 first-degree relatives with migraine. It would also be interesting to investigate the susceptibility of induced migraine attacks in different large families with many affected individuals, because different families may have different causes to their migraine. By studying specific families, we will examine a more homogenous genetic cause. Lastly, we could also investigate the contribution of familial predisposition to other signaling pathways that are implicated in migraine e.g. the pathway of cyclic guanosine monophosphate (cGMP)-signaling [87] by provocation with GTN or sildenafil.

Despite the strengths of GWAS, our results of the currently known SNPs indicate that their clinical effects may be very small and that better DNA technologies like exome sequencing and whole-genome sequencing might be the next step to get a better insight in migraine genetics. Conversely, we have not identified all migraine-associated SNPs. It would therefore be plausible to investigate the relationship between SNPs and migraine response again in a larger sample size when more migraine-associated SNPs are discovered. In addition, besides investigating the additive effect of SNPs, dominant, recessive or combinative effects should also be examined. A recent study in schizophrenia showed that different genotypic networks of SNP sets cause distinct clinical syndromes or phenotypes [88]. Since it is likely that complex heterogeneous disorders such as migraine and schizophrenia share similar genetic architectures, this approach may be applied on migraine as well. Another approach using SNPs as an empirical measure of genetic risk is the so-called polygenic risk scores (PRS). PRS is the sum of effect for an ensemble of risk-associated alleles that do not individually achieve significance in a large-scale association study. Family history can be incorporated in these SNP approaches as well to reveal genotype-phenotype associations.

The lack of PS symptoms after CGRP infusion, suggest peripheral mechanisms of CGRP-induced migraine attacks and that CGRP is not useful model for studying PS. This is possibly because CGRP pass the blood brain barrier (BBB) poorly [32]. In contrast, PACAP38 showed a tendency to induce PS in migraine patients (48%), which is comparable with the incidence of PS induced by GTN (36%, 12 out of 33) [1]. This could be because PACAP38 is able to cross the BBB modestly by a specific saturable transporter [12] and thus exerting some central effects [36]. Therefore, further investigations are warranted and it would be interesting to use advanced imaging techniques comparing the effects of PACAP38 with GTN. It is possible that the underlying mechanisms of PACAP38-induced attacks are more similar to GTN than CGRP.

However, it is important that future studies of PS assess each non-headache symptom prospectively by a specific questionnaire or by direct interview, because other methods are unreliable in the study of such complex symptoms. Moreover, it is important to have a healthy control group to separate side effects of trigger substances from actual PS, and to include the incidence of PS among those who did not develop migraine attack as well. It would also be preferable to ask the patients whether the induced PS mimicked their usual PS prior their spontaneous attacks.

In conclusion, the present thesis suggests that genetics factors such as family load and genetic variants do not contribute to susceptibility of migraine attacks induced by CGRP or PACAP38. In addition, our findings suggest that CGRP provoke migraine attacks without premonitory symptoms indicating migraine induction via peripheral mechanisms, whereas PACAP38 induced premonitory symptoms in 48% of patients indicating a possibly central effect.

LIST OF ABBREVIATIONS

AUC = Area under the curve
 BBB = Blood brain barrier
 cAMP = Cyclic adenosine monophosphate
 cGMP = Cyclic guanosine monophosphate
 CGRP = Calcitonin gene-related peptide
 CNS = Central nervous system
 GTN = Glyceryl trinitrate
 GWAS = Genome-wide association studies
 HR = Heart rate
 MA = Migraine with aura
 MAP = Mean arterial pressure
 MO = Migraine without aura
 NO = Nitric oxide
 PACAP38 = Pituitary adenylate cyclase-activating peptide-38
 PS = Premonitory symptom
 SNP = Single nucleotide polymorphism
 TNF- α = Tumor necrosis factor alpha
 VIP = Vasoactive intestinal peptide
 VRS = Verbal rating scale

SUMMARY

Migraine has a strong genetic component and is characterized by multiphasic events including an initial premonitory phase with premonitory symptoms (PS). Calcitonin gene-related peptide (CGRP) and pituitary adenylate cyclase-activating peptide-38 (PACAP38) are endogenous neuropeptides that can trigger migraine attacks and have in recent years gained considerable interest in the migraine field. Yet, the exact pathophysiological mechanisms underlying CGRP and PACAP38-induced attacks are not fully clarified. Human provocation models have shown that these peptides induce attacks in only two-third of migraine patients. Whether this diverse migraine response after CGRP or PACAP38 may be explained by genetic factors is unknown.

The present thesis includes four studies that explore different factors that may be associated with the CGRP- and PACAP38-induced migraine response. In study I-II we investigated the role of familial predisposition (family load) and number of risk conferring gene variants on migraine attacks induced by CGRP or PACAP38. In study III, we investigated biochemical changes of CGRP, vasoactive intestinal peptide (VIP), S100B and TNF-alpha in the blood after PACAP38. Finally in study IV, we studied whether CGRP or PACAP38 may induce PS.

Study I-II demonstrated that PACAP38 and CGRP induce migraine attacks in 63% and 72% of the patients, respectively. Moreover, we showed that patients with high family load or a high number of migraine associated gene variants did not report more migraine attacks after CGRP or PACAP38 than those with no familial predisposition or few gene variants. Study III showed that PACAP38 infusion caused changes in plasma concentrations for VIP and S100B, but not CGRP and TNF-alpha, suggesting activation of parasympathetic nerve endings. Study IV showed absence of PS after CGRP and lack of statistical difference in PS between patients who reported and not reported attacks after PACAP38 suggesting peripheral mechanisms of induction.

In conclusion, the present thesis suggests that genetics factors such as family load and genetic variants do not contribute to susceptibility of migraine attacks induced by CGRP or PACAP38. Additionally, our data indicate that CGRP and PACAP38 primarily have a peripheral site of action. We believe that the acquired knowledge from this thesis on how CGRP and PACAP38 might be involved in migraine pathophysiology would contribute to the development of novel and better migraine treatments in the future.

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