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Clinical and Genetic Aspects on Cluster Headache

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'Suddenly - a huge phantom bird sank three talons of its angry claws deeply into my head and face and tried to lift me. Nor warnings, nor preliminary signs. Just wham! A massive, killing pain came over my right eye --- I clutched my head, stumbled out to the broad lawns, and skulked along oleander hedges to the deserted tennis courts. And there in the darkness, I moaned, I panted, ballooned my cheeks, blew out short bursts of air, licked my hot lips, wiped tears that poured out of my right eye, and clawed at my head, trying to uproot the fiendish talons from their iron grip. One racking hour later, the talons let go. The paroxysm eased as suddenly as it had convulsed. Euphoria set in --- It's gone, darling! A whopping headache, but it's gone!'

Frank Capra

For my family

SUMMARY

Cluster headache (CH), a primary neurovascular headache syndrome, is characterized by recurrent, unilateral, short-lasting attacks of excruciating pain in the temporal region. The pain is considered one of the most severe pain conditions known to humans. This thesis has focused on studies of the clinical picture, especially in relation to headache classification criteria, studies of the genetic background and a search for pathophysiological mechanisms through gene expression analysis.

In **Study I** we prospectively evaluated the prognosis after one typical cluster period. We found that 13 (26.5%) had had one cluster period only during a mean observation time of 8.9 years, and conclude that some patients may suffer from one cluster period only. In the new IHS classification (2004) only one period is required for a CH diagnosis.

In **Study II** we identified 55 affected in 21 different CH families of whom 12 had atypical symptoms. The atypical cases did not fulfil the IHS (2004) diagnostic criteria for CH, but had clinical symptoms resembling CH. We suggest that the clinical spectrum of familial CH may be broader than previously thought and that these atypical cases in CH families may represent an expanded spectrum of the disease.

Mutations of the CACNA1A gene have shown to cause several, mainly episodic, neurological disorders. In **Study III** we studied the impact of the CACNA1A gene on CH by performing an association analysis of two polymorphic markers in 75 sporadic CH patients and 108 matched controls. We found no significant differences between the patients and controls and we conclude that a great importance of the CACNA1A gene in our sporadic CH patients is unlikely.

Nitric oxide (NO) has been regarded as an important mediator in vascular headache pathophysiology. In **Study IV** we analyzed five polymorphic markers of the three different NOS genes (NOS1, NOS2a and NOS3 coding for iNOS, nNOS and eNOS respectively) in 91 CH patients and 111 matched controls. However, this study offers no support for an association between these markers and our CH patients.

In **Study V**, an international collaboration, a genome wide scan of extended CH pedigrees was performed, without identifying a single disease locus for CH. An association analysis of two polymorphisms of the HCRTR2 gene in a Danish, Swedish and British sporadic case-control cohort could not confirm a recently reported association of CH to this gene. Complete HCRTR2 sequencing in 8 independent familial CH cases could not detect any mutations. Genetic predisposition to CH is likely to be complex and compounded by heterogeneity.

Finally, in **Study VI**, by using Affymetrix microarray technology, we obtained gene expression profiles from peripheral blood from 3 CH patients during attacks, between attacks and in remission, and from 3 matched controls. An upregulation of several S100 proteins was seen during the active phase of the disease compared to remission, which could be confirmed for the calcium-binding S100P gene with quantitative RT-PCR in 6 CH patients. These findings might indicate an inflammatory process during the active phase of CH.

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MAIN REFERENCES

This thesis is based on the following articles, which will be referred to by their Roman numerals:

- I. **Sjöstrand C**, Waldenlind E, Ekbom K. A follow-up study of 60 patients after an assumed first period of cluster headache. *Cephalalgia*. 2000 Sep;20(7):653-7.
- II. **Sjöstrand C**, Russell MB, Hillert J, Ekbom K, Waldenlind E. Familial cluster headache: Is atypical cluster headache in family members part of the clinical spectrum? *Cephalalgia Online* Early June 1, 2005 /in press.
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- IV. **Sjöstrand C**, Modin H, Masterman T, Ekbom K, Waldenlind E, Hillert J. Analysis of nitric oxide synthase genes in cluster headache. *Cephalalgia*. 2002 Nov;22(9):758-64.
- V. Baumber L, **Sjöstrand C**, Leone M, Harty H, Bussone G, Hillert J, Trembath RC, Russell MB. Genomewide scan and HCRTR2 candidate gene analysis in a European cluster headache cohort. Manuscript.
- VI. **Sjöstrand C**, Duvefelt K, Steinberg A, Nilsson Remahl I, Waldenlind E, Hillert J. Gene expression profiling in cluster headache indicates upregulation of S100 proteins during active phase. Manuscript.

ABBREVIATIONS

bp	basepairs
CACNA1A	calcium channel, voltage-dependent, p/q type, alpha-1 subunit
CGRP	calcitonin gene related peptide
CH	cluster headache
EA	episodic ataxia
FHM	familial hemiplegic migraine
GTN	glyceryltrinitrate
HCRTR2	hypocretin receptor 2
HLA	human leukocyte antigen
IHS	International Headache Society
LD	linkage disequilibrium
MA	migraine with aura
MELAS	mitochondrial myopathy, encephalopathy, lactic acidosis and stroke
MO	migraine without aura
MR(T), MRI	magnetic resonance (tomography), magnetic resonance imaging
NO	nitric oxide
NOS	nitric oxide synthase
eNOS	endothelial NOS
iNOS	inducible NOS
nNOS	neuronal NOS
PCR	polymerase chain reaction
PET	positron emission tomography
PBMC	peripheral blood mononuclear cells
RT-PCR	reverse transcription-polymerase chain reaction
SCA	spinocerebellar ataxia
SNP	single nucleotide polymorphism
SUNCT	short-lasting unilateral neuralgiform headache with conjunctival injection and tearing
TAC	trigeminal autonomic cephalgias
VIP	vasoactive intestinal peptide

BACKGROUND

Introduction

Cluster headache (CH) is a primary headache syndrome characterized by recurrent, unilateral, short-lasting attacks of excruciating pain in the orbital/supraorbital/temporal region, associated with ipsilateral autonomic symptoms and signs. The attacks commonly recur with a clock-wise regularity and appear in clusters, so called cluster periods. The pain is considered one of the most severe pain conditions known to humans.

The pathophysiological background to CH has for many years been obscure, although several important discoveries during the recent years have clarified the picture. Today it is generally accepted that there is a central and peripheral activation in CH, and CH is thus considered to be a neurovascular (1) disorder.

In the present studies we have performed a prognostic clinical evaluation of patients after a first period of headache as regards the natural course of the disease. We have also defined the phenotypes in CH families as regards the clinical pattern. Two candidate gene association studies of CH patients and matched controls have been performed, in addition to a genome scan of CH families and an associated candidate gene association analysis. We have, finally, analyzed the gene expression pattern in peripheral blood from CH patients during different disease stages.

Cluster headache

Historical aspects

CH has been identified in early publications, with clinical symptoms described in detail by the Dutch physician Nicolaas Tulp already in 1641 (2). In 1745 Gerard van Swieten, from Leiden and professor at Vienna School, gave a full description of a case of episodic CH in his textbook of clinical medicine (3). It was then described by some authors during the late 19th century and in the beginning of the 20th century. However, not until Horton described the syndrome in more detail it became more generally known (4). The periodicity of the disease was first described by Ekbom (5). Several names have been used for the disorder, for example Horton's neuralgia, ciliary neuralgia, migrainous neuralgia and histaminic cephalgia. The term cluster headache, due to its periodicity with attacks appearing in clusters, was introduced by Kunkle (6).

Some prominent persons are supposed to have been suffering from CH, among others Franz Kafka (1883 – 1924). Ekbom and Ekbom described in 2004 that Kafka, according to his diary, suffered from severe headache attacks between 1913 and 1917 (7). Kafka described pain so intense that he felt to be 'tortured by agonies like a martyr'. Several features seem to be indicative of CH and his experience from suffering from severe pain might have had some impact on his literature production. President Thomas Jefferson (1743 - 1826) has been regarded as having suffered from migraine, but it has been proposed by Diamond and Franklin that he suffered from CH as he had periodical headaches, according to his diaries and letters (8) .

Clinical description

CH displays, in most cases, a very characteristic clinical picture with attacks of very severe, unilateral pain localized orbitally, supraorbitally and/or temporally. The attacks last for 15-180 minutes and occur with a frequency of once every other day to 8 times/day. The attacks are accompanied with ipsilateral symptoms of autonomic dysfunction, such as lacrimation, conjunctival injection, nasal congestion, rhinorrhoea, ptosis, miosis, eye-lid edema, forehead and facial sweating. However, up to 3% display no autonomic symptoms (9). Most CH patients have a feeling of restlessness during attacks, with an urge to move and walk around. The description of a CH attack by Frank Capra, on one of the front pages in this thesis, captures the classical symptoms of an attack with clear precision (10).

Interestingly, patients often describe the pain in a very conform manner. The pain is described as extremely intense and citations to describe the pain as 'it feels like a red hot poker behind my eye' or 'it feels as if the eye is being pushed out of its socket' are common. The pain is sometimes radiating in two different directions; either supraorbitally to the forehead and/or temple (upper syndrome) or infraorbitally towards the teeth and jaw, or even to the neck (lower syndrome) (11). Some patients also have a

feeling of a milder, dull pain between attacks, i.e. they are not totally symptom free between attacks.

The cluster periods, i.e. the active periods of attacks, usually last for weeks to months, which on average last 6-12 weeks (12, 13). The series of attacks are separated by remission periods usually lasting for months to years, on average about 12 months. The majority of patients have this course with headache-free intervals – episodic CH. However, some patients (about 10%) develop a chronic course with continuous attacks without remission periods – chronic CH (14, 15). During active periods CH patients are often very sensitive to vasodilators such as alcohol, and attacks can be provoked by nitroglycerin (16), which is sometimes used as a diagnostic tool.

The attacks often display a clockwise regularity, with attacks recurring at certain times of the days, or nights. Furthermore, it is common that periods recur at certain times of the year. Thus CH displays a typical circadian and also a circannual rhythmicity.

Despite the classic clinical picture there are also several cases presenting an atypical picture (17-19). Sometimes attacks last longer than for 3 h occur, and this is supposed to be more common in females (20). Autonomic symptoms may lack in otherwise typical CH (9, 21). Attacks with prodromal signs of aura have been described (22, 23). There are also a few cases described with attacks of autonomic symptoms without pain (24, 25). The former classification of the International Headache Society (26) required autonomic symptoms in order to diagnose CH. However, the new revised version of this classification, i.e. The International Classification of Headache Disorders (27) does not have this requirement if restlessness and/or agitation are present. The IHS classification will be discussed in more detail later.

Epidemiology

In a survey in 18-year-old men from Sweden, in the middle 70's, a prevalence of CH of one per 1000 people, or more exactly 0.09% was found (28). However, onset of CH is usually after 20 years of age and men are more often affected than women. The Swedish data was extrapolated by Kudrow (29) to the distribution of age at onset from headache clinic data to yield a prevalence rate of approximately 0.4%. A study from San Marino yielded a prevalence of 0.07% (30). In an American investigation from Olmsted County the diagnosis was based on case records and was not confirmed by a clinical interview. In this latter study a prevalence of about 0.4% was found (31).

CH is, for unknown reasons, more common in men than in women. The male:female ratio used to be about 5-6:1. However, there has been a change in the sex ratio over the years with an increasing amount of women affected (32, 33). A recent British study found a male:female ratio of 2.5:1 (15). There have been speculations as whether this is due to changes in lifestyle (smoking and working habits) over the years (32). It could also be due to a better understanding and knowledge about the disorder and thereby a correct diagnosis. The age at onset is usually between 20 and 40 years of age, with a mean age of onset at about 28 years (32, 33). CH is rare in children.

Natural history

There is limited information regarding the natural history of CH (34-38). In an investigation by Kudrow approximately one-third of patients who had CH for 20 years or longer reported complete remission (35). Another third presented attenuation on severity and yet another third remained unchanged. A series of 189 consecutive patients who had had a disease duration of over 10 years were evaluated (14). About 13% of episodic CH patients converted to a chronic pattern and the remaining continued to have an episodic pattern. On the other hand, 33% of the chronic cases evolved into an episodic or intermediate (subchronic) pattern. A Japanese study of 68 CH patients that were followed over an 18-year period revealed no change in attack severity, frequency, duration or associated symptoms but the remission periods lengthened from a mean of 1.1 to 3.3 years (37). A follow-up study of 123 patients with episodic CH and of 9 patients with chronic CH was undertaken after 10-25 years (36). A significant but low remission rate for both episodic and chronic CH was seen. There is no former information about the prognosis after a first period. In the former IHS classification (26) at least 2 periods were used for a definitive CH diagnosis.

Migraine versus cluster headache

Migraine features such as accompanying nausea and photophobia are common in patients with CH (12, 20, 39, 40) while aura symptoms are rare in CH (22, 23). CH features may occur in migraine and vice versa. Thus, an eventual relationship between migraine and CH has been discussed by several authors (41-43). Migraine coexists in CH patients (44, 45). However, in migraine an activation of the brainstem is seen (46) in contrast to the activation of hypothalamus in CH (1).

Pathophysiology

The pathophysiology of CH is still in many aspects unknown, but several important findings have been revealed during recent years. The typical CH features of circadian and seasonal rhythmicity of attacks, as well as the trigeminal distribution of pain and ipsilateral autonomic symptoms, have pointed to certain structures and systems. CH is today considered a neurovascular headache due to the involvement of hypothalamic and trigeminovascular activation (1).

Hypothalamic activation

As CH attacks tend to occur with a clockwise regularity it has for several years been proposed to be a chronobiological disorder (47-53). The biological clock is located in the nucleus suprachiasmaticus in the hypothalamus. Levels of melatonin, prolactin, testosterone, growth hormones (GH) and cortisol have been monitored and seem to be

different in CH patients compared to controls. In all, these hormonal changes have pointed to an involvement of central structures in the hypothalamic region.

Further evidence for this central hypothesis was when May et al in 1998 showed activation of the ipsilateral hypothalamic grey matter during an acute attack of CH, studied by PET (1). Initially this activation was observed in nitroglycerin induced attacks, but it has later been reproduced in spontaneous attacks (54). This activation had not been seen in experimental induced pain or in migraine (55). Voxel-based morphometric analysis of MRI's of patients with CH and controls revealed an increase of the hypothalamic volume in CH patients (56). Additionally, in contrast to migraine, no activation was seen in the brainstem and this finding further emphasized the impression of migraine and CH being separate entities.

Trigeminovascular activation

Cranial vascular tone is regulated by the trigeminovascular, parasympathetic and sympathetic systems. The trigeminal system is the only known pain-sensitive innervation of the cranial vessels. The periorbital pain in CH indicates involvement of the trigeminal nerve and its central pathways. Episodic CH patients were examined during spontaneous attacks to determine cranial release of neuropeptides and markedly raised levels of CGRP and VIP were seen (57). This finding was seen as evidence for activation of the trigeminovascular system (CGRP) and the cranial parasympathetic nervous system (VIP). Additionally, elevated levels of CGRP were found in the external jugular vein on the affected side during active periods and it was raised further during attacks.

Vasodilatation

Various vasodilators, such as alcohol, histamine and nitroglycerine, are known to cause attacks during active periods of CH. Nitroglycerine is a donor of nitric oxide (NO) that has very powerful vasodilator effects. Histamine is also supposed to have its vasodilator effects through NO, see more below. Investigations with carotid angiography and MR-angiography have shown a local orbital vasodilatation during CH attacks (58). Studies of blood flow (rCBF) and transcranial Doppler have shown dilatation of the middle cerebral artery on the ipsilateral side during CH attacks (59-61). However, dilatation of cranial vessels seem not to be specific to any particular headache syndrome as significant vasodilatation in the region of the major basal arteries has also been seen in both CH, spontaneous nitroglycerin- and capsaicin induced headache (55).

Inflammation

Some authors have suggested that CH could be due to a transient recurrent inflammation in the cavernous sinus – a transient periorbital venous vasculitis (62, 63). It has been suggested that if the venous outflow is disturbed it might result in stasis, dilatation and oedema of proximal vessels. Pathological findings at orbital

phlebography has been shown (63, 64), but seem not be exclusive for CH, since the same findings have been seen in other headache disorders. Furthermore, no pathologic abnormalities have been seen in cavernous sinus region with MR (65). It has also been suggested that a venous vasculitis is a part of a systemic inflammation (63). However, in another investigation, no evidence for a systemic inflammation was seen when studying conventional inflammation blood parameters (66). Nevertheless, there are still other signs of inflammation in CH since changes of several immunocompetent cells (67-70) have been seen in CH patients indicating that a sterile inflammation might exist.

HLA antigen frequencies have been reported in CH, mainly in Italian studies. An increased frequency of HLA-DR5 as well as a decreased frequency of HLA-B14 was seen (71, 72). In patients corresponding well to lithium treatment, HLA-B18 was reported to be increased (73). However, the studied material was small and the finding in need of confirmation.

Nitric oxide

Nitric oxide (NO) is a small messenger molecule, a potent short-lived and highly reactive free radical, with many different physiological effects in the body (74, 75). NO is formed by the action of different isoforms of the enzyme nitric oxide synthase (NOS), which converts L-arginine to L-citrulline. There are three different forms of NOS encoded by three different genes: nNOS, iNOS and eNOS. The gene coding for nNOS (neuronal NOS, expressed by neurons and involved in neurotransmission and neuroendocrine functions) is called NOS1. Inducible NOS (iNOS) is up-regulated by several cytokines (including IFN- γ , tumour necrosis factor-alpha and IL-1 β) in a complex manner, and NO itself induces the production of several cytokines. The gene encoding iNOS is called NOS2A. The gene coding for eNOS (endothelial NOS, expressed by endothelial cells and implicated in vascular smooth muscle relaxation through a CGMP-mediated signal transduction pathway) is designated NOS3.

NO has been suggested as the common mediator in vascular headache pathophysiology (76). NO is involved in vasodilation of cranial arteries, see above, and it can also promote neurogenic inflammation. Activation of NO leads to relaxation in vascular smooth muscle, which leads to vasodilation, and subsequently gives migraine in migraineurs and attacks in CH patients during periods. Histamin and glyceryltrinitrate (GTN) also produces headache, histamine probably through activation of NOS and GTN by delivering NO directly.

NO plasma concentrations have been shown to be significantly higher in CH patients, both during active phase and during remission (77). Nitrite accumulation in PBMC has been seen during bouts, but not during remission (78). In all, NO plays a critical role in vasodilation, neurotransmission and inflammation, thus combining the central and the peripheral activation systems.

Treatment

CH attacks are most often extremely intense and there is a need to abort, or at least reduce, the pain. Patients need to be instructed to avoid things that might trigger an attack, for example alcohol if this triggers attacks during active periods. Some patients experience periods after changes in the sleep-wake cycle, i.e. for example after vacation trips and work-shift changes. Apparently, many patients experience attacks after a short time of sleep and some patients seem to be able to avoid attacks by changing the sleeping pattern.

There are today several pharmacological options for treatment, both treatment of attacks and prophylactic medication. Today sumatriptan is probably the most effective and widely used acute medication. About 74% of patients experience complete relief after 15 minutes after subcutaneously self-administered sumatriptan as compared with 26% of placebo-treated patients (79). There seems to be no rebound during long-term frequent use of this medication (80-82). Today some other triptans have been used for the treatment of CH attacks, also case reports of prophylactic effects (83-85), but injections of sumatriptan seem to be the most effective choice for acute attacks.

Oxygen is another often effective treatment in CH attacks (86) and approximately 70% of patients will obtain relief within 15 minutes (87). Dihydroergotamine and ergotamine tartrate have been frequently used in CH and can be used as both attack- and prophylactic medication.

For prophylaxis verapamil is probably the most frequently used medication today. An open-label study has shown that 69% improved by more than 75% during treatment with verapamil (88). Dosage is often very high compared to when used in other conditions and CH patients usually tolerate, for unknown reasons, very high doses. The mechanism behind the efficacy of verapamil in CH is yet not known. Verapamil is a calcium antagonist, an L-channel blocker, and speculations whether high doses of verapamil might have influences on other calcium channels have been suggested (89). Lithium carbonate is another effective prophylactic treatment in CH. Good to excellent results were found in 78% of 304 patients with chronic CH (90, 91). Lithium has also induced remission in patients with episodic CH. Other prophylactic choices are, for example, methysergide, topiramate and melatonin.

There are several surgical procedures that can be tried on refractory chronic CH patients. The ones directed toward the sensory trigeminal nerve seem to have been the most successful. However, the important finding of activation in hypothalamus on the ipsilateral side during CH attacks (1) has prompted surgeons to try a new surgical procedure, deep-brain hypothalamic stimulation, which has shown to be very effective in refractory chronic CH cases (92-94).

Hereditary factors in cluster headache

Hereditary factors were previously regarded as being of minor importance for the etiology of CH. During the last decades this impression has changed since several studies have reported a positive family history.

Genetic epidemiology

Four genetic epidemiological surveys have provided more complete information about CH cases and their relatives (41, 95-97). Kudrow found, in American survey, a 45.6-fold increased risk of CH in 1st degree relatives. The study was done on the basis of reports by probands, and there was no confirmation by direct interviews with the possibly affected relatives. Russell showed in a Danish genetic epidemiological survey a 14.1-fold increased risk in 1st degree relatives and a 2.3-fold increased risk in 2nd degree relatives. Possibly affected relatives were interviewed by a physician in this Danish study. In an Italian investigation, a 39-fold increased risk for CH in 1st relatives and an 8-fold increased risk in 2nd degree relatives was found. The Italian survey was based on report from the proband and possibly affected relatives were interviewed by physicians. However, 11 of the 57 affected relatives had probable CH, i.e. they fulfilled all but one criterion for CH. A French study showed an 18-fold increased risk for CH in 1st degree relatives and this study is maybe the most accurate, since all 1st degree relatives were directly interviewed by a physician.

A complex segregation analysis of Danish families confirmed an autosomal-dominant mode of inheritance in these families (98). However, no common clear mode of inheritance has been seen in all the reported families, although CH seems to be an autosomal-dominant inherited disorder in some families, but probably with incomplete penetrance.

CH has been reported in seven concordant monozygotic twin pairs (99-104). This also indicates the importance of genetic factors, although publication itself introduces selection bias (105). The latter is emphasized by the only published large twin survey based on the Swedish Twin Registry and the Swedish Inpatient Registry (106). In this study two monozygotic and nine dizygotic twin pairs were all discordant for CH, and they had been discordant between 10 and 31 years. Nevertheless, the results from these genetic epidemiological studies indicate a genetic influence of the disease.

Clinical picture of familial CH

Even though there are several reports on familial CH there is little detailed information about the clinical characteristics of these families.

In a Danish study 18 CH families for intra- and interfamilial variability of symptoms were analysed (107). A lower age at onset in children than in parents with CH was

found. One family with high frequency of attacks (1-8 attacks/day) was observed. Another proband diagnosed as CH lacked autonomic symptoms while the 2nd degree relative had autonomic symptoms. A family with CH in three generations with worsening of symptoms in each successive generation was seen. Both chronic and episodic CH was seen within the same families. Despite the fact that a few families displayed a certain clinical pattern it is also well known that episodic CH and chronic CH may change over the years. Also, different forms of CH were described in the same family. Thus, genetic heterogeneity has not been regarded as likely on clinical grounds (108).

In an Italian survey a family history of CH in 44 of 220 probands was found (97). Twelve (21%) of the 57 affected relatives had CH with undetermined periodicity. In addition to this, another 11 (19%) had CH-like disorders not fulfilling IHS criteria (26). The authors of this study discussed about the fact that they personally interviewed all possibly affected relatives and thus uncovered a high number of difficult-to-diagnose forms of CH, thereby increasing the incidence of CH in relatives. In a second Italian investigation the clinical characteristics of sporadic and familial CH in 76 sex- and age-matched patients was compared (109). Females with familial CH had a lower age at onset than females with sporadic CH, but otherwise there were no specific clinical differences. In yet another Italian report two cases who experienced attacks of autonomic symptoms without headache was described (24).

In a French investigation a high frequency of females in familial CH compared to sporadic CH cases was found (95).

In a Dutch study 75 CH families in 1720 CH patients comprising a total of 162 affected relatives were identified (110). Twenty-four evidently had probable CH, mainly caused by long attack duration, i.e. duration of more than 3 hours.

Molecular genetic studies of CH

The molecular genetic background of CH has been an uninvestigated field, probably mostly due to the fact that CH until recently was regarded as a sporadic disorder. However, there are a few studies, or rather reports, including very small numbers of patients.

In a Japanese man with sporadic CH and no family history of MELAS a point mutation was reported in mitochondrial transfer RNA(Leu(UUR)) gene at nucleotide pair 3243 (111). This mutation could not be detected in neither an Italian nor in a German study of CH patients (112, 113). Another report described multiple deletions of mitochondrial DNA in a Japanese man with CH and chronic progressive external ophthalmoplegia (114). However, this patient did not fulfill the IHS criteria for CH due to too long attacks. Since there is often a transmission of the disorder from father to offspring a mitochondrial inheritance is not very likely.

Molecular genetics of migraine

Migraine is an inherited disorder in most cases and this is supported by several facts; for example increased risk in first-degree relatives and a higher concordance of migraine in monozygotic twins. There are two major forms of migraine; migraine with aura (MA) and migraine without aura (MO). Genetic factors are supposed to play a major role in MA, whereas a combination of genetic and environmental factors are supposed to be of importance in MO (115).

It was a breakthrough for genetic studies within the migraine field when a genetic background for the rare migraine form familial hemiplegic migraine (FHM) was found. FHM is characterized by migraine headache, but they are preceded by hemiparesis which may last from minutes up to several weeks. Some patients also develop progressive cerebellar ataxia.

Linkage studies of FHM families showed that about 50% of the families were linked to chromosome 19p13 markers. In 1996 the voltage-gated P/Q-type calcium channel alpha1A-subunit (CACNA1A) gene was identified in this region (116). The gene is mainly expressed in the CNS, primarily in the cerebellum, thalamus, hypothalamus and cerebral cortex. Mutations in this gene were later identified in several FHM families from different countries (117-119). Mutations of the CACNA1A gene result in a wide clinical spectrum varying from mild and fully reversible symptoms to severely progressive symptoms, both FHM, episodic ataxia (EA2) and spinocerebellar ataxia (SCA6) (116, 120-122). Another locus in FHM families has been identified on chromosome 1q23 (123, 124), with mutations of the ATP1A2 gene encoding the alpha2 subunit of the Na⁺, K⁺-ATPase (125).

An involvement of the CACNA1A gene in the more typical forms of migraine has also been suggested (126-128). An affected sib-pair analysis showed that the CACNA1A gene is likely to be involved in the more common forms of migraine, but with a stronger effect in MA (126). A linkage analysis of one large typical migraine family also demonstrated linkage to the same chromosome 19 region (129). However, subsequent studies in several populations of MA and MO did not verify an involvement of the CACNA1A gene in the more common forms of migraine (130-132). Thus, it seems difficult to draw any definitive positive or negative conclusions about an involvement of the CACNA1A gene from these studies. Serotonin receptor genes, dopamine receptor genes, serotonin transporter gene, ACE gene have all been studied, but with inconsistent results between studies (133-140).

According to the hypothesis that NO plays a major role in vascular headache pathophysiology two association studies and linkage analysis of migraine and NOS have been performed, endothelial and inducible NOS respectively, but no clear association was found between markers of these genes and migraine (141, 142).

Gene expression in CH

Almost no former gene expression studies have been performed in CH. However, in one study investigations whether changes in the expression of G-proteins underlie altered cell signaling in migraine and CH were performed (143). The background for this assumption was that altered physiological responses are seen in migraineurs and that differences in cell signaling are found in various cell types isolated from peripheral blood in migraineurs. A smaller reduction of Gs alpha and Gq alpha mRNA was seen in CH patients, most marked in those without medication. However, levels of Gs alpha mRNA were significantly reduced in CH patients compared to migraine patients.

Methodological aspects

International Headache Classification

Since there are, so far, no diagnostic markers for CH, the diagnosis most often relies exclusively on the headache history from the patient, unless an attack is being observed. Attacks may be provoked by administration of nitroglycerin during active periods (16) or attacks may be observed when the patient seeks medical care during an attack. Still, the diagnosis is made according to the history as regards duration of attacks and periods and frequency of attacks.

Not until the beginning of the 60's there was a classification for headache disorders, the Ad-Hoc Committee of The National Institute of Health who published a headache classification (144). The basis for this classification was the assumed etiology of the headache, and it became controversial from the start. It was clear that it was difficult for headache researchers and experts to communicate without a more precise classification (145).

The International Headache Society was founded in 1982. A committee was set up for classification of headache disorders with Professor Jes Olesen as chairman. In 1988 the first international diagnostic criteria for headache disorders was published, The Headache Classification Subcommittee of the International Headache Society (IHS) Classification and Diagnostic Criteria for Headache Disorders, Cranial Neuralgias and Facial Pain (26). Criteria were based on empirical findings and consensus among headache experts. Today there is a new classification from 2004, The International Classification of Headache Disorders (27). The former and new classifications for CH are showed in Tables 1 and 2.

In the new edition CH belongs to the group of TAC's – trigeminal autonomic cephalgias. TAC is a grouping of headache syndromes used for primary headaches with similar clinical pattern, i.e. trigeminal distribution of pain and ipsilateral cranial autonomic features, and thus presumably similarities as regards pathophysiology (146). The TAC's include CH, paroxysmal hemicrania, and short lasting unilateral neuralgiform headache attacks with conjunctival injection and tearing (SUNCT).

Table 1. Classification and Diagnostic Criteria for Headache Disorders, Cranial Neuralgias and Facial Pain. Headache Classification Committee of the International Headache Society (1988).

3.1 *Cluster headache*. Diagnostic criteria:

- A. At least five attacks fulfilling B–D
- B. Severe unilateral orbital, supraorbital and/or temporal pain lasting 15–180 minutes if untreated
- C. Headache is associated with at least one of the following signs which have to present on the pain side:
 - (1) Conjunctival injection
 - (2) Lacrimation
 - (3) Nasal congestion
 - (4) Rhinorrhoea
 - (5) Forehead and facial sweating
 - (6) Miosis
 - (7) Ptosis
 - (8) Eyelid edema
- D. Frequency of attacks: from one every other day to 8 per day.
- E. Not attributed to another disorder.

3.1.1 *Cluster headache periodicity undetermined*

- A. Criteria for 3.1 fulfilled.
- B. Too early to classify as 3.1.2 or 3.1.3

3.1.2 *Episodic cluster headache*

Description: Occurs in periods lasting 7 days to one year separated by pain free periods lasting 14 days or more.

- A. All the letter headings of 3.1.
- B. At least 2 periods of headaches (cluster periods) lasting (untreated patients) from 7 days to one year, separated by remissions of at least 14 days.

3.1.3 *Chronic cluster headache*

Description: Attacks occur for more than one year without remissions lasting less than 14 days.

- A. All letter headings of 3.1.
- B. Absence of remission phases for one year or more or remissions lasting less than 14 days.

3.1.3.1 *Chronic cluster headache unremitting from onset*. (Formerly primary chronic.)

- A. All letter headings of 3.1.3
- B. Absence of remission periods lasting 14 days or more from onset.

3.1.3.2 *Chronic cluster headache evolved from episodic*. (Formerly secondary chronic.)

- A. All letter headings of 3.1.3
- B. At least one interim remission period lasting 14 days or more within one year after onset, followed by unremitting course for at least one year.

Table 2. The International Classification of Headache Disorders, 2nd Edition, Headache Classification Subcommittee of the International Headache Society (2004).

3.1 *Cluster headache*. Diagnostic criteria:

- A. At least five attacks fulfilling B–D
- B. Severe or very severe unilateral orbital, supraorbital and/or temporal pain lasting 15–180 minutes if untreated
- C. Headache is accompanied by at least one of the following:
 - (1) Ipsilateral conjunctival injection and/or lacrimation
 - (2) Ipsilateral nasal congestion and/or rhinorrhoea
 - (3) Forehead and facial sweating
 - (4) Ipsilateral eyelid oedema
 - (5) Ipsilateral forehead and facial sweating
 - (6) Ipsilateral miosis and/or ptosis
 - (7) A sense of restlessness or agitation
- D. Attacks have a frequency from one every other day to eight per day
- E. Not attributed to another disorder

3.1.1 *Episodic cluster headache*

Description: Occurs in periods lasting seven days to one year separated by pain free periods lasting one month or more.

Diagnostic criteria:

A. All fulfilling criteria A–E of 3.1

B. At least two cluster periods lasting from 7 to 365 days and separated by pain free remissions of one month or more

3.1.2 *Chronic cluster headache*

Description: Attacks occur for more than one year without remission or with remissions lasting less than one month

Diagnostic criteria:

A. All alphabetical headings of 3.1

B. Attacks recur for more than one year without remission periods or with remission periods lasting less than one month

Comments: In a large series with good follow-up, 27% of patients had only a single cluster period. These should be coded 3.1 Cluster headache.

Genetic studies

Mendel initiated the investigation of modern genetics by studies of the texture, color and length of garden peas. Based on this he suggested that a factor was transmitted from parent to off-spring, and this factor produced an observable trait. His findings were applied to humans and there are 5 major different mendelian patterns of inheritance; autosomal dominant, autosomal recessive, x-linked recessive, x-linked dominant and y-linked inheritance (148).

Are genetic factors of importance?

There are several approaches to find genes causing human diseases. However, the first step before going any further with time-consuming sample collection and laboratory work is to define whether there is a genetic component of the disease. This is mainly done by determination whether there is a familial aggregation of the disease. If there is a familial aggregation it has to be calculated whether this can not only be due to environmental or cultural factors.

In epidemiological surveys the term RR (relative risk) is often used. The RR is the ratio of the incidence of the disease among relatives to affected persons and the general population. A familial aggregation is implied if the ratio exceeds 1, i.e. the disease is more likely in relatives compared to the general population.

$$RR = \frac{\text{Proband (relative is affected/proband is affected)}}{\text{Proband(random member of population is affected)}}$$

Definition of the phenotype

The definition of the phenotype is a critical step for all genetic studies. The phenotype is the outward appearance of an individual with a disorder. The clinician has to determine which inclusion criteria to use for the present study. For many diseases there might be certain clinical criteria to fulfill on examination, certain investigations might have to be done, to determine whether a person is affected or not. For other diseases, such as headache disorders, there is only the history from the patients unless the patient, for example, is being observed during a CH attack. A thorough knowledge of the clinical pattern of the disease and detailed interview is therefore of great importance. Results from genetic studies might be misinterpreted if affected are defined as unaffected and vice versa.

For the diagnosis of headache disorders the IHS classification is most often used in research. To receive diagnostic information the patients are most often interviewed directly, and undergo physical examination. However, in genetic epidemiological surveys, especially with the purpose to find familial cases, it is common to use questionnaires. After this, suspected cases are interviewed, and suspected 1st- and 2nd-degree relatives are contacted in the same way or the information relies only on the proband. Different results from genetic epidemiological studies in CH could probably partly be due to these methodological differences. Difficult-to-diagnose forms of CH were found when relatives were interviewed directly in an Italian study (97). On the other hand, in another investigation from the same group, it was shown that only one extra case of CH was found when first-degree relatives were interviewed directly compared to only selective interviews with probands (147).

Other factors, such as age at onset, are of importance when defining the phenotype for genetic studies in CH. Since we have no markers or methods to predict a CH diagnosis later in life we might include subjects as unaffected who might eventually develop the disease.

Basic concepts in genetics

A **gene** is a specific instruction, consisting of bases (nucleotides) in a certain order interpreted to aminoacids, encoding for a specific protein. The physical site or location of a gene is called **locus**. At any particular gene site there can exist different forms of the gene, **alleles**. An individual has two alleles at each locus, one of the alleles is inherited from the father and the other from the mother. If a person has the same alleles on both loci it is referred to as homozygosity, or the person is **homozygous** for the allele. Heterozygosity is referred to when the alleles can be distinguished from each other, i.e. the person is **heterozygous** for the allele. Very subtle differences are sometimes seen between alleles, sometimes only one base pair change. On the other hand, they can also be large deletions or insertions resulting in different gene function and disease. Any locus that has more than one form (allele) occurring with a frequency of at least 1% is referred to as a **polymorphism**. Polymorphisms are regarded as a normal part of genetic variability and polymorphisms of the same gene may or may not have different functions. (148)

Genetic homogeneity refers to diseases, conditions or other characteristics explained by the same genetic background.

Genetic heterogeneity refers to diseases, conditions or other characteristics that appear similar but whose genetic basis is different in different populations or individuals. The term locus heterogeneity is used when a single disorder is caused by mutations in genes at different chromosomal loci. Population heterogeneity means that a disease is caused by the same genes, but the effect is of different magnitude in different populations.

Complex genetic disease

Many common diseases are believed to have an underlying genetic predisposition, due to familial aggregation, but do not exhibit a clearly recognizable inherited pattern. In addition to a genetic predisposition other factors such as environmental factors might be of importance. Several, maybe interacting, loci with unknown penetrance might be involved. These diseases are considered to be genetically complex and the term “complex genetic disease” is often used. Genes that are supposed to be involved in complex diseases are often referred to as **susceptibility genes** as they can not be regarded as causative genes, since several genes are supposed to be involved.

Search for disease causing/susceptibility genes

The two main approaches in genetic studies are linkage studies and allelic association studies. Very briefly **linkage** is about **loci** and **association** is about **alleles**.

Linkage studies

Linkage is the traditional method used for the search of genes responsible for diseases with a mendelian inheritance, since the affected within a family are supposed to carry the disease gene. Linkage analysis is, briefly, the gene-hunting technique that traces patterns of heredity in families, in an attempt to locate a disease-causing gene mutation. Thus, linkage studies are used for the study of chromosomal regions segregating with the phenotype, i.e. cosegregation of two or more loci is examined in a family to determine whether these loci segregate independently or remain in close physical relationship to each other.

Recombination fraction - theta Θ - the measure of genetic linkage, i.e. that a parent will produce a recombinant offspring. Recombination occurs when homologous chromosomes cross-over, and in a nonrecombinant offspring the parental type is intact. Theta ranges from 0 for linked loci on the same chromosome and 0.5 for unlinked loci, and 2 loci are supposed to be linked if theta is less than 0.5 (149).

LOD-score - the logarithm of the ratio of the odds that two loci are linked with a recombination fraction equal to or greater than 0, and less than 0.5. Thus, it measures the probability of two loci being close together and consequently being inherited

together. A lod score of 3 or higher is accepted as evidence of linkage between two markers.

For the studies of complex diseases, linkage analysis requires increased number of families. Additionally, it is of great value to have extended pedigrees, i.e. many affected within a large family.

Allelic association studies

The aim of association studies is to test whether a certain allele is more frequent in a group with affected compared to a group of unaffected individuals. The study of allelic association is a method for identifying disease gene loci, especially the search for susceptibility genes in complex diseases. It is often used for verification of the most interesting loci found in a linkage analysis, but it is also often used in a ***candidate gene*** approach in studies of complex diseases. Candidate genes are genes supposed to be of functional importance for the pathophysiology of the disease. Association to a certain allele can be explained by either the action of the polymorphism itself or if the polymorphism is in linkage disequilibrium with other disease causing mutations. ***Linkage disequilibrium*** is, briefly, described as when alleles at two different loci are inherited together more often than what could be expected by chance, given the known allele frequency and recombination fraction between the two loci. There are two different methods used for allelic association studies; family-based studies and case-control studies.

With ***family-based studies*** the possibility of genetic differences between case and controls within a family is studied. The controls are here unaffected individuals within the same family. The most commonly used method is the TDT- transmission disequilibrium test. The trio, or the TDT-triad, consists of the affected individual and his/hers unaffected parents and several trios are studied.

In ***case-control studies*** allele frequencies in a group of unrelated affected individuals with a certain disease are compared to a group of matched controls. It is of great importance to have the controls matched for ethnicity, gender and age, since one of the largest problems in case-control association studies is population stratification, i.e. subgroups within the population regarded as affected.



Genetic markers

Genetic markers reflect any segment of DNA that can be identified, or whose chromosomal location is known, so that it can be used as a reference point. The two

most commonly used markers are microsatellites and SNP's. Today SNP's are more commonly used.

Microsatellites are repeated sequences of DNA present in everyone, of a set length. The dinucleotide is the most common repeat, with a highly polymorphic form of repeat. Since the dinucleotide repeats are very short it may sometimes be difficult to distinguish one allele from each other. Several trinucleotide repeats are known to cause diseases, but is most often quite stable. Tetranucleotide repeats are also quite polymorphic, and more easily viewed and distinguished from one another on the acryl amide gel electrophoresis.

SNP's (single nucleotide polymorphisms) are DNA sequence variations that occur when a single nucleotide in the genome sequence is altered. Each individual has many single nucleotide polymorphisms that together create a unique DNA. SNP's are most commonly part of the human variation and they are regarded as the most abundant polymorphic genetic markers.

Gene expression

Gene expression is the process of gene transcription and translation, i.e. the reading of the DNA sequence to produce mRNA. The mRNA is then decoded to produce its protein product. In any cell, only genes required for the current function of the cell are 'switched on' or 'expressed'. Thus, studies of gene expression are of great importance for cell function and disease function.

RT-PCR

RT-PCR (reversed transcriptase polymerase chain reaction) is a powerful method to quantify gene expression. First, complementary DNA (cDNA) is made from an RNA template, using a reverse transcriptase enzyme, and then some of it is used in a PCR reaction to produce large quantities. In real-time PCR the PCR products can be detected in real time, meaning that the accumulation of PCR products could be visualized at each cycle. The measurements of amplified sequences are made in the exponential phase of the amplification which gives an accurate result.

There are two major methods to analyze data from real-time quantitative PCR experiments; absolute quantification and relative quantification (150).

Absolute quantification: The input copy number of the actual transcript (target gene) is determined with the use of a calibration curve (or standard curve), i.e. the PCR signal is related to a standard curve comprised of samples with different amount of the target transcript. With this method an absolute copy number is determined.

Relative quantification: The change in gene expression is related to a reference and there is no need for a standard curve. It is based on the expression levels of a target

gene versus a housekeeping gene (reference or control gene). Thus, there are no units used to express relative quantities. By using an endogenous control as an active reference, quantification of an mRNA target can be normalised for differences in the amount of total RNA added to each reaction. The most common choices are 18S RNA, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and β -actin. The $2^{-\Delta\Delta C_T}$ method is a calculation for analysis of real-time quantitative PCR data (150).

Gene expression using microarray technology

Classical research is usually driven by a specific hypothesis. Due to the technical possibilities today there are new methods to use in clinical and other research. Instead of a hypothesis driven approach, the microarray technology can be used for gene expression studies of thousands of genes at the same time.

The term microarray technology refers to an automated, high-throughput technique for simultaneously analyzing thousands of different DNA sequences affixed to a thumbnail-sized “chip” of glass or silicon. This is still a quite new method and it is most often used to study changes in gene expression. Today it can also be used for DNA genotyping and thus it can even be used for linkage analysis, association studies and population genetics. The term gene chip technology is often used and refers to GeneChip® from the manufacturer Affymetrix, which is probably the leading brand for prefabricated chips for large scale expression or genotyping analysis.

GeneChip microarrays consist of small DNA fragments, oligonucleotides. An array usually refers to the miniaturized array of a large number of unique DNA sequences, i.e. the microarray chip (151). The oligonucleotides are chemically synthesized at specific locations on a coated quartz surface. For the Affymetrix prefabricated gene chips the synthesis is made by a method called photolithography, a light-directed oligonucleotide synthesis on the chips (152). Each gene is represented by 11 probe pairs of 25 bp oligonucleotides. Each probe pair consist of a perfect match (PM) and a mis-match (MM) to the target. One chip is used for one sample, unless samples are pooled. One of the advantages of the Affymetrix system is that multiple distinct oligonucleotide probes on each chip represent every gene (www.affymetrix.com). It is, for example, possible to analyze more than 56 000 gene transcripts per chip, covering the whole human genome.

Nucleic acids from experimental samples are extracted, amplified, labeled and then hybridized to the array.



Affymetrix GeneChip® probe array. Image courtesy of Affymetrix.

Dataprocessing and analysis of microarray experiments

The analysis of the microarray experiment is divided into several steps. Due to the extreme amount of data and the many approaches for statistical calculations and analytical methods it is an issue of controversies. The microarray analysis will just be very briefly summarized here. Several software programs are available for analysis. For Affymetrix GeneChips analysis the Microarray Suite software (MAS) or today's GeneChip Operating Software (GCOS) from Affymetrix may, for example, be used for transcript quantification and array data comparison for relative expression level changes. However, there are also other probe level analysis methods, for example dChip (153) and RMA (151). **Robust Multichip Average (RMA)** consists of three steps; a background adjustment, quantile normalization and summarization. Data is analyzed for a set of chips using only PM and ignoring MM. Results are received as log values.

Normalization is the general term used to describe the process of removing systemic variation, technical non-biological influences, in microarray experiments that may affect the measured gene expression levels (154). Normalization classically means to make the data more normally distributed. However, normalization in microarray analysis also means standardization and centralization methods. There are several normalization procedures that can be performed; per-chip, per-gene, global and local normalization.

Scaling is an important step where an arbitrary target signal scales the average intensity of all genes on each array, within a data set, to a specified target signal. This process enables comparison of multiple arrays within a data set.

Signal values are made from the raw hybridization data. A quantitative algorithm calculates signal and signal Log ratio directly from the hybridization intensities of probes. The other set of confidence algorithms provide p-values and calls (absent, present or marginal) that measure the confidence in **detection** of the specific target RNA (www.affymetrix.com/support/help/faqs).

It is also possible to use several other software programs with statistical program for further analysis – for example Genespring, Spotfire, SAM (Statistical Analysis of Microarrays) or simply conventional t-tests.

Principal component analysis (PCA) is a statistical method of analyzing multivariate data in order to express their variation in a minimum number of principal components, i.e. an unsupervised dimension reduction technique in which points in a multidimensional space are projected into fewer dimensions. In a typical microarray experiment the expression of thousands of genes is measured across many conditions. PCA reduces the data dimensionality by performing a covariance analysis between factors.

Pairwise analysis (or pairwise comparison) is when two measurements are being compared, for example two different time-points in a single individual or treated versus untreated subjects. Results are described as increased, decreased or no change. Also, a figure of fold-change is received for information about the degree of up- vs downregulation.

Advantages with micro array gene expression technology

There are many advantages with the microarray technology, and some of them are apparent. For example it is possible to receive information from many thousands of genes in the same experiment, instead of time-consuming several experiments. It is thus possible to understand complex mechanisms and networks involved in biological processes and diseases. When comparing expression pathways it can be possible to, for example, detect new regulatory pathways, find disease causing mechanisms and validate mechanisms of treatment. Unexpected results from microarray experiments may function as hypothesis generators instead of conventionally hypothesis driven studies.

Problems and disadvantages in microarray studies

Despite the apparent advantages there are several challenges in microarray experiments and the subsequent analysis and some of them have slightly been discussed above.

- The price – the method is still expensive which may cause not enough replicates in the experiments.
- High quality RNA is requested for reliable results, i.e. this might also result in not enough replicates
- There might be variability in chips quality. A small scratch on a chip might cause problems with the analysis.
- There is risk of high rate of false positives in the analysis if not multiple comparisons are taken into account.
- It might be difficult to discover trends in a multidimensional expression pattern.
- The complexity of analysis – the method is still new and there are still problems in the general analysis procedure.

- Due to the advanced information received there might not be enough biological knowledge today for the obtained results
- Annotations might not be updated or referred to the actual probe ID's.

Ontologies

An ontology defines a common vocabulary for researchers who need to share information. Gene Ontology (GO) was developed by the Gene Ontology Consortium to help annotate information on gene products (not the genes) using the following three organizing principles of *molecular function*, *biological process* and *cellular component*. This was the first and is currently the main ontology in genomics (155). GO is now often used as a tool when analyzing data obtained from microarray experiments. Information for everyone is available online on www.geneontology.org.

AIMS OF PRESENT STUDIES

The overall aim of the present project was to better understand the clinical picture and the genetic background as well as pathophysiological mechanisms in CH, with the ultimate aim to, hopefully, contribute to diagnosis and treatment of CH patients.

I. To evaluate the prognosis after one typical CH period as regards the risk of catching a second period and the time to recurrence.

II. To evaluate and determine CH affection status in family members of CH families, for clarification of the clinical picture in familial CH.

III. To determine whether polymorphisms of the CACNA1A gene are of importance for CH susceptibility.

IV. To determine whether polymorphisms in the three different NOS-genes are of importance in CH aetiology.

V. To search for a CH gene through linkage analysis. Furthermore, to investigate whether a formerly reported association of the HCRTR2 gene could be replicated in another European cohort, or if a mutation of the HCRTR2 could be detected in familial CH cases.

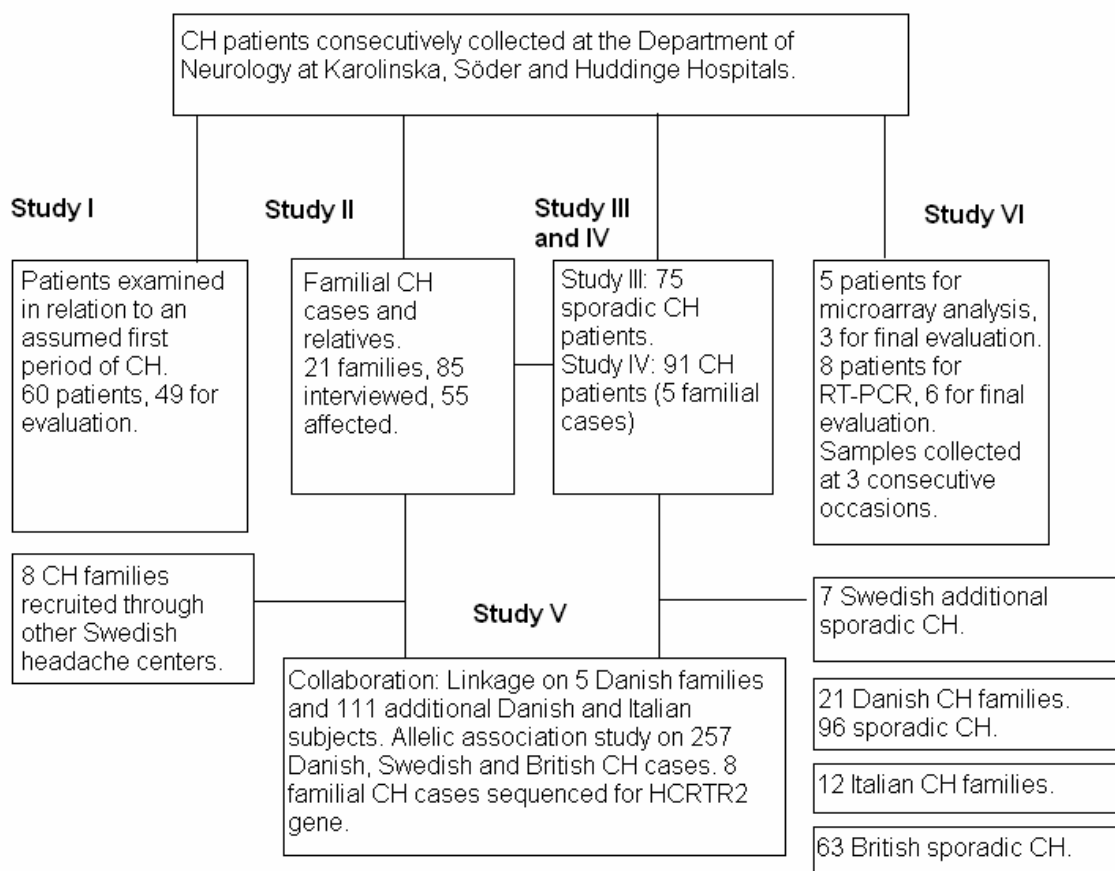
VI. To identify genes that are differentially expressed in peripheral blood during different stages of CH, assuming that changes in gene expression of pathophysiological interest would also be seen in peripheral blood.

MATERIALS AND METHODS

CH has been studied for almost four decades at our Department of Neurology; initially at Karolinska Hospital and Söder Hospital in Stockholm, after 1997 at Huddinge University Hospital and today referred to as Karolinska University Hospital, Huddinge. The clinical information today comprises 604 CH patients (33). The patients participating in the present studies, except for participants from other countries within an international collaboration, have been selected from this group of CH patients. However, some families were recruited through kind information from other headache specialists and a few were recruited through a national headache website.

All studies have been ethically approved and all patients have given their informed consent.

Figure 1. Overview of patient collection.



Patients and controls

Study I

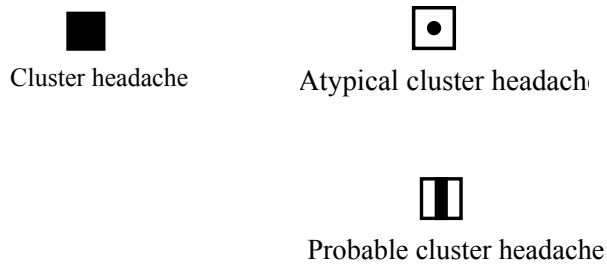
During 1981-1996 a consecutive series of 60 patients was examined at the department of Neurology at Söder Hospital in Stockholm, in relation to an assumed first period of CH. In 1998 their clinical record were reviewed according to the actual IHS criteria (26) and it was again established that they had CH except that periodicity could not be defined. Of these patients we found that 28 patients had received a definitive CH diagnosis over the years. The remaining 32 patients had only had contact with the Department in relation to this first period and thus we had no further documentation as regards the subsequent course of their headaches.

Study II

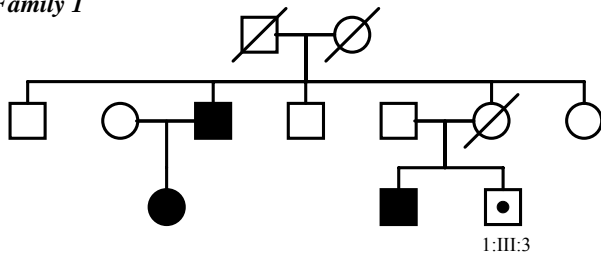
Probands with CH who reported one or more possibly affected relatives with CH were consecutively collected from our larger group of CH patients. Twelve affected subjects belonged to five families. By informing headache specialists on meetings we also received another eight families from other departments and clinics. We got an ethical permission to have an advertisement about our study on Svenska Migränförbundet's (a network for headache patients in Sweden) website, www.migran.org. Two families were identified via this advertisement. Thirty families with at least two cases of suspected CH were identified. Nine families were excluded because only one affected was still alive in six families, two families declined to participate and in one family it was impossible to reach the affected relative. A total of 21 families were available for evaluation. Pedigrees of all 21 families are shown in Figure 2.

Four deceased persons with possible CH are labelled affected in the pedigrees (Families 9, 12 and 14), but they were not included in the final analyses, since their diagnoses were not verified by clinical documentation. One person with CH was not included in the analysis, since the clinical record could not be found and relatives regarded her as being too old for an interview (Family 9). All families were of Caucasian origin except for family 14, which was of Afroamerican origin except for the unaffected Caucasian mother in the second generation.

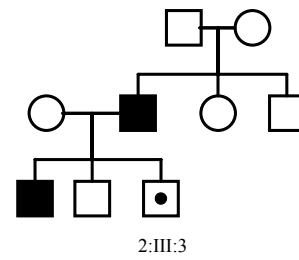
Figure 2. Pedigrees of cluster headache families.



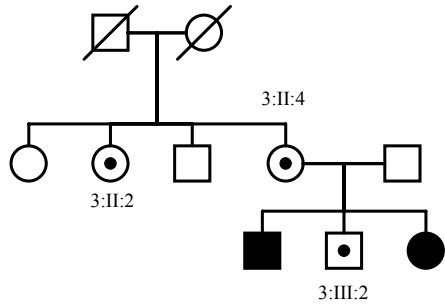
Family 1



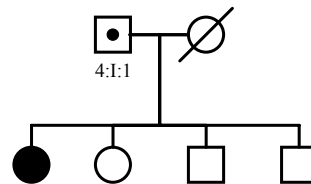
Family 2



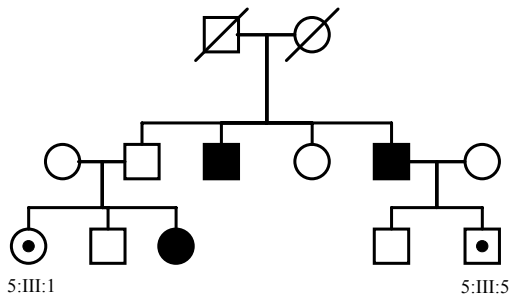
Family 3



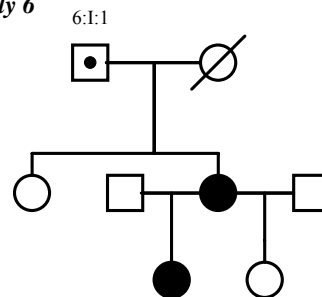
Family 4



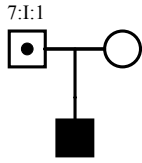
Family 5



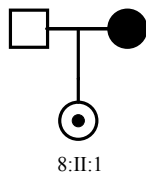
Family 6



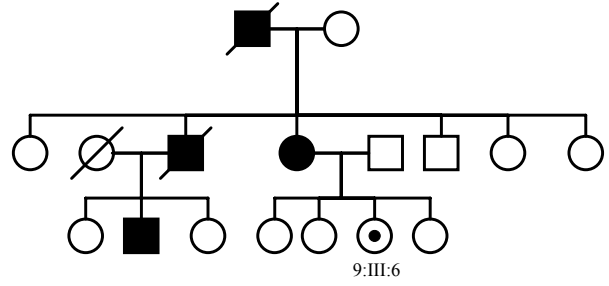
Family 7



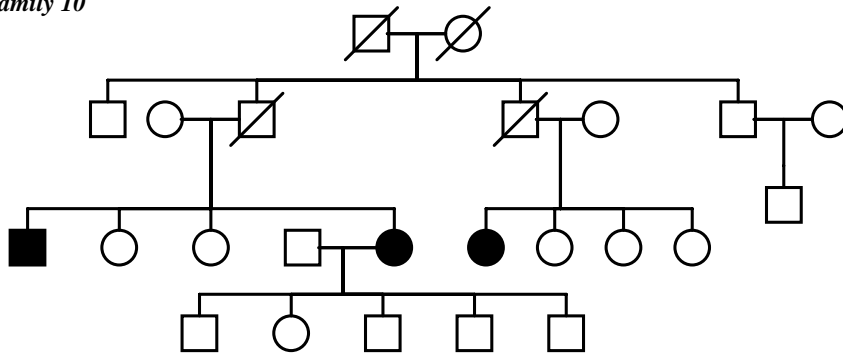
Family 8



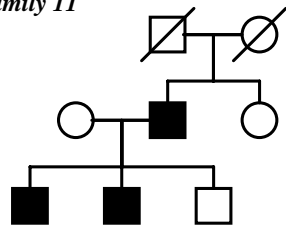
Family 9



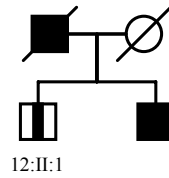
Family 10



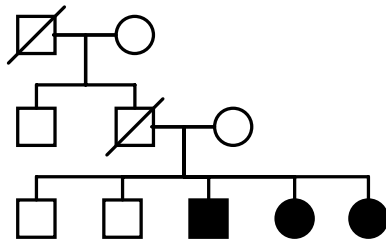
Family 11



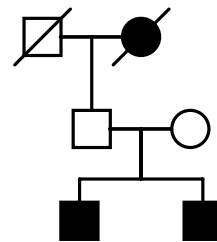
Family 12



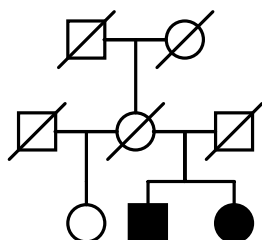
Family 13



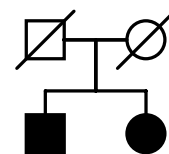
Family 14



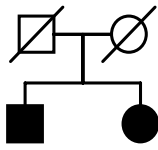
Family 15



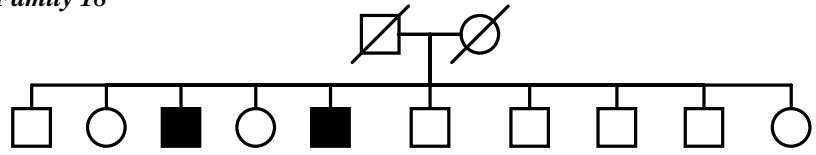
Family 16



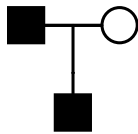
Family 17



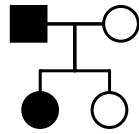
Family 18



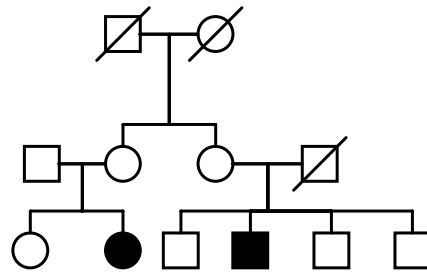
Family 19



Family 20



Family 21



Study III

In the first genetic study 75 Caucasian outpatients with sporadic CH were investigated, 56 (74.7%) males and 19 (25.3%) were females. The patients had consecutively visited our department or were contacted for participation in a genetic study. The mean age was 49.4 years (range 24–74 years). Six patients had chronic CH and 69 patients had episodic CH. They had all been seen by us and clearly fulfilled the International Headache Society (IHS) criteria for CH (26). The control group consisted of 108 Caucasian persons, 74 (68.5%) males and 34 (31.5%) females. The mean age of the controls was 48.9 years (range 25–72 years). The control group, drawn from a larger group of controls to match for age and gender, consisted of blood donors, healthy volunteers and spouses of patients.

Study IV

For the second genetic study 91 CH patients (23 females and 68 males) were investigated, 75 of these patients were also studied in Study III. The additional patients in this study were approached in the same way as the others. In the patient group there were 92.3% episodic cases and 7.7% chronic cases. Four patients were familial cases. They all clearly fulfilled the IHS (26) criteria for CH, and all had attended our department. The control group consisted of 111 healthy individuals (35 females and 76 males). Even in this case the controls were drawn from a larger group of controls (consisting of blood donors, healthy volunteers and spouses of patients) to match for age and gender. Both patients and controls were of Caucasian origin.

Study V

Danish CH families and case-control dataset

A total of more than 800 probands with CH were recruited from a neurological clinic, two departments of neurology and from the Danish patient organization for CH. Probands with a positive family history were interviewed by telephone. Subsequently, all possibly affected relatives were also interviewed by telephone. Non-affected relatives were interviewed in families with two or more affected. The sporadic cases of CH were recruited from the above mentioned 800 probands with CH. The control group consisted of 40 year old individuals without CH from the general population.

Italian case-control dataset

Familial CH cases were ascertained from a sample of 220 CH patients (163 men, 57 women; mean age 44 years) attending the Headache Centre of the Carlo Besta National Neurological Institute, Milan. All were of Italian origin. A total of 57 affected relatives were identified. Of these, 50 were ascertained from directly interviewed probands and the remaining seven from probands replying to the questionnaire.

Swedish CH families and case-control dataset

CH families, sporadic CH cases and controls were recruited as described above for study II, III and IV. Seven additional sporadic cases were collected for this study. No families containing atypical CH cases were included in the genome scan.

British CH families and case-control dataset

The UK patient cohort was ascertained through Departments of Neurology and Neurosurgery, and at the Organisation for the Understanding of Cluster Headaches in the UK (OUCH (UK); www.clusterheadaches.org.uk) annual conference. A collection of British patients affected with primary open angle glaucoma, not known to suffer from CH, was used for the UK control population.

The combined European sporadic cohort is shown in Table 3.

Table 3. Studied population for HCRTR2 SNP's.

	c.224-26A>C *	c.922G>A *
Danish cohort		
CH patients, n	96	96
Controls, n	69	72
Swedish cohort		
CH patients, n	99	98
Controls, n	84	106
British cohort		
CH patients, n	63	63
Controls, n	90	94

Eight patients were sequenced for the HCRTR2 gene, 2 familial cases from each country. The Swedish familial CH patients (SWE13 and SWE14 in the paper) were members of CH families number 5 and 20, see Figure 2.

Study VI

CH patients in active period were consecutively collected at our department. They were followed during active period, during an attack and during remission. From a larger collection of more than 10 patients we decided to study 5 patients with microarray gene expression technique. All had episodic CH diagnosis according to IHS criteria (2004) and they were of Caucasian origin. Two out of 5 patients, a 64-year old male and a 55-year old woman, were later withdrawn from this study due to poor quality in one of the three samples from each one of these 2 patients.

Three CH patients were thus finally studied during 3 different disease stages; 1) a 46-year old male, 2) a 40-year old male and 3) a 38-year old male. They all used sumatriptan subcutaneously as attack medication. Patient number 1 had episodic CH and periods with depressions, no prophylactic medication prior to the study. He had 2 attacks/day during the active phase. Patient number 2 had been splenectomized at the age of 23. He had episodic CH for many years but was otherwise healthy, no prophylactic medication prior to the study. He had 3-4 attacks/day during active phase. Patient number 3 had episodic CH and had been taken verapamil 640 mg/day as CH prophylaxis p.o. that was withdrawn 3 days prior to the study. He had 2-4 attacks/day.

Three healthy headache free males of Caucasian origin aged 47, 42 and 40 were used as matched controls (age \pm 2 years). RNA from two additional healthy controls, a 60-year old male and a 52-year old female were labelled for hybridisation, but they were not analysed in the present study as the matched patients were withdrawn, see above.

For the quantitative RT-PCR experiment we collected samples from 8 patients as described above, i.e CH patients were consecutively collected during attack, period and remission as described above. However, due to technical problems we had to exclude 2 patients and finally the group consisted of 4 males and 2 females, mean age 48 years, range 36-64 years. The control group consisted of 14 individuals, 9 males and 5 females, mean age 44 years, range 31-55 years. One of these patients as well as two of the controls were also included in the microarray experiment.

Methods

In *Study I* the clinical records for all patients, observed in relation to a first interview, were reviewed according to the actual IHS criteria (28 patients, referred to as Group 1 in the publication). Thirty-two individuals had not had any further contact with our department (Group 2) and they were checked against the National Registry of Population as regards life status and present address. Individuals in the latter group were sent a letter of information telling them that they would be contacted by telephone for a structured interview as regards the possible recurrence of headache attacks. The letter presented a number of structured diagnostic questions as regards the clinical course after the first period to be answered in the telephone interview. If not reached by the first letter or by telephone, a second letter with the same questions was mailed, with the instruction to return the questionnaire by mail. The time to recurrence and observation time was noted.

In *Study II* we contacted probands with a supposed familial occurrence of CH by mail and/or by telephone, and they were informed about the study. The probands provided names and addresses of possibly affected relatives and unaffected 1st - or 2nd -degree relatives who would be willing to participate in the study. They all received a questionnaire regarding CH and other types of headaches, and were also asked to report about affected relatives, like the probands. Those not responding were contacted a second time by telephone or mail. Probands, affected relatives, possibly affected and unaffected willing to participate filled out a form concerning clinical aspects on headache. All were in a structured way instructed to inform about other related persons with headaches.

All possibly affected were personally interviewed. Only those with clinical documentation (through case records, questionnaire and/or personal interview) were included in the analyses. The 2nd edition of The International Classification of Headache Disorders was used for headache diagnosis with respect to CH, other TAC's and migraine (27). If two or more of the CH criteria (A-E) were not fulfilled patients were classified atypical CH, if the headache could not clearly be classified as migraine, tension-type headache or another type of headache described in the classification. Only families with at least two cases of suspected CH were included in the study as we considered CH to be familial if this criterion was met.

Retrospectively ranked worst pain intensity during an attack was scored on a pain scale from 0-10. These numbers were transformed to pain characteristics of the IHS classification as follows; 0 = no pain, 1-3 = mild pain, 4-6 = moderate pain, 7-9 = severe pain and 10 = very severe pain.

For *Study III and IV* peripheral blood samples were collected from patients and controls. DNA was extracted by using a modified salting out method (156).

For *Study III* two highly polymorphic intragenic markers were chosen, the (CA)_n-repeat marker D19S1150 (forward 5'-GGAGAAGCATAGAAAAGCCA-3' and reverse

5'-CCTGTTGAAAACCTCCTGACC-3') and the (CAG)_n triplet repeat sequence in the 3-prime-UTR (exon 47) with forward primer sequence S-5-F1 (5'-CACGTGTCCTATTCCCCTGTGATCC-3') and reversed primer sequence S-5-R1(5'-TGGGTACCTCCGAGGGCCGCTGGTG-3') as described by Zhuchenko et al (122). I will not go into detail about PCR-conditions for the CACNA1A markers here.

In *Study IV* we studied two polymorphic markers of the NOS1 (nNOS) gene; a dinucleotide repeat here referred to as NOS1a and a trinucleotide repeat referred to as NOS1b. The primer sequences and polymerase chain reaction (PCR) conditions for NOS1a and for NOS1b have been described by Xu et al (157). For NOS2A (iNOS) we studied two polymorphisms located in the promotor region of the gene. One of these markers is a biallelic AAAT/AAAAT repeat sequence extending from 756 to 716 bp 5' of the main TATA-directed initiation site. For the biallelic repeat we used primer sequence and PCR conditions described by Bellamy et al (158). The other NOS2A marker, a (CCTTT)_n pentanucleotide repeat, has also been mapped to the promotor region. For the pentanucleotide repeat we used primer sequence and PCR conditions described elsewhere (159). For NOS3 (eNOS) we genotyped the polymorphic (CA)_n repeat in intron 13 of the NOS3 gene using the primers and PCR conditions described by Nadaud et al (160).

For both these studies, PCR fragments surrounding the microsatellites were amplified in PCR reactions, separate conditions for different primers, before fluorescent labeling. Electrophoresis of the amplified DNA fragments was performed on polyacrylamide gels on an ABI 377 DNA sequencer. Fragment sizes were determined by comparison with internal lane size standards. The results were analyzed using the GENESCAN/GENOTYPER software (version 1.1; PE Biosystems).

In the *Study V* genomic DNA was extracted from 10 ml of peripheral blood by standard techniques from the Danish, Italian and British participating subjects. Genomic DNA from the Swedish material was extracted using a modified salting-out procedure (161). A genomewide screen was performed for individuals from five multigenerational Danish families, using fluorescently labelled microsatellite markers with an average spacing of 9 cM (from the 404-marker screening set 10, Marshfield Centre for Medical Genetics). PCR amplifications were performed, no described details in this section. PCR products were pooled and electrophoresed through 6% polyacrylamide gels (Flowgen) on an ABI 377 DNA sequencer. Genotype data were generated using GeneScan v3.0 and Genotyper v2.1 software (Applied Biosystems). Data for 21 markers that failed to optimise were excluded from further analysis. Typing was successful for 95% of genotypes at the remaining 383 markers. For those loci reaching a LOD and/or NPL score of 2.0 or above, four additional markers flanking each locus at an average spacing of 2 cM were analyzed in 111 additional individuals (from 16 Danish and 12 Italian families).

As for the HCRTR2 allelic association analysis SNP genotyping on two polymorphisms of the HCRTR2 gene on chromosome 6p11-q11(rs3122169 and rs2653349 from dbSNP; www.ncbi.nlm.nih.gov) were amplified from plated DNA samples. Sequence-analysis primers were used for exons 2 and 5 respectively, and resultant products were blotted onto Hybond N (Amersham). For each SNP, a pair of

allele-specific oligonucleotides (ASOs) were synthesized. All probes were 18-mers, with the polymorphism located 8 nucleotides from the 5' end. ASOs were end-labelled with γ^{32} -dCTP to probe dot blots of the corresponding PCR products, as described by Jeffreys et al (162). Filters were washed in 3 M TMAC, 0.6% SDS, 10 mM sodium phosphate (pH 6.8), and 1 mM EDTA at 56°C for 20 min, rinsed in 3 × saline sodium citrate at room temperature, and followed by direct examination of autoradiographs compared for each allele.

An affected subject from each of the independent families D42, D162, ITA6, ITA48, SWE13, SWE14, UK1 and UK5 plus one normal control individual was sequenced for the complete protein-coding region and intron/exon boundaries of the HCRTR2 gene. Amplification of the gene was performed by PCR in 40 μ l reactions, and products were separated by electrophoresis through 2% LE agarose (FMC Bioproducts), followed by purification using the QIAquick PCR purification kit (Qiagen®). Purified PCR products were sequenced with the Applied Biosystems BigDye terminator kit, and were electrophoresed through 5% LongRanger (Cambrex) polyacrylamide gels on an ABI 377 sequencer (Applied Biosystems). Sequence traces were analyzed with Sequence Analysis v3.2 and SeqEd v1.0.3 computer software (Applied Biosystems®).

In **Study VI** the PAXgene Blood RNA System (PreAnalytix, Qiagen®), a standardized vacutainer technology for stabilization and purification of intracellular RNA, was used for RNA extraction. Peripheral blood was drawn into PAXgene tubes that were left in room temperature for 2-12 hours before extraction. RNA was extracted according to the manufacturer's directions.

Total RNA quantity was determined by ultraviolet absorbance at 260nm. The purity of the isolated RNA was determined by measuring the ratio of absorbance at 260 and 280 nm. The ratio for all samples was between 1.9 and 2.3. To determine the integrity of the RNA for microarray analysis, i.e. RNA quality control, an analysis was performed on the Agilent® 2100 Bioanalyzer according to the manufacturer's instructions.

The laboratory work, after RNA extraction, was performed at Affymetrix Core Facility at Karolinska Institute, Novum. Sample preparation and processing procedure was performed following the manufacturer's protocols. cDNA was synthesized according to the the Instruction Manual MessageAmp™ aRNA Kit Ambion (version 1785). The Instruction Manual MessageAmp™ aRNA Kit Ambion (version 0309 b) was used for the labeling procedure. The Affymetrix Human U133 Plus 2.0 GeneChip® Array with more than 54 000 transcripts covering almost 22 000 genes was used. The labelled cRNA samples were hybridized to the Human U133 Plus 2.0 GeneChip® Array from Affymetrix following the GeneChip® Expression Analysis Technical Manual (701021 rev 3). The GeneChips® were incubated for 16 hours at 45° C. They were washed and stained in the Affymetrix Fluidics Station (Fluidics Protocol EukGE-WS2v4, Wash A Low stringent 6X SSPE 0.01% Tween 20, Wash B High stringent 0.1M MES ,0.1M Na,0.01% Tween 20). The microarrays were scanned by the Affymetrix GeneChip® Scanner 3000 (2004).

As for the RT-PCR experiment RNA was extracted as described above using the PAXgene blood collection system, and converted to cDNA by using the protocol in

chapter 2.10 in the manual TaqMan® Gold RT-PCR Kit. PCR was performed in a 25 ul volume reaction by using the ABI Prism 7700 Sequence Detection System. GAPDH (Applied Biosystem) was used as internal control. The S100P gene was used for evaluation by using the TaqMan® Gene Expression Assays, Assay Hs00195584_m1, from Applied Biosystems.

Statistical analysis

In *Study I* only simple calculations were made, for example mean and percentage.

Statistical analyses in *Study II* were performed using GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego California USA, www.graphpad.com. Wilcoxon match-pairs signed rank test, Fisher's exact test and standard deviation were used. All tests of significance were two tailed using 5 % significance level.

For the allelic association studies, *Study II* and *Study IV*, allele and phenotype frequencies for patients and controls were compared using Fisher's exact test. Phenotype frequencies refer to total numbers of alleles. The level of significance was chosen as $p < 0.05$. The large number of comparisons made in the study should be considered in evaluating deviations observed. For *Study V*, SNP data were analysed by chi-square test, to compare both allele and phenotype frequencies between case and control populations. A Fisher's exact test was also used to assess ratios between the two allele frequencies. Statistical significance in this latter study was established at $p < 0.01$.

In *Study III* we also performed a Monte Carlo simulation test to assess the likelihood of our data as described by Sham & Curtis (163). Linkage disequilibrium (LD) was assessed by Fisher's exact test and by chi-square analysis between allele pairs of the two markers. The sample size allows 80% power (with a two-sided CI of 95%) to detect a RR of 2.5 of an allele with a frequency of 13% in controls.

In *Study IV* we also calculated probability values for 2 x k contingency tables using a chi-square test in which allele frequencies for patients and controls were compared. We estimate that we had at least 80% power to detect a factor with a relative risk of 2.5 with a phenotype frequency of 12% in controls.

In *Study V* the simulation program SLINK (164, 165) was used to establish the cumulative linkage power contained in the five largest Danish families, and to ascertain whether they carried the potential to achieve statistical significance. These data indicated that LOD scores of statistical significance could be reached in this particular cohort. The computer program GAS (Genetic Analysis System v2.0; Alan Young, Oxford 1993-1995) was used to format genotype data for linkage analysis, to verify inheritance and to calculate marker allele frequencies. Single- and multipoint linkage analyses were performed using Genehunter v2.1 (166). LOD scores were calculated on

the assumption that CH was segregating as an autosomal dominant trait with 30% penetrance. A disease allele frequency and phenocopy rate of 0.001 were assumed. Due to potential ambiguity for the mode of transmission in CH, model-free non-parametric NPL scores were also generated for each analysis. For those loci reaching a LOD and/or NPL score of 2.0 or above, four additional markers spanning each locus with an average spacing of 2 cM were next analysed.

HCRTR2 SNP data was analyzed using a chi-square test, to compare both allele and genotype frequencies between case and control populations. Statistical significance was established at $p < 0.01$.

The Affymetrix GeneChip® Operating Software (GCOS) Version 1.0 was used for initial raw data processing in **Study VI**. The GCOS Expression algorithm was used for transcript quantification and array data comparison for relative expression level changes. For comparison of different normalisation methods we also used the Robust Multichip Average (RMA) method (167) implemented in the module Affy of the BioConductor microarray analysis software (www.bioconductor.org). Principal Component Analysis (PCA) was performed separately after both GCOS and RMA normalisation using the Spotfire® (version 7.3) software.

We used the comparison analysis for identifying up- and downregulated genes. Pair-wise comparisons on signal values from different disease stages and controls were performed using the Affymetrix GCOS software. Results were obtained by increased, decreased or no change in expression signal values and also described as a 2-log fold-change, as well as a detection p-value. We filtered (GCOS) for increased and decreased respectively in all subjects, with present in all increased and present in all compared when decreased. Thus, a specific transcript was defined as regulated when the corresponding probe sets fulfilled the following criteria: Signal detected as present (“P”) in 3/3 replicates for “increased”, or baseline replicates for “decreased”, change (“increase”/“decrease”) in 3/3 of the chip comparisons. This is regarded as a robust method based on the knowledge that microarray measurements are more reliable for transcripts of high abundance. We also performed paired t-tests which generated gene lists of hundreds of significant genes. The p-values from these calculations are used for the comparison of methods, besides the pairwise comparisons.

Additionally, we divided the data for studies of gene expression patterns of presumably functionally interesting genes, classified according to ontology. The following ontologies were studied; Biogene amine metabolism, Calcium channel activity, G-protein coupled receptor activity, G-protein coupled receptor protein signalling pathway, Immune response, Lipid metabolism, Nitric oxide metabolism, Rhythmic behaviour and Synaptic transmission. These groups were defined according to the Gene Ontology database, www.geneontology.org.

Statistical analysis on expression levels from the RT-PCR experiment during period, attack and remission was performed by paired ANOVA, Repeated Measures Analysis of Variance, followed by Tukey-Kramer post test for multiple comparisons. Unpaired t-test was performed for comparison between different disease stages and controls. Fisher’s exact test was used for comparison of selected genes within a group of genes

(upregulated S100 proteins) compared to all genes represented on the chip, by using R from Bioconductor (version 2.0.1). The level of significance was chosen $p < 0.001$.

All probe ID's were checked against Affymetrix annotations, Netaffx analysis and annotation center, at www.affymetrix.com/analysis/netaffx. Just recently, all probesets on the Affymetrix U133 Plus GeneChip® array was re-identified by a research group (168). All up- and downregulated probe ID's in the pairwise comparisons model were checked against this new lists, downloaded from <http://mriweb.moffitt.usf.edu/mpv/>. We chose to describe results in gene names, although it is important to keep in mind that these are related to the probe ID's.

RESULTS

Study I

The patients were initially divided into 2 groups; group 1 with only one documented period and group 2 who had been diagnosed with CH over the years. Altogether six patients were found to be deceased. Six patients from group 2 were lost to follow up because they could not be reached by telephone or by letter. The final group for evaluation of prognosis thus included 49 patients, including the deceased patient with CH diagnosis from group 1.

In total 13 (26.5%) out of 49 patients had not suffered from any further cluster periods during a mean observation period of 8.9 years (median 8.0 years, range 2.5–17 years). The only female had her first cluster period at the age of 66 years, and she had no further bouts during the following 9 years. The mean age at onset among the 13 patients was 40.8 years (median 40 years, range 23–66 years) and the mean duration of the first period was 3.7 weeks (median 4 weeks, range 1–8 weeks).

Altogether 36 patients in groups 1 and 2 had a definitive CH diagnosis according to the IHS criteria on follow up. Four of them had primary chronic CH and one had secondary chronic CH. The remaining 31 patients suffered from episodic CH.

In conclusion, we found that some patients with CH may suffer from only one period during many years or even in their lifetime, and that most patients with a definitive CH diagnosis probably recur within 3 years. The incidence for a second period after 3 years in our material was about 17%. The patients with only one period on follow up had a shorter duration of the first period (mean 3.7 weeks) than the deceased patients with only one documented period (mean 9.5 weeks) and the patients with episodic CH on follow up (mean 7.6 weeks). Furthermore, the patients with one period only were slightly older at onset, but otherwise the symptomatology was similar to those with a definitive CH diagnosis.

Study II

We identified 42 subjects with CH (27 men and 15 women) in 21 families. One subject had probable CH. Twelve subjects (7 men and 5 women) from 9 families had clinical symptoms that closely resembled CH, but they did not fulfil all the diagnostic criteria for CH. They were classified atypical CH. We also received information from 31 unaffected 1st - and 2nd- degree relatives (13 men and 18 women, mean age 60 years, range 14-86 years) by personal telephone interview. Thus we personally interviewed 86 subjects (55 sufferers of typical, probable or atypical CH and 31 unaffected).

The male:female ratio was 1.9:1 in CH and 1.4:1 in atypical CH, while the combined male:female ratio was 1.8:1. The mean age at onset was similar among those with CH

and atypical CH (27 years (range 12-56 years, SD \pm 11) versus 27 years (range 12-58 years, SD \pm 12, $p=0.76$). The combined mean age at onset in CH and atypical CH was significantly lower in the 2nd and 3rd generation than in the 1st generation (31 vs. 22 years; $p<0.01$).

All CH sufferers had strictly unilateral pain. Six had unilateral pain with side changing location; four had a side of pain that was more dominant. The change of side occurred within cluster periods in two subjects and between cluster periods in the remaining four. Two of those with side changing symptoms belonged to the same family as well as another subject with atypical CH without side-changing symptoms.

Twenty-four of the 86 interviewed subjects had migraine (28 %) according to the International Classification of Headache Disorders 2004 (27). Twenty-one had MO and three had MA. Nine of the 42 with CH also had migraine (21 %), seven had MO and two had MA. The subject with probable CH had no migraine. Four with atypical CH had migraine (33 %), of whom three had MO and one had MA. Altogether 13 out of 55 (24 %) with typical or atypical CH had migraine.

Atypical CH

We found 12 cases with headache of similar type as CH, who did not fulfil diagnostic criteria for CH (27). These are referred to as atypical CH. They all had strictly unilateral pain in the orbital/periorbital area, but in two persons the side of pain could change. The temporal pattern of headaches was different, i.e. for example there were 1-2 attacks per week or only infrequent sporadic attacks a couple of times/year. This along with one other missing feature was the reason why they could not be classified neither CH nor probable CH. Four subjects described moderate to severe pain for several days, which was aggravated one or more times during the period (1:III:3, 5:III:1, 7:I:1 and 9:III:6). One person (3:III:2) had continuous mild pain in the orbital/periorbital area with infrequent attacks of more intensive pain in the same area. All subjects except one (8:II:1) reported one or more autonomic symptoms during attacks. Five were restless and eight were agitated during attacks. Only two of the 12 atypical CH cases were neither restless nor agitated during attacks, one of them had autonomic symptoms, while the other had no autonomic symptoms. Pain was aggravated by alcohol in 5 of the 12 subjects.

The mean age of the atypical CH sufferers was 50 years (range 21-75) and the mean duration of disease was 25 years (4-45 years).

Familial CH

Among the 42 CH cases 39 had episodic CH and 3 had chronic CH. The mean duration of episodic cluster periods was 6.4 weeks ($n=37$). Two persons could not describe the exact duration of periods of their episodic CH.

The mean attack frequency per day during active periods of CH was 3.2 (SD \pm 2, $n=42$). However, all affected in families 6, 13 and 21 reported a high daily attack frequency; the two with typical CH in family 6 had 6-8 and 2-6 attacks per day, the three affected in family 13 had 5-8, 4 and 1-6 attacks per day, respectively, and the two

affected in family 21 had 5 and 3-8 attacks per day. Of these three families only family 6 had an affected with atypical CH.

All patients with typical CH described one or more associated autonomic symptom on the pain side during attacks; 35 (83 %) had conjunctival injection, 37 (88 %) had lacrimation and 33 (79 %) had nasal congestion and/or rhinorrhoea. Fifteen (36 %) described miosis on the affected side, but 20 persons were uncertain about miosis. Twenty-eight (67 %) described ptosis, but 7 persons did not know if they had ptosis during attacks. Twenty-seven (64 %) described increased sensitivity to light and 15 (36 %) had nausea during attacks. Twenty-three (55 %) reported provocation of attacks by alcohol.

Furthermore, not mentioned in the paper, we received information about frequent tobacco use ever, and here we found a significant difference ($p < 0.005$) as regards smoking habits between typical and atypical CH compared to healthy relatives. Thirty-three of the 43 (77 %) with typical and probable CH had ever used tobacco regularly. Fifteen persons were still smokers, of whom 8 were heavy smokers, i.e. they consumed more than 15 cigarettes/day and 3 were chewing tobacco (grounded tobacco placed under the upper lip). Among those with atypical CH, 7 had ever been using tobacco (58 %) and one was still a heavy smoker. The difference in smoking habits between CH and atypical CH was not significant ($p = 0.27$). In the unaffected group 13 of 31 (42 %) had ever used tobacco and 5 were current smokers, 3 were heavy smokers.

Study III

In the study of CACNA1A polymorphisms in CH and controls we found 11 alleles of the dinucleotide repeat polymorphisms (D19S1150), ranging in size from 144 to 168 bp. The 158 bp allele was somewhat more frequent in CH patients and the 156 bp allele was somewhat more frequent in controls, but none of the comparisons were statistically significant.

For the CAG-repeat in the 3' coding region we detected alleles ranging in size from 112 to 142, with estimated numbers of repeats ranging from 4 to 14. The reported repeat lengths in control populations in other studies vary from 4 to 20 repeats (116, 122, 169, 170). We observed fragments varying from 112 to 142 base pairs, corresponding to 4–14 repeats. The 14 repeat allele was somewhat more frequent in the control group, but differences were not significant.

Chi-square comparisons of phenotype frequencies with Monte Carlo simulation confirmed the lack of significance ($p = 0.398$ for D19S1150 marker and $p = 0.365$ for the CAG-repeat).

We analyzed the extent of LD between specific alleles of the two markers. The distribution of genotypes was similar in patients and controls, with a minor increase of the combined alleles, 162/139, in patients (23% vs. 13% in controls, OR = 1.8, $p = 0.16$). The 154 allele occurred more often together with the 136 allele in both patients

and controls (OR = 2.39, $p < 0.01$). This indicates a slight LD between the two markers that appeared to be similar in patients and controls.

Study IV

In the NOS-study we found 13 alleles ranging in size from 186 to 214 bp for the dinucleotide NOS1a marker.

For the NOS1b marker we found nine alleles ranging in size from 395 bp to 419 bp. As for the bi-allelic polymorphism in the NOS2A (iNOS) promotor region, we found two different alleles with sizes 313 bp and 317 bp. Homozygosity for 313 was the most common genotype. Genotypes were distributed according to the Hardy–Weinberg equilibrium.

For the NOS2A pentanucleotide (CCTTT)_n marker nine alleles were detected ranging in size from 179 bp to 219 bp, corresponding to 8–16 repeats. The carriage of NOS2A 194-bp allele was significantly more common in controls ($p = 0.01$, odds ratio (OR) = 0.45, 95% confidence interval (CI) = 0.25–0.82).

For the dinucleotide marker in NOS3 20 alleles were found ranging in size from 146 bp to 184 bp.

In all, we found that the distribution of phenotype and allele frequencies was similar in patients and controls except for the carriage of the NOS2A 194-bp allele, which was significantly more common in controls. Genotypes for the 194 marker allele were distributed according to the Hardy–Weinberg equilibrium in both groups. Our patient group has about the same frequency of this allele as control groups in previously studied Caucasian populations (159, 171–174), and thus we are hesitant as to the significance of this result, but we cannot totally exclude that it could be a negative association.

The familial CH cases did not show any specific pattern, but the number was small, which makes it difficult to draw firm conclusions.

Study V

The results from the complete genome screen on the Danish extended pedigrees gave no significant support for linkage to a single chromosome. However, LOD and NPL scores either potentially interesting or suggestive of linkage (≥ 2.0) were obtained for two distinct loci on chromosome 2 (markers D2S1353; 164.51 cM, and D2S1363; 227.0 cM), and one locus on each of chromosome 8 (GATA151F02; 27.4 cM) and chromosome 9 (D9S2169; 14.23 cM).

Of the four loci examined, inclusion of additional markers maintained support of potential linkage at the GATA151F02 locus on chromosome 8 alone. Multipoint linkage analysis generated NPL scores over 2.0 for three of the four flanking markers

($NPL_{\max} = 2.32$ for distal marker D8S1827). Single point analysis failed to support linkage to newly typed markers flanking any of the initial loci investigated.

When analyzing genotype data for each of the four distinct potentially linked loci in 60 further subjects, subdivided into Danish and Italian cohorts, the Danish cohort (consisting of families D42 to D799 plus 16 new pedigrees) provided no evidence of linkage to any of the identified loci. A maximum combined multipoint NPL score of 1.82 at D8S1827, and a maximum single point NPL of 1.41 for GATA151F02 was generated. In contrast, the comparatively small Italian cohort generated compelling support for linkage to chromosome 9, achieving multipoint NPL scores of 2.19 and 2.41 ($p = 0.0098$) for markers D9S2169 and D9S281, respectively. Despite producing a relatively small multipoint LOD_{\max} of 1.53 for this region, SLINK simulations for this cohort reached a maximum LOD score of only 1.51 under heterogeneity, with a 0.2% likelihood of achieving a LOD score greater than 1.5 by chance. Subsequent linkage analyses using the combined Danish and Italian populations did not approach a LOD score of statistical significance at any of the four loci, and drastically reduced the chromosome 9 scores achieved previously in the Italian cohort.

No sequence variants of the HCRTR2 gene were detected by comparison to wild-type control sequence. Furthermore, no significant differences in allele or genotype frequencies between sporadic patient and control populations were detected. The 922G/A polymorphism in exon 5 [rs2653349] has previously been reported to be associated with CH. However, there were no difference in either phenotype or allele frequencies in our diverse CH cohort, when compared to the matched control population. The dataset was also stratified into distinct populations, but still no differences were seen.

Study VI

Overall quite small intraindividual changes between different disease stages were seen. On the other hand, large interindividual differences were seen between patients and controls.

Pairwise comparisons on signal values from different disease stages and controls, comparisons within patients and between patients and controls, generated relatively few regulated genes. The most obvious finding was the upregulation of several calcium binding S100 proteins; S100A8, S100A12, S100P and also annexin 3 (S100 protein like), during active period and attack (except S100A8) vs remission. RASEF and RARA seem to be upregulated during attack vs active period. Also, TNFRSF10C, a member of the tumor necrosis factor (TNF) family of cytokines, and ICAM3 also regarded as being involved in the immune system, showed upregulation during attack vs remission. BIRC1, baculoviral IAP repeat-containing protein, also named neuronal apoptosis inhibitory protein (NAIP) was upregulated during both period and remission in relation to controls. Charcot-Leyden crystal protein, considered to be a morphologic hallmark of eosinophil-related disease (175) was downregulated in patients during attack and remission compared to controls. CREB5, cAMP responsive element binding protein 5, was upregulated during attack vs controls. EIF5A, eukaryotic translation

initiation factor 5A, showed downregulation in relation to controls. Integrin β 2 with probe ID 236988_x_at was upregulated in patients compared to controls, most apparently during active period, but the expression was upregulated in all CH phases in the three patients compared to controls. However, the probe ID is not specific for Integrin β 2 and other probes referring to the same gene did not show the same pattern.

This finding of upregulation of several S100 proteins (a group comprising 24 proteins) was significant for both groups (both periods and attack), i.e. out of almost 22 000 genes only a few were upregulated, but of these several were S100 proteins, $p < 0.0001$ for period vs remission and $p = 0.00037$ for attack vs remission. There were no significant differences for these transcripts in relation to controls.

The upregulation of S100P during active phase could be confirmed, since quantitative RT-PCR of S100P gene expression showed significant differences between attack and remission, $p = 0.048$ (repeated measures analysis of variance and Tukey-Kramer correction for multiple comparisons). Two patients had a higher expression level during period compared to attack, but still it was higher compared to remission. Comparison of means between period and remission and controls was non-significant, $p = 0.664$ and 0.146 respectively. Comparison of means between attack and controls was considered not quite significant, $p = 0.089$. Thus, these results were also consistent with the microarray analysis.

DISCUSSION

Clinical aspects

Maybe due to the rarity of CH there are limited amount of studies as regards the natural clinical course of CH.

Our follow-up study is to our knowledge the first follow-up study of consecutive CH patients examined in relation to an assumed first period of headaches. Commonly, patients come to the neurologist for a confirmation of a CH diagnosis after several cluster periods. It is also well known that there is a diagnostic delay as many patients are seeing several doctors before a correct diagnosis (15, 176-178). According to the former IHS criteria two headache periods were required for a definitive diagnosis of episodic CH (26).

After a mean observation period of 8.9 years, 27% (13/49) of our patients still had had one cluster period only. Our study also showed that 83% of the patients with a definitive CH diagnosis had their second cluster period within 3 years and 94% within 5 years. The time to recurrence for those 31 patients who had definitive episodic CH varied from a few months to about 11 years, while the observation period for those with one period only varied between 2.5 and 17 years. We can therefore not exclude that some of the patients with the shortest observation period still may show up with a second cluster. On the other hand, most patients with a definitive CH had their second period within 3 years, as mentioned above. We also found that the patients with one period only had a shorter duration of first period than the deceased patients with only one documented period and the patients with episodic CH on follow up. It is somewhat difficult to draw any firm conclusions from these findings as the sample size is small and it is also common that the duration of periods may vary greatly within one individual.

The possibility to diagnose CH already in relation to the first attacks might be an advantage when choosing treatment options, but also for prognostic information to our patients. Patients with a first period may now get the information that CH *might*, in some cases, disappear after one period only.

This follow-up study might be criticized for the limited number of cases studied. However, it takes years to collect patients that are observed in relation to a first period since most patients come to see a neurologist first several years after onset of the disease, see above.

Obviously, this study has had some impact on the revised IHS classification from 2004 (27). In the new classification CH should be diagnosed already in relation to the first period, i.e. no further periods or continuous attacks are needed for the diagnosis, but still for a diagnosis of episodic or chronic CH. This change in the classification was made with referral to our study. However, we believe that these patients, met during

their first periods, are probably unsuitable for any research study except for natural history studies.

In our study on familial CH, we found 12 cases with headache of similar type as other affected within the same family, but with somewhat different pattern and thus not fulfilling CH diagnostic criteria (27). We have named these patients atypical CH. They all had clinical CH characteristics, but the attacks occurred in a sporadic way rather than in clusters even though some had a tendency towards “mini-bouts” (179). Some had pain described to be somewhat milder than in typical CH. A few described very severe pain for many hours. Some reported episodes with moderate to severe headache lasting for some days, but less than a week, with more intense pain for several hours during these days. One subject had continuous mild and dull pain in the orbital/periorbital area with infrequent attacks of moderate to very severe pain associated with autonomic symptoms in the same area. All atypical cases described strictly unilateral orbital/periorbital pain. All but one had autonomic features during attacks. Some of the cases could in theory be classified as MO or episodic tension-type headache. However, features as combinations of ipsilateral autonomic symptoms, restlessness and agitation are not common clinical symptoms of migraine without aura or tension-type headache.

The probands in this study were asked to report all 1st - and 2nd - degree relatives that had CH or migraine. They were also asked to report healthy relatives that could be willing to participate in a study on clinical and genetic aspects of familial CH. Contacted persons in second hand were also asked to give the same information on their relatives. Thus, we interviewed everybody in the family suspected of having CH and hopefully we received information on most affected individuals. However, it is today well known that there is a risk of overestimation of CH in relatives, if the relatives are not personally interviewed. Our study definitely confirms this, since several individuals were supposed to have CH but had atypical CH or migraine. On the other hand, we also found a case suffering from atypical CH, of which the proband was unaware of.

Former studies have indicated that some familial CH cases might differ from the clinical pattern of sporadic CH. Also, in former studies of familial CH, cases not fulfilling IHS diagnostic criteria (26) have been commented, but with no further detailed information (97, 110). In the former classification the term “cluster headache like disorder” was an entity and in the Italian study several cases were referred to this group. In the Dutch investigation a group of patients were referred to as fulfilling all criteria minus one, most often due to too long attacks. Sjaastad has reported on so-called ‘mini-bouts’ and one of the cases, with this kind of temporal pattern, also had a brother with CH (179). It is therefore very likely that atypical CH does not only exist in Swedish CH families, although it has not been reported in a structured way before.

On the other hand, atypical pattern of CH in sporadic patients has been described in several reports. Several of our patients describe a somewhat ‘milder’ form of the disease and a few had not seen a doctor for their symptoms. This could thus be the same with atypical variants of sporadic CH. Sjaastad has reported on mild CH before (18) and just recently a report on CH cases without autonomic symptoms was published

(180). The only difference the authors found compared to those with autonomic symptoms was the intensity of pain, which was reported to be somewhat milder in those without autonomic symptoms. The authors conclude that these cases might represent a milder form of CH.

We therefore suggest that atypical CH represents an expanded spectrum of the disease, and that this might be the case in both sporadic and familial CH cases. We also suggest that the atypical cases in our families are phenotypical variations of the disease, representing one disorder with the same genetic background within the families. The identification of these patients could be of importance since we might be able to treat patients with these atypical symptoms. It is obvious, from discussions with our atypical cases, that sumatriptan works for many of these patients too. On the other hand, it is also obvious that some patients have a milder form of CH and they are thus not in the same urgent need for effective treatment.

The incidence of migraine was quite high in our interviewed individuals in our CH families (28%). The high prevalence of migraine (35%) among our series of relatives without CH is likely to be caused by selection bias, as a few were thought to have CH and for that reason included. The incidence of migraine was also quite high in our typical and atypical CH (24%). However, they were all able to clearly differentiate between the different headache types, which further emphasizes that the atypical CH was of another type than migraine.

Smoking is very frequent in CH patients (181, 182) and an interesting, formerly unpublished, observation from this study is the high incidence of former or actual tobacco use in the affected group compared to their unaffected relatives. In both the CH and the atypical CH group the use of tobacco was significantly higher than in the unaffected group. However, we would have liked to have information from all 1st and 2nd degree relatives as our unaffected relatives may not be representative for all closely related relatives. Nevertheless, this finding could be an example of gene environment interaction, i.e. tobacco use being an environmental trigger for CH in genetically predisposed persons.

A very recent published observation of eleven non-smoking CH sufferers showed that eight cases had been exposed to significant second-hand tobacco smoke from parents during childhood (183). The age of onset of CH in these individuals appeared to be earlier than for the average CH sufferer. Obviously, the potential relevance of smoking in CH needs further studies.

It would also, for other reasons, have been of interest to have had personal contact with all first- and second degree relatives to the probands, not only suspected affected or healthy individuals. Calculations of RR in relatives to CH could thus have been performed. However, this was not the initial intention of the study.

Genetic aspects

We have performed 2 candidate gene studies on CH cases and controls, one linkage study combined with an allelic association study and one microarray study on different disease stages of CH.

At the beginning of our genetic research on CH we were, probably, the first group to study candidate genes in CH. The molecular genetic background had not formerly been studied except for studies with a very small sample size, or rather case reports. This could partly explain the small sample size of our dataset as this was something new and even small studies were of interest. Also, we started this genotyping in 1999 and at that time it was not uncommon with studies on sample sizes around 100 individuals and about 100 controls. However, research within the genetic field has expanded enormously during these past years. Association studies were criticized due to studies with small sample size that never were replicated in other populations. Therefore, for some years, linkage studies seemed to be the clue for genetic research in complex diseases. Today, it is becoming more popular again to perform allelic association studies on genes supposed to be of functional importance, but also in the search of finding genes within loci that have been indicated in linkage studies. Furthermore, it is today well known that studies are best performed in large, well characterized, clinical and control population of up to 2000 individuals in both groups. If smaller, it is of great importance to replicate in other populations.

At the end of the 90's there was a break through in migraine genetics due to the findings of mutations in the CACNA1A-gene that were responsible for several episodic neurological disorders, especially its importance in FHM (116). At this time several studies were performed for the search for a plausible relevance of this gene even in the more common forms of migraine.

Thus, we decided to study the CACNA1A gene, assuming that it could also be of importance in CH pathophysiology. We did not find any significant differences as regards phenotype and allele frequencies between patients and controls. Our conclusion was that, lacking other direct indications of an importance of this gene in CH, we considered the data sufficient at that time. We emphasized that a minor contribution to sporadic CH or a major contribution in a subgroup, for example familial cases of CH, could not be ruled out. We can, though, not be certain of our conclusions about the CACNA1A gene and CH. We can, only, conclude that differences in the polymorphisms studied not seem to be of any importance in our studied CH population.

In the same issue of Cephalalgia where paper III was published, another paper on the CACNA1A gene in CH was presented. A linkage study had been performed in an extended Dutch CH pedigree (184). Haplotype analysis did not reveal an obvious disease haplotype, and SSCP analysis of all 47 exons of the CACNA1A gene did not reveal a causative mutation. The authors concluded that CH in this family was not caused by mutations in the CACNA1A gene. In addition, the editorial in this issue discussed the negative finding in this latter study (185). Obviously, despite our negative

result, studying the CACNA1A gene in CH was relevant and not far-fetched at this stage.

We then decided to study polymorphic markers of the NOS-genes assuming that they would be of importance in CH pathophysiology. NO was also very popular at this time, and the importance of NO in the pathophysiology of neurovascular headache disorders was well established (16, 76, 77, 186, 187). NO had been proposed to be the final promoting factor in CH, since it is involved in both the central and the peripheral activation systems; NOS function in the hypothalamus can itself be modulated by NO-ergic routes, and NO is also involved in vasodilation (78). We assumed that there could be a genetically determined predisposition for abnormal function of the NO system in CH patients.

We analyzed five polymorphic markers of the three NOS genes assuming that they should be of importance in CH aetiology. We did not find any significant differences between patients and controls. We concluded that genetic variations of the three NOS genes do not contribute greatly to CH susceptibility. NO may still be the common mediator in CH, but it might be due to other mechanisms than genetic variations within the NOS genes. However, we concluded that we could not completely rule out that these genes have a small influence for the risk of CH. However, again, this is a matter of sample size and the risk for false negative results in studies with too small datasets. It should also be mentioned that our negative results not have been studied in another population.

However, after two association studies on relevant candidate genes we started the collection of CH families, with the aim to join an international collaboration - a linkage study on CH families from several countries. This work was initiated on the Danish largest families with a genome scan with almost 400 microsatellite markers covering the whole genome at 9 cM intervals. Four loci showing LOD scores of potential significance were analyzed with additional markers in additional individuals of Danish and Italian origin. When individuals were added, the formerly positive LOD scores were deflated. Thus, linkage analysis combining data from the ethnically diverse groups of Danish and Italian origin caused a drop in LOD score at all four loci by comparison to original calculations. Thus, a single disease locus for CH was not identified.

Recently, in the Nov issue 2004 of *Neurology*, a new allelic association study on CH was published (188). This was, together with a negative genetic study on CH and trace amines (189), the first published molecular genetic studies on CH after our last genetic investigation on the NOS-genes. In this particular Italian study several polymorphisms of the hypocretin/orexin system genes were evaluated in 109 CH patients and 211 controls. A G>A polymorphism of the gene was significantly different between cases and controls. Homozygosity for the G allele was associated with an increased disease risk for CH (OR: 6.79, 95% CI, 2.25 to 22.99). The authors conclude that their data suggest that the HCRTR2 gene or a linked locus significantly modulates the risk for CH. Interestingly, this study also had a quite small sample size just like our former studies, at least as regards the affected individuals studied.

The finding of an association to a hypocretin receptor gene, of relevance in sleep disorders, was of great interest since this moves us back towards the hypothalamus. Hypocretin-1 and -2 (also called orexin-A and -B) are neuropeptides located exclusively in the posterolateral hypothalamus. They act on G protein receptors, named HCRTR1 and HCRTR2, respectively. Hypocretins are important in regulating the sleep-wake cycle and a mutation in hypocretin has been reported to cause narcolepsy (190).

However, this Italian group has just published (Online Early, June 2005, in *Cephalalgia*) another association study on an Italian cohort of CH patients, also with focus on the biological clock but this time with negative results (191). A *Clock* gene polymorphism was studied, but phenotype and allele frequencies were the same in patients and controls.

We now have the results from an allelic association study on the HCRTR2 gene in a Swedish, Danish and British cohort comprising 259 patients and 272 controls. Despite the lack of a locus with high LOD score in relation to the gene, an association study was performed on SNP's in the HCRTR2 gene. In our cohort the HCRTR2 association could not be confirmed. After stratification of SNP data into different populations it was still not confirmed. This finding needs to be confirmed in another larger population before it is possible to draw any final conclusions about whether the HCRTR2 gene is of importance in CH pathophysiology.

The combined results from the genome scan and the HCRTR2 association analysis indicate that there might be multiple disease genes segregating in this particular European cohort, there might even be population-specific genes.

It could also be that genetic factors in CH are of less importance than we thought some years ago. In the beginning of our genetic research in CH we had other prevalence figures, i.e. CH was supposed to be less common. However, during the last years there have been new investigations regarding CH prevalence. A recently published study from Vågå (Norway) yielded a prevalence of CH of 0.38% (192). In this survey all participants were interviewed by a neurologist according to the criteria of the International Headache Society (26). However, three of the seven affected participants did not fulfill the strict criteria for CH. An Italian survey recently revealed a prevalence of 0.2%, and thus also pointing to a higher CH lifetime prevalence than previous reports (193). Possible CH cases were screened from a sample of 10, 071 patients registered at seven general practitioners in Parma. A CH diagnosis was confirmed in 21 patients. Even in this study the probably affected had been interviewed by an experienced neurologist. Thus, it is likely that the true prevalence of CH is higher than previously thought, i.e. one per 500 rather than one per 1500, and in many ways corresponding to the old extrapolated figures from Ekbom (28, 29). Recalculations of former results in relation to these new prevalence data gives a lower RR in relatives than from previous prevalence data (194), i.e. the RR for CH in 1st degree relatives is 5 to 18 and in 2nd degree relatives 1 to 3. However, yet another investigation from USA has recently been performed by counting the incidence of medically recognized CH within Olmsted County. The results were compared with previously published

incidence data from 1979 through 1981. The overall age- and sex-adjusted incidence decreased from 9.8/100,000 person-years in 1979-1981 to 2.07/100,000 person-years in 1989-1990 (195). Also, my personal impression, without any scientific data, is that a prevalence of 1/500 is a rather high figure.

Thus, to summarize the results from this collaboration, genetic predisposition to CH is likely to be complex and compounded by locus heterogeneity and variation in genes conferring only a small effect size.

Our last study, on gene expression profiling in CH, was probably the most difficult to interpret and evaluate, although we finally got some very interesting results. CH is a disease with well defined disease stages and thus ideal for gene expression studies for a better understanding of pathophysiological mechanisms. There has been a tradition at our lab, at least for a few years, to study gene expression with real time-RT-PCR methodology, which is supposed to be a quite robust method for studies of gene expression. Our initial aim of this study was to study the regulation of clock genes. Ideally this should be performed on hypothalamic tissue. However, this is not, of course, feasible. We thus decided to study gene expression in peripheral blood, although it would have been of interest to be closer to the brain. Still, we regarded it as of great interest to study gene expression patterns in peripheral blood during different phases, especially since inflammatory mechanisms have been proposed to be of relevance in CH pathophysiology. We also had knowledge in our group on how to perform microarray studies on animals. However, performing microarray analysis on CH patients showed out to be quite complicated for several reasons.

Microarray studies offer a great opportunity for studies of large scale gene expression. However, the technique still deals with some problems; the methodology is still new and the analysis procedure is an issue of controversy. Even if we have statistical methods for analysis, we do not have all the answers on how to interpret the results received and whether we can trust the findings. Microarray experiments are also still an expensive procedure. On the other hand, a lot of expression information is generated in one experiment only. Furthermore, unexpected results from microarray experiments may function as hypothesis generators instead of conventionally hypothesis driven studies.

Gene expression might differ due to circadian rhythms, ethnicity, gender, age and more. We therefore decided to collect samples at the same time of the day, except for attack samples, to reduce normal gene expression changes and variations over the day. Pooling of samples is tempting, mainly with the aim to minimize costs, but we knew from experience that this could be crucial if we had outliers. Thus we decided not to pool any samples, rather to collect fewer samples but analyze them individually. We decided to use a new RNA extraction technique, mainly for the convenience of being able to collect samples during nocturnal attacks.

We experienced some pitfalls during the different steps of this gene expression study. The RNA extraction method had not been widely used before and today there are reports on how to manage and receive better quality by using modified protocols (196). If we had better RNA quality from the very beginning we probably would have had

received better samples for the microarray analysis. We also received large differences between samples interindividually, but small differences between samples intraindividually. After having tried several methods for analysis, we decided to use the most robust method, and probably the least controversial for Affymetrix data, i.e. the pairwise comparison analysis. In studies with more replicates than in our study it is common to also filter, for example, for genes that are increased in 4 out of 5. Due to the small sample size we chose to only report those that were increased/decreased in all, with present in all increased.

However, we hereby received relatively small gene lists with quite small differences as regards fold-change, but on the other hand we got some interesting results. Out of only 11 upregulated genes during period versus remission 3 were S100 proteins, S100 A8, S100A12 and S100P. This was also seen when comparing attack versus remission, S100A8, S100P and also annexin A3, calcium-binding and S100 protein like, were upregulated. ICAM3 also showed upregulation during active phase. BIRC1 (neuronal apoptosis inhibitory protein) and CREB5 were upregulated in patients compared to controls.

The finding of several upregulated S100 proteins was clearly significant, when analyzing the fact that several genes from the same group (comprising 24 genes) were upregulated from a selection of 54 000 transcripts corresponding to almost 22 000 genes. Upregulation of S100P during active phase of CH was confirmed with quantitative RT-PCR in six CH patients.

The family of S100 proteins is the largest groups of calcium binding proteins and S100 proteins are of interest as mediators of calcium-associated signal transduction. The usefulness of calcium binding proteins of the S100 family, especially S100A8 (calgranulin A) and S100 A12 (calgranulin C), has been demonstrated as diagnostic markers of inflammation, at low grade inflammation not detected by other diagnostic tests (197). These proteins have also been described as pro-inflammatory proteins activating endothelial cells (198). The role of S100P is yet not completely understood, but overexpression of S100P has been reported in pancreatic cancer (199-201). In a recently published study S100P was, together with several other S100 proteins, found to be upregulated during the acute phase of Kawasaki disease (202).

Whether there is an inflammatory component in CH has been debated, both a local venous inflammation and a systemic inflammation have been discussed (62, 63, 68-70, 203, 204). In a study from our group no signs of systemic inflammation, studied with conventional blood parameters, was seen (66). However, the results from this present study indicate that S100 proteins of pro-inflammatory type are upregulated during the active phase of the disease. The fact that these proteins are calcium-binding might also be of relevance in relation to the prophylactic treatment with verapamil, a calcium-antagonist. We cannot be certain whether this upregulation is a primary or secondary phenomenon in CH, and the relevance of this finding. However, pro-inflammatory proteins of other types are known to be involved in the complex interaction between hypothalamus and the immune system.

Concluding remarks

We have performed studies on CH with quite different approaches, both from a clinical and a genetic view.

As for the clinical studies we found atypical variants that had not been formerly reported. We found, in our prospective study, that some patients might suffer from one period only, and in the new IHS classification from 2004 CH should be diagnosed in relation to the first period. This might be of clinical importance as some patients may only have one period during lifetime.

In our CH families we found that several individuals obviously had an atypical form of CH. This atypical CH did not present as a new homogenous type of headache, rather several varieties of CH not fulfilling diagnostic criteria for CH. Several with atypical CH also had migraine, and they could clearly differentiate between the headache types. These findings suggest that atypical CH in CH families may represent an expanded spectrum of the disease with a common aetiology, i.e. a common genetic background within families, although environmental factors cannot be ruled out. We also noticed that actual and former tobacco use was higher in the affected compared to the unaffected group, which might be an indication of gene-environment interaction. Our plan is to study this in further detail by collecting information from additional relatives.

Today it is generally known and accepted that large, well-defined, case-control cohorts are needed for reliable genetic association analysis. However, we chose a candidate gene approach to study polymorphic markers of the *CACNA1A* genes and also for the three different NOS-genes in CH patients and matched controls, assuming that they would be of functional importance in CH themselves or in LD with other disease causing mutations. We did not find any clear association to these markers and CH in our limited case-control cohort, and thus conclude that these genes do not play a major role in CH pathophysiology. However, only studies of the same genes in another cohort could clearly rule out whether our conclusions were reliable.

In the genome wide scan in 5 Danish extended pedigrees suggestive linkage indicated four possible disease loci, but there was a marked drop in LOD score when further Danish and Italian families were added. Thus, no single locus for CH was identified. As for the *HCRTR2* gene, which recently was shown to be associated with CH in an Italian case-control cohort, an association analysis of SNP's in this gene was performed in a Danish, Swedish and British cohort. However, the formerly reported association could not be replicated in our European cohort, not even when the data was stratified into distinct populations. Eight familial cases were also sequenced, but no mutations within this gene were seen. We conclude, from these studies, that genetic heterogeneity in CH is likely.

We performed a global gene expression analysis in peripheral blood assuming that we could find changes in gene expression of pathophysiological interest also in peripheral blood. We found upregulation of several calcium-binding S100 proteins, mainly of proinflammatory type, during the active phase of the disease. The potential role of these genes in CH needs further studies.

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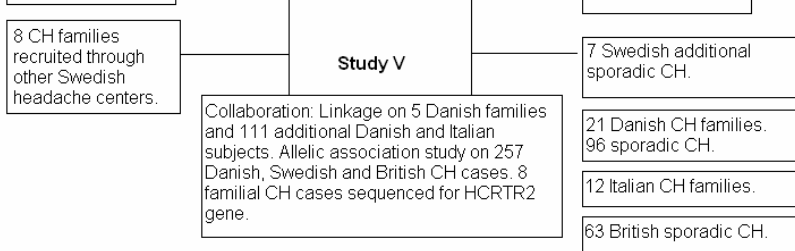
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Family 4

