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DECIPHERING THE ROLE OF GENETICS AND CIRCADIAN RHYTHM IN CLUSTER HEADACHE

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DECIPHERING THE ROLE OF GENETICS AND CIRCADIAN RHYTHM IN CLUSTER HEADACHE

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By

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To my mother

POPULAR SCIENCE SUMMARY OF THE THESIS

Cluster headache is considered one of the most painful conditions in humans and is notoriously known as “suicide headache”. The pain has been described as a sharp, stabbing, or throbbing sensation on one side of the head, and is often accompanied by, for example, tearing, a runny nose, or restlessness. The headache attacks come in periods with up to eight attacks per day, and these active periods can be interrupted by symptom-free intervals of several years. One out of ten cluster headache patients have a close relative also suffering from cluster headache. Thereby there seems to be a heritable component, even though most likely many factors contribute to this very complex disorder. A striking feature of cluster headache is that for a majority of patients the headache attacks can recur at specific times of the day. This periodicity of the attacks has led to the hypothesis that the biological clock may be involved in cluster headache. Our inner biological clock is steered at a molecular level by several key genes which coordinate the activity of the cell, depending on the time of day. The production of certain hormones, such as melatonin which regulates the sleep-wake cycle, is for example higher at night. The interval of this inner clock lasts normally slightly more than 24 hours, which is why it is also referred to as ‘circadian rhythm’ (*circa diem* = approximately one day). On a systemic level, the clocks of different cells and organs are synchronized by a small brain region which receives, among others, information about light via the eyes.

For this thesis, I have studied the genetic material of cluster headache patients in comparison to healthy individuals for possible risk factors, so-called gene variants. If a certain variant occurs significantly more often in patients than in individuals without cluster headache, this gene variant is likely to be linked to the disease. I have focused on genes involved in circadian rhythm but have even investigated genes in connection to trigger factors (e.g. alcohol) or treatment for cluster headache. In a large screening of gene variants over the entire genome of patients and healthy study participants, I could detect several new genes that may play a role in cluster headache. In addition to genetic studies, I have analyzed questionnaire data from cluster headache patients in order to learn more about their symptoms, the location and severity of the pain, the frequency and length of their headache attacks, lifestyle, triggers, and use of treatment. Cluster headache is more common in men than in women, and therefore I was also interested in differences between genders. My main findings revealed that most patients have recurrent cluster headache attacks at night between 2:00 and 4:00 am, and patients who have been smoking had a later disease onset compared to non-smokers. When comparing male with female patients, more women than men had a close relative also diagnosed with cluster headache, suffered from longer active headache periods, and more often reported a diurnal pattern of their attacks.

These findings contribute to increasing the understanding of the disease which may help to improve treatment for patients or even to find the causes for cluster headache.

POPULÄRVETENSKAPLIG SAMMANFATTNING AV AVHANDLINGEN

Hortons huvudvärk beskrivs som en av de mest smärtsamma tillstånd hos människan och kallas även för "självordshuvudvärk". Smärtan beskrivs som en pulserande, stickande eller dunkande känsla på ena sidan av huvudet och det förekommer oftast andra symtom, t.ex. tårflöde, rinnsnuva, eller rastlöshet i kroppen. Huvudvärksattackerna kommer i perioder med upp till åtta attacker per dag, och dessa aktiva perioder avbryts av symtomfria intervaller som kan vara i flera år. En av tio patienter med Hortons huvudvärk har en nära släkting som också lider av Hortons huvudvärk. Det verkar därmed finnas en ärftlig komponent vid sidan om flera andra okända faktorer som bidrar till denna komplexa sjukdom. En anmärkningsvärd egenskap vid Hortons huvudvärk är att många patienter uppger att huvudvärksattackerna återkommer vid specifika tidpunkter på dygnet. Denna regelbundenhet har lett till hypotesen att den biologiska klockan har betydelse för sjukdomen. Vår inre biologiska klocka styrs på molekylär nivå av flera nyckelgener som koordinerar cellens aktivitet beroende på tiden på dygnet. Produktionen av vissa hormoner, bl.a. melatonin som reglerar sömncykeln, är till exempel högre på natten. Intervallet av denna inre klocka brukar vara lite längre än 24 timmar, därför kallas det också för dygnsrytm eller "circadisk rytm" (*circa diem* = ungefär en dag). På kropps nivå synkroniseras olika celler och organs klockor av en liten hjärnregion som mottar information om t.ex. ljus från ögonen.

För denna avhandling har jag studerat genetiskt material från patienter med Hortons huvudvärk samt friska individer för att hitta möjliga riskfaktorer, så kallade genvarianter. Om en viss variant är mycket vanligare hos patienter jämfört med individer utan Hortons huvudvärk, så finns det stor risk att genvarianten är kopplad till sjukdomen. Jag har lagt fokus på gener kopplade till dygnsrytm men har även undersökt gener knutna till triggerfaktorer (t.ex. alkohol) och behandling av Hortons huvudvärk. Dessutom gjorde jag en storskalig screening av genvarianter över hela arvs massan från patienter och friska studiedeltagare där vi upptäckte flera nya gener med tänkbar roll vid Hortons huvudvärk. Utöver dessa genetiska studier har jag också analyserat enkätdata från Hortonpatienter för att utöka kunskapen om deras symtom, lokalisering och intensiteten av smärtan, förekomst och längd av huvudvärks-attacker, livsstil, triggerfaktorer och behandling. Eftersom Hortons huvudvärk är vanligare hos män än kvinnor har jag vidare tittat på könsskillnader. Mina huvudsakliga fynd visar att de flesta patienter får återkommande attacker på natten mellan kl. 2:00 och 4:00, och patienter som har rökt eller snusat har senare sjukdomsdebut jämfört med dem som inte röker eller snusar. När man jämför manliga och kvinnliga patienter har fler kvinnor än män en nära släkting som också har fått diagnosen Hortons huvudvärk, de lider av längre aktiva perioder och rapporterar oftare ett rytmiskt mönster av sina attacker.

Dessa fynd bidrar till ökad kunskap om sjukdomen som kan hjälpa att förbättra behandling för patienter och även att hitta orsakarna till Hortons huvudvärk.

POPULÄRWISSENSCHAFTLICHE ZUSAMMENFASSUNG DER DOKTORARBEIT

Cluster-Kopfschmerz ist eine der schmerzhaftesten Zustände im Menschen und hat den Beinamen „Selbstmord-Kopfschmerzen“. Der Schmerz wird als ein stechendes, bohrendes oder reißendes Gefühl auf der einen Seite des Kopfes beschrieben und ist oftmals von z.B. einem tränenden Auge, einer laufenden Nase oder Rastlosigkeit im Körper begleitet. Die Kopfschmerzattacken kommen in Phasen mit bis zu acht Attacken pro Tag, und diese aktiven Phasen sind von symptomfreien Intervallen von bis zu mehreren Jahren unterbrochen. Einer von zehn Patienten mit Cluster-Kopfschmerz hat einen nahen Verwandten, der auch an Cluster-Kopfschmerz leidet. Daher scheint es eine erbliche Komponente zu geben, obwohl vermutlich viele Faktoren zu dieser sehr komplexen Krankheit beitragen. Ein auffälliges Merkmal dieser Krankheit ist, dass die Mehrheit der Patienten ihre Attacken zu bestimmten Tageszeiten erleiden. Diese ausgeprägte Rhythmik hat zu der Theorie geführt, dass die biologische Uhr bei diesen Patienten möglicherweise fehlreguliert ist. Unsere innere biologische Uhr wird auf molekularer Ebene von mehreren wichtigen Genen gesteuert, die die Aktivität der Zelle abhängig von der Tageszeit koordinieren. Die Produktion von bestimmten Hormonen wie Melatonin, das den Schlaf-Wach-Rhythmus reguliert, ist z.B. in der Nacht höher. Das Intervall dieser inneren Uhr dauert normalerweise etwas mehr als 24 Stunden, daher wird es auch als „circadianer Rhythmus“ (*circa diem* = ungefähr ein Tag) bezeichnet. Auf Körperebene werden die Uhren der verschiedenen Zellen und Organe von einer kleinen Gehirnregion synchronisiert, die unter anderem Informationen zu Tageslicht über die Augen erhält.

Für diese Doktorarbeit habe ich das Erbmaterial von Cluster-Kopfschmerzpatienten und gesunden Probanden auf mögliche Risikofaktoren, sogenannte Genvarianten, untersucht. Tritt eine bestimmte Variante häufiger in Patienten auf als in Personen ohne Cluster-Kopfschmerz, so ist diese Genvariante vermutlich mit der Krankheit assoziiert. Ich habe Fokus auf solche Gene gelegt, die den Tag-Nacht-Rhythmus steuern, aber auch Gene untersucht, die eine Verbindung zu Auslösern (z.B. Alkohol) oder Behandlung von Cluster-Kopfschmerz haben. In einem umfassenden Screening von verschiedenen Genvarianten im gesamten Erbmaterial von Patienten und gesunden Probanden konnte ich mehrere neue Gene finden, die möglicherweise eine Rolle bei Cluster-Kopfschmerz spielen. Zusätzlich zu den genetischen Studien haben wir Daten einer Fragebogenstudie ausgewertet, um mehr über die Symptome, die Lokalisation und den Schweregrad der Schmerzen, die Häufigkeit und Länge der Kopfschmerzattacken, den Lebensstil, Auslöser sowie die Anwendung von Medikamenten bei Cluster-Kopfschmerzpatienten zu erfahren. Cluster-Kopfschmerzen treten häufiger bei Männern als bei Frauen auf, daher habe ich auch Unterschiede zwischen den Geschlechtern analysiert. Meine Untersuchungen zeigen, dass die meisten Patienten wiederkehrende Attacken in der Nacht zwischen 2:00 und 4:00 Uhr haben und dass Patienten, die rauchen oder früher geraucht haben, ein späteres Krankheitsdebüt haben als jene, die nicht rauchen. Bei dem Vergleich zwischen männlichen und weiblichen Patienten mit Cluster-Kopfschmerz zeigt sich, dass mehr Frauen als Männer einen nahen Verwandten mit der Diagnose Cluster-

Kopfschmerz haben, sie an längeren aktiven Phasen leiden und häufiger ein regelmäßiges Muster wiederkehrender Kopfschmerzattacken aufweisen.

Diese Funde tragen zum besseren Verständnis dieser Krankheit bei und könnten dabei helfen, die Behandlung von Patienten zu verbessern oder sogar die Ursachen von Cluster-Kopfschmerzen zu finden.

ABSTRACT

Cluster headache (CH) is a complex neurovascular disorder with a distinct circadian attack pattern. Although many aspects of the disease's pathophysiology remain to be elucidated, it is likely caused by a combination of different genetic and environmental risk factors. Making use of an extensive CH biobank established by our lab, genetic material from patients and controls were screened for several single nucleotide polymorphisms (SNPs) in different candidate genes. In addition, gene expression was analyzed in fibroblast cell lines from patients and healthy controls. Using a hypothesis-free approach, a genome-wide association study (GWAS) was performed on the Swedish material as well as in a combined analysis with a CH cohort from the UK. To characterize the Swedish CH population in terms of clinical patterns and sex differences, two observational studies were conducted based on questionnaire data from CH patients.

In **study I**, we could demonstrate a clear diurnal attack pattern for a majority of patients and that tobacco consumption delays the onset of CH. Pronounced gender differences were detected in **study II**, where we showed that a significantly higher proportion of female patients suffered from the chronic form of CH, had a positive family history for the disorder, and reported diurnal rhythmicity of their attacks to a larger extent than male patients. Because of evident circadian attack patterns in CH, **study III-V** focused on circadian rhythm genes. We found a link between one SNP in the hypocretin receptor 2 (*HCRTR2*) gene and the disorder, but could not confirm previously reported associations of other *HCRTR2* SNPs with CH. However, a SNP in the core clock gene circadian locomotor output cycles kaput (*CLOCK*) was associated with CH and led to increased *CLOCK* gene expression. Another core clock gene, cryptochrome circadian regulator 1 (*CRY1*), included a variant that was less common in patients, and was more highly expressed in patients compared to controls.

Alcohol, nitric oxide (NO), and calcitonin gene-related peptide (CGRP) are all vasodilators which may induce CH attacks, therefore genes connected to these molecules have been of interest in genetic studies of CH. The alcohol dehydrogenase 4 (*ADH4*) gene was previously linked to CH in smaller case-control studies, however in our much larger **study VI**, we could not confirm this association with *ADH4*. In **study VII**, we investigated SNPs in the different NO synthase (*NOS*) genes but could not identify a clear role for these variants in the disorder. In **study VIII**, we demonstrated a link between CH and a SNP in the receptor activity modifying protein 1 (*RAMP1*) gene, encoding a CGRP receptor component, as well as increased *RAMP1* gene expression in CH patients compared to controls. The first-line prophylactic treatment for CH is verapamil, a calcium-channel blocker and vasodilator. The anoctamin 3 (*ANO3*) gene encodes for a calcium-activated ion channel, and in **study IX** we found an association between an *ANO3* gene variant and CH.

Previous GWAS on migraine have yielded two interesting SNPs in the Swedish migraine population. In **study X**, we reported that the variant in the metadherin (*MTDH*) gene was also associated with CH, while the variant in the PR/SET domain 16 (*PRDM16*) gene was migraine-specific. The first GWAS on CH was performed on a very small Italian cohort, and

in **study XI**, we could not confirm the findings for PACAP receptor 1 (*ADCYAP1R1*), membrane metalloendopeptidase (*MME*), and a *14q21* variant. When performing a GWAS on our Swedish CH material in **study XII**, we detected two significant loci near the genes *MER* proto-oncogene, tyrosine kinase (*MERTK*) and special AT-rich sequence-binding protein 2 (*SATB2*), which could be consolidated in a UK CH cohort.

These studies demonstrate an involvement of the circadian rhythm in the pathophysiology of CH, and revealed some possibly dysregulated pathways in relation to treatment of CH. The GWAS findings underline that there is a genetic component to CH which needs to be investigated further.

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- II. **Fourier C**, Ran C, Steinberg A, Sjöstrand C, Waldenlind E, Belin AC. In-depth analysis of gender differences in the Swedish cluster headache population. *Manuscript*
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**These authors contributed equally*

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LIST OF ABBREVIATIONS

ADCYAP1R1	PACAP receptor 1
ADH4	Alcohol dehydrogenase 4
ANAPC1	Anaphase promoting complex subunit 1
ANO3	Anoctamin 3
BDNF	Brain-derived neurotrophic factor
BMAL1/2	Brain and muscle ARNT-like 1/2
BMI	Body mass index
CACNA1A	Calcium voltage-gated channel subunit alpha1 A
CADD	Combined annotation dependent depletion
CCG	International consortium for cluster headache genetics
CCH	Chronic cluster headache
cDNA	Complementary DNA
CGRP	Calcitonin gene-related peptide
CH	Cluster headache
CHMS	Cluster headache maximum severity
CHSS	Cluster headache severity scale
CI	Confidence interval
CLOCK	Circadian locomotor output cycles kaput
CRCP	CGRP receptor component protein
CREB	cAMP-responsive element binding protein
CREM	cAMP-responsive element modulator
CRLR	Calcitonin receptor-like receptor
CRY1/2	Cryptochrome circadian regulator 1/2
Ctrl	Controls
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DUSP10	Dual specificity phosphatase 10
ECH	Episodic cluster headache
EDTA	Ethylenediaminetetraacetic acid

eQTL	Expression quantitative trait loci
FBLN7	Fibulin 7
FBS	Fetal bovine serum
FHL5	Four and a half LIM domains 5
GDPR	General data protection regulation
GERP	Genomic evolutionary rate profiling
GNB3	G protein subunit beta 3
GRCh37/38	Genome reference consortium human 37/38
GWAS	Genome wide association study
HCRT1/2	Hypocretin receptor type 1/2
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPA	Hypothalamic-pituitary-adrenal (axis)
HRC	Haplotype reference consortium
HWE	Hardy-Weinberg equilibrium
ICHD	International classification of headache disorders
IMSE	Immunomodulation and multiple sclerosis epidemiology study
ITGAL	Integrin subunit alpha L
LD	Linkage disequilibrium
LINC01705	Long intergenic non-protein coding RNA 1705
LINC01877	Long intergenic non-protein coding RNA 1877
MAF	Minor allele frequency
MERTK	MER proto-oncogene, tyrosine kinase
MME	Membrane metalloendopeptidase (= neprilysin)
mRNA	Messenger RNA
MTDH	Metadherin
MTHFR	Methylenetetrahydrofolate reductase
NO	Nitric oxide
NOS	Nitric oxide synthase
NPAS1-4	Neuronal PAS domain protein 1-4
NR1D1/2	Nuclear receptor subfamily 1 group D member 1/2
OR	Odds ratio

P2/3	Passage 2/3
PACAP	Pituitary adenylate cyclase-activating polypeptide
PCA	Principal component analysis
PCDHB6	Protocadherin beta 6
PCR	Polymerase chain reaction
PER1-3	Period circadian protein homolog 1-3
PLCE1	Phospholipase C epsilon 1
PRDM16	PR/SET domain 16
QC	Quality control
qPCR	Quantitative (real-time) polymerase chain reaction
RAMP1	Receptor activity-modifying protein
RBM3	RNA binding motif protein 3
REM	Rapid-eye movement
REV-ERBs	= NR1D1/2
RFX2	Regulatory factor X2
RNA	Ribonucleic acid
RORA/B/C	Retinoic acid receptor-related orphan receptor A/B/C
RORE	ROR element
SAIGE	Scalable and accurate implementation of generalized mixed model
SATB2	Special AT-rich sequence-binding protein 2
SCN	Suprachiasmatic nucleus
SNP	Single nucleotide polymorphism
TMEM87B	Transmembrane protein 87B
UTR	Untranslated region
VIP	Vasoactive intestinal peptide
ZT	Zeitgeber

1 INTRODUCTION

1.1 CLUSTER HEADACHE

Cluster headache (CH) is classified as a neurovascular disease, characterized by strictly unilateral, severe pain attacks commonly located around one eye. The intensity of the pain is excruciating and the affected is usually unable to lie down or sit still. In fact, CH is notoriously called ‘suicide headache’ because the pain can be so severe that the suffering individual may attempt to end his or her life [1]. The head pain is often accompanied by a sense of restlessness and/or ipsilateral, autonomic symptoms, such as conjunctival injection, lacrimation, nasal congestion, or ptosis. The headache attacks last between 15–180 minutes and usually come in clusters, which last several weeks to months with a frequency of one attack every other day up to eight attacks per day. These active clusters are separated by remission periods lasting months to years where the affected is completely symptom-free. Depending on the length of the remission period, CH is divided into the two subtypes episodic CH (ECH) and chronic CH (CCH). In CCH patients, who make up 10–15% of all CH patients, the attacks occur for one year or longer without remission, or with remission periods lasting less than three months [2]. However, up to 33% of the patients shift from one subtype to the other during their lifetime [3, 4]. CH is diagnosed according to the guidelines of the International Classification of Headache Disorders (ICHD) (Box 1) [2]. The global prevalence for CH is between 0.05–0.1%, and it has been estimated that between 7–20% of the patients have a first- or second-degree relative also diagnosed with CH [5–7]. The age at onset is typically between 20–40 years and, interestingly, men are afflicted three times more often than women, although this ratio has shifted over the years [8]. This shift may be due to lifestyle changes in both men and women but could also be attributed to an increased recognition of the disease, especially in women [9]. Several studies have compared the clinical presentation of CH in male and female patients, and differences in age at onset, attack duration, pain location, associated symptoms, chronobiology, and comorbid conditions, such as depression, have been observed between the sexes [10–14].

Box 1: ICHD-3 diagnostic criteria for cluster headache

- A. At least five attacks fulfilling criteria B–D
- B. Severe or very severe unilateral orbital, supraorbital and/or temporal pain lasting 15–180 minutes (when untreated)
- C. Either or both of the following:
 1. At least one of the following symptoms or signs, ipsilateral to the headache:
 - Conjunctival injection and/or lacrimation
 - Nasal congestion and/or rhinorrhoea
 - Eyelid oedema
 - Forehead and facial sweating
 - Miosis and/or ptosis
 2. A sense of restlessness or agitation
- D. Occurring with a frequency between one every other day and 8 per day
- E. Not better accounted for by another ICHD-3 diagnosis

A remarkable feature observed in CH is the clocklike rhythmicity by which the headache attacks often occur. In the vast majority of patients, 67–82%, the attacks recur at specific times of the day, predominantly at night between midnight and 4:00 am [15, 16]. Between 41–56% of the patients additionally report circannual rhythmicity of their cluster periods [15–17]. Although many studies suggest a connection of the occurrence of headache bouts to photoperiods (length of daylight) and a number of patients have recurring cluster bouts during specific seasons or months on an individual level, there is not a distinct time of year that stands out [18, 19]. While several studies found most bouts to occur in two peaks at the time of the solstices during spring and autumn, others report one peak during months with the least daylight [15, 18–21]. All these studies concur that the fewest bouts arise during the summer.

The pathophysiology of CH is largely unknown. Although the trigeminovascular system plays a central role during the headache attacks, there is no evidence that mere vascular changes lead to pain [22]. Neurotrophins, such as the brain-derived neurotrophic factor (BDNF), may be involved, as they are known pain mediators and modulators, and BDNF peripheral levels have been shown to be altered in CH patients [23]. Previously, it was believed that CH was a purely vascular disease. However, current research suggests that CH is rather a complex brain network disorder involving multiple cortical, subcortical, and brainstem regions. A number of studies were able to demonstrate widespread dynamic functional and structural changes in the brain of CH patients during different phases of the disorder [24]. At least three systems are hypothesized to be involved in the pathophysiology: the trigeminovascular system which is responsible for the pain perception, the cranial autonomic system which generates the observed autonomic symptoms, and the hypothalamus [25]. The anterior hypothalamus, including the suprachiasmatic nucleus (SCN), may be conductor for the striking circadian rhythmicity of the attacks, while the posterior hypothalamus possibly contributes to the restlessness that many CH patients experience during their attacks [26, 27].

Several trigger factors have been reported for CH, of which alcohol is the most prominent. More than 50% of CH patients state that alcohol can elicit a headache attack during an active phase [15, 28–30]. Other less common triggers include stress (or relaxation after stress), weather changes (heat/cold), certain odors, bright lights, histamine, and the nitric oxide (NO) donor nitroglycerin [2, 15, 30–33].

1.2 LIFESTYLE AND COMORBIDITIES IN CLUSTER HEADACHE

Regarding lifestyle habits, there is a large consensus that there are significantly more smokers/tobacco users among CH patients [15, 29, 34–36]. However, it is not clear whether smoking is a risk factor for CH or rather a form of self-medication. In one of our studies, we found that tobacco users had a significantly later disease onset than patients without any history of tobacco use which was confirmed by Rozen et al. [30, 37]. This suggests that these patients may possibly delay their CH onset by smoking. In older studies, it has also been

observed that CH patients have a higher tendency for alcohol abuse [29, 34]. These studies only included male patients, and recent studies could not confirm this observation. On the contrary, many patients report reducing their alcohol consumption drastically during an active bout [15, 30, 35]. There have been indications for an unhealthy lifestyle in CH patients with respect to weight, for example studies report a higher body mass index (BMI) compared to controls and obesity as an accompanying symptom in 12.1% of CH patients [36, 38].

Very few comorbidities have been found for CH suggesting this patient group to be rather healthy apart from their dire headache attacks. On the other hand, two recent studies report significantly increased sickness absence and disability pension days for CH patients compared to matched references [39, 40]. About 16% of CH patients also suffer from migraine, another primary headache disorder, and this is similar to the migraine prevalence in the general population [30, 41]. An increased risk of cardiovascular disease has been suggested for CH, but the results are conflicting [36, 42, 43]. Several studies have reported that CH patients are more likely to be diagnosed with depression, deviated septum, or dental/temporomandibular joint problems [14, 15, 36, 44, 45]. However, these reports need to be considered with caution since these may reflect previous misdiagnoses, or secondary diseases, rather than true comorbidities [43, 46, 47]. Interestingly, the prevalence of diabetes is reported to be lower in CH patients as compared to the general population [15, 43, 44]. It has also been proposed that CH patients may have a comorbidity with sleep disorders because of generally poor sleep quality and nocturnal headache attacks [48]. However, studies on, for example, sleep apnea have been opposing, and a temporal relationship between nocturnal attacks and rapid-eye movement (REM) sleep could not be confirmed [21, 49].

1.3 TREATMENT OF CLUSTER HEADACHE

Since there are no treatments specifically developed for CH, patients either use common pain killers, such as paracetamol, ibuprofen, and aspirin, which rarely give sufficient pain relief, or they are prescribed migraine medication, for example triptans. CH and migraine share some pathological features, including activation of the trigeminovascular system, neurogenic inflammation, recurrence of headache attacks, lateralized pain, and associated autonomic symptoms [2, 50, 51]. However, in migraine these symptoms typically include nausea and vomiting rather than the previously mentioned autonomic symptoms for CH. In addition, migraine attacks last much longer than CH attacks, between 4–72 hours, and are generally less painful than CH [2, 52]. This fundamental difference in headache burden makes it challenging to treat CH in a similar way as migraine because CH requires fast-acting medication for immediate attack abortion. Yet, the majority of CH patients uses triptans as acute treatment and up to 70% report them to be effective [28, 30]. However, per recommendation the use of triptans is limited to a certain number of doses per month, depending on the route of administration. In addition, they can have considerable side effects, such as headache, chest or neck pain, fatigue, paresthesia, and coronary vasoconstriction [53]. Triptans act as serotonin receptor agonists on nociceptive trigeminal nerve endings and are

also potent vasoconstrictors [54]. Another commonly used, but not widely available, acute treatment is oxygen, with an efficacy in around 76% of all CH patients [28, 55]. The advantage with oxygen is that no or only minimal side effects are reported compared to triptans [56]. The mechanism of action and why oxygen is so effective specifically in CH is not yet fully understood, but one study suggests that it acts via cranial autonomic pathways rather than on trigeminal afferents [57]. For prophylactic treatment during cluster periods the most frequently used is verapamil, a calcium-channel blocker generally used to treat high blood pressure by dilating the blood vessels. Therefore, it cannot be used in patients with hypotension. Curiously, CH patients receive double the dose that is used in cardiovascular disease, possibly because access for verapamil to the brain is limited where it most likely acts in the hypothalamus [25, 58]. Other preventive treatments used by CH patients during a bout are corticosteroids, such as prednisolone, and lithium. The mechanisms of action are not clear for either of them, but they have been proven to be effective in a smaller portion of patients [28, 59, 60].

A recently developed treatment makes use of antibodies against calcitonin gene-related peptide (CGRP) or its receptor. CGRP is a neuropeptide and potent vasodilator with a variety of functions, including transmission of nociception in cerebral blood vessels and activation of the trigeminal system, [61, 62]. It is released by central as well as peripheral neurons and binds to a receptor complex consisting of calcitonin receptor-like receptor (CRLR), receptor activity-modifying protein 1 (RAMP1), and CGRP receptor component protein (CRCP) [63]. Several studies could link CGRP and its receptors to both migraine and CH, and demonstrated that anti-CGRP antibodies may prove to be a new effective preventive treatment for both disorders [64–67]. In addition, CGRP levels are increased in CH patients during an attack, and an infusion of CGRP could induce a CH attack in patients during the active phase [51, 68].

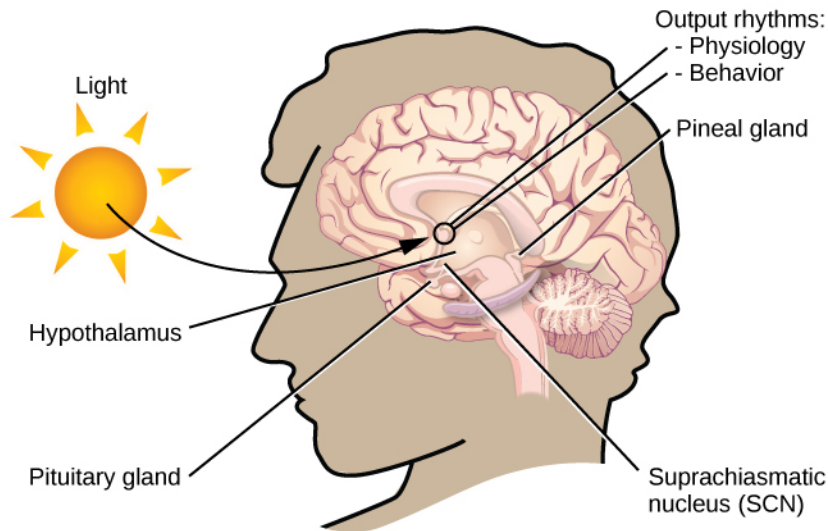
1.4 CIRCADIAN RHYTHM

In a wide range of organisms, including cyanobacteria, plants, fungi, flies and mammals, biological activities are organized into daily cycles which are driven by an endogenous molecular clock [69]. This clock runs in cycles of roughly 24 hours, therefore it is also referred to as the circadian clock (*circa diem* = approximately one day). It operates independently of external cues, such as light, social behavior (including feeding), or temperature variations throughout the day [70]. However, the clock needs to be synchronized regularly by environmental stimuli to maintain this circadian rhythm in accordance with the natural light/dark cycle.

In mammals, these environmental stimuli are processed centrally in the brain by the suprachiasmatic nucleus (SCN), located in the hypothalamus (Figure 1). The SCN is subdivided into a core and a shell region. The former is essential for coupling within the SCN which is needed for a coherent output signal from the SCN. This coupling becomes evident

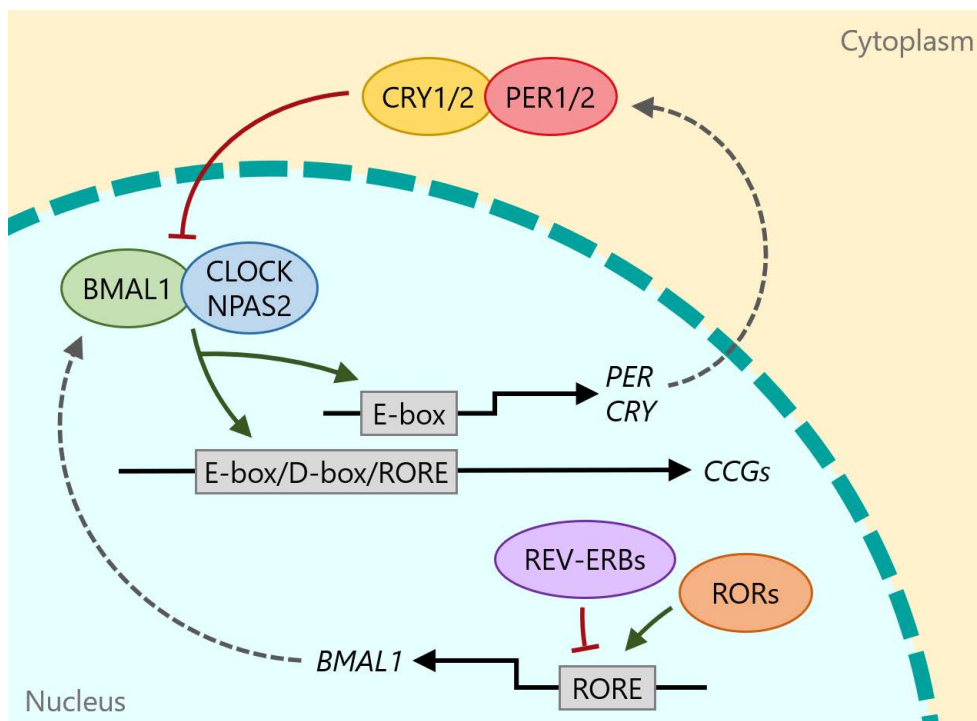
during jet lag where the core can shift more quickly to the new light/dark cycle while the shell shifts only after receiving coupling signals from the shifted core. Hence, there is a lag in adjusting to a new time zone [71].

Figure 1. Brain regions important for the body's internal clock



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Figure 2. The circadian core clock transcriptional-translational feedback loop



BMAL1, brain and muscle ARNT-like protein 1; *CCGs*, clock-controlled genes; *CLOCK*, circadian locomotor output cycles kaput; *CRY*, cryptochrome circadian regulator; *E-box/D-box*, promoter elements; *NPAS2*, neuronal PAS domain protein 2; *PER*, period circadian protein homolog; *REV-ERBs*, nuclear receptor subfamily 1 group D members; *ROR*, retinoic acid receptor-related orphan receptor, *RORE*, ROR response element

The core region of the SCN receives afferent input from photosensitive retinal ganglion cells in the eyes via the retinohypothalamic tract. When the eyes are exposed to a light stimulus, the axons of these cells release glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP), which enhances the effect of glutamate, at synaptic contacts with SCN neurons. Glutamate triggers membrane depolarization and calcium influx in the SCN core cells which triggers a signaling cascade leading to vasoactive intestinal peptide (VIP) release in the synaptic cleft between SCN core neurons and shell neurons, and ultimately to the transcription of genes, such as *PER1* and *PER2* (*period circadian protein homolog 1* and *2*) which are directly involved in the core clock feedback loop [72]. By this mechanism, phase delays and phase advances can be induced in order to calibrate the endogenous clock to the external environment [71].

After receiving input, the SCN sends efferent projections to local primary targets, such as the lateral hypothalamus (site of orexinergic neurons), the pineal gland (site of melatonin release), the periventricular nucleus and the nucleus of the vagus nerve (connections to the autonomic system), and the pituitary gland (master gland of hormone secretion) [73, 74]. What is more, the SCN regulates the hypothalamic-pituitary-adrenal (HPA) axis, a major neuroendocrine system which modulates, for example, stress response and releases glucocorticoids like cortisol in a circadian fashion [75]. In turn, the HPA axis communicates with the circadian system [76]. Interestingly, it has been shown that not only endogenous but also external glucocorticoids, such as prednisolone, suppress circadian oscillations in the SCN as well as in peripheral tissues [77, 78]. In addition to photic input, there is a non-photoc regulation of circadian rhythms via projections from the serotonin-containing raphe nuclei in the midbrain to the SCN. When the organism is exposed to light, serotonin released inside the SCN can, for instance, block or modulate the resetting of circadian oscillations at subjective night, i.e. while the organism is kept in darkness [79].

Most cell types and tissues have their own circadian rhythm. The SCN plays a crucial role in coordinating circadian rhythmicity by synchronizing the independent cellular clocks in the brain and peripheral tissues. It has been shown that in the absence of the SCN, the rhythmic oscillations dampen out in most tissues [80]. This strongly underlines the pacemaker function of the SCN and its importance in maintaining the circadian rhythm within the organism. On a cellular level this rhythm is controlled and maintained by five core clock protein families which are encoded by *CLOCK* (*circadian locomotor output cycles kaput*), *NPAS1-4* (*neuronal PAS domain protein 1-4*), *BMAL1/2* (*brain and muscle ARNT-like protein 1/2*), *CRY1/2* (*cryptochrome circadian regulator 1/2*), and *PER1-3* in humans. In a negative feedback loop, *CLOCK*, or its paralog *NPAS*, and *BMAL1* form heterodimers and activate transcription of other genes, including *PER* and *CRY*, by binding to specific promoter elements called E-boxes. The protein products, *PER* and *CRY*, dimerize and inhibit their own transcription by binding to the *CLOCK/BMAL1* complex (Figure 2) [74, 81]. Additional regulators of the molecular clock are two nuclear receptor families encoded by *REV-ERB-alpha/beta*, also called *NR1D1/2* (*nuclear receptor subfamily 1 group D member 1/2*), and *RORA/B/C* (*retinoic acid receptor-related orphan receptor A/B/C*). In a second feedback

loop, REV-ERBs are activated by CLOCK/BMAL1 and in turn repress *BMAL1* expression, competing with RORs, which activate *BMAL1*, for binding at the *BMAL1* promoter [82].

There are several studies showing relevance of circadian timing in disease. For example, transgenic mice with a mutant *Clock* gene develop metabolic syndromes. Acute sleep deprivation, as seen in shift workers, can alter circadian clock gene expression in peripheral tissue [83]. What is more, this circadian disruption is associated with increased risk for metabolic dysfunction, cardiovascular disease, and cancer [84]. It is suggested that SCN function is impaired in patients with disorders of the nervous system, such as Alzheimer's disease, schizophrenia, Huntington's disease, and bipolar disorder [85–88]. In addition, several clock genes, including *CRY2*, *NR1D1*, *PER2*, *BMAL1* and *NPAS2*, have been implicated in psychiatric disorders, for example depression, bipolar disorder, psychosis, and seasonal affective disorder [89–92].

1.5 CIRCADIAN RHYTHM INVOLVEMENT IN CLUSTER HEADACHE

It has long been proposed that circadian rhythm has a role in CH. Not only do CH patients generally have headache attacks at the same time every day during a cluster period, studies have also shown that the hypothalamic region, which is crucial for the central regulation of the circadian rhythm, is activated during these attacks [93]. Interestingly, a circadian rhythmicity in pain thresholds of the nociceptive flexion reflex could be demonstrated in ECH patients [94]. Furthermore, a lacking habituation of the trigeminal reflex was observed in CH patients which could be driven by hypothalamic dysfunction during a bout [95]. Another study demonstrated that the modulation of nociceptive input is affected by the activation of hypocretin (orexin) receptors 1 and 2 (HCRTR1 and HCRTR2) in the posterior hypothalamus [96]. These receptors are activated by neurotransmitters called orexins which are selectively synthesized in the hypothalamus and regulate different neuroendocrine and autonomic functions, such as the circadian sleep/wake cycle process or feeding behavior. In addition, the circadian secretion of melatonin and cortisol was altered, and abnormal levels of the SCN neuropeptide VIP were found in CH patients [62, 97].

Several treatments that are used as prophylactic treatment in CH with variable efficacy have been shown to affect the circadian system. For example, corticosteroids and melatonin are believed to reset the body clock [98, 99]. Lithium lengthens the circadian period and enhances *PER2* expression, while valproic acid shortens the circadian period [100]. In mice, verapamil appears to lead to a dose-dependent period-shortening and to altered expression of several clock genes, including *Clock*, *Bmal1*, *Per3*, and *Cry2*, in the trigeminal ganglion and hypothalamus, but not the SCN [101].

1.6 GENETICS OF CLUSTER HEADACHE

It is hypothesized that CH is a complex genetic disorder and, similar to migraine, genetic factors likely influence the risk of developing CH [102]. There are few studies on the heritability of CH, but it has been established that having a first- or second-degree relative diagnosed with CH increases the risk of also developing the disease [103]. One study estimates CH heritability to be $h^2 = 0.26$, but because only 2.3% of the patients in the cohort reported familial occurrence, this heritability is probably an underestimation [104]. It is not entirely clear how familial CH is inherited, but a majority of studied pedigrees are consistent with an autosomal dominant pattern [105]. Interestingly, two systematic reviews on the family history in CH detected a significantly higher preponderance of familial CH in females [105, 106].

Although CH research has just recently expanded tremendously, there have been large efforts to increase insights into the genetics of CH [107]. Several candidate genes have been proposed, including the previously mentioned *HCRTR2*, *PER3*, *CLOCK* as well as *ADH4* (*alcohol dehydrogenase 4*), *NOS* (*nitric oxide synthase*), *CACNA1A* (*calcium voltage-gated channel subunit alpha1 A*), and *MTHFR* (*methylenetetrahydrofolate reductase gene*). Many studies have focused on *HCRTR2*, and while several studies reported an association of the exonic variant rs2653349 with CH, others could not confirm these results [108–111]. Regarding other circadian clock genes, no association for *PER3* was found, and for *CLOCK* the results are opposing [112–115]. Because alcohol is such a pronounced trigger for CH attacks, the *ADH4* gene has been investigated, and results vary between different study cohorts [116–118]. Nitric oxide is a potent vasodilator, therefore it has been hypothesized that dysfunctional NOS could play a role in CH. However, a strong genetic link could not be confirmed so far [119]. Studies reported associations between migraine and the genes *CACNA1A* and *MTHFR*, but these associations were not found for CH [120, 121].

Another approach is to look at genetic variants which might affect treatment efficacy. A common variant in *GNB3* (*G protein subunit beta 3*) is suggested to modulate responder rate to triptans in CH patients [122, 123]. A study on differential gene expression in CH patients responding to lithium showed the genes *NR1D1* and *RBM3* (*RNA binding motif protein 3*) to be significantly altered in CH patients [124]. *RBM3* is essential for the temperature-entrained circadian gene expression.

Large genetic screenings and hypothesis-free approaches for CH have been scarce. However, for migraine, which is much more common than CH, several genome-wide association studies (GWAS) and meta-analyses have been published [125–129]. A GWAS on Swedish migraine patients could replicate associations with *MTDH* (*metadherin*) and *PRDM16* (*PR/SET domain 16*) [130]. *MTDH* is involved in angiogenesis but even has a role in the glutamate pathway [131]. The potential function of *PRDM16* in migraine is still unclear, but it has been reported that *PRDM16* may be associated with triptan response [132]. Another interesting gene discovered via genome-wide studies in migraine is *FHL5* (*four and a half LIM domains 5*) which encodes a transcription factor activating cAMP-responsive elements

CREM and *CREB* with a role in synaptic plasticity and memory formation [129]. Because of some shared features in migraine and CH, it is possible that some of these genes may also be involved in the pathophysiology of CH. In 2016, the first GWAS on CH has been published for a small sample of 99 patients and 360 controls [133]. This Italian study suggests a role for the PACAP receptor 1 gene *ADCYAP1R1* and the neprilysin gene *MME* in CH, both of which have a pivotal role in pain processing. However, larger studies must confirm these results.

The challenge in genetics research is the discrepancy between different cohorts because the frequency of genetic variants and the impact of genetic risk and protective factors can vary immensely between populations in different geographic locations. Another concern introduced by the low incidence of CH has been to assemble enough patients to perform genetic screening with sufficient statistical power.

In conclusion, many genetic studies point towards the involvement of genetic components in the etiology of CH, although more and larger studies are needed in order to conclude on the importance of specific variants in relation to CH.

2 RESEARCH AIMS

Multiple genetic factors can combine in different ways to increase or decrease the risk for disease. The larger objective of this research project was to identify some of these factors for CH in order to increase the understanding of its pathophysiology. The three main aims of this thesis were to:

1. Identify genetic markers and candidate genes associated with CH
2. Characterize cellular mechanisms and expression of candidate genes as well as how genetic markers affect the normal function of these genes
3. Characterize clinical manifestations and circadian rhythm in relation to CH and treatment.

These objectives were achieved by using and further extending a unique Swedish CH biobank with biological tissue and clinical data from patients with CH as well as from neurologically healthy controls. It is hypothesized that CH is a complex genetic disorder and that multiple genetic factors, in combination with environmental factors, will modify the risk for the disease. To test this hypothesis, genetic risk factors were identified and validated by characterizing cellular mechanisms and expression of relevant genes. Additionally, it was studied to which degree identified genetic markers affect the normal function of these candidate genes in biological samples from CH patients, in active as well as in remission periods, and in controls. For example, differences in mRNA folding, transcription factor binding prediction, or mRNA expression levels were investigated. Today, little is known about trigger factors for CH attacks, and a systematic characterization of active and remission periods is lacking. One aspect of the general objective of this thesis is the involvement of circadian rhythm regulation in relation to CH as well as treatment. To address this, specifically genes regulating circadian rhythm were characterized. Results from all of these studies may aid the development of more efficient drugs with fewer side effects as well as more individualized treatments.

3 MATERIALS AND METHODS

3.1 MATERIALS

The materials used for the different projects of this thesis are part of a Swedish CH biobank which was established by the lab in 2014. The biobank includes DNA samples, primary fibroblast cell lines, and extensive questionnaire data from validated CH patients as well as neurologically healthy control individuals. The study material was obtained after approval of the local ethics committee in Stockholm and informed consent given from both patients and controls. CH patients were recruited at the neurology clinics of the Karolinska University Hospital, Stockholm, Sweden, as well as in collaboration with neurologists at clinics in other parts of Sweden. A minority of patients and controls contacted us via advertisements for the study. Each diagnosis was validated by an experienced neurologist according to the International Classification of Headache Disorders 3rd version beta (ICHD-3b) [134]. A majority of the controls for the genotyping studies were anonymous, healthy blood donors between the age of 18 to 60 years from the Stockholm area. Only sex was known for these individuals.

Patients were asked to give a blood sample either at the outpatient clinic or at a health center. In connection to the visit at the clinic, the patient was also asked to fill in an extensive questionnaire regarding clinical features, treatment, triggers, and lifestyle. Patients giving a blood sample at the health center were able to send their completed questionnaire by mail. A subset of patients and controls also had a skin biopsy taken at the clinic which was used for culturing fibroblast cell lines.

For the GWAS, additional genotype data from neurologically healthy controls was obtained from the Immunomodulation and Multiple Sclerosis Epidemiology (IMSE) study at Karolinska Institutet.

3.2 METHODOLOGY

3.2.1 Observational Studies

The extensive questionnaire data from the CH biobank were used to perform cohort studies on the Swedish CH population in order to clinically characterize patients and subgroups of patients, such as those with CCH versus ECH, or male versus female patients. In addition, the effects of different lifestyle factors, treatments, and triggers on clinical features were investigated by statistically comparing the different subgroups.

3.2.2 DNA Extraction

Blood samples were stored at -20°C prior to DNA extraction. For DNA purification from human whole blood, the Gentra Puregene Blood Kit (QIAGEN, Hilden, Germany) was used according to the manufacturer's instructions with slight modifications: Instead of centrifuging the DNA threads to form a pellet, the threads were collected using an inoculation loop, washed in ice-cold 70% ethanol, air-dried and then added to a cryotube with DNA Hydration solution (10 mM Tris, 1 mM EDTA, pH 7–8). In order to achieve RNA-free DNA, the samples were incubated with RNase A enzyme (QIAGEN, Hilden, Germany) after cell lysis. The DNA samples were incubated with gentle shaking at room temperature overnight up to two days to completely dissolve the DNA threads. DNA concentration was measured with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies LLC, Wilmington, DE, USA). As part of the CH biobank, the DNA samples were stored at -20°C .

3.2.3 Genotyping

The SNPs analyzed in the present studies were chosen because they were located in genes of interest and had either been previously investigated in CH or were associated with treatment used in CH, other brain disorders, or relevant conditions. For genotyping of single variants in candidate genes, two different methods were applied. For the majority of SNPs, quantitative real-time polymerase chain reaction (qPCR) was performed using predesigned TaqMan® SNP genotyping assays and TaqMan® genotyping master mix (Thermo Fisher Scientific, Waltham, MA, USA) on an ABI 7500 FAST Real-Time PCR instrument (Applied Biosystems, Foster City, CA, USA). This genotyping method is based on the use of two fluorescently labeled probes specifically designed for each allele of the SNP of interest in addition to the forward and reverse primers. For most assays, it was sufficient to use only half of the assay volume for each reaction, the missing volume was replaced by water (total volume of 10 μL per well on a 96-well plate). 2–5 ng dried DNA was used for each reaction, water served as negative control on each plate. The cycler was generally programmed as follows: pre-PCR read at 60°C and enzyme activation at 95°C for 10 minutes, 40–55 cycles of 95°C for 15 seconds and 60°C for 1 minute, and post-PCR read at 60°C for 1 minute. Allelic discrimination was determined using the 7500 software v2.3 supplied with the instrument.

Where the TaqMan® assays did not yield reliable results, a second method called pyrosequencing was used for validation. Pyrosequencing is a technique where a short DNA fragment of approximately 10 bp is sequenced by detecting the energy that is released when a nucleotide is incorporated into a predefined DNA strand [135]. Primers were designed using the software Primer3 or ApE v2.0.49, with melting temperatures between 58 – 60°C [136]. Primer folding energy, optimally higher than -2 kcal/mol, was assessed by the web-based RNA/DNA folding software Mfold [137]. To confirm specificity, the online NCBI Blast tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used. DNA fragments were amplified by PCR using one regular and one biotinylated primer. The biotinylated PCR products were fixed onto streptavidin-coated Sepharose® beads (GE Healthcare, Uppsala, Sweden) using a

PyroMark® vacuum prep tool (Biotage AB, Uppsala, Sweden), then denatured and purified in 70% ethanol, 0.2 M NaOH, and washing buffer according to manufacturer's instructions. Finally, the single-stranded PCR fragments were annealed to the pyrosequencing primer for 2 minutes at 80°C and then sequenced using PyroMark® Gold reagents (QIAGEN, Hilden, Germany) on a PSQ 96 system (Biotage AB, Uppsala, Sweden). The results were manually reviewed and analyzed with the software provided with the instrument.

3.2.4 Fibroblast Cell Culture

Skin biopsies were taken from the inner side of the upper arm from each study participant after a topical anesthetic was applied. A 2x4-mm skin piece was removed and placed in a tube with chilled PBS until further processing. To establish fibroblast cultures from skin, a slightly modified protocol by Takashima was adopted [138]. The skin biopsy was cut into several smaller pieces without removing the subcutaneous tissue and placed in the center of a small petri dish with a sterile coverslip on top. 5 mL growth medium (85% DMEM, 13% FBS, 1% 1 M HEPES buffer solution, and 1% 100x penicillin/streptomycin solution) was added before the petri dish was placed in a humidified incubator (37°C, 5% CO₂). When confluency was reached, the fibroblasts were detached with trypsin/EDTA and passaged to larger culture flasks. After the P2 generation reached confluency, the fibroblast cells were frozen in 10% DMSO/FBS with 500,000 cells per cryotube, and as part of the CH biobank stored at -150°C until further use.

3.2.5 mRNA Expression Studies

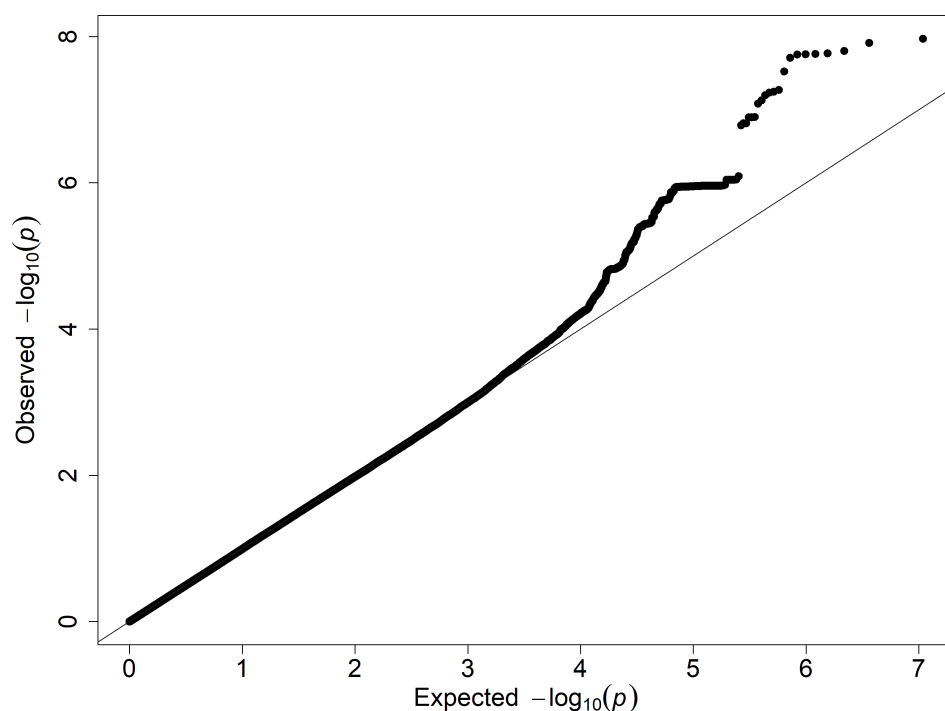
The frozen fibroblast cell lines were quickly thawed and transferred to a large culture flask with growth medium. When P3 reached about 80% confluency, the cells were given a serum shock to reset the circadian clock. By using this method, it can be assured that the cells are synchronized when looking at the gene expression, especially of molecular clock genes. Normal growth medium was exchanged with a starvation medium only containing 0.5% FBS. After 24 hours, the shock medium containing 30% FBS was given and this time point is marked as *zeitgeber* (ZT) 0. The high FBS medium was exchanged for normal growth medium after 2 hours (ZT+2). Cells were then harvested at the time point of interest. For that, the fibroblasts were detached via trypsinization and centrifuged at 500 × g for 5 minutes in a 15-mL tube. The supernatant was removed, and the cell pellet was immediately frozen on dry ice and then kept at -150°C until further processing. RNA extraction was performed using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. RNA concentration was measured using the NanoDrop ND-1000 and the RNA was immediately frozen at -80°C. Using the QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany), the RNA was converted to cDNA according to the manufacturer's instructions. The cDNA was then used to perform gene expression studies using qPCR and SYBR Green technology. Depending on the study, different instruments were used for carrying out the qPCR; either the ABI 7500 FAST Real-Time PCR instrument (Applied Biosystems, Foster City, CA, USA) or the Bio-Rad CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA).

3.2.6 Genome-Wide Association Study

CH patients were genotyped at the SNP&SEQ Technology Platform, Uppsala, Sweden, and controls from the IMSE study were genotyped at deCODE genetics, Reykjavik, Iceland. For both materials the Illumina Infinium 24v1.0 Global Screening Array (Illumina Inc., San Diego, CA, USA) was used. Before the two datasets were merged, the control data was lifted over from the GRCh38/hg38 reference genome to the GRCh37/hg19 build, so that the positions of all markers matched between the control and the patient dataset. Quality control (QC) was performed according to standard guidelines using the open-source whole genome association analysis toolset PLINK v1.9 [139, 140]. QC included filtering for low genotyping call rate per variant/individual, monomorphic variants, deviation from the Hardy-Weinberg equilibrium (HWE), heterozygosity, and duplicates, as well as a principal component analysis (PCA) to control for population stratification. Prior to imputation the HRC/1000G Imputation Preparation and Checking Tool v4.2.9 (<https://www.well.ox.ac.uk/~wrayner/tools>) was applied to check for errors related to strand, reference allele assignment, and allele frequency differences against the Haplotype Reference Consortium panel v1.1. Subsequently, the merged dataset was phased using Eagle v2.3 and imputed on the Michigan imputation server [141]. Single variant association testing was performed using the R package “SAIGE” implementing the Scalable and Accurate Implementation of Generalized mixed model which accounts for case-control imbalance and sample relatedness [142]. Monomorphic SNPs and markers with an imputation quality score $R^2 < 0.3$ or minor allele frequency (MAF) < 0.05 were excluded from further analysis. Plots were generated using the R package “qqman” and the web-based plotting tool LocusZoom [143, 144]. A quantile-quantile (Q-Q) plot, mapping expected against observed p -values obtained from the association analysis, shows that the values follow a straight line and then form a tail at the end with high p -values, illustrating true disease associations (Figure 3). In addition, the genomic inflation factor of $\lambda = 0.99$ indicates that our data is of high quality and not influenced by, for example, population structure, relatedness, or genotyping errors.

In addition, for a mega-analysis the Swedish dataset was combined with a CH dataset from the UK after separate imputation. SAIGE association analysis was run on the merged material and variants with $R^2 < 0.3$ and $MAF < 0.01$ were excluded. A lower MAF threshold was chosen here because of the considerably larger sample size of the merged material. Several downstream analyses, including functional variant annotation and prediction, gene-based association testing, gene expression and expression quantitative trait loci (eQTL) analysis, as well as a pathway analysis, were performed on the combined material. Reported metrics include the combined annotation dependent depletion (CADD) score, where scores >10 are predicted to be the 10% and >20 the 1% most deleterious possible substitutions in the human genome, as well as the genomic evolutionary rate profiling (GERP) score, with scores >2 indicating high evolutionary constraint [145, 146].

Figure 3. Q-Q plot for the Swedish cluster headache association analysis



Quantile-quantile (Q-Q) plot showing the observed p -values of 5,427,757 markers versus expected p -values. The continuous line indicates the distribution of variants under the null hypothesis, i.e. when there are no genetic associations. Genomic inflation factor is $\lambda = 0.99$.

3.2.7 Statistical and *In Silico* Analyses

Power calculations were performed with the power and sample size calculation software PS v3.0, and deviation from HWE was tested for using the previously available Online Encyclopedia for Genetic Epidemiology Studies software [147, 148]. Data from the observational studies, genotyping, and mRNA expression studies were analyzed with GraphPad Prism v5.04 or v8.0.1 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com), depending on the study. Details on which tests were used can be found in the individual publications. For some studies, a logistic regression analysis with sex as covariate was performed using PLINK v1.07 [140]. For haplotype analysis of genotyping results, either PLINK v1.07 or HaploView v4.2 was used, depending on the study [149]. In HaploView, permutation testing with 10,000 permutations to correct for multiple testing was run in addition to the multimarker analysis.

Secondary mRNA structure and folding energy were predicted using Mfold to evaluate possible effects of disease-associated variants on, for example, RNA stability [137]. A partial RNA sequence of the gene of interest, consisting of the minor allele of the SNP and a flanking sequence of 70 nucleotides on each side of the SNP, was analyzed and compared to the RNA sequence containing the major allele. To evaluate the effect of associated variants on predicted transcription factor binding site affinity, we used the web-based computational tool SNP2TFBS [150].

3.3 ETHICAL CONSIDERATIONS

Because this project had a focus on human genetics, and human material was collected for the presented studies, the use of animals for experimental research could be averted. In addition, there are currently no satisfying animal models for headache.

Most of the experiments involved human DNA or skin cell samples, which were obtained from the individual causing only minimal discomfort. There are some ethical questions that need to be addressed when working with human material and questionnaire data. The ethical permit allows us to contact specialists across Sweden in order to receive information on CH patients. Each individual recruited to the study is informed about the objective of the project and the possibility to terminate participation at any time, and they give consent to participating in the study before we proceed with the sample collection. The individual fills out a questionnaire and we take blood and/or skin samples by minimally invasive standard procedures which do not harm the individual.

Confidentiality of personal information is a major concern when collecting this type of data, therefore the data must be stored securely with only few authorized persons having access, in accordance with GDPR (General Data Protection Regulation). Each study participant is assigned a sample identifier, and during handling and analysis of the data, researchers will only work with this sample identifier. This way, the individual data, such as name, personal number, and contact information, cannot be tracked by unauthorized people. In addition, only relevant information that is needed for the study was collected.

An often-raised ethical dilemma is the possibility of finding a pathogenic high-penetrance mutation in the material. Because researchers are not genetic counselors and should adhere to their obligation of handling all data anonymously, the individual should not be informed about the increased risk of disease which would lead to a breach of anonymity. Additionally, by informing the individual their choice of whether or not they want to know about the risk is taken away. In this research project, the investigated genetic variants are more or less common in the general population and represent risk factors rather than disease-causing mutations. CH is most likely a complex genetic disorder with low penetrance, and the combination of different genetic factors, which increase the risk for this disorder, needs yet to be elucidated. Hence, there is no need to pass on such information to study participants.

In summary, all ethical concerns for this project have been evaluated carefully by the research group and the regional ethical review board. Because CH is such an understudied disorder, it is important to do research in this field. Considering that participation in the study bears no health risks and data integrity is well maintained, we see this study as fully justified and as a step closer to improving the health of CH patients.

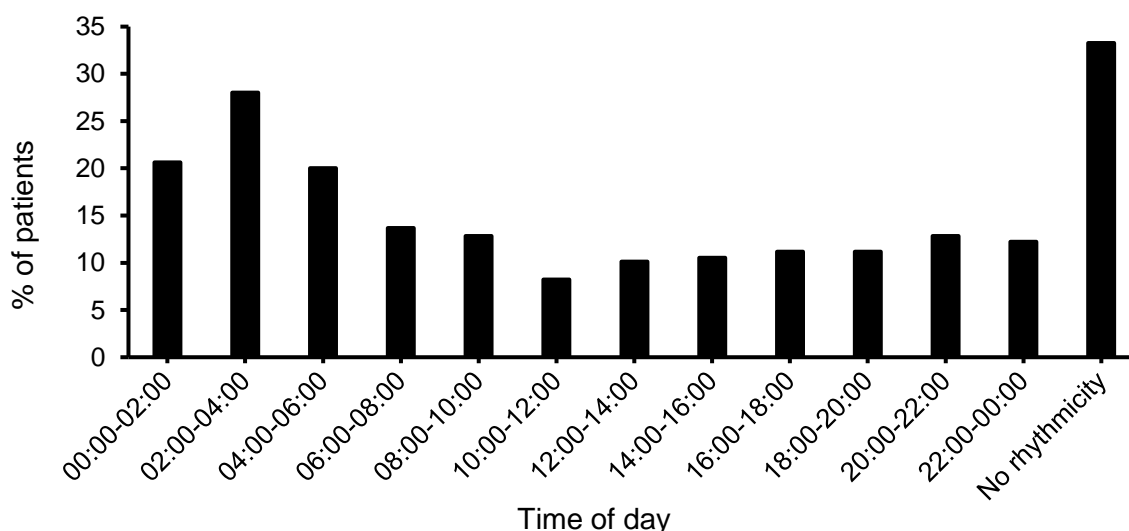
4 RESULTS

4.1 OBSERVATIONAL STUDIES (STUDY I AND II)

In order to get a better understanding of the clinical burden of CH, we have performed two observational studies on questionnaire data from CH patients.

In **study I**, we have analyzed data from 500 patients, of which 68% were male and 11% suffered from the chronic subtype. Patients with CCH had later disease onset of about 4.5 years on average than patients with ECH. Similar to previous studies on lifestyle of CH patients, we could confirm that there was a larger proportion of smokers among patients compared to the general Swedish population; the same was found for the use of *snus* (snuff) which is a popular tobacco product in Sweden. Interestingly, CH patients currently using or with a history of using tobacco (cigarettes or *snus*) had a delayed disease onset by almost three years on average compared to those who did not smoke or consumed *snus*. Tobacco use did not differ between ECH and CCH patients, however we could show that CCH patients consumed much less alcohol than ECH patients. When asked about possible trigger factors for their attacks, alcohol was the most prominent for all patients, followed by stress, weather/temperature, triggers related to food/drink, and relaxation/sleep. Significantly more CCH patients slept less than five hours per night than patients with ECH, and regarding chronotype, we found that more CCH than ECH patients were larks (morning persons). Two thirds of the patients reported that their attacks may occur at specific times of the day, and the most stated time interval for attack arrival was 02:00–04:00 at night (Figure 4).

Figure 4. Distribution of recurring attacks in cluster headache patients



Attack distribution over 24 hours divided in two-hour intervals for 317 cluster headache patients who reported diurnal rhythmicity (more than one interval could be chosen). The remaining 183 patients did not exhibit a diurnal attack pattern (no rhythmicity).

Figure adapted and slightly modified from Steinberg et al. (*Cephalalgia*, 2018) [30]

Regarding treatment, there was a trend for a more frequent use of acute treatment, namely sumatriptan injections, in episodic compared to chronic patients. Not surprisingly, significantly more CCH patients used prophylactic treatment than patients with ECH. Verapamil was used most by these two patient groups, whereas lithium and glucocorticoids were slightly more common among chronic patients, and the serotonin antagonist pizotifen was more commonly used by episodic patients.

For this study, we developed a cluster headache severity scale (CHSS) taking into account the attack duration and number of attacks per day during a bout as well as the bout duration; the more frequent or longer the attacks and bout, the higher the CHSS score. Not unexpectedly, CCH patients had a much higher mean score than ECH patients. In addition, there was a significant difference between male and female patients with women having a higher CHSS mean score than men. Patients who exhibit a diurnal attack pattern had a lower CHSS mean score than those who did not experience diurnal rhythmicity for their attacks. From the CHSS score, we defined a subgroup of CH patients with maximum severity (CHMS). When comparing the CHMS to the non-CHMS group, we could detect similar differences as between chronic and episodic patients, namely a later disease onset, greater use of prophylactic medication, higher number of alcohol abstainers, and more patients sleeping less than five hours at night.

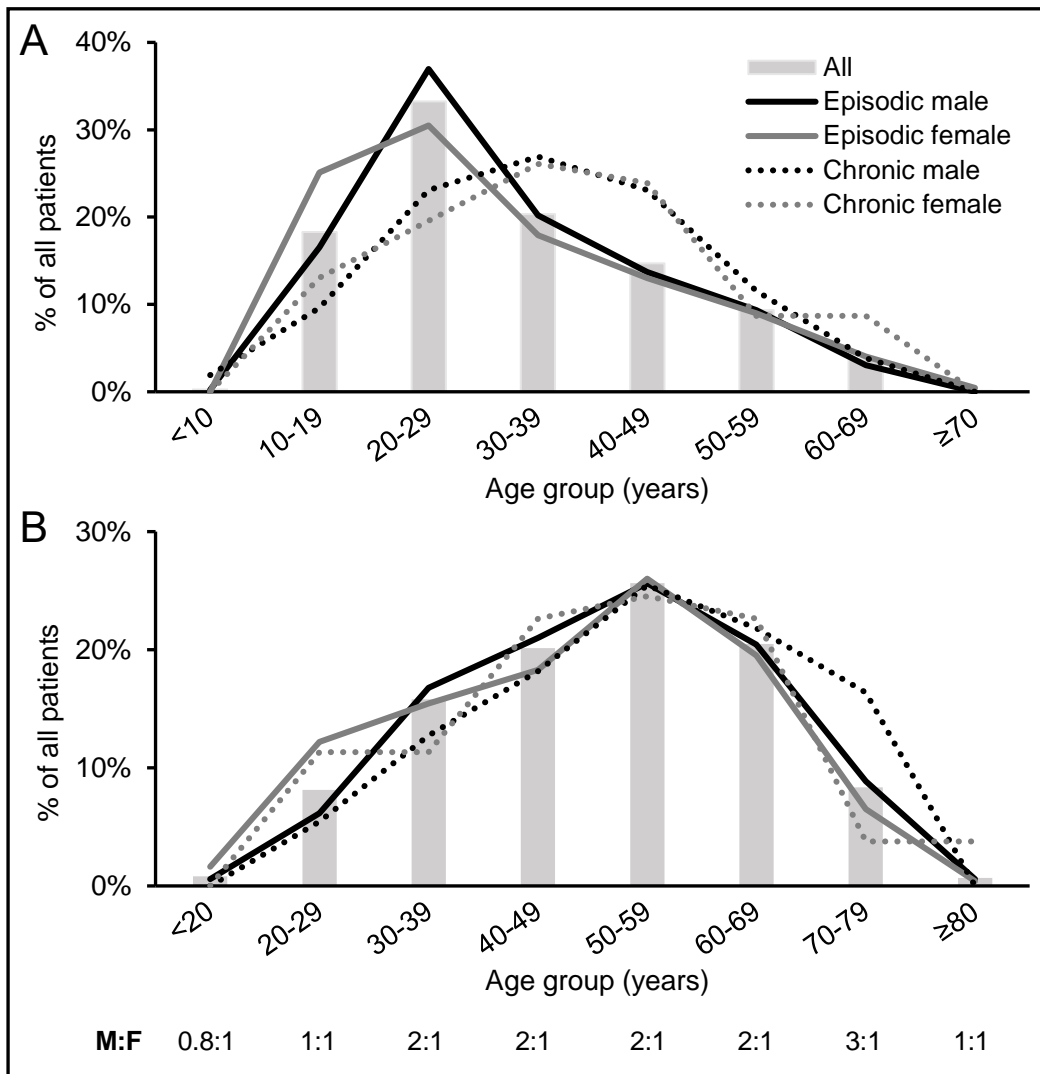
Study II included 874 patients and focused on gender differences in CH. Similar to the first study, the male to female ratio was 2:1. Men and women did not differ in age at disease onset, although a higher proportion of women than men seemed to have an early onset below the age of 20 years. The female CH population was generally younger than the male patient group at the start of study participation, and when looking at the age distribution of CH patients, the male to female ratio increased with age (Figure 5). As has been reported before, significantly more female than male CH patients had a close relative who also suffered from CH, so although more men are diagnosed with CH, women appear to have a higher heredity for the disease. In addition, the proportion of female patients with CCH is higher than for male patients, which is reflected by significantly longer cluster bouts in women. Female CH patients more often used prophylactic treatment than men, and this difference was only significant for episodic patients, whereas male and female CCH patients used preventive treatment with similar frequency. There was no difference in the perception of pain; for both sexes CH attacks were rated close to unbearable. However, associated symptoms like drooping eyelid and restlessness were more common in females than in males.

Because of our interest in circadian rhythm in relation to CH, we investigated differences in chronobiology between male and female patients. A higher number of women with CH experienced a diurnal attack pattern compared to men. Although half of all CH patients reported to have active bouts at certain times of the year, the sexes did not differ in annual rhythmicity. Regarding chronotype, there was an indication of eveningness in men and morningness in women. Studies on the general population have shown that this preference may change over time, therefore we also looked at the age distribution for the different

chronotypes. For middle-aged and older groups, the CH cohort resemble the general population, however for the age group below 35 years, both male and female patients tended towards eveningness which contradicts findings indicating that younger women rather are morning persons. Analysis of nocturnal sleep shows that male and female patients differ in the amount of sleep they get. Specifically, we found that more women than men slept for less than five hours per night.

Similar to the general population, more female than male CH patients were also diagnosed with migraine. In an attempt to compare the prevalence of migraine in our CH cohort to the general Swedish population, we found in this study that CH patients may have an increased risk for suffering from migraine in addition to CH.

Figure 5. Age distribution in male and female cluster headache patients



Male and female patients subdivided by cluster headache subtype for (A) age at disease onset, and (B) age at study recruitment. Male to female (M:F) ratio is stated for each age group in B.

We asked patients to list trigger factors for their CH attacks in a free-text answer, which have been mentioned in study I. For several of these triggers, there was a difference in frequency between male and female patients, for example alcohol and food or non-alcoholic beverages were more common trigger factors in men, while stress/worry, weather/temperature, and lack of sleep were more likely to elicit an attack in women.

When studying different lifestyle factors in our CH cohort, we found that the BMI for male patients was significantly higher than for the general male population, while no such difference could be seen for females. As indicated in the first study, CH patients have a higher tobacco use than the general population, and this is true for both sexes. Especially smoking in female patients is exceptionally high, which is highlighted by the fact that women in the general population smoke significantly less than men, while female and male patients smoke equally much. For alcohol consumption, we were unable to do a direct comparison to the general population, but generally we could observe that both male and female CH patients were to a larger extent abstaining from alcohol compared to the general population.

4.2 GENES RELATED TO CIRCADIAN RHYTHM (STUDY III–V)

Due to the striking rhythmicity of CH attacks, candidate genes related to circadian rhythm have been of specific interest for this thesis. A list of genetic variants in clock genes that were studied here and were associated with CH in Sweden is presented in Table 1.

Table 1. Significant variants in circadian rhythm-related genes

Gene	SNP	MAF Ctrl %	MAF CH %	OR (95% CI)	p-value
<i>HCRTR2</i>	rs3122156	29.9	25.9	0.82 (0.68–0.99)	0.042
<i>CLOCK</i>	rs12649507	30.1	35.6	1.29 (1.08–1.54)	0.007
<i>CRY1</i>	rs8192440	42.0	36.7	0.80 (0.68–0.94)	0.006

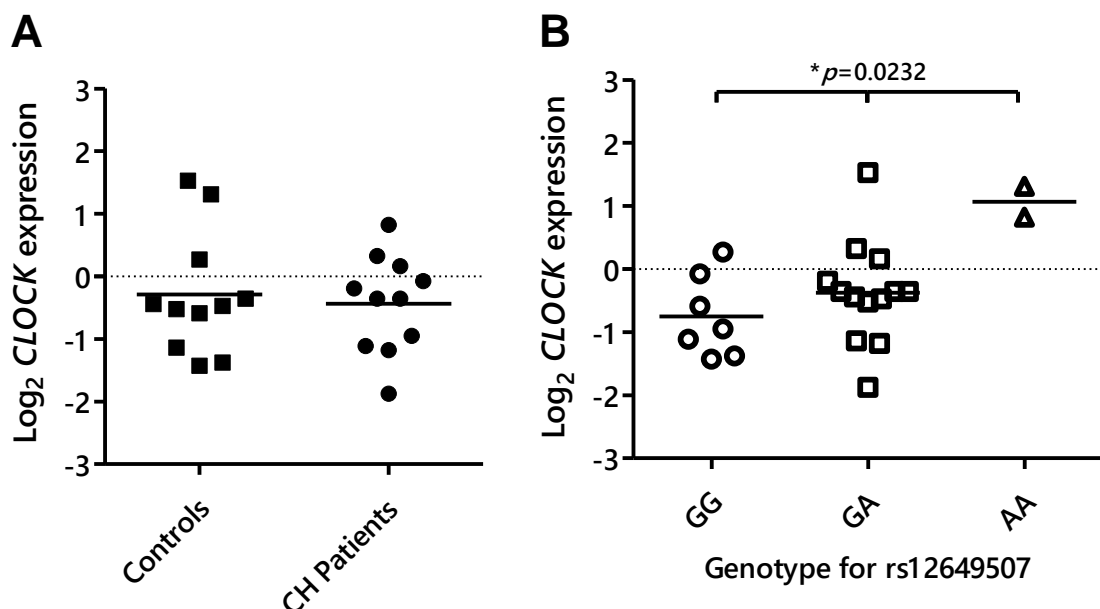
SNP: single nucleotide polymorphism; MAF: minor allele frequency; Ctrl: controls; CH: cluster headache; OR: odds ratio; CI: confidence interval; p-value < 0.05 was considered significant.

One of the most studied candidate genes in CH is *HCRTR2* with conflicting results. We investigated three SNPs, rs3122156, rs2653342, and rs2653349, in this gene and could not consolidate association of these variants with CH (**study III**). There was a trend for significance for rs3122156, where the minor allele was more common in controls than in patients, suggesting a protective effect of this variant. However, this trend did not hold after

correction for multiple testing using Bonferroni correction. A haplotype analysis of the three SNPs revealed a significant haplotype for CH which is most likely driven by the rs3122156 variant, as it was again more common in control individuals than patients. After performing permutation testing, another way to correct for multiple testing by resampling, this haplotype lost significance. An *in silico* analysis on the effect of the exonic *HCRTR2* variant rs2653349 on the RNA structure pointed to slight changes in predicted folding energy of *HCRTR2* mRNA and may therefore affect *HCRTR2* mRNA stability.

Three SNPs in the *CLOCK* gene that were either implicated in diurnal preference (rs1801260) or sleep duration (rs11932595, rs12649507) were screened for in the Swedish CH material and compared to controls (**study IV**) [151, 152]. The minor allele of the rs12649507 variant was significantly more common in patients than in controls, and the MAF was even higher in a subset of patients reporting diurnal rhythmicity of their attacks. A haplotype containing the major allele of each of the three SNPs was much more common in controls than patients, and this difference was even more significant than the association with rs12649507 alone, indicating a synergistic effect of these three variants. When comparing *CLOCK* gene expression between eleven patients and eleven controls, we did not detect a difference which may be due to the small number of samples. However, when analyzing *CLOCK* expression with regard to rs12649507 genotype, we could detect a significantly higher expression in individuals who were homozygous for the minor allele (Figure 6).

Figure 6. *CLOCK* mRNA expression in correlation to disease status or genotype



(A) Quantification of *CLOCK* mRNA levels in human fibroblasts from controls ($n = 11$) and cluster headache (CH) patients ($n = 11$). (B) *CLOCK* mRNA expression levels in the same individuals grouped by the three different rs12649507 genotypes GG ($n = 7$), GA ($n = 13$), and AA ($n = 2$). * p -value < 0.05 was considered significant.

Figure adapted from Fourier et al. (*Cephalalgia*, 2018) [86]

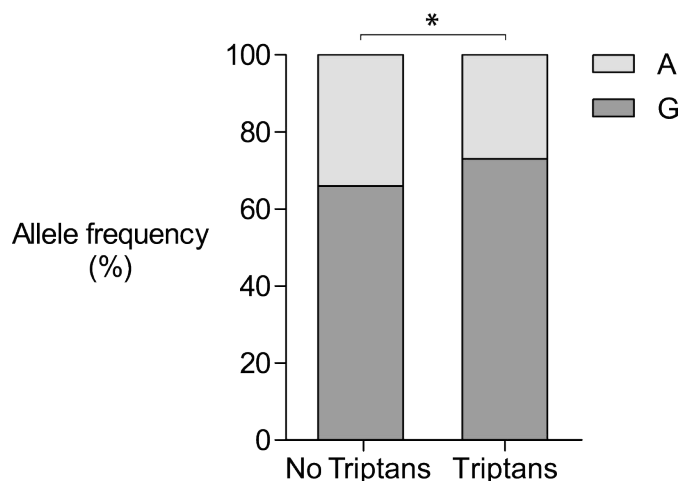
The *CRY* genes have been implicated in several neurological and psychiatric disorders, and in **study V**, we analyzed two variants each in *CRY1* (rs2287161, rs8192440) and *CRY2* (rs10838524, rs1554338). The genotyping yielded an association between the exonic *CRY1* variant rs8192440 and CH, where the minor allele was more common in controls than in patients, pointing to a protective role of this variant for CH. This difference was even more pronounced when comparing controls to only a subset of patients with a circadian attack pattern. The predicted secondary *CRY1* mRNA structure containing the rs8192440 major allele differed slightly from the structure containing the minor allele when performing an *in silico* analysis. In addition, we could show a trend for increased *CRY1* gene expression in CH patients compared to controls, however we did not find an effect of the associated SNP rs8192440 on *CRY1* expression.

4.3 GENES WITH A LINK TO THE VASCULAR SYSTEM (STUDY VI–IX)

Alcohol, NO, and CGRP are all vasodilators and inducers of CH attacks. Different genes involved in pathways of these molecules have been considered intriguing candidate genes for CH. The *ADH4* gene has been studied in several different cohorts with conflicting results. We investigated two previously associated *ADH4* variants (rs1126671, rs1800759) in our Swedish case-control material (**study VI**) and could not replicate the results published for a smaller Italian CH cohort for neither SNP nor haplotype analysis of the two SNPs.

The neurotransmitter NO has been discussed to play a role in CH pathophysiology, therefore we investigated eight genetic variants in members of the NOS enzyme family which were previously studied in relation to migraine (**study VII**). Neuronal NOS (NOS1) and endothelial NOS (NOS3) are dependent on calcium, while cytokine-inducible NOS (NOS2) is calcium-insensitive. We detected a trend for association of *NOS2* variant rs2779249 with CH, which did not hold after correction for multiple testing. Because multimarker analyses in migraine yielded interesting associations, we performed a haplotype analysis for each of the three *NOS* genes. We could not identify disease-associated haplotypes for *NOS1* and *NOS3*, but for the two *NOS2* gene variants, a haplotype containing the minor allele of each SNP was slightly more common in patients than controls. In a stratified analysis, we investigated these SNPs in relation to vasoactive substances, such as coffee, tobacco, alcohol, triptans and verapamil, by comparing the MAF of the different *NOS* markers between patients reporting to consume or use these substances versus those that do not. This analysis revealed an overrepresentation of the minor allele of *NOS1* variant rs2682826 in CH patients not using triptans whereas the MAF of this SNP for patients using triptans was similar to the MAF of controls (Figure 7).

Figure 7. Allele frequencies for rs2682826 in patients using and not using triptans



Allele frequency of *NOS1* rs2682826 in triptan users ($n = 388$) vs. triptan non-users ($n = 140$).

* $p = 0.039$, where p -value < 0.05 was considered significant.

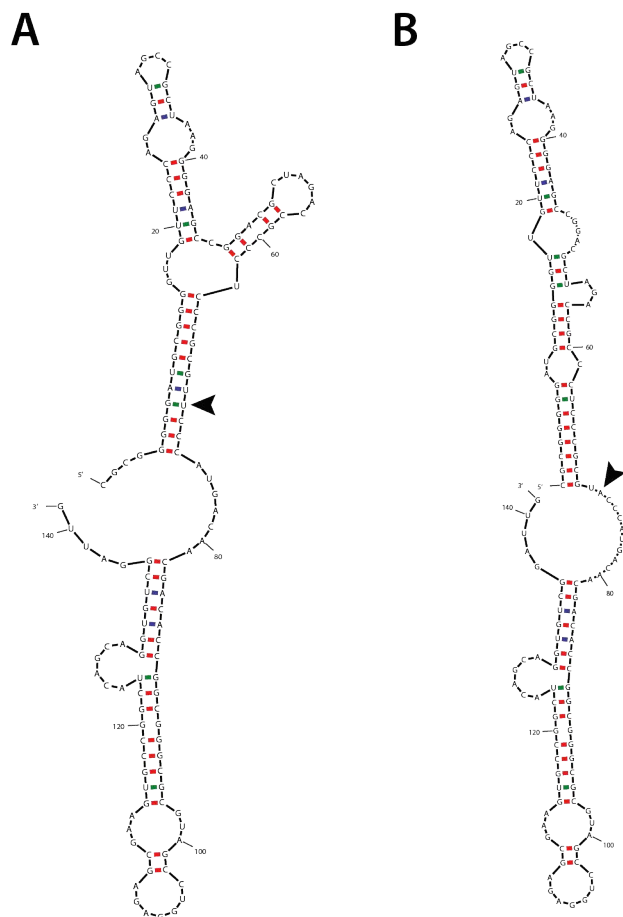
Figure adapted from Ran et al. (*Brain Sciences*, 2021) [117]

CGRP is a promising neuropeptide with clear involvement in headache. New treatment strategies for migraine and CH making use of monoclonal antibodies target either CGRP or its receptor. The objective of **study VIII** was to investigate a possible genetic association between the CGRP receptor component *RAMP1* and CH. Two SNPs (rs3754701, rs7590387) in the *RAMP1* gene were previously implicated in headache [153, 154]. Genotype analysis showed significantly higher MAF for rs3754701 in CH patients compared to controls, and interestingly, this association was only true for ECH patients in our material. In addition, patients displayed significantly higher *RAMP1* gene expression than control individuals, independent of CH subtype. We could not identify an effect of the CH-associated SNP rs3754701 on *RAMP1* expression, but demonstrated that the homozygous genotype of the minor allele (CC) for the other SNP rs7590387 led to increased *RAMP1* gene expression compared to the other two genotypes GC and GG.

Verapamil is the first-line prophylactic treatment in CH. Curiously, it is not only a calcium-channel antagonist inhibiting calcium influx but also a vasodilator. A previously conducted study on migraine aiming to correlate genetics to verapamil response found eight markers in seven different genes which were associated with good treatment response [155]. Of these, we selected four SNPs with a direct or indirect connection to calcium signaling in order to screen our CH material (**study IX**): rs17844444 in *PCDHB6* (*protocadherin beta 6*), rs10882386 in *PLCE1* (*phospholipase C epsilon 1*), rs1531394 in *ANO3* (*anoctamin 3*), and rs2230433 in *ITGAL* (*integrin subunit alpha L*). For rs2230433 in *ITGAL*, the MAF was higher in controls than in patients, but this difference did not reach significance. There was a clear overrepresentation of individuals with the AA genotype for the *ANO3* marker rs1531394 in CH patients., however not a distinct difference in MAF for the A allele between

patients and controls. We then performed a stratified analysis under a recessive model with subsets of patients either using or not using verapamil. Both patient groups had a higher frequency for the risk genotype AA compared to controls but did not differ significantly from each other. An *in silico* analysis studying the effect of rs1531394 on *ANO3* mRNA structure predicted a 5.5% increase in initial folding energy for the minor allele-containing RNA fragment (Figure 8). Since this SNP is located in the 5'UTR of *ANO3*, we further investigated possible overlap with transcription factor binding sites in this region. The computational tool SNP2TFBS predicted the transcription factor RFX2 (Regulatory Factor X2) to bind to the *ANO3* promoter and the minor allele A of rs1531394 to lower the affinity of RFX2 to this region compared to the major allele T. Gene expression analysis of *ITGAL* and *ANO3* did not yield reliable results due to low expression in fibroblasts.

Figure 8. *In silico* analysis of mRNA fragments with the T or A allele at SNP rs1531394



Effect of rs1531394 on the predicted ANO3 mRNA folding of a 141 bp partial sequence: rs1531394 is indicated by an arrowhead with the T allele in (A) with the initial folding energy $\Delta G = -63.9$ kcal/mol, and the A allele in (B) with $\Delta G = -60.4$ kcal/mol.

Figure adapted from Ran et al. (Brain Sciences, 2019) [118]

The results on significant SNPs located in genes with a connection to the vascular system that were presented here have been summarized in Table 2.

Table 2. Significant variants in genes related to the vascular system

Gene	SNP	Allele/Genotype	Ctrl %	CH %	<i>p</i> -value
<i>NOS2</i>	rs2779249	C	71.4	67.6	0.049
		A	28.6	32.4	
<i>RAMP1</i>	rs3754701	A	63.4	57.5	0.009
		T	36.6	42.5	
<i>ANO3</i>	rs1531394*	TT + TA	86.3	80.5	0.009
		AA	13.7	19.5	
<i>MTDH</i>	rs1835740	C	81.5	77.9	0.043
		T	18.5	22.1	

SNP: single nucleotide polymorphism; *Ctrl*: controls; *CH*: cluster headache; *p*-value < 0.05 was considered significant. *rs1531394 was analyzed under a recessive model.

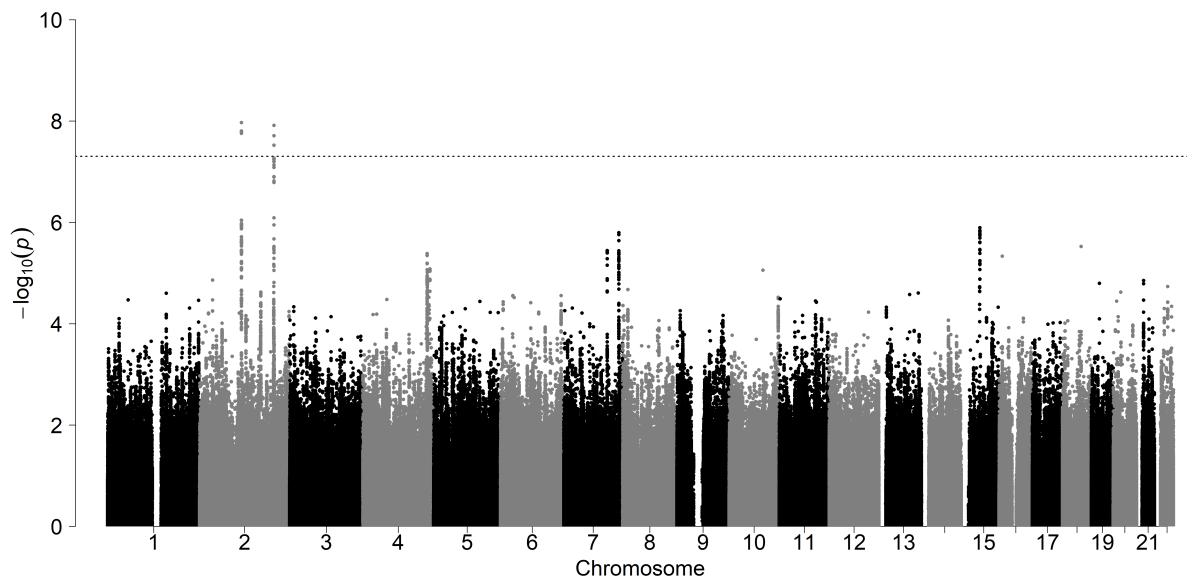
4.4 GENES DETECTED BY GWAS (STUDY X–XII)

Migraine is a primary headache disorder that shares certain features with CH, such as the activation of the trigeminovascular system. Large GWAS and meta-analyses could consolidate *MTDH* to increase the risk for migraine, and even *PRDM16* has been established as a candidate gene. One variant close to the *MTDH* gene (rs1835740) and one variant in *PRDM16* (rs2651899) could be confirmed in a Swedish GWAS replication study and were therefore interesting candidates to be studied in Swedish CH patients (**study X**). The minor allele of the *MTDH* SNP was significantly enriched in CH patients compared to controls (Table 2). Interestingly, this difference was even more pronounced when comparing controls to a subgroup of CH patients who also suffered from migraine. The first GWAS on migraine reported that rs1835740 may affect *MTDH* gene expression. When we studied *MTDH* mRNA levels in a subset of CH patients and control individuals, we did not find a difference in gene expression between cases and controls but could confirm that rs1835740 altered *MTDH* expression.

The first GWAS for CH was performed on an Italian cohort of 99 patients and 360 controls. The authors found two suggestive hits, rs1006417 in the gene-poor chromosomal region *14q21* and rs12668955 in the *ADCYAP1R1* gene, as well as the rare variant rs147564881 in the *MME* gene detected by a gene-based analysis. To see whether we could reproduce these results in our Swedish CH cohort, we genotyped our much larger case-control material for these three variants (**study XI**). We could not find a significant difference in genotype nor allele frequency between CH patients and controls for the two suggestive hits, rs1006417 and rs12668955. For the rare *MME* variant rs147564881, only wild-type carriers were detected among Swedish CH patients, therefore genotyping of controls for this SNP was discontinued. These findings suggest that the three SNPs found in the Italian study do not contribute to the risk of developing CH in the Swedish population.

Because there has been a lack of larger genetic studies on CH with sufficient material in the literature, we performed a GWAS on our Swedish case-control material. After QC, the material consisted of 591 CH patients and 1,134 controls. Results from the association analysis visualized in a Manhattan plot (Figure 9) identified two loci on chromosome 2. Regional plots of chromosome 2 (Figure 10) show how numerous variants cluster in the proximity of *MERTK* (*MER proto-oncogene, tyrosine kinase*), or *SATB2* (*special AT-rich sequence-binding protein 2*) and *LINC01877* (*long intergenic non-protein coding RNA 1877*).

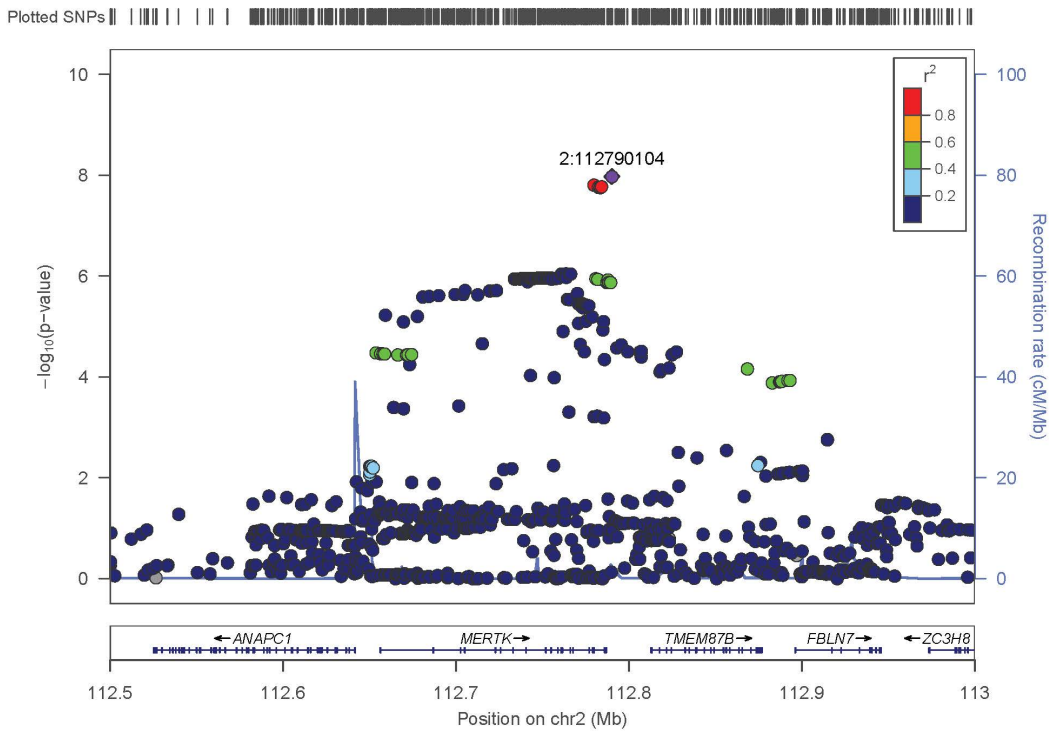
Figure 9. Manhattan plot for the Swedish cluster headache association analysis



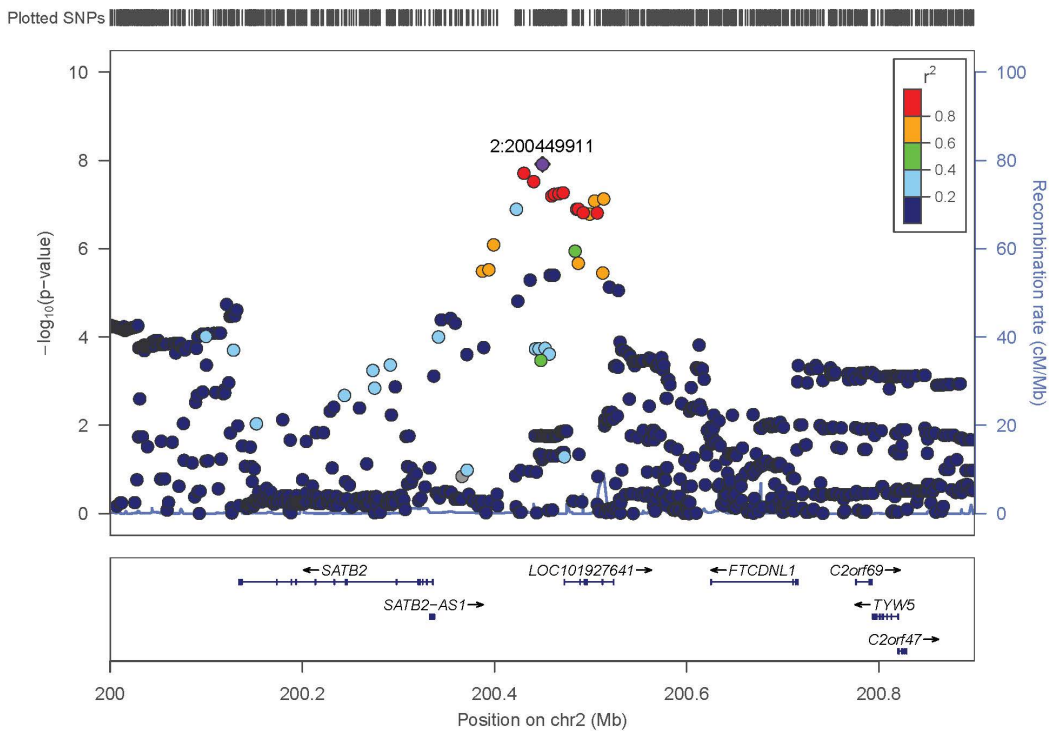
Manhattan plot showing the $-\log_{10}$ of the p -value for individual SNPs on chromosomes 1-22. Association analysis was performed on 591 cases and 1,134 controls. The dotted line indicates genome-wide significance level $p < 5 \times 10^{-8}$.

Figure 10. Regional plots for the Swedish cluster headache association analysis

A



B



LocusZoom plots for two loci on chromosome 2 reaching genome-wide significance. Variant position, recombination rates, and gene boundaries are based on GRCh37/hg19. (A) Lead variant rs72825689 ($p=1.07 \times 10^{-8}$) is located near the MERTK gene. (B) Lead variant rs4675692 ($p=1.22 \times 10^{-8}$) is located near the SATB2 gene and the long non-coding RNA LINC01877 (= LOC101927641).

In **study XII**, we combined our Swedish GWAS data with those from a UK CH cohort, resulting in a dataset of 1,443 cases and 6,748 controls of European ancestry. The UK-only GWAS independently replicated our findings on chromosome 2, and the combined analysis gave rise to two additional loci on chromosome 1 and 6. The locus in region *1q41* on chromosome 1 does not comprise any known genes; the nearest gene is the long non-coding RNA *LINC01705*, and the closest coding gene is *DUSP10* (*dual specificity phosphatase 10*). The locus on chromosome 6 contains the *FHL5* gene which, as previously mentioned, has been identified as a susceptibility locus for migraine in a large GWAS meta-analysis.

Furthermore, we performed a number of downstream analyses on the results from the combined material. All lead SNPs, meaning variants with the highest *p*-value in each locus, were located in non-coding regions, and functional variant annotation detected two *MERTK* and two *FHL5* missense variants with moderate impact to be in high linkage disequilibrium ($r^2 > 0.9$) with the lead SNP in the respective locus. One of these *MERTK* variants and both *FHL5* variants had high CADD scores >15 , and all four missense variants showed a high level of mammalian conservation with GERP scores above 2. Gene-based association testing identified five genes significantly associated with CH: *TMEM87B* (*transmembrane protein 87B*), *ANAPC1* (*anaphase promoting complex subunit 1*), and *FBLN7* (*fibulin 7*) in addition to *MERTK* and *FHL5*. All five candidate genes are expressed in the human brain, with *ANAPC1* and *FBLN7* most highly expressed in neurons, *MERTK* and *TMEM87B* highly expressed in brain support cells, such as microglia and astrocytes, and *FHL5* highly expressed in brain endothelial cells. Via eQTL mapping, variation in mRNA expression for eleven genes, including *DUSP10*, *MERTK*, *TMEM78B*, *FBLN7*, and *SATB2*, could be linked to genome-wide significant SNPs in our material, with expression in brain, vascular, and immunological tissues. For the pathway analysis, genes within a specific window around each lead SNP were included. In total, 74 pathways were significantly ($p < 0.05$) enriched for 46 genes in the candidate regions. Pathways of particular interest included positive regulation of glial cells and regulation of gliogenesis in the central nervous system. In addition, many pathways related to the differentiation and activation of immune cells, or cell adhesion involved *MERTK*.

5 DISCUSSION

Because CH is still under-recognized, underdiagnosed, and undertreated, it is of utmost importance to increase the understanding of the clinical features and the pathophysiology of this dreadful disease in order to improve the treatment and life of CH patients. To be able to do meaningful research, a well-characterized CH cohort is needed which poses a challenge because to date CH is diagnosed merely by specific symptoms and classification criteria that exclude other diseases. We feel confident that our Swedish CH biobank consists of material of high quality from CH patients with expert-validated diagnosis. The number of patients included in this biobank, with extensive questionnaire data, genetic material, and fibroblast cell lines, makes it an invaluable resource for our research on this rarely heard of and yet so common disease, and one of the largest CH biobanks in the world.

We thoroughly characterized the Swedish CH population in two cohort studies, showing clear differences between CH subgroups, namely ECH and CCH patients, or men and women. As expected, chronic patients generally have a higher disease burden than episodic patients, which affects several aspects of their health and daily life, for example constant lack of sufficient hours of sleep, more frequent use of prophylactic medication which may have considerable side effects, and high tobacco consumption. Curiously, CH patients with a history of smoking or taking *snus* had a delayed disease onset, which could point to nicotine as some sort of self-medication. We could see similar differences for male and female CH patients, where women generally appear to be more debilitated by the disease than men. On top of a higher disease burden from CH, female patients have a higher comorbidity for migraine and a higher heredity for CH. Interestingly, more women report a diurnal pattern of attacks with a tendency to more nighttime attacks than men, although recall bias between the two groups cannot be excluded. We also developed a scale to assess the severity of CH, CHSS, which as we demonstrated in our study could be a useful tool for clinicians to classify CH patients into other subgroups than the conventional ECH and CCH, or do intra-individual comparisons of patients between different periods. Also, treatment could be adjusted for better efficiency according to the CHSS score.

Deciphering genetic relationships in complex disorders, such as CH, is challenging because the combination of genetic variants with low effect, involvement of multiple genes, and additional environmental risk factors complicates the interpretation of results. Nevertheless, looking at the genetic component of CH gives a valuable insight into its pathophysiology and may bring us one step closer to solving the puzzle of what causes this disorder. We have used different approaches on tackling the genetics of CH; hypothesis-driven candidate gene studies and hypothesis-free genome-wide association studies. Both methods have their advantages and disadvantages, and either one tries to answer a specific scientific question, or to find previously unknown associations and generate new hypotheses. Although a hypothesis-free approach appears to lead to more robust associations, especially when conducted on a large dataset, some small but relevant connections may stay undetected because of polygenic inheritance. Therefore, I think both approaches are of great value in genetic research.

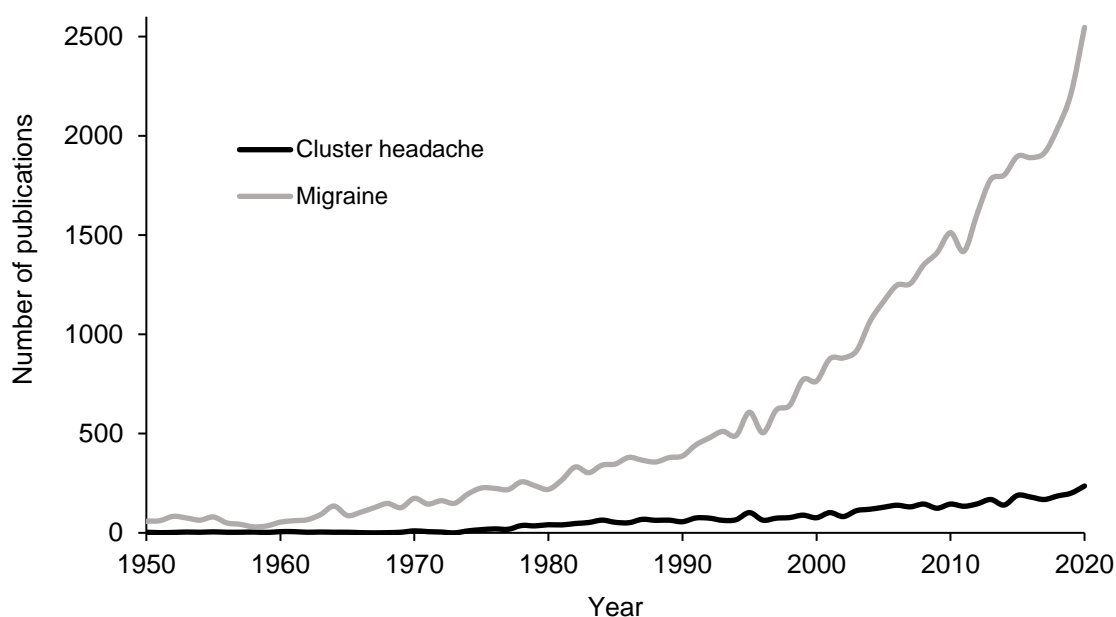
We have performed several case-control studies on candidate genes with involvement in circadian rhythm, and were able to find genetic variants in *HCRTR2*, *CLOCK* and *CRY1* that were linked to CH. Interestingly, the link was usually even stronger when looking at the subset of patients with diurnal attack patterns. These findings support the hypothesis that circadian rhythm plays indeed a role and is possibly somehow dysregulated in CH. In addition, we have shown associations of variants in candidate genes that may play a direct or indirect role in treatment mechanisms. As mentioned previously, most current medications used for CH were originally developed for migraine, and the mechanism of action for many is not entirely clear. Our data could show that certain genetic variants may pose a susceptibility for CH and could influence treatment response. For example, we could link a *NOS1* variant to the use of triptans. Because monoclonal anti-CGRP antibodies are a relatively new treatment for headache, we did not have sufficient data on the use of this medication among our CH patients to perform an analysis. However, the *RAMP1* variant that we could link specifically to ECH may be relevant for this new treatment, also due to the interesting fact that anti-CGRP antibodies have proven to be ineffective in CCH patients [156]. A variant in *ANO3* points to calcium involvement in CH pathophysiology but does not appear to be linked to verapamil response in CH which was previously reported for migraine.

The first GWAS in migraine has been published in 2010, and the forming of large headache consortia has increased the number of individuals for analysis and strengthened the results. GWAS in different populations and meta-analyses in combined European data concluded independently that *MTDH* may be involved in migraine. An association study on our CH case-control material could link a well-established migraine-associated variant in *MTDH* to CH, which makes it a possible general marker for neurovascular headaches. GWAS in CH with a sufficient number of cases have been more challenging due to the much lower prevalence compared to migraine. The first GWAS in CH was published in 2016 by an Italian group and was quite underpowered. The suggestive hits that were reported in this study could not be replicated in our Swedish CH material, indicating that these may have been population-specific SNPs. Although even our material is quite small for a proper GWAS, we have performed genome-wide screening for genetic variants, and in collaboration with a group in the UK, we could combine and increase our GWAS dataset. The confidence in our data increased immensely, when the newly discovered CH-associated loci near *MERTK* and *SATB2* could be replicated independently in the UK and Swedish cohorts, and remained significant in the combined analysis. Particularly, *MERTK* is an intriguing new candidate gene for CH because it is involved in neuroinflammation and is an activator of *CREB* (*cAMP-responsive element binding protein*). *CREB* encodes a transcription factor which regulates the expression of molecular clock genes and is critical for light entrainment of the circadian clock [157]. What is more, *CREB* activation contributes to the sensitization of nociceptive cells and meningeal pain hypersensitivity [158]. Finally, triptans used for treatment of CH reduce the activity of *CREB* in the trigeminal system [159]. Taken together, these exciting findings urge CH researchers to investigate the CREB pathway as well as the involvement of *MERTK* and neuroinflammation in CH pathophysiology further.

6 CONCLUSIONS AND PERSPECTIVES

Although CH is known to be one of the most painful conditions in humans, still relatively little research is done on the disorder. However, compared to two decades ago, the number of newly published research articles on CH has tripled from 76 in the year 2000 to 236 new publications in 2020, which indicates that awareness for CH has increased, and gradually more research is conducted (Figure 11). Compared to publications on migraine, this number is still incredibly low.

Figure 11. Number of new publications per year on headache disorders



Source: NCBI PubMed (May 2021)

The work of this thesis could contribute to increasing the understanding of clinical as well as genetic aspects of CH. We highlighted in which ways chronic CH patients may have a higher disease burden compared to episodic patients but demonstrated with the CHSS that even certain ECH patients can have a high severity score, for example due to a frequent number of bouts per year. We could also show that female patients are generally struck harder by their CH, although the disorder is more common in men. However, our research also shows that the male to female ratio has decreased across the age groups, implying that CH may be more recognized in women nowadays, although an increased number of female patients may in part also be due to changes in lifestyle among both sexes over the decades. Because we have seen clear differences in the chronobiology of CH subgroups, namely circadian rhythm, chronotype, and sleep, future studies will include a thorough investigation of, for example, sleep and diary-led recordings of attack occurrence throughout the day in CH patients, both in active and remission phase, to minimize recall bias.

With our genetic studies on CH, we could advance the field either by determining the reproducibility, or lack thereof, of candidate gene studies in other CH cohorts, or by introducing new potential candidate genes that could help decipher the CH pathophysiology or lead to improved treatment. For example, studies on genetic variants that are linked to treatment response may in the future be used to quickly identify which drug(s) would be the most effective in a specific CH patient. This emerging field within personalized medicine is referred to as pharmacogenomics.

As a recent article on the genetics of CH concludes, candidate gene studies have dominated the field [160]. This is most likely due to the small size of the different cohorts as a result of the relatively low prevalence of CH, as well as the cost for doing larger GWAS, and exome or whole genome sequencing. In order to increase the sample size for broader genetic screening of sufficient statistical power, it is essential for researchers to join forces. We have been part of a collaboration with several groups in Europe and USA, who formed the International Consortium for Cluster Headache Genetics (www.clusterheadachegenetics.org). Given our promising results from our Swedish GWAS which were consistent with findings in a CH cohort from the UK, the consortium is planning on performing a large meta-analysis with roughly 5,000 cases and 500,000 controls in the near future to consolidate these findings and possibly detect new associations. With this immense project, the field of CH genetics will move a considerable step closer to elucidating possible disease mechanisms that contribute to CH manifestation.

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